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Ulcerative colitis-associated colorectal cancer

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Abstract

The association between ulcerative colitis (UC) and colorectal cancer (CRC) has been acknowledged. One of the most serious and life threatening consequences of UC is the development of CRC (UC-CRC). UC-CRC patients are younger, more frequently have multiple cancerous lesions, and histologically show mucinous or signet ring cell carcinomas. The risk of CRC begins to increase 8 or 10 years after the diagnosis of UC. Risk factors for CRC with UC patients include young age at diagnosis, longer duration, greater anatomical extent of colonic involvement, the degree of inflammation, family history of CRC, and presence of primary sclerosing cholangitis. CRC on the ground of UC develop from non-dysplastic mucosa to indefinite dysplasia, low-grade dysplasia, high-grade dysplasia and finally to invasive adenocarcinoma. Colonoscopy surveillance programs are recommended to reduce the risk of CRC and mortality in UC. Genetic alterations might play a role in the development of UC-CRC. 5-aminosalicylates might represent a favorable therapeutic option for chemoprevention of CRC.

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Key words: Ulcerative colitis-associated colorectal cancer;

Risk factor; Dysplasia; Surveillance colonoscopy; Chemoprevention

Core tip: Colorectal cancer (CRC) is more frequent in patients with long-term ulcerative colitis (UC), and is one of the most serious and life threatening consequences of UC. Knowledge of risk factors for CRC is important to identify UC patients who need surveillance. Colonoscopy surveillance programs are recommended to reduce the risk of CRC and mortality in UC. Genetic alterations might play a role in the development of CRC in UC patients. 5-aminosalicylates might represent a favorable therapeutic option for chemoprevention of CRC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease showing mucosal inflammation from the rectum to the oral side. Crohn and Rosenberg reported the first case of adenocarcinoma complicating UC in 1925^[1]. The risk of developing colorectal cancer (CRC) is found to be high in patients with long-term UC^[2,3]. UC-CRC is considered to develop from a non-neoplastic inflammatory epithelium to dysplasia to cancer. Therefore, colonoscopic surveillance in patients with long-standing UC has been recommended. UC-CRC shows characteristic clinicopathological features of CRC. In this paper, the characteristic properties of UC-CRC are reviewed.

CLINICAL FEATURES OF COLITIS-ASSOCIATED CRC

Clinicopathological features

UC-CRC patients are younger, more frequently have mul-

multiple cancerous lesions, and a macroscopically permeating pattern of spread, including mucinous or signet ring cell carcinomas, compared with sporadic CRC^[1-3]. The advanced stage at presentation causes less favorable outcome of UC-CRC in IBD patients.

Incidence of UC-CRC

An inflammatory environment is believed to play an important role in the pathogenesis of UC-CRC in patients with chronic colitis^[4]. UC-CRC accounts for about 1% of all CRC^[5]. The risk of CRC begins to increase 8 or 10 years after the initial diagnosis^[6-8]. Table 1 summarizes representative reports on the risk of developing colorectal cancer in patients with UC. Eaden *et al*^[9] conducted a meta-analysis of 116 studies, and found that the probability of CRC in patients with UC increased with the duration of disease; 1.6% at 10 years after a onset of UC, 8.3% at 20 years after, and 18.4% at 30 years after. This increased incidence of UC-CRC is four to ten times greater than that for sporadic CRC, and the average age of onset is 20 years earlier. Several other studies reported that the risk of UC-CRC in UC patients was 5%-7% at 20 years after onset of disease^[10-14], 7%-14% at 25 years^[15,16] and 7.5%-18%^[9,17] at 30 years. Eaden *et al*^[9] confirmed that there is an increased risk for UC-CRC in pancolitis (5.4%), while the incidence in all patients with UC was 3.7%. In some countries, patients with UC have not been found to be at increased risk of CRC development. Winther *et al*^[18] reported that the probability of CRC in Denmark was 0.4% by 10 years, 1.1% by 20 years, and 2.1% by 30 years of disease, suggesting that neither the overall cancer risk nor the UC-CRC risks increased after a median of 19 years of follow-up evaluation. This low rate of CRC development may reflect the high rates of surgical approach and chemoprevention for UC in Denmark. Taken together, the 5-aminosalicylic acid (5-ASA) treatment and frequent surveillance colonoscopy with proctocolectomy for dysplasia could explain the reduction in the incidence of CRC in UC patients. Moreover, current studies indicate that the risk of CRC seems to be lower. Rutter and co-workers reported cumulative incidences of UC-CRC of 2.5% at 20 years of colitis duration, 7.6% at 30 years, and 10.8% at 40 years^[19] indicating only a 1.5 to 2-fold increased risk for CRC (5%) in comparison with the non-UC population. Söderlund *et al*^[20] indicated that the overall cumulative incidence of CRC at 10, 20, and 30 years after the inflammatory bowel disease (IBD) diagnosis was 1%, 1.5%, and 2.7%, respectively. Manninen *et al*^[21] reported only slightly increased risk for UC-CRC in UC patients in a Finnish cohort. Hata *et al*^[22] reported that the cumulative risks for the development of invasive cancer at 10, 20, and 30 years were 0.5%, 4.1%, and 6.1%, respectively, while those for the development of definite dysplasia at 10, 20, and 30 years were 3.1%, 10.0%, and 15.6%, respectively. A further current systematic review with meta-analysis in 2014, based on 81 studies and 181923 patients, reported that the risk of UC patients

developing colorectal cancer has decreased steadily, and the incidence rate decreased from 4.29/1000 patient-years in the 1950s to 1.21/1000 patient-years in the last decade^[23]. The risk of developing CRC with longstanding Crohn's colitis is considered to be similar to that of UC, while the incidence of CRC in Crohn's disease showed various ranges in cancer risk^[24-27].

Patients with UC who have undergone proctocolectomy have a very small risk of dysplasia in the ileal pouch^[28]. Anal transitional zone dysplasia after ileal pouch-anal anastomosis is infrequent. Anal transitional zone preservation did not lead to the development of cancer in the anal transitional zone after five to ten years of follow-up^[29].

Patients with UC who develop CRC have a worse prognosis than for CRC patients without UC^[30-33], and the long-term prognosis of UC-CRC is even worse when patients with the same tumor stage are compared^[32]. UC-CRC is frequently diagnosed at an advanced stage^[33]. These findings emphasize the importance of knowledge of risk factor for UC-CRC and surveillance for patients with UC.

RISK FACTORS OF UC-CRC

Knowledge of risk factor for CRC is important to categorize subgroups of UC patients who need frequent surveillance or intense treatment. Risk factors for CRC in UC patients include, anatomical extent, young age at diagnosis, duration of disease, concurrent primary sclerosing cholangitis (PSC) and family history of CRC. In addition, smoking, pseudopolyps, persistent inflammation of the colon and backwash ileitis are also risk factor for CRC^[34,35]. These UC patients with risk factors should be enrolled in an intensive surveillance program.

Pancolitis

The anatomical extent of colitis is an independent risk factor for the development of CRC. A meta-analysis showed that the incidence of CRC in patients with extensive UC was 5.4%^[36]. Patients with pancolitis are at high risk of CRC, left-sided colitis is moderate risk, and proctitis and proctosigmoiditis are low risk, being similar to the non-UC population^[20,37,38]. Ekbom *et al*^[38] reported that UC patients with pancolitis had a 15-fold higher risk of CRC compared with the non-UC group, in contrast to an increased risk of 2.8 for patients with left-sided colitis and no significant increased risk for those with proctitis, and reported an overall risk of 4.8 for UC patients with extensive disease.

Young age

Young age at onset of colitis has been reported as an independent risk factor for CRC^[6]. CRC risk varied by age at initial diagnosis of UC; patients diagnosed at childhood (0-19 years old) had a relative risk of 43.8 followed by those diagnosed in young (20-39 years old) with a relative risk of 2.65^[39].

Table 1 Risk of developing colorectal cancer in patients with ulcerative colitis

Ref.	Year	Years after UC						Country
		10	15	20	25	30	40	
Gilat <i>et al</i> ^[14]	1988	0.2%	2.8%	5.5%		13.5%		Israel
Lennard-Jones <i>et al</i> ^[13]	1990		3%	5%				United Kingdom
Langholz <i>et al</i> ^[7]	1992				3.1%			Denmark
Eaden <i>et al</i> ^[9]	2001	1.6%		8.3%		18.4%		United Kingdom
Hata <i>et al</i> ^[22]	2003	0.5%		4.1%		6.1%		Japan
Winther <i>et al</i> ^[18]	2004	0.4%		1.1%		2.1%		Denmark
Lakatos <i>et al</i> ^[17]	2006	0.6%		5.4%		7.5%		Hungary
Rutter <i>et al</i> ^[107]	2006			2.5%		7.6%	10.8%	United Kingdom
Söderlund <i>et al</i> ^[20]	2009	1%		1.5%		2.7%		Sweden

UC: Ulcerative colitis.

Long disease duration

Duration of UC is an important risk factor for CRC development. Among patients with IBD, the median time from diagnosis of IBD to CRC was 17 years; 21% of IBD patients developed tumors within 10 years after onset^[40].

PSC

IBD patients with PSC, a chronic cholestatic liver disease, have an increased risk of CRC^[41]. Broomé *et al*^[42] revealed a cumulative risk of CRC in UC patients with PSC of 9% after 10 years duration of symptoms, 31% after 20 years and as high as 50% after 25 years; compared with 2%, 5% and 10% in patients with UC alone matched for each duration. A meta-analysis found that 21% of UC patients with PSC developed CRC compared with 4% of UC patients without PSC. The risk of CRC in UC patients with PSC was 4.8-fold higher than that in patients with UC without PSC^[43].

Family history of colorectal cancer

A family history of CRC in UC patients increases the risk of CRC, irrespectively of the type and extent of IBD, as compared with patients with UC without positive family history for CRC^[44-46].

MOLECULAR FEATURES OF UC-CRC

Genetic characteristics detected in sporadic CRC, such as genetic mutations, microsatellite instability (MSI), and DNA hypermethylation, were also recognized in UC-CRC^[4,33,47-50]. Mutations in *p53* occur early in the adenoma-carcinoma sequence and are often detected in non-dysplastic or indefinite dysplasia in UC, while *p53* mutations occur in the late phase in sporadic adenoma^[51]. MSI is also relatively frequent in non-dysplastic inflamed epithelia, and transforming growth factor β receptor type II (*TGF β RII*) is one of genes targeted by the MSI process in UC-CRC^[50]. Hyper-methylation of *hMLH1*^[50,52], *p16INK4a*^[53], and *p14ARF*^[54] seems to precede dysplasia and contribute to the genetic alterations in UC-CRC^[55]. MicroRNAs (miRNAs) play a critical role in regulating key pathogenic mechanism in IBD^[56]. The miRNA-124a

gene has a tumor-suppressive function and is methylated during carcinogenesis in UC patients, and the methylation level of miR-124a-3 is a promising marker for estimating individual risk for CAC^[57]. By contrast, miRNA-155 overexpression is particularly associated with MSI in CA-CRC^[58]. These molecules might be useful biomarkers for early detection and treatment response of CRC in IBD patients.

Inflammatory stresses, such as reactive oxygen species and some free radicals, may cause these genetic changes^[59-61] and are considered to be factors in the pathogenesis of UC-CRC^[62,63].

SURVEILLANCE COLONOSCOPY

Cancer surveillance is based on the high-risk factors that identify patients who are likely to develop cancer. The management of UC has changed with biological therapies, surgical treatment, and surveillance tools, which have reduced the risk of CRC in patients with UC^[9,20,23]. Surveillance is recommended during the remission state to reduce the difficulty of differentiating reactive change from dysplasia^[64]. Data from an 18-year surveillance program demonstrated that cancer was detected at an early stage in 80% of surveyed patients, compared with only 41% of non-surveyed UC patients^[65]. There is evidence that surveillance colonoscopy reduces the risk of CRC and mortality in UC: The overall 5-year survival rate was 77% for the surveillance group, compared with only 36% for the control group^[33,35,65]. It has been reported that a prior history of surveillance colonoscopy reduces the odds of developing CRC by 60%-80%^[35,66].

These guidelines, commissioned by the Clinical Services' Committee of the British Society of Gastroenterology for clinicians and allied professionals caring for patients with IBD in the United Kingdom, provide an good clinical practice for surveillance and treatment^[67]. The guidelines state that UC patients should be advised to have a review colonoscopy 8-10 years after disease onset to check the extent of colitis. Current recommendations are for regular surveillance every 1-2 years in the second decade of the disease to yearly by the fourth decade. The recommended guidelines for the surveillance of CRC in

Table 2 Timing of surveillance colonoscopy for colorectal cancer in ulcerative colitis

Ref.	Year	Guidelines	Beginning of surveillance (years after onset of symptoms)	Surveillance schedule
Van Assche <i>et al</i> ^[73]	2013	European Crohn's and Colitis Organization (ECCO)	8 yr	High risk ¹ ; 1-2 yr Low risk ¹ ; 3-4 yr
Farraye <i>et al</i> ^[72]	2010	American Gastroenterological Association (AGA)	8 yr	Extensive colitis or left-sided colitis; 1-2 yr Patients with PSC; 1 yr High-grade or low-grade dysplasia; colectomy or repeat colonoscopy within 6 mo Indefinite dysplasia; 3 to 12 mo No dysplasia; 1-2 yr
Kornbluth <i>et al</i> ^[70]	2010	American College of Gastroenterology (ACG)	8-10 yr	1-2 yr
Cairns <i>et al</i> ^[68]	2010	British Society of Gastroenterology (BSG)	10 yr	lower risk ² ; 5 yr intermediate risk ³ ; 3 yr higher risk ⁴ ; 1 yr
Leighton <i>et al</i> ^[69]	2006	American Society for Gastrointestinal Endoscopy (ASGE)	8-10 yr	1-2 yr (indefinite dysplasia: 3 to 6 mo)
Eaden <i>et al</i> ^[71]	2002	United Kingdom	8-10 years (pancolitis) 15-20 yr (left-sided colitis)	3 yr (second decade) 2 yr (third decade) 1 yr (fourth decade)

¹Low-risk is 0-2 points and high-risk is 3-4 points; Risk factor: Pancolitis, endoscopic and/or histological inflammation, pseudopolyps, and family history of CRC; each risk factor is counted with one point; ²lower risk: extensive colitis with no active inflammation or left-sided colitis; ³intermediate risk: extensive colitis with mild active inflammation or post-inflammatory polyps or family history CRC in FDR aged ≥ 50 ; ⁴higher risk: active inflammation or stricture in past 5 years or dysplasia in past 5 years declining surgery or PSC/transplant for PSC or family history CRC in FDR aged < 50 . PSC: Primary sclerosing cholangitis; CRC: Colorectal cancer; FDR: First-degree relatives.

UC by some societies^[67-75] are summarized in the Table 2. These recommend surveillance programs are summarized as follows: (1) surveillance colonoscopy should be performed during remission state; (2) initial surveillance colonoscopy for CRC should be performed 8-10 years after onset; (3) regular surveillance should be performed annually or biannually; (4) surveillance colonoscopy for patients with PSC should be performed annually from the beginning of PSC diagnosis; (5) random biopsy of four lesions might be taken every 10 cm through the colon; and (6) if dysplasia is detected, the biopsies should be reviewed by a second gastrointestinal pathologist.

The main aim of surveillance programs is to detect dysplastic alterations. The cumulative probability of developing dysplasia or CRC in UC patients was 7.7% at 20 years and 15.8% at 30 years^[13]. CRC incidence was 14 of 1000 UC patients-years' duration and the incidence of any advanced lesion was 30 of 1000 person-years' duration. When low-grade dysplasia (LGD) is detected on surveillance, there is a 9-fold risk of developing cancer and 12-fold risk of developing any advanced lesion^[76]. Among patients with LGD who undergo colectomy, 19% will already harbor CRC or high-grade dysplasia (HGD) and 30%-50% will develop advanced neoplasia over the following 5 years^[77-79]. HGD carries a 43% risk of synchronous cancer^[80].

The guidelines described random biopsy^[67]. A study

of multiple biopsies taken at colonoscopy suggested that 33 biopsies are required to give a 95% chance of detecting dysplasia^[81]. In contrast, targeted biopsies are recommended to increase the frequency of dysplasia detection, compared with random biopsies. Chromoendoscopy might improve the imaging of subtle mucosal changes that are suggestive of neoplasia, compared with standard endoscopy^[82]. Indigo carmine contrast dye highlights irregularities in the mucosal architecture, improving the precision of endoscopic diagnosis. Methylene blue stains the normal epithelium of the colon; the absence of staining might indicate the presence of neoplastic changes in the intestine. Magnifying endoscopy could assist us to further visualize the delicate surface patterns^[83].

On the other hand, some studies have highlighted the failures of surveillance colonoscopy by the guidelines^[39,84,85]. In 50%-80% of cases with colitis-associated neoplasms, the lesions are not visible upon endoscopy^[38]. It would be necessary to clarify that the surveillance systems could contribute to the decline of the mortality of UC patients.

TREATMENT FOR DYSPLASIA

Histopathological diagnosis of polypoid mucosa of UC is important with respect to clinical treatment for dysplasia. UC with HGD usually leads to a total colectomy because

of the high incidence of adenocarcinoma (42%-67% of the colectomy specimens)^[75,79,86]. When HGD in flat mucosa is the initial discovery, surgery or polypectomy is done. Polypectomy should be performed along with biopsies taken from the surrounding mucosa. If the polypectomy is confirmed as complete and biopsies of the adjacent mucosa are negative for dysplasia, a follow-up examination within 6 mo should be performed^[75,78]. If the dysplastic lesion persists or “dysplasia associated lesions or masses” (DALM) exists, a proctocolectomy should be performed^[75].

In contrast, the management of LGD is controversial^[87]. About 30%-50% of patients with LGD progressed to HGD or CRC; an unrecognized synchronous CRC may already be present in up to 20% of UC patients with LGD^[77,79], which indicates that LGD is a risk factor for CRC. In contrast, some studies have shown that patients with LGD have a lower rate of CRC than previously reported^[88].

Dysplasia found in DALM or in areas without any macroscopically visible mucosal alteration is believed to be the origin CRC^[89,90]. The guidelines also state that particular attention should be paid to DALM that harbor a high risk of progression to CRC^[71]. In addition, patients with DALM are recommended to undergo prophylactic proctocolectomy with an ileoanal pouch. By contrast, some polyps, such as adenoma-like mass (ALM), are unrelated to colitis and can be managed by endoscopic polypectomy because of less carcinogenic potential^[91].

The serrated neoplasia pathway was recently proposed in CRC^[92]. Serrated epithelial changes and sessile serrated polyps are uncommonly detected (0.2%-1%) by colonoscopy in chronic ulcerative colitis and Crohn's disease patients^[93], while Bossard *et al*^[94] found that serrated lesions, such as hyperplastic polyps and sessile serrated polyps/adenomas, accounted for approximately 7% of premalignant lesions in the inflamed mucosa of patients with IBD.

CHEMOPREVENTION

Chemoprevention refers to the use of an anti-inflammatory therapy or other substance to reduce or prevent the development of cancer. The current decreased incidence of CRC might be due to a better control of inflammation by improved medical therapy and higher rates of mucosal healing^[95]. Intervening before the development of neoplasia might be promising method to decrease cancer and prevent colectomy.

5-ASA

5-ASA, the nuclear kappa-B pathway inhibitor, is a first line agent for anti-inflammatory therapy^[96]. Continuing inflammation is a plausible mechanism causing malignant transformation; therefore, anti-inflammatory therapy might be useful for chemoprevention in UC patients. 5-ASA reduces oxidative stress, inhibits cell proliferation and promotes apoptosis^[96]. Most reports indicated that

5-ASA reduces the risk of CRC in chronic ulcerative colitis^[34,35,97,98], however, a few did not. A meta-analysis performed by Herrinton *et al*^[85] showed a protective association between the use of 5-ASA and CRC or a combined end point of CRC/dysplasia: in a pooled analysis of 334 CRC cases among patients with UC, regular use of 5-ASA reduced the risk of CRC by approximately 50%, similar to the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) in patients without UC^[99]. In contrast, several studies did not find any chemopreventive effect of 5-ASA^[100-102].

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) may be a practical chemoprevention against colonic exposure to bile acid in patients with PSC^[103]. UDCA use was closely associated with decreased prevalence of neoplasia because UDCA reduces the colonic concentration of the secondary bile acid as a carcinogen^[104,105]. It has been reported that UDCA reduced the risk of CRC in PSC patients with IBD by 80%^[103].

Steroids, aspirin, NSAIDs

There are several studies that suggest steroids, aspirin, and NSAIDs may reduce the incidence and mortality of CRC in UC^[34,35,106].

Total colectomy

The cumulative CRC risk in patients with UC is 30%-40% at 20-30 years after onset of disease, which might suggest that total colectomy is recommend after 15 years of disease in patients with UC. However, the role for prophylactic colectomy in patients with IBD remains controversial.

FUTURE DIRECTION

Accumulating studies about UC-CRC suggest that control of long-term background inflammation and mucosal damage is vital. The use of maintenance chronic ulcerative colitis therapies could be an important strategy for reducing CRC risk in UC patients. Inflammatory stresses, such as reactive oxygen species and some free radicals, have been considered to cause genetic damages of UC epithelium. UC-CRC shows characteristic clinicopathological features. Analysis of the correlation between these genetic features and clinicopathological features might be useful to develop new therapies and to reduce the risk of UC-CRC in the future.

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Recent applications of chemosensitivity tests for colorectal cancer treatment

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Abstract

The evaluation of therapeutic efficacy is necessary to predict the outcome of patients with metastatic colorectal cancer (CRC). In these patients, there is a critical need for predictive chemosensitivity assays and biomarkers to optimize efficacy and minimize toxicity. The introduction of targeted agents has improved the progression-free survival and overall survival of patients with metastatic disease. However, approximately 50% of patients do not show a positive response to chemotherapy and the selection of patients likely to respond to a specific regimen remains challenging. Cell culture-based chemosensitivity tests use autologous viable tumor cells to evaluate susceptibility to specific agents *in vitro* and predict their direct effects. Adenosine triphosphate-based assays and methyl thiazolyl-diphenyl-tetrazolium bromide-based assays are used widely as

sensitivity tests because of their short assay period, technical simplicity, and the requirement of small amount of specimen. Among protein- and gene-based chemosensitivity assays, assessment of KRAS mutation status predicts the response to epidermal growth factor receptor-targeted therapy in CRC patients. The validation of predictive and prognostic markers enables the selection of therapeutic regimens with optimal efficacy and minimal toxicity for each patient, which has been termed personalized treatment. This review summarizes currently available predictive and prognostic chemosensitivity tests for metastatic CRC.

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Key words: Colorectal adenocarcinomas; Colorectal cancer; Chemotherapy; *In vitro* assays; Molecular targeted therapy; Individualized therapy

Core tip: This review summarizes currently available predictive and prognostic chemosensitivity tests and biomarkers in terms of cell culture, protein, and gene. Cell culture-based chemosensitivity tests are used widely in clinical practice because of their short assay period, technical simplicity, and the requirement of small amount of specimen. Among protein- and gene-based chemosensitivity assays, assessment of KRAS mutation status predicts the response to epidermal growth factor receptor-targeted therapy in colorectal cancer patients.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common can-

cer and the fourth most frequent cause of cancer death worldwide^[1]. CRC develops as a consequence of accumulated genetic and epigenetic alterations that result in the loss of tumor suppressor genes and activation of oncogenes.

The response to chemotherapy varies among patients, with objective tumor response rates to standard chemotherapy regimens of 30%-40% in patients with metastatic CRC. Therefore, a reliable method to determine the sensitivity or resistance of tumors to specific chemotherapy agents would be useful in clinical practice. For this purpose, cell culture-based chemosensitivity tests have been investigated for more than 30 years; however, their use is limited by technical issues, a low success rate for primary culture, length of time required, and poor correlation with clinical response. To overcome these obstacles, gene- and protein-based chemosensitivity tests have been investigated, and certain gene alterations have been identified that are predictive of clinical drug response.

In the present review, we discuss recent advances in cell culture-based chemosensitivity tests and the identification of genomic alterations as biomarkers for the design of efficient chemotherapy regimens for CRC patients.

CELL CULTURE-BASED CHEMOSENSITIVITY TESTS

In cell culture-based chemosensitivity tests, autologous viable tumor cells are evaluated to determine the susceptibility of that tumor to specific agents *in vitro* and to predict the response to therapy. Although cell culture-based chemosensitivity tests have been investigated extensively, they are not widely used because of technical problems, a low success rate for primary culture, length of time required, and poor correlation with clinical response^[2]. In 2004, the American Society of Clinical Oncology (ASCO) stated that the use of *in vitro* drug response assays to select chemotherapeutic agents for individual patients is not recommended outside of the clinical trial setting^[3]. In a 2011 update, no changes were made to the original ASCO guidelines because of insufficient evidence to support the use of these assays in clinical practice^[4]. Several *in vitro* chemosensitivity and drug resistance assays have been developed, including the human tumor cloning assay, differential staining toxicity, adenosine triphosphate (ATP)-based and methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) assays, histoculture drug response assay (HDRA), and extreme drug response assay (EDRA)^[3,5]. Among these assays, ATP-based and MTT assays are commonly used as simple sensitivity tests. The advantages of these assays are a short assay period, technical simplicity and the requirement of a relatively small amount of specimen^[6,7]. Table 1 describes cell culture-based *in vitro* assays that have been recently used in clinical trials of human solid cancers.

ATP-based assay

The ATP-based assay is a sensitive cytometric assay that

evaluates tumor cell viability by measuring the intracellular ATP levels of drug-exposed cells and untreated controls^[8]. This test has several advantages over other cell-based assays, including higher sensitivity for predicting cell viability, accurate distinction between cancer cells and normal cells and the requirement of a small number of cells^[9]. The ATP-based chemotherapy response assay (ATP-CRA) is an improved method in which the proliferation of normal cells in tumor tissues can be inhibited through the use of ultralow attachment culture plates; this assay does not require large amounts of specimen and has a relatively short test turnaround time^[10]. Several preclinical and clinical studies have shown the feasibility and good treatment outcomes of ATP-CRA-guided chemotherapy in ovarian, breast, stomach, and lung cancer^[11-14].

Differences in the chemosensitivity of CRC patients to several anticancer drugs, including 5-fluorouracil (5-FU), oxaliplatin, and irinotecan have been investigated in preclinical studies^[15,16]. The only clinical study was reported by Hur *et al*^[6] who showed that ATP-CRA could improve treatment response and resectability in initially unresectable colorectal liver metastasis. In their study, the authors showed that the ATP-CRA guided chemotherapy group showed better treatment response (48.4% *vs* 21.9%, $P = 0.027$) and a higher rate of resectability of hepatic lesions (35.5% *vs* 12.5%, $P = 0.032$). However, multi-institutional randomized controlled trials are needed to validate the use of ATP-CRA for individualized chemotherapy in CRCs.

MTT assay and histoculture drug response assay

The MTT assay is a high throughput (96-well plates) method for the quantification of viable cells without the need for elaborate cell counting. It is commonly used to determine the cytotoxicity of drugs at different concentrations. The principle of the MTT assay is that mitochondrial activity remains constant in viable cells, and therefore an increase or decrease in the number of viable cells is correlated with changes in mitochondrial activity. The mitochondrial activity of cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilized. The absorbance of the resulting solution, which indicates cell viability, is quantified by measuring the optical density (OD) at 540 and 720 nm using a plate reader. Presently, clinical correlation studies using the MTT assay have been reported for breast and stomach cancers^[17,18].

Hoffman *et al*^[19] developed the HDRA and applied it to the three-dimensional culture of tumor tissue fragments using a collagen gel matrix and an MTT end point^[20]. Several conventional drug sensitivity tests use isolated tumor cells obtained after enzymatic digestion. By contrast, the HDRA technique uses cancer tissue fragments in which cells maintain their native architecture and can grow in three dimensions. This enables the maintenance of intercellular contacts and interactions with stromal cells. The HDRA thus enables assessment of the sensitivity of tumor cells to anticancer drugs un-

Table 1 Overview of the cell culture-based chemosensitivity tests

Name	Studied tumor type	Description
MTT ^[17,18]	Breast and stomach	The MTT assay measures mitochondrial activity and is most often used to detect loss of cell survival/cell viability in response to a drug or toxin. Tumor cell suspensions are cultured with various chemotherapy agents for 3-4 d and then exposed to the MTT reagent; because it reduces intracellularly to a blue dye, the intensity of uptake yields an estimate of the number of viable cells to determine drug sensitivity
HDRA ^[5,21,22]	Stomach, breast, ovary, and colon	The HDRA uses cancer tissue fragments and three-dimensional cell culture, in which intercellular contacts and interactions with stromal cells are maintained. Tumor specimens are cut into 1-mm ³ pieces and put on a gelatin sponge infiltrated with culture medium containing a test drug. After incubation for 3-7 d, cell viability is assessed using the MTT assay
ATP ^[6,11-14]	Ovary, breast, stomach, and colon	The quantification of intracellular concentrations of ATP as a measure of cell survival has gained wide acceptance for the evaluation of the medium and long-term cytotoxic effects of drugs (2-3 d). The assay is based on the bioluminescent detection of cellular ATP and is extremely sensitive, allowing the measurement of ATP levels in a single adherent or non-adherent mammalian cell
EDRA ^[26,31]	Ovary, breast, lung, and colon	After 3-5 d of culture, tumor cells obtained from fresh biopsy specimens are labeled with tritiated thymidine. The level of uptake is tracked after exposure to chemotherapy drug concentrations that approximate the peak level achieved clinically. Extreme resistance is identified when thymidine incorporation is inhibited in the presence of the drug by less than one standard deviation of the median cell inhibition measured for several hundred reference tumor samples

MTT: Methyl thiazolyl-diphenyl-tetrazolium bromide; HDRA: Histoculture drug response assay; ATP: Adenosine triphosphate bioluminescence; EDRA: Extreme drug resistance assay.

der conditions that mimic those of the *in vivo* environment^[21]. The correlation rate of the HDRA to clinical response was reported to range from 74% to 92.1% in several studies of head and neck, gastric, and colorectal cancers^[5,22,23].

Recently, our group compared chemosensitivity assessed using the HDRA with the clinical response to different treatment regimens in patients with advanced CRC^[7]. HDRAs were performed to assess the effect of seven combinations of anticancer drugs, including 5-FU with leucovorin (FL), FL with oxaliplatin (FOLFOX) and with irinotecan (FOLFIRI), and their combinations with bevacizumab and cetuximab. The results of 324 HDRAs showed that tumor inhibition rates were higher for FOLFOX (34.2%-39.2%) than for FOLFIRI (24.2%-32.7%, $P < 0.001$). Evaluation of 86 chemotherapeutic regimens showed that the correlation rate of HDRA to the clinical response to chemotherapy was 66.3% (57/86), with sensitivity and specificity values of 72.7% (40/55) and 54.7% (17/31), respectively. Despite variations in accuracy, HDRA might be a feasible and useful technique to predict chemosensitivity in individual patients. Similar to the ATP-CRA, further randomized multi-institutional studies are necessary to support the routine clinical application of the HDRA.

Extreme drug response assay

The EDRA was developed by Kern *et al*^[24] as an exclusion test to identify drugs unlikely to elicit a response. According to the Bayesian theory, any laboratory assays will be accurate only when the assays are extremely (> 98%) specific for drug resistance, concurrently with high overall response rates. The EDRA measures inhibition of DNA synthesis by calculating the rate of proliferating tumor cells plated in agar medium using a thymidine incorporation methodology. The percent inhibition of cellular thymidine incorporation (PCI) comparing the quadruplicate negative and duplicate positive controls is

calculated for each drug using a liquid scintillation counter. Tumor specimens are classified as exhibiting extreme drug resistant (EDR) to an agent when the PCI result is more than one standard deviation below the median PCI for examining drug^[25]. Kern *et al*^[24] reviewed 450 correlations between EDRA results and clinical response over an 8-year period and identified EDR with > 99% specificity. The EDRA has been used to identify patients with therapeutic failure and relapse in various types of tumors including ovary, breast, and lung cancers^[26-29].

In CRC, Fan *et al*^[30] analyzed the outcomes of EDRA in 102 CRC patients treated with 5-FU single chemotherapy using cell viability and ATP assays. In the clinical correlation of 25 Dukes' D patients with EDRA, the sensitivity and specificity of the assay were 100% and 95%, respectively. Recently, Mechetner *et al*^[31] analyzed the results of EDRA performed in 4854 CRC specimens and showed that primary and metastatic tumors showing EDR to FL had up to 58% cross-resistance to a variety of chemotherapy agents, with the lowest percentages for oxaliplatin (11% and 8%, respectively) and irinotecan (16% and 14%, respectively). Approximately 20% of tumors showed EDR to either FOLFOX or FOLFIRI. They concluded that the results of the EDRA obtained at initial diagnosis may be useful for the selection of therapeutic regimens for metastatic disease.

GENE-AND PROTEIN-BASED CHEMOSENSITIVITY TESTS

Molecular markers of fluoropyrimidines

Thymidylate synthase: The gene expressions of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and thymidine phosphorylase (TP) play a key role in 5-FU resistance. TS, an essential enzyme for DNA synthesis, is the target of 5-FU. Despite controversial results^[32,33], many studies and meta-analyses have shown that the downregulation of intra-tumoral TS protein

Table 2 Protein and gene-based chemosensitivity tests in colorectal cancer

Marker	Target chemotherapy drug	Function	Change	Consequence
TS ^[34,35]	5-FU	Essential enzyme for DNA synthesis	TS expression ↓	¹ Chemotherapy response ↑
DPD ^[33,35]	5-FU	Degradation of 5-FU	DPD expression ↓	¹ Chemotherapy response ↑
TP ^[39]	5-FU	Activation of 5-FU (from 5'-DFUR to 5-FU)	Stromal TP expression ↑	¹ Chemotherapy response ↑
UGT1A1 ^[49]	Irinotecan	Degradation of the active metabolite of irinotecan (SN-38)	Polymorphism of UGT1A1 (UGT1A1*28)	Irinotecan toxicity ↑
ERCC1 ^[54]	Oxaliplatin	Excision nuclease that repairs platinum-induced DNA adducts	ERCC1 expression ↓	¹ Chemotherapy response ↑
KRAS ^[65-69]	Anti-EGFR	Proto-oncogene in the EGFR signaling pathway	Mutation of the KRAS gene	Chemotherapy response ↓
NRAS ^[72]	Anti-EGFR	Proto-oncogene in the EGFR signaling pathway	Mutation of the NRAS gene	Chemotherapy response ↓
BRAF ^[74-77]	Anti-EGFR	Signaling gene acting downstream of KRAS	Mutation of the BRAF gene (V600E)	Chemotherapy response ↓

¹Chemotherapy responses of these markers are generally inconsistent without strong evidences. TS: Thymidylate synthase; 5-FU: 5-fluoropyrimidine; DPD: Dihydropyrimidine dehydrogenase; TP: Thymidine phosphorylase; 5'-DFUR: 5'-Deoxy-5-fluorouridine; UGT1A1: Uridine diphosphate glucuronosyltransferase 1A1; ERCC1: Excision repair cross-complementation group 1; anti-EGFR: Anti-epidermal growth factor receptor (cetuximab or panitumumab).

and mRNA expression is a strong prognostic marker for the response to 5-FU based chemotherapy regimens in CRC^[34,35] (Table 2).

DPD: DPD catalyzes the first and rate-limiting step of the pyrimidine catabolic pathway. DPD is also responsible for the degradation of 5-FU and influences the antitumor and adverse effects of 5-FU. High intratumoral DPD activity markedly decreases the cytotoxic effect of 5-FU. Despite its low incidence, DPD deficiency is associated with severe adverse effects after 5-FU-based chemotherapy and can result in death mainly from infectious disease due to neutropenia^[36]. DPD protein and mRNA expression is a strong prognostic marker of the response to 5-FU based chemotherapy regimens in CRC^[33,35].

TP: TP is a key enzyme involved in the synthesis and degradation of pyrimidine nucleotides. The antiapoptotic and angiogenic effects of TP are closely related to the growth and metastasis of CRC. In addition, TP is a key enzyme in the activation pathway of the 5-FU prodrug 5'-deoxy-5-fluorouridine (5'-DFUR) to 5-FU^[37]. The expression of TP in CRC has a dual function. High expression of TP is associated with poor prognosis in patients with CRC, as indicated by increased infiltration, growth, and tumor metastasis. However, the upregulation of TP expression in CRC tissues improves the curative effect of 5-FU, which is important in the treatment of CRC. Therefore, the up- and down-regulation of TP expression in tissues plays an important role in the emergence and development of tumors and may affect prognostic and therapeutic indices. Despite conflicting results regarding the association between TP expression and prognosis^[37-39], TP serves as an indicator of angiogenic potential and plays an important role in cancer chemotherapy as a target for antiangiogenic agents and as an activating enzyme of 5-FU prodrugs^[40].

Single-nucleotide polymorphisms: Genome-wide single nucleotide polymorphism (SNP) analysis may represent a promising approach for the identification of

new predictive biomarkers for clinical application. The chemosensitive SNP markers GPC5 rs553717 (AA), SSTR4 rs2567608 (AA) and EPHA7 rs2278107 (TT) were identified through a three step process consisting of *in vitro* screening, identification, and validation^[41]. These candidate markers are significantly correlated with recurrence or chemoresponsiveness in patients receiving fluoropyrimidine-based adjuvant chemotherapy. Recently, our group identified two chemosensitive SNP markers for chemoradiation (CRT) therapy in patients with low lying rectal cancer. Two candidate markers, CORO2A rs1985859 and FAM101A rs7955740, may be of value for the prediction of radiosensitivity to preoperative CRT, although further validation is needed in large cohorts^[42].

Molecular markers of irinotecan

Uridine diphosphate glucuronosyltransferase 1A1: Irinotecan (CPT-11) is an inhibitor of DNA topoisomerase I that is widely used in the treatment of CRC. Irinotecan is metabolized to its active metabolite, SN-38, which is 1000 times more active than the unmodified drug. The major route of SN-38 elimination is *via* glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1), an essential enzyme involved in the complex metabolism of irinotecan. UGT1A1*28 is a common allele with seven TA repeats in the promoter of UGT1A1 compared with the wild-type allele (UGT1A1*1) with six repeats. A seven-repeat allele is associated with decreased transcription and expression of UGT1A1 and reduced enzymatic activity, which lead to higher or more prolonged exposure to SN-38. Investigation of the variant UGT1A1*28 showed that the homozygous variant allele is associated with a significantly increased risk for myelosuppression and gastrointestinal toxicities in patients treated with irinotecan^[43]. The frequency of UGT1A1*28 is very low in Asians compared with that in Caucasian^[44,45]. Another polymorphism, UGT1A1*6, characterized by replacing single nucleotide in exon1 of UGT1A1, has been also considered to be related with reduced SN-38 glucuronidation activity and bears a higher allele frequency in Asians than Caucasians^[45]. A recent

meta-analysis of 11 studies revealed that UGT1A1*6 polymorphisms is also potential biomarkers predicting irinotecan-induced severe toxicity in Asians in addition to UGT1A1*28^[46]. Studies investigating the efficacy of irinotecan in CRC patients bearing different UGT1A1*28 genotypes have yielded conflicting results that are difficult to interpret because of small sample sizes and the associated poor statistical power^[47]. Overall, UGT1A1 genotypes predict severe neutropenia and diarrhea, but not treatment efficacy^[48,49].

Molecular markers of oxaliplatin

Excision repair cross-complementation group 1: Oxaliplatin is a platinum analogue that differs from cisplatin by the presence of a diaminocyclohexane ligand in its chemical structure. Excision repair cross-complementation group 1 (ERCC1) is an excision nuclease within the nucleotide excision repair (NER) pathway that plays a major role in the repair of platinum-induced DNA adducts. Overexpression of ERCC1 has been reported in cisplatin-resistant cancer cell lines^[50]. Downregulation of ERCC1 expression in tumor tissues is associated with favorable overall survival in advanced CRC patients treated with oxaliplatin-based chemotherapy^[51]. However, clinical correlations between ERCC1 polymorphisms and poor oncologic outcomes have been reported^[52,53]. In a recent study, immunohistochemical analysis showed a correlation between negative expression of the ERCC1 protein and favorable overall survival and low recurrence rates^[54]. However, the precise role of ERCC1 expression needs to be validated in further studies.

Molecular markers of EGFR-targeted treatment

The monoclonal antibodies cetuximab and panitumumab, which target the epidermal growth factor receptor (EGFR), have expanded the range of treatment options for metastatic CRC^[55]. EGFR and downstream signaling pathways are activated by several mechanisms, including overexpression of the receptor, overexpression of the ligand, activating mutation of the receptor, or inactivation of tumor suppressor genes. EGFR ligands, the receptor itself, and the downstream signaling molecules, such as KRAS, NRAS, BRAF, PIK3CA, and its suppressor PTEN, have all been examined as potential effectors of resistance to EGFR-targeted therapy^[56]. The mutation status of signaling molecules downstream of the EGFR target may predict the clinical response to EGFR-targeted therapies.

EGFR ligands: EGFR ligands, such as amphiregulin and epiregulin, may stimulate EGFR through an autocrine or paracrine loop with positive feedback^[57]. Amphiregulin and epiregulin are coregulated by binding to the same receptor. Accordingly, similar prognostic or predictive effects would be expected. Despite inconsistent results^[58], many studies reported that increased expression of genes encoding amphiregulin and epiregulin strongly associated with increased therapeutic benefit from cetuximab in

metastatic CRC patients with KRAS wild-type^[59-62]. Although previous data have shown similar results for amphiregulin and epiregulin, epiregulin is recently favored as a better predictor^[58,63]. Further researches confirming the usefulness of these candidate markers are needed.

KRAS: Activating mutations in the KRAS oncogene, located on the short arm of chromosome 12, are commonly associated with progression from a benign adenoma to a dysplastic adenocarcinoma and occur in 30%-40% of CRCs^[64]. The value of KRAS as a predictive biomarker for anti-EGFR therapy has been demonstrated, as mutations of this gene result in the activation of the EGFR pathway. In 2006, Lièvre *et al.*^[65] showed that whereas all patients who responded to cetuximab presented with wild-type KRAS, 68% of non-responders showed mutations in this gene. Phase III CRYSTAL and phase II OPUS trials showed the benefit of cetuximab in metastatic CRC patients treated with FOLFIRI and FOLFOX, respectively^[66,67]. These findings were confirmed in many other studies^[68,69]. Currently, the presence of the wild-type form of KRAS is considered a positive predictive marker of response to EGFR inhibitor therapy. KRAS mutations are associated with lack of treatment response and a reduction in median progression-free survival (PFS) in patients treated with cetuximab/panitumumab alone or in combination with chemotherapy. The results of several clinical trials have led to the recommendation of KRAS mutational screening of codons 12 and 13 in patients with metastatic CRC^[70].

NRAS: NRAS mutations, which occur in a smaller percentage (approximately 5%) of patients than KRAS mutations, arise at a later stage in the development of CRC and suppress apoptosis^[71]. A recent PRIME trial showed that extended RAS mutations including NRAS exons 2, 3, or 4 were associated with inferior PFS and overall survival after panitumumab-FOLFOX4 treatment^[72]. Therefore, NRAS mutational screening should be considered in terms of its low incidence and time-cost benefits.

BRAF: An activating mutation (V600E) of the KRAS downstream signaling protein BRAF is present in 3%-12% of CRC patients^[73]. BRAF mutations are mutually exclusive of KRAS mutations in CRC^[68]. The negative prognostic value of BRAF mutations in KRAS wild-type patients treated with anti-EGFR therapy was demonstrated in several studies^[74-77]. A recent pooled analysis of the CRYSTAL and OPUS trials confirmed that BRAF mutation is not a predictive marker for response of cetuximab in combination chemotherapy but shows as a negative prognostic marker^[78]. Although evidence is still insufficient to demonstrate an actual association of BRAF mutations with non-responsiveness to anti-EGFR therapy, BRAF genetic screening is recommended by National Comprehensive Cancer Network in patients with KRAS wild-type before anti-EGFR therapy.

PI3K/PTEN: Another major downstream signaling pathway activated by EGFR in addition to the KRAS-BRAF-MAPK pathway is the PI3K/PTEN/AKT signaling pathway. PIK3CA can be dysregulated by activating mutations in the PIK3CA p110 subunit or through inactivation of the tumor suppressor phosphatase and tensin homologue (PTEN) phosphatase. PIK3CA and PTEN mutations can coexist with KRAS and BRAF mutations^[79,80]. The clinical impact of PTEN protein expression and PIK3CA mutations remains controversial. Sartore-Bianchi *et al.*^[81] showed that PIK3CA mutations and PTEN loss in CRCs are significantly associated with lack of response to panitumumab or cetuximab treatment. However, Prenen *et al.*^[82] reported no strong rationale for using PIK3CA mutations as a single marker for sensitivity to cetuximab in chemotherapy-refractory metastatic CRC. A recent randomized controlled trial also showed that neither PIK3CA mutation status nor PTEN expression are prognostic or predictive of response to cetuximab^[83]. Further studies are needed to confirm the usefulness of these candidate markers.

SNP: Patients carrying the GG genotype at DFNB31 rs2274159 or LIFR rs3729740 are more sensitive to cetuximab-containing regimens than those carrying at least one A allele^[84]. Cell lines transfected with the G allele at LIFR rs3729740 and the C allele at ISX rs361863 showed higher sensitivity to cetuximab-containing regimens than those with the A and T alleles. Recently, a clinical association study conducted by our group showed that patients homozygous for the wild-type alleles (GG) of LIFR rs3729740 exhibited a 1.9 times greater overall response rate and 1.4 mo longer PFS than those homozygous or heterozygous for the mutant allele^[85].

Molecular markers for VEGF targeted treatment

Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1, VEGFR-2, and VEGFR-3 are intimately involved in cell migration and proliferation and promote endothelial cell survival and protection against endothelial cell apoptosis and senescence^[86]. Bevacizumab is a recombinant humanized monoclonal IgG1 antibody against VEGF-A that decreases the availability of free circulating VEGF-A, preventing receptor activation. Hurwitz *et al.*^[87] showed that bevacizumab significantly improved overall survival in patients with metastatic CRC^[87]. Although certain candidate markers have been identified, no efficient chemotherapy marker for bevacizumab-based regimens has been established.

Plasma VEGF-A: The measurement of concentrations of circulating protein is an attractive biomarker strategy, as blood is easily accessible, the assays are inexpensive, and the proteins may be readily and quantitatively measured by automated methods^[88]. Plasma VEGF levels have been proposed to reflect VEGF-dependent tumor angiogenesis, and might predict benefit from bevacizumab^[89]. Although increased plasma VEGF-A levels are

well established as indicators of poor prognosis^[90], data regarding the predictive effect of baseline VEGF-A levels have largely been inconsistent^[91]. A recent study demonstrated that an early increase of plasma VEGF-A level after the initial decrease is a potential predictive marker of a poor response and reactive resistance to bevacizumab^[92]. The predictive value of VEGF-A to bevacizumab will be evaluated in the phase III MERiDiAN trial (opening in 2012).

Neuropilin-1: Neuropilin-1 (NRP1) is a VEGF co-receptor that enhances VEGF binding to VEGFR-2, VEGFR-2 phosphorylation, and VEGF-induced signaling and migration^[93]. Preclinical data suggest that NRP1 is a valid anticancer target which has roles in both the proliferation of tumor cells and pathological angiogenesis^[94,95]. In gastric and breast cancers, tumor NRP1 expression was identified as a potential predictor of bevacizumab efficacy^[96,97]. Despite insufficient data for CRC, tumor NRP1 expression appears as a promising biomarker for anti-angiogenic therapy.

SNP: Several SNP markers have been identified as markers of chemosensitivity to bevacizumab therapy. Koutras *et al.*^[98] reported that the VEGF-1154 GG genotype was a significant adverse prognostic factor for overall survival in patients with metastatic CRC receiving irinotecan-based chemotherapy plus bevacizumab. Recently, Loupakis *et al.*^[99] showed that VEGFR-2 rs12505758 C-variants were associated with shorter PFS [HR = 1.36 (1.05-1.75), $P = 0.015$] compared to T/T variants. Our group found that patients carrying the TT genotype at ANXA11 rs1049550 or at least one G allele at LINS1 rs11247226 were more sensitive to bevacizumab therapy than those carrying at least one C allele or the AA genotype^[84]. In a recent clinical association study, we showed that the TT genotype at ANXA11 rs1049550 was correlated with increased sensitivity to bevacizumab^[85]. These data indicate that SNP analysis may represent a promising approach for the identification of novel predictive biomarkers for clinical application.

CD133: CD133, a surface protein widely used for the isolation of colon cancer stem cells, is associated with tumor angiogenesis and recurrence. Pohl *et al.*^[100] showed that patients with high gene expression levels of CD113 (> 7.76) showed a significantly greater tumor response (RR = 86%) than patients with low expression levels (≤ 7.76 , RR = 38%, adjusted $P = 0.003$), independent of the expression of VEGF or its receptor. Combined analyses of two CD113 polymorphisms (rs2286455 and rs3130) showed a significant association with PFS (18.5 mo *vs* 9.8 mo, $P = 0.004$) in multivariate analysis as an independent prognostic factor for PFS (adjusted $P = 0.002$)^[100].

CONCLUSION

Although cell culture-based chemosensitivity tests have

been investigated extensively, consistent results have not been achieved mainly because of technical problems and variable clinical correlations. Certain *in vitro* sensitivity tests, including ATP- and MTT-based assays, have recently been used in clinical practice, although validation in large and well-controlled cohorts is necessary. Further development of these chemosensitivity assays may enable the accurate prediction of sensitivity or resistance to chemotherapy drugs. Regarding protein- and gene-based chemosensitivity assays, assessment of KRAS mutation status to predict the efficacy of antibodies targeting EGFR in patients with metastatic CRC is an important step. In addition, gene expression-based panels aimed at determining the risk of relapse in elderly and marginal patients who are more sensitive to chemotherapy may represent a valuable clinical tool^[10]. Although many potential biomarkers have recently been reported, few have emerged as clinically useful, mainly because of limited reproducibility, technical faults, and their assessment in small and heterogeneous cohorts. The two types of chemosensitivity tests, *in vitro* assays and molecular markers, could be used in combination for an accurate prediction of the clinical response to chemotherapy. The development of novel cell-based assays and genomic technologies could usher in an era of personalized molecular medicine in which patients will be accurately stratified based on their specific molecular profile.

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WJG 20th Anniversary Special Issues (7): Liver transplant

Management of recurrent hepatitis C virus after liver transplantation

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active HCV viremia after antiviral treatment have better survival. Many studies published over recent years have shown that antiviral treatment of post-transplant HCV hepatitis carried out during the late phase is the best option for improving the prognosis of these patients. Until 2011, PEGylated interferon plus ribavirin was the standard of care, resulting in a sustained virological response in around 30% of recipients. The addition of protease inhibitors, such as boceprevir or telaprevir, to the standard of care, or the use of other direct-acting antiviral drugs may involve therapeutic changes in the context of HCV recurrence. This may result a better prognosis for these patients, particularly those with severe recurrence or factors predicting rapid progression of fibrosis. However, the use of these agents in LT still requires clarification in terms of safety and efficacy.

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Key words: Hepatitis C virus; Liver transplantation; Recurrence; Treatment

Abstract

Chronic hepatitis C virus (HCV) infection is the leading cause of death from liver disease and the leading indication for liver transplantation (LT) in the United States and Western Europe. LT represents the best therapeutic alternative for patients with advanced chronic liver disease caused by HCV or those who develop hepatocarcinoma. Reinfection by HCV of the graft is universal and occurs in 95% of transplant patients. This reinfection can compromise graft function and patient survival. In a few cases, the histological recurrence is minimal and non-progressive; however, in most patients it follows a more rapid course than in immunocompetent persons, and frequently evolves into cirrhosis with graft loss. In fact, the five-year and ten-year survival of patients transplanted because of HCV are 75% and 68%, respectively, compared with 85% and 78% in patients transplanted for other reasons. There is also a pattern of recurrence that is very severe, but rare (< 10%), called fibrosing cholestatic hepatitis, which often involves rapid graft loss. Patients who present a nega-

Core tip: Chronic hepatitis C virus (HCV) infection is the reason for about 50% of liver transplants in the western world. Reinfection of the graft is universal and can compromise graft function and patient survival. The development of an efficient antiviral therapeutic strategy has been the focus of clinical research in recent years, including when, how much and at what point this treatment should be applied. The introduction of new drugs for the treatment of chronic HCV hepatitis may involve therapeutic changes and, perhaps, a better prognosis for these patients, particularly those with severe recurrence or factors predicting rapid progression of fibrosis.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the leading cause of death from liver disease and the leading indication for liver transplantation (LT) in the United States and Western Europe^[1,2]. LT represents the best therapeutic alternative for patients with advanced chronic liver disease because of HCV or those who develop hepatocarcinoma.

HCV reinfection of the graft is universal and occurs in 95% of transplant patients. This reinfection can compromise graft function and patient survival. In a few cases, the histological recurrence is minimal and non-progressive; however, in most patients, it follows a more rapid course than in immunocompetent persons, and frequently evolves to cirrhosis with graft loss. The five-year and ten-year survival of patients transplanted because of HCV is 75% and 68%, respectively, compared with 85% and 78% in patients transplanted for other reasons^[3]. There is also a pattern of recurrence that is very severe, but rare (< 10%), called fibrosing cholestatic hepatitis, which often involves rapid graft loss^[4]. Those patients who present a negative HCV viremia after antiviral treatment have better survival^[5].

Many studies published over recent years have shown that antiviral treatment of post-transplant HCV hepatitis carried out during the late phase is the best option for improving the prognosis of these patients. Until 2011, PEGylated interferon plus ribavirin was the standard of care, resulting in a sustained virological response (SVR) in around 30% of recipients^[6].

The addition of protease inhibitors (PI), such as boceprevir or telaprevir, to the standard of care or the use of other direct-acting antiviral (DAA) drugs may involve therapeutic changes in the context of HCV recurrence. This may result in a better prognosis for these patients, particularly those with severe recurrence or factors predicting rapid progression of fibrosis^[7]. However, the use of these agents in LT still requires clarification in terms of safety and efficacy.

NATURAL HISTORY OF HEPATITIS C RECURRENCE AFTER LT

Recurrence of HCV post transplantation

Viral infection recurs in almost all cases and occurs immediately after the graft reperfusion phase. The diagnosis of viral recurrence is purely virological and is established by detection in serum of HCV RNA using polymerase chain reaction (PCR) techniques. The levels of viremia are generally far higher than those existing before the transplant^[8]. However, the diagnosis of relapse of hepatitis or disease in the graft is based on histological findings.

Pathophysiologically, two patterns of recurrence can be distinguished: (1) a pattern of chronic HCV hepati-

tis similar to that seen in non-transplanted patients, but with a faster course, reaching states of advanced fibrosis or cirrhosis in a shorter time (9-12 years *vs* 20-50 years); and (2) fibrosing cholestatic hepatitis, which is less common (3%-5%) but very severe, and generally appears in the context of intense immunosuppression. It can present as an initial manifestation of disease relapse or, less commonly, in the context of recurrent chronic hepatitis. Fibrosing cholestatic hepatitis is characterized by marked jaundice with cholestasis and high titers of viremia. This form usually progresses rapidly to acute liver failure, with graft loss soon after.

Histological confirmation is necessary to establish the diagnosis of HCV recurrence, as well as enabling assessment of the degree of activity and a periodic follow-up of histological disease progression. This not only provides information about the prognosis, but also establishes the differential diagnosis with other complications, such as rejection, biliary disease or vascular problems^[4,9-11].

A new non-invasive technique, hepatic elastography, has become available recently, which appears to correlate well with the stage of fibrosis. This technique can detect an important degree of fibrosis ($F \geq 2$) from the sixth month after transplantation, and has an excellent diagnostic capacity at 12 mo post-transplantation^[12].

Clinical course of HCV recurrence

The histological involvement of the graft and the natural history of recurrence both vary, with different presenting forms. Post-transplant reinfection with HCV is associated with greater aggressiveness than in immunocompetent patients^[13,14].

At around the fifth month after transplantation, acute hepatitis occurs, which is generally asymptomatic in 50% of patients. Histologically it presents characteristics of lobular hepatitis with varying degrees of inflammatory infiltrate in the portal space, mainly of lymphocytes and macrovesicular steatosis, similar to the histological pattern found in acute hepatitis in immunocompetent patients.

Of those patients who experience relapse of their HCV infection after LT, 20% have histological lesions compatible with mild chronic hepatitis 5 years post-transplantation. The others experience a more important chronic evolution. The progression to hepatic cirrhosis occurs in 30% of these patients after 5 to 7 years post-transplant, and is much faster than in immunocompetent persons^[15].

The progression of fibrosis is much more accelerated in those patients who receive their transplants because of HCV infection and who have a recurrence of the disease: up to five times faster than in immunocompetent persons. Accordingly, the cirrhosis evolves earlier, with an average of 10 years compared with 20-30 years for immunocompetent persons with chronic HCV infection^[15,16].

Once cirrhosis is reached, 40%-50% of transplanted patients will experience their first decompensation within one year. Survival after this first episode of decompensa-

tion is 50%^[14,16].

Factors influencing the recurrence of HCV and graft survival

The course of post-transplant hepatitis C is determined by the interaction of different factors that affect the severity and timing of HCV recurrence.

Pre-transplant factors - donor and host related: Certain pre-transplant factors in the recipient are associated with worse evolution, including female sex, older age, and the presence of diabetes or metabolic syndrome^[17-21]. HCV has a reciprocal relation with insulin resistance, in both transplanted and non-transplanted persons: HCV predisposes to insulin resistance, but insulin resistance itself contributes to increasing the morbidity and mortality associated with HCV infection. Other pre-transplant factors depend on the virus; for example, the genotypes HCV 1b and 4, which are factors predicting a poor response to standard antiviral therapy, or a high pre-transplant viral load (especially above 1 million IU/mL)^[22]. The absence of response to antiviral therapy and coinfection with HIV are associated with a worse prognosis^[23].

Other factors related with the donor and the peri-operative period can also affect the severity and the time to relapse of post-transplant HCV infection, such as an older donor age (> 50 years), a high degree of steatosis in the donor liver, a prolonged ischemia time, a non-heart beating donor, a living donor, preservation lesion, a partial split graft or anti-HCV positive donors, all of which have been associated with a worse evolution^[24-27].

Recent studies appear to show that polymorphisms in the interleukin 28 B gene (*IL-28B*), in both the donor and the recipient, may influence not only the response to antiviral therapy, but also the evolution of hepatitis C from post-transplant HCV reinfection, with a worse evolution in those with the genotypes CT and TT (of the polymorphism rs12979860) compared with the genotype CC^[28,29].

Post-transplant metabolic syndrome: Patients who received LT because of HCV who develop metabolic syndrome (50% during the first year) present a greater risk for fibrosis if they experience a recurrence of their HCV in the graft. This is why it is necessary to start preventive measures, as well as maintain a strict control of the post-transplant metabolic complications, particularly diabetes^[18,30,31].

Immunosuppression: Immunosuppression is one of the factors that can cause recurrence of HCV, although no direct relation has been found with any particular therapeutic regimen^[11]. Immunosuppression is associated with greater replication of HCV, especially during the early post-transplant period. It also results in a reduced activation of the immune cellular system, vital for the defense against the virus, and a weakened response in cases of severe recurrence, as in fibrosing cholestatic hepatitis^[32,33].

Steroids: Steroid administration, particularly high doses in the form of a bolus to control severe rejection, is associated with greater severity of HCV recurrence^[11,34]. This explains why some authors defend the use of steroid-free regimens, which also reduces the incidence of metabolic complications, especially hyperglycemia^[35,36]. However, most transplant groups still use steroids in recipients who have HCV, although with optimized doses. Rather than a rapid taper, a slow taper is preferred in general practice^[34].

Calcineurin inhibitors: The course of post-transplant HCV is not related to the type of calcineurin inhibitor given. Data are contradictory, and though no concrete recommendation has yet been established, a valid option is to begin tacrolimus immediately after transplantation, converting to cyclosporine if treatment for HCV is required^[37-39].

Other immunosuppressive agents: No evidence-based recommendations exist concerning the influence of other immunosuppressive drugs, such as azathioprine, mycophenolate or mTOR inhibitors.

In conclusion, the principal aim is to optimize the treatment, avoiding over-immunosuppression^[34]. On the other hand, in the era of the new direct acting antiviral (DAA) drugs against HCV, the choice of immunosuppressive agent will be affected not only by these considerations, but also by potential drug interactions.

ANTIVIRAL THERAPY STRATEGIES IN RECURRENT HCV INFECTION

The main goal of antiviral treatment is the permanent eradication of HCV and the achievement of a SVR. Additionally, antiviral therapy can also provide stabilization of disease progression and prevention of graft loss even in the absence of a virological response^[40].

The treatment of HCV recurrence is similar to that for non-transplanted patients, including the use of new antiviral agents. However, there is no overall agreement on patient selection or the treatment regimen, though it may be similar to that used in immunocompetent persons^[41].

Up to 50% of patients require treatment modification, with 25% even requiring withdrawal, as a result of side effects, mainly a marked reduction in hemoglobin (60%-80%), alterations in mood (10%) and asthenia (60%-70%). Acute rejection occurs in around 6% of treated patients, triggered mainly by PEGylated interferon. Less than 1% experience chronic rejection, which occurs most commonly in recipients who have a better response to antiviral treatment. In this situation, the rejection has been attributed to improved hepatic function with the resulting change in metabolism of the immunosuppressive drugs, which could determine a reduction in their blood levels^[41,42]. Accordingly, close vigilance and monitoring of the immunosuppression are necessary during treatment, as well as a histological study in the event

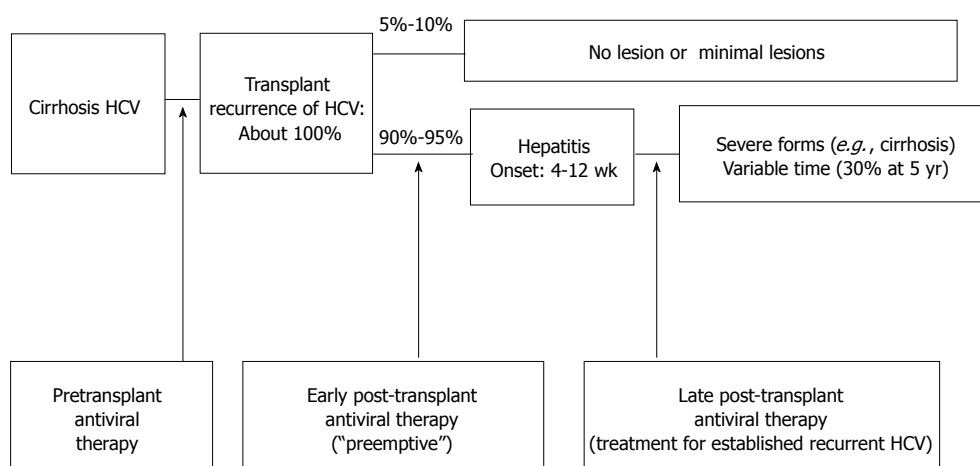


Figure 1 Antiviral therapy strategies in recurrent hepatitis C virus infection. HCV: Hepatitis C virus.

of unexplained laboratory findings.

Overall, different points of therapeutic intervention have been used to attempt to prevent or eradicate HCV infection in liver transplant patients (Figure 1).

Pre-transplant antiviral therapy

The aim of pre-transplant antiviral therapy is to inhibit viral replication before transplantation, and lower HCV viremia and its recurrence after LT. Nevertheless, many patients with HCV on the transplant waiting list have advanced disease precludes them from antiviral treatment. Thus, this treatment is only indicated in 50% of cases, with just 40% of these able to have the optimal dose and duration, which is associated with lower rates of viral response. Nonetheless, this treatment results in 30% of recipients reaching the transplantation process with no detectable viral load, a state that minimizes the risk of recurrence, although it does not completely prevent the reappearance of the virus in the graft^[43]. Accordingly, before transplantation, all patients should be treated if they have no liver decompensation, are in Child-Pugh A and have a MELD < 18, provided there is no contraindication^[44,45]. This poor tolerance to treatment in cirrhotic patients has led to the concept of the low accelerating dose regimen (LADR), which contemplates the introduction of antiviral treatment at minimal doses, increasing the dose every 2 wk depending on tolerance, attempting to reach the full dose^[44].

HCV treatment after LT

Two treatment strategies can be adopted once the patient has received the transplant.

Pre-emptive therapy: The aim of this strategy is to eliminate HCV before the appearance of hepatic lesions. The potential advantage of treating recipients at an early stage, usually with from the first month, is the absence of severe graft involvement or fibrosis. However, during this stage, the patients are still recovering from the

surgery, are receiving multiple drugs and high doses of immunosuppressors, and have a greater risk of rejection, so that postponing antiviral therapy is recommended^[46-49]. Although this treatment is effective in 1%-13% of cases, 35% of the patients who have this option require drug withdrawal because of intolerance or side effects^[49]. Recipients with a history of an aggressive infection or who are coinfecting may be candidates for early treatment, provided the presence of rejection is excluded.

Treatment delayed until after the recurrence of the HCV:

The most widely used strategy involves initiating antiviral therapy once the histological consequences of HCV recurrence are detected by a histopathological study of the graft. In this later state the recipient receives fewer immunosuppressive agents and usually has a better clinical and analytical status, which permits antiviral treatment to be optimized and is efficient in 20%-40% of cases^[5,42,50,51]. Even so, 28% of recipients require early withdrawal of the treatment and 73% require the dose of the antivirals to be minimized. This reduced exposure to the treatment, together with a greater viral replication and unfavorable genotypes, explain the reduced treatment response compared with non-transplanted patients^[6]. Thus, treatment strategies should be individualized, considering patient comorbidity (renal failure, hyperglycemia), graft function or a history of rejection, and the characteristics of the HCV^[52].

Factors predicting antiviral response in a patient who undergoes LT because of HCV infection are similar to those seen in immunocompetent patient. Factors associated with a worse response include advanced donor age, advanced fibrosis, the presence of genotype 1, a high initial viral load and the presence of metabolic syndrome. Obtaining a rapid viral response (4 wk after starting antiviral therapy) and an early viral response at 12 wk of treatment predict a sustained viral response, as seen with HCV treatment in non-transplant patients^[53,54].

Polymorphisms in interleukin (*IL-28B*) related with

response to antiviral therapy in immunocompetent patients^[55] are also related to response in transplant patients, with similar results. The CC genotype is associated with higher SVR rates^[56]. Interestingly, a donor with the CC genotype may partially restore sensitivity to treatment in an unfavorable *IL28B* genotype recipient. This could explain the lack of association between pre- and post-transplant treatment outcome^[57]. Based on these findings, a lack of response to antiviral treatment before a transplant should not prevent an attempt to re-treat HCV in the same patient, particularly if the donor genotype is different to that of the recipient^[28].

Another important factor associated with a greater sustained viral response concerns treatment adherence; at least 80% compliance should be aimed for. The role of baseline immunosuppression on viral response is still under debate. The only prospective, randomized study reported to date did not find significant differences in SVR in patients treated with cyclosporine *vs* those treated with tacrolimus^[58].

The PHOENIX study observed an SVR in 22% of patients treated with an early regimen as opposed to 21% of patients who started treatment after confirmation of the recurrence in the graft, with the former experiencing a higher incidence of adverse reactions and treatment withdrawal^[59].

On the other hand, the role of maintenance therapy in virological non-responders has not been adequately assessed, especially in those who achieve clinical or histological benefit on hemodynamic, histological or elastography study, or in patients who normalize transaminases during treatment^[60].

RETRANSPLANTATION FOR RECURRENT HCV

In the United States, 30% of all liver retransplants occur because of recurrence of HCV^[1]. The International Liver Transplantation Society Expert Panel indicated that the age of the recipients and the donors, a bilirubin ≥ 10 mg/dL, renal dysfunction and early recurrence of HCV-related cirrhosis after transplant are all associated with a worse prognosis after retransplantation^[61]. The development of fibrosing cholestatic hepatitis also has an unfavorable prognosis after retransplantation^[62].

Models predicting survival after retransplantation have been validated. These include the Markmann score^[63] and the Rosen score^[64], which are the most accepted and enable prediction of prognosis in the retransplant patient, thus improving associated survival. Generally speaking, a retransplant is indicated in recipients with an estimated 1-year survival of at least 55%, which includes patients with a Rosen score < 20.5 ^[62].

Although retransplantation is generally associated with worse survival, it is not clear whether HCV-positive patients have significantly worse results. Whatever the case, it is important to personalize each case and just select those patients with favorable clinical characteristics.

DIRECT-ACTING ANTIVIRALS IN RECURRENT HCV INFECTION

The advent of new drugs for the treatment of HCV infection, as well as polymerase and protease inhibitors, will considerably change the management of HCV infection because of their high antiviral power^[65,66]. Around 50% of non-transplant patients who are difficult to treat because of the presence of factors predicting a lack of response experience a greater sustained viral response^[52]. However, little information is available about the use of these drugs in liver transplant patients.

Protease inhibitor triple therapy in patients on waiting lists

The DAAs telaprevir and boceprevir, approved in 2011 for the treatment of genotype 1 HCV, increase the SVR in both naive and previously treated patients^[67]. However, their use in cirrhotic patients is limited, and they have not been approved for patients with decompensated cirrhosis, a situation common to many patients on waiting lists. These patients can only receive these DAAs as off-label therapy in selected cases and with great caution given the limited information available. The concentration of boceprevir in patients with advanced cirrhosis (Child-Pugh C) is between 45% and 62%^[68] higher, whereas the concentration of telaprevir may be reduced by 46% in patients in Child-Pugh B^[69]; no recommendations currently existing for adjusting the dose in these cases. The lead-in of PEG-IFN and RBV could prove useful to check the tolerability in these patients before adding a protease inhibitor.

Although few studies are available, the use of triple therapy in patients on the liver transplant waiting list is associated with high rates of early viral response; however, in up to 25% of cases, early withdrawal is necessary because of secondary effects and 10% of patients experience decompensation of their disease^[70,71]. Accordingly, there is currently no general recommendation for the use of triple therapy in patients on the liver transplant waiting list, although it can be contemplated in select non-decompensated cirrhotic patients and under close control.

Protease inhibitor-based triple therapy post-LT

In transplanted patients, the increase in efficacy, applicability and tolerance of this therapy, and the possible interactions with other drugs, remain unknown and more studies are required.

Although the evidence available for the efficacy of triple therapy in transplanted patients is scarce, it has shown increased rates of rapid and early viral response with DAAs. The main limitation of triple therapy, however, is interaction with immunosuppressive drugs. Telaprevir and boceprevir are inhibitors of cytochrome P450 3A, responsible for the metabolism of both cyclosporine and tacrolimus. Studies in healthy volunteers showed that boceprevir and telaprevir increase the area under the curve of cyclosporine and tacrolimus by 2.7- and 17-fold,

respectively^[72]. Some authors recommend reconversion to cyclosporine in all possible patients before starting triple therapy. The levels of other immunosuppressors, such as everolimus or sirolimus, may also rise because of the same mechanism.

Only a few, small studies have assessed real-life experience with DAAs in post-transplant recurrence. The preliminary data, obtained from the experience of single centers^[73-77], show an increase in early viral response compared with double therapy, although there is a need to reduce the dose of cyclosporine, particularly tacrolimus, and a greater rate of secondary effects.

Coilly *et al.*^[7] undertook the first multicenter study comprising 37 patients with recurrence of HCV. The end-of-treatment virological response rate was 72% in the boceprevir group and 40% in the telaprevir group. The cyclosporine dose was reduced 1.8-fold with boceprevir and 3.4-fold with telaprevir. The use of tacrolimus necessitated reducing the dose 5-fold with boceprevir and 23-fold with telaprevir.

Another multicenter study^[78], this time involving 60 patients treated with triple therapy (35 with telaprevir and 25 with boceprevir), showed early SVR rates that were better than those with double therapy. Most patients needed a reduction in their immunosuppressive drugs from the first day of antiviral therapy, with strict control of the blood levels. The results concerning efficacy coincided with those of the individual study reported initially.

The secondary effects of triple therapy also constitute a limitation to its use in transplant patients, who frequently have a reduction in the doses of PEGylated interferon and ribavirin, high rates of early withdrawal from treatment and the requirement for colony-stimulating factors.

The main secondary effect is medullary toxicity, with a greater incidence of cytopenia than in non-transplant patients, particularly anemia, requiring a reduction in the dose of ribavirin and the addition of erythropoietin, in up to 95% of cases according to some series^[73]. The requirement for transfusions is also more common. Other secondary effects reported include skin symptoms, anorectal syndrome and dysgeusia.

Both the multicenter studies mentioned above reported the presence of severe infections, with sepsis being the main cause of death in some series^[7,78].

Rejection, described with the standard treatment in relation to interferon, may be more common in patients treated with triple therapy, particularly at the end of this treatment, because of the recovery of cytochrome p450 activity and therefore of the metabolism of the immunosuppressors, with a sudden and severe reduction in plasma levels.

Thus DAAs open up a hopeful new era in the treatment of post-transplant HCV relapse, especially that caused by the increase in viral response rates, which could even warrant the consideration of anticipatory treatment in this group of patients. Nonetheless, further studies and evidence are required, from both clinical trials and real-life experience, particularly concerning tolerability and

safety. Precisely because of this limitation, the future perspectives, such second generation DAAs and interferon-free treatment, are also promising.

CONCLUSION

Hepatitis C recurrence continues to present a major challenge in LT. Despite recent advances, the results in patients with HCV infection are not satisfactory, mainly because of recurrence of the primary disease and a lack of availability of an efficient prophylactic therapy. Likewise, antiviral therapy still presents important limitations, particularly its poor tolerance, which hinders its use at full doses or for a sufficient duration to achieve an adequate response. The most recommended attitude is to attempt antiviral therapy before the transplant, particularly for those patients with maintained liver function, in an attempt to avoid disease progression; however, if this is not possible, at least reach transplantation with a negative viremia. Once recurrence is established, the principles of management include optimal donor selection, early identification of HCV recurrence, diligent and aggressive use of antiviral therapy, and close attention to immunosuppression management. Strict monitoring of the progression of the fibrosis by serial biopsies and/or elastography will enable early identification of those patients who might benefit from antiviral therapy to delay the advance of the disease and thus avoid the need for a retransplant. The introduction of DAAs provides hope for the development in the near future of new protocols with novel antiviral drugs for LT that are safer and more effective.

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WJG 20th Anniversary Special Issues (7): Liver transplant

Rationale for the potential use of mesenchymal stromal cells in liver transplantation

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osteocytes. For several years now, MSCs have been evaluated for their *in vivo* and *in vitro* immunomodulatory and 'tissue reconstruction' properties, which could make them interesting in various clinical settings, and particularly in organ transplantation. This paper aims to review current knowledge on the properties of MSCs and their use in pre-clinical and clinical studies in solid organ transplantation, and particularly in the field of liver transplantation. The first available clinical data seem to show that MSCs are safe to use, at least in the medium-term, but more time is needed to evaluate the potential adverse effects of long-term use. Many issues must be resolved on the correct use of MSCs. Intensive *in vitro* and pre-clinical research are the keys to a better understanding of the way that MSCs act, and to eventually lead to clinical success.

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Key words: Mesenchymal stem cells; Organ transplantation; Complication; Immunosuppression; Tolerance

Core tip: For several years now, mesenchymal stromal cells (MSC) have been evaluated for their *in vivo* and *in vitro* immunomodulatory and 'tissue reconstruction' properties which could make them interesting in various clinical settings, and particularly in organ transplantation. This paper aims to review current knowledge on the properties of MSCs and their use in pre-clinical and clinical studies, and particularly in the field of liver transplantation.

Abstract

Mesenchymal stromal cells (MSCs) are multipotent and self-renewing cells that reside essentially in the bone marrow as a non-hematopoietic cell population, but may also be isolated from the connective tissues of most organs. MSCs represent a heterogeneous population of adult, fibroblast-like cells characterized by their ability to differentiate into tissues of mesodermal lineages including adipocytes, chondrocytes and

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent and self-renewing cells that reside essentially in the bone marrow as a non-hematopoietic cell population. MSCs represent a heterogeneous population of adult, fibroblast-like cells characterized by their ability to differentiate into tissues of mesodermal lineages including adipocytes, chondrocytes and osteocytes. In addition to the bone marrow, MSCs have been isolated from various other tissues such as adipose tissue^[1], skin^[2], heart and spleen^[3], placenta^[4], umbilical cord blood^[5] as well as lung and liver^[6,7], and it appears that MSCs reside in the connective tissues of most organs^[8].

No specific marker for MSCs has yet been found. Presently, MSCs are identified using a number of features defined by the International Society for Cellular Therapy which states three minimal criteria^[9]: (1) adhesion to plastic in standard culture conditions; (2) expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and Human Leukocyte Antigen (HLA)-DR surface molecules; and (3) *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts.

For several years now, MSCs have been evaluated for their *in vivo* and *in vitro* immunomodulatory and “tissue reconstruction” properties that could make them interesting in various clinical settings such as organ transplantation. This paper aims to review current knowledge on the properties of MSCs and their use in pre-clinical and clinical studies in solid organ transplantation, and particularly in the field of liver transplantation.

IMMUNOMODULATORY EFFECTS OF MSCS

A large number of *in vitro* and *in vivo* studies have documented the anti-inflammatory and immunoregulatory properties of MSCs on both the adaptive and innate immune system. However, there is strong evidence that MSCs are not constitutively immunosuppressive, they have to be “activated” or primed by local inflammatory conditions. Tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and interferon (IFN)- γ are the key cytokines to allow MSC immunomodulation by regulating their immunophenotype^[10,11]. The high dependence on environment settings could also explain conflicting data in some *in vitro* and *in vivo* studies. These settings must be further studied and considered in clinical trials.

MSC immunogenicity

Both human MSCs (hMSCs) and murine MSCs (mMSCs) show low immunogenicity and do not lead to alloreactive T lymphocyte-mediated immune response *in vitro*. Indeed, under normal conditions, MSC membranes express low levels of human leukocyte antigen (HLA) class I molecules and do not express HLA class II (major histocompatibility complex (MHC)-II) nor co-stimulatory

molecules^[12,13]. MSCs were thus considered as immune privileged cells. However, more recent data with mMSCs has suggested that MHC-I on MSCs could present antigen to CD8+ T cells^[14]. In addition, a narrow window of IFN- γ could induce MSCs to upregulate MHC-I and MHC-II and thus, induces an “antigen presenting cell-like” function. This finding has been observed with both mMSCs and hMSCs^[10,15-17]. Furthermore, it has been demonstrated in an animal model of bone marrow^[18] and skin transplantation^[19] that donor-derived MSCs could be immunogenic and could promote graft rejection.

MSC interaction with immune cells

It is important to highlight that, in some experimental conditions, effects of mMSCs and hMSCs have been evaluated on murine immune cells. Results are not always transposable to human clinical conditions, especially as it is well known that tolerance is more easily achieved in animal models than in humans.

It has been demonstrated *in vitro* and *in vivo*, that MSCs may exert their immunomodulatory effects by acting on many types of immune cells including T cells, B cells and natural killer (NK) cells. The ability of MSCs to inhibit T cell proliferation has been shown in various experimental settings both with mMSCs and hMSCs. *In vitro*, hMSCs highly inhibit proliferation and cytokine production^[20] as well as the development of human cytotoxic CD8+ T cells in mixed-lymphocyte reactions (MLRs)^[21,22]. Moreover, it has been observed that MSCs promote human T cell anergy and inhibit alloreactive T cells through a Th2 pathway^[23]. Nevertheless, it appears that the effect of MSCs on T cells is dependent on the dose used. While a high MSC/T cell ratio exert strong inhibitory effects, low MSC/T cell ratios enhance T cell proliferation^[24].

MSC-induced T-regulatory (T-reg) cell recruitment and generation probably play an important role in MSC-mediated immunomodulatory effects. This has been observed both *in vitro*^[25,26] and *in vivo*^[27,28] both on murine and human immune cells. Additionally, previous studies have shown that T-reg induced production requires cell contact and some MSC released factors such as prostaglandin (PG)-E2 and tumor growth factor (TGF)- β 1^[29] or HLA-G^[30,31]. It has been suggested that this effect could also be partially mediated by an interaction between MSC chemokine (c-c motif) ligand 1 (CCL1) and its receptor on T cells, chemokine (c-c motif) receptor 8 (CCR8)^[23]. More recently, it has been demonstrated that mMSCs could promote T-reg expansion by their effects on immature dendritic cells^[32].

Published results on the effects of hMSCs on B cells and NK cells are contradictory. Some studies have demonstrated that MSCs could inhibit the proliferation and immunoglobulin secretion of B cells^[33-35] while others have found no effect of MSCs on human B cell proliferation^[11,21]. Some researchers have even found that MSCs could stimulate human B cell proliferation and antibody secretion^[36,37]. MSCs have shown an ability to inhibit the proliferation of IL-2 or IL-15 stimulated human NK

cells^[38,39] and their IFN- γ production^[38]. The effects of MSCs on the cytotoxic activity of NK cells are even more controverted. While some studies failed to find such an effect^[40] (especially in freshly isolated NK cells^[41]), others have demonstrated that MSCs could inhibit NK-cell cytotoxicity^[30,39]. As MSCs express HLA-1 antigens, even at a low level, it appears that they may be vulnerable to activated NK-cell lysis^[42].

Many studies have shown that MSCs can prevent the differentiation, maturation and functions of antigen-presenting cells (APCs), such as human or murine dendritic cells (DC)^[17,43,44], and thus indirectly modulate T and B cell functions. In addition, it was shown that mMSCs may induce murine mature DC into a Jagged-2-dependent regulatory DC population^[45]. MSCs may also exert effects on innate immune cells, for example through increased IL-10 secretion by macrophages in mice^[46].

Mechanisms

The mechanisms of immunosuppression by MSCs remain unclear. Whereas MSCs exert their effect by direct cell contact *via* the expression of adhesion molecules, it has also been shown that the immunomodulatory and anti-inflammatory properties of MSCs mainly involve the production of secreted soluble factors. It has been observed that MSCs are still immunosuppressive without cell contact^[22]. It should be noted that the mechanisms of MSC-mediated immunosuppression seems to vary from one species to another^[47].

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyses the degradation of tryptophan. The resulting depletion of tryptophan and the accumulation of its metabolites have shown strong inhibitory properties on immune cells, including human T cells^[48], activated B cells^[11] and NK cells^[39]. MSCs do not constitutively express IDO, but IDO can be upregulated under inflammatory conditions, for example after exposure to IFN- γ , TNF- α and IL-1^[47,48]. IDO could play an important role regarding transplantation given that it has been shown to partially inhibit allo-responses of T cells *in vitro*, and to enhance tolerance towards the graft and allogeneic T cell transfer *in vivo*^[49,50]. IDO seems to be predominant in human MSC-mediated immunomodulatory properties^[47]. However IDO does not seem to be the only mechanism implicated as in some conditions where MSCs do not express IDO they keep their immunomodulatory properties^[51]. A high concentration of nitric oxide (NO) is known to inhibit the immune response in both *in vitro* and *in vivo* studies. It has been shown to inhibit the proliferation of T cells in murine models. NO is synthesized by the inducible NO synthase (iNOS) that is induced in murine MSCs by interaction with CD4+ or CD8+ lymphocytes in inflammatory conditions involving IFN- γ and TNF- α or IL-1^[52,53]. As in the case of IDO for human MSCs, iNOS appears to play a major role in murine MSC-mediated immunomodulation^[47,52]. Both tryptophan depletion and NO are expected to have an exclusively local action^[54,55].

The HLA-G protein is a non-classical human MHC-I molecule. Initially found in trophoblasts, where it plays a crucial role in maternal-fetal tolerance^[56], HLA-G has recently been involved in immunomodulation by MSCs^[57]. HLA-G has shown tolerogenic properties *inter alia* due to its interactions with inhibitory receptors on dendritic cells, NK, and T cells. Selmani *et al.*^[30] have demonstrated that hMSCs, by secreting the soluble isoform HLA-G5, are capable of inhibiting human allo-activated T lymphocytes, NK-cell cytotoxicity and IFN- γ secretion, and of promoting the expansion of CD4⁺CD25^{high}FoxP3⁺ regulatory T cells. Likewise, HLA-G can promote CD3⁺CD4^{low} and CD3⁺CD8^{low} immunosuppressive T cells. It seems that HLA-G expression is IL-10-dependent and needs close cell contact with alloreactive T cells^[30]. It has been suggested that co-injection of HLA-G and MSCs could be used to prevent rejection in organ transplantation.

Another candidate mechanism involves the role of PGE2 (Prostaglandin E2) secreted by MSCs. It appears that MSC-derived PGE2 is involved in MSC-mediated immunomodulation by acting on murine and human T cells (in both Th1 and Th2 responses), NK cells and macrophages^[46,58]. Prostaglandins have a short half-life. This suggests that they play their role using a paracrine or autocrine action mechanism. Furthermore, it has been observed in human MSCs that IDO and PGE2 have a synergistic inhibitory effect on T cell proliferation, and on the proliferation and cytotoxicity of NK cells^[39,59]. However, other studies suggest that PGE2 could in fact have an immunostimulatory role by facilitating Th1 cell differentiation and Th17 cell expansion^[60].

IL-10 plays an important role in MSC-mediated immunosuppression through the induction of IL-10 production in APCs^[61]. Nevertheless, no direct secretion of IL-10 by MSCs has yet been proven.

Blocking each of these factors alone does not restore immune cell function and proliferation, indicating that multiple factors are involved.

Other factors are also secreted: TGF- β and Hepatocyte growth factor (HGF)^[20] (inhibition T-lymphocyte proliferation), IL-1 receptor Antagonist^[62] (anti-inflammatory), Peptide LL-37^[63] (anti-inflammatory and antibacterial), Matrix Metalloproteinase (MMP) 3, MMP9^[64] (acting on neoangiogenesis), angiopoietin-1^[65] (acting on protein permeability). TNF- α and insulin-like growth factor-binding proteins^[51] also seem to be implicated.

On the other hand, MSCs also have the ability to secrete pro-inflammatory chemokines and cytokines, such as monocyte chemo-attractant protein 1 (MCP-1 or CCL2)^[66], IL-6, IL-8, soluble ICAM-1, Interferon gamma-induced protein 10 (IP-10 or CXCL10) and MCP-2 (or CCL8). The secretion of these factors is dependent on inflammatory conditions and could enhance immune response *via* immune cell attraction^[67]. Therefore, MSCs appear to have a dual immunomodulatory capacity depending on the above-identified secreted factors.

The mechanisms involved in the immunomodula-

tory capacity of MSCs are complex and remain largely unknown. Their properties seem to be highly dependent on many parameters in which local immunologic conditions seem to play a crucial role. Finally, it is important to know that there is currently no single standard method to isolate MSCs. It is thus conceivable that changes in the culture medium used to increase and select MSC population may influence their properties.

TISSUE REPAIR/"ORGAN RECONSTRUCTION" EFFECT

In addition to their ability to differentiate into cells of the mesenchymal lineage, it has been demonstrated that MSCs can also differentiate *in vitro* into other cells such as neurons^[68], cardiomyocytes^[69], tubular epithelial cells in kidneys and hepatocytes^[70-72]. They are also capable of differentiating and engrafting into many tissues, especially if an inflammatory signal is present^[73]. These data have motivated further research in the field of MSCs as potential "tissue repairers". Cultured MSCs have shown strong evidence of "tissue repair" properties in response to tissue injury or disease in many animal models with myocardial infarction^[74], kidney disease^[75,76], lung injury or some neurological disorders^[64]. In clinical trials, MSCs have been used successfully to treat bone and cartilage diseases^[77] (e.g., osteogenesis imperfecta), as well as acute and chronic myocardial infarction^[78-80].

MSCs have shown the ability to home in on injured tissue after intravenous infusion. It has been demonstrated that MSCs can express several chemokine receptors such as CCR1, CCR7, CXCR4, CXCR6, CX3CR1^[81], CCR4, CCR10, CXCR5^[82], c-Kit, c-Met^[83], VEGF receptors^[84] and PDGF receptors^[85]. This variety of receptors and the chemotactic migration they have shown in response to the stimulating chemokines and cytokines could partially explain their ability to migrate to sites of inflammation. This hypothesis assumes that the injured tissue also expresses specific receptors facilitating the adhesion and migration of MSCs. However, the exact mechanism of homing in on injured tissue remains largely unknown.

Nevertheless, many studies have observed that MSCs are significantly trapped in the lung after intravenous infusion^[86,87]. Despite their ability to migrate to inflammation sites and to differentiate into many tissues, MSCs exhibit very low and transient levels of engraftment *in vivo*^[86,88]. For example, in a mouse model of acute myocardial infarction, a significant improvement of myocardial function was observed after human MSC injection, while no donor cell could be detected 3 wk after infusion. In a rat model, no MSC could be found in the liver within 7 d after injection of syngeneic rat MSCs in recipient livers through the portal vein^[89]. Contradictorily, in a clinical trial treating myocardial infarction with intracoronary injection of MSCs, the MSCs were still viable 3 mo after transplantation^[90]. In another study, MSCs were detected in various tissues of baboons 19 mo after intravenous

injection^[88].

In fact, it is thought that MSCs are likely to act through the secretion of soluble factors and change of the tissue microenvironment with paracrine interactions, rather than through their transdifferentiation capacity^[91,92]. However, current *in vivo* data are not sufficient to define the exact mechanism. It has been demonstrated that MSCs could facilitate tissue repair by stimulating angiogenesis^[93] and inhibiting apoptosis, as well as fibrosis, in the site of injury^[94].

Furthermore, there is much evidence supporting the protective effect of MSCs in acute kidney injury models^[95]. It appears that MSCs could increase the proliferation of tubular cells and reduce apoptosis^[96,97]. There is a lack of data on the treatment of liver injury with MSCs, but their properties and regenerative potential mentioned above have encouraged researchers and clinicians to investigate further in this field. They could play a therapeutic role in the replacement of diseased hepatocytes, and the stimulation of their regeneration through the action of trophic molecules^[98].

In a study on acute liver injury, rats were successfully treated with MSC infusion, with a decrease of biochemical markers of liver injury and an improved survival rate. Hepatocyte replication was enhanced while apoptosis decreased by 90%^[98]. Similarly, it has been demonstrated that MSCs are efficient in treating fulminant hepatic failure in rats^[99]. Otherwise, it has been suggested that MSCs could only be efficient in a therapeutic window, indicating that higher doses could paradoxically be inefficient or even induce liver fibrosis^[98].

Although it is hoped that MSCs could potentially be an alternative to liver transplantation in end-stage liver disease, or a potential temporary solution to maintaining liver conditions of patients waiting for a graft, MSCs have been tried in only a small number of clinical trials to treat cirrhosis.

In a phase I - II trial, 8 patients with end-stage liver cirrhosis were treated with the infusion of autologous MSCs *via* a peripheral or portal vein. The treatment was well tolerated, with no significant adverse effects and the liver function was significantly improved^[100]. A randomized placebo-controlled trial using MSCs to treat decompensated cirrhosis has recently been published^[101]. Out of 27 patients, 15 received autologous bone marrow MSCs *via* a peripheral vein and 12 received a placebo. The results were evaluated using the Model for End-Stage Liver Disease (MELD) score, Child-Pugh score, liver function tests and liver volume. In this study, there was no beneficial effect of MSC infusion in cirrhotic patients. It is clear that other studies with larger cohorts are necessary to clarify the therapeutic potential of MSCs in cirrhosis.

ANTI-OXIDATIVE EFFECT/TREATMENT OF ISCHEMIA REPERFUSION INJURY

Ischemia reperfusion injury (IRI) is caused by the blood supply returning into a tissue after an ischemic period.

This sudden reperfusion and oxygenation paradoxically impairs the endothelium with a dilatation in arterioles, increased fluid filtration and plasma protein extravasation from post-capillary venules, as well as an increased production of oxygen radicals and a reduction of nitric oxide generation. This imbalance leads to the release of inflammatory mediators (*e.g.*, TNF, platelet activating factor) and the expression of adhesion molecules that cause leukocyte adhesion to the endothelium^[102]. This results in the stimulation of both innate and adaptive immune responses with an accumulation of immune cells, followed by organ damage. The release of danger-associated molecular patterns (DAMPs) and the complement system are also implicated^[103].

Solid organ transplantation is impacted by IRI, which contributes to acute graft rejection, delayed graft function and enhanced immunogenicity. IRI represents a major concern in liver transplantation, and use of MSCs in IRI has been studied for solid organ transplantation in animal models and in clinical trials.

MSCs seem to be recruited by hypoxic and injured tissues that express adhesion molecules and a SDF-1 gradient stimulating CXCR4 and CXCR7 on these cells^[104]. Furthermore, it has been demonstrated that MSCs can transmigrate through TNF-alpha activated endothelium to join the inflamed tissue^[105]. Lately, Pan *et al.*^[106] found that the inactivation of the MEK/ERK signalling pathway by MSCs plays a major role in the improvement of hepatic IRI in rats.

Prevention and treatment of liver IRI in animal models

MSCs have shown therapeutic effects for the treatment of IRI in the kidney, heart and lung in a significant number of studies^[107]. Only a few studies have been published for IRI in the liver, and the exact role of MSCs has not yet been defined.

Jin *et al.*^[108] recently evaluated the effect of allogeneic bone marrow (BM)-derived MSCs to attenuate IRI in rats during the first 24 h after liver reperfusion. In their model partial ischemia was obtained by vascular clamping during 60 min. BM-MSCs were injected through the portal vein. Injury severity, oxidative stress response and apoptosis of the liver was regularly evaluated during the first 24 h and compared to a sham-transplanted control group. The conclusion of this study is that allogeneic BM-MSCs partially protect the liver from IRI when injected *via* the portal vein due to their ability to suppress oxidative stress and to inhibit apoptosis. Another related model using adipose-derived MSC injections *via* a peripheral vein in mice also showed a significant protective effect against liver IRI^[109].

In addition to liver IRI, research has also focused on the potential beneficial effect of MSCs in partial liver transplantation. In a recent study 50% reduced-size liver transplantations in rats were used to examine whether MSC-conditioned medium (MSC-CM) could protect hepatocytes and sinusoidal endothelial cells (SEC) and enhance their regeneration^[110]. MSC-CM was injected in rats

via a peripheral vein directly after orthotopic partial liver transplantation. Compared with the control group, the MSC-CM group showed a significantly lower release of liver injury biomarkers and a clear survival benefit. More proliferating hepatocytes and SECs, and less apoptosis were observed. Many inflammatory cytokine levels and the infiltration by neutrophils and Kupffer cell activation were decreased. VEGF and MMP-9 expression was increased in the graft. All these facts suggest that MSC-CM could have potential in prevention of liver injury, and to enhance its regeneration in partial liver transplant. Kanazawa *et al.*^[111] also found in a model of IRI with major hepatectomy that MSCs protected the liver from IRI and that liver regeneration was enhanced.

However, it has been demonstrated in a liver IRI model that intravenously injected MSCs are short-lived, that viable MSCs do not go beyond the lungs, and that they remain in the circulation for a very limited period^[112]. It has thus been suggested that other cells should be implicated to mediate the powerful immunomodulatory and regenerative properties of MSCs on target organs.

POTENTIAL USE OF MSCS IN LIVER TRANSPLANTATION

Liver transplantation represents the unavoidable treatment of end-stage liver diseases. Despite satisfactory long-term results, transplantation success mostly relies on immunotolerance, *via* acceptable graft-host immune matches and immunosuppressive measures. The latter unfortunately exposes the patient to the classical consequences of a down-regulated immune system, such as opportunistic infections and the typical outbreak of neoplasms. Due to their immunomodulatory properties, MSCs could prove highly effective in obtaining sufficient immunotolerance to reach even higher success rates while avoiding excessive immunosuppression, and thus severe and life-threatening side effects.

MSCs as immunomodulation therapy in transplantation
MSCs for graft-vs-host disease after hematopoietic cell transplantation: A clinical success? Graft-vs-host disease (GVHD) is a major complication frequently observed after hematopoietic cell transplantation (HCT), resulting from the attack of recipient organs by donor lymphocytes. MSCs might play a role in the treatment of GVHD through their immunomodulatory effects rather than their regenerative properties. Although pre-clinical studies for the prevention or treatment of GVHD by MSCs gave rise to conflicting results, MSCs have shown a clear efficacy in clinical trials, especially in steroid-resistant GVHD^[113]. In a phase II study, 68% of patients with acute steroid-resistant GVHD showed a complete response to MSC infusion with a significant decrease in mortality^[114]. A series of other studies have shown similar results with varying degrees of GVHD, suggesting that MSCs have a serious potential future in GVHD management^[115-117].

MSCs in solid-organ transplantation

Animal models: MSC infusion has shown the ability to prolong graft survival in heart^[118-120], skin^[121] and kidney^[122-124] animal transplantation models. However, one group found no effect of MSCs alone on heart allograft survival in a mouse model^[125], and another group found that MSCs infused after kidney transplantation could cause premature graft dysfunction^[122].

Only a few studies have been published in liver transplantation models. In one such study, it was demonstrated that adipose-derived MSCs significantly decreased acute rejection after orthotopic liver transplantation in rats^[126], based on serum rejection markers and on hepatocyte apoptosis. Serum levels of IL-2 were reduced and those of IL-10 were increased. In this model, MSC were infused intravenously 7 d before and 3 d after liver transplantation as well as during the operation *via* the portal vein. MSCs also played a role in a discordant liver xenotransplant model by alleviating acute rejection^[127].

Another group studied the ability of BM-MSC infusion to inhibit acute graft rejection after allogeneic liver transplantation in rats^[128]. MSCs were derived from the recipient, the liver donor or a third party, and infused intravenously at the time of surgery as well as once daily for 3 d thereafter. MSC-treated recipients survived significantly longer compared with the control group. Furthermore, there was no significant difference between the 3 groups receiving MSCs from various origins. Histological analysis showed severe acute graft rejection at day 7 in rats without MSC infusion, while acute graft rejection was significantly decreased in the other groups. These observations were associated with a marked increase in the number of T-reg cells in recipients receiving MSCs. This suggests an important role of T-reg cells in MSC-mediated immunosuppression.

Available data in humans (kidney transplantation)

Results of a phase I clinical trial studying the treatment of allograft rejection after kidney transplantation by autologous BM-MSCs, have recently been published^[129]. The MSC-based treatment was well-tolerated and no related serious adverse effects were reported. Two MSC infusions were performed after a biopsy-proven rejection or interstitial fibrosis/tubular atrophy (IF/TA). In this study, MSCs showed their ability to reduce IF/TA. In addition, a donor-specific down-regulation of the peripheral blood mononuclear cell proliferation was shown. However, a potentially increased susceptibility to opportunistic infections was observed, with the development of viral infections in 3 out of 6 MSC-treated patients.

In a randomized controlled trial in living donor kidney transplantation, Tan *et al.*^[130] demonstrated that, in comparison with antibody induction therapy, induction by autologous MSCs significantly correlated with fewer acute rejections, a lower risk of opportunistic infections and a better renal function at 1 mo. Furthermore, fewer adverse effects were seen in both autologous MSC groups compared to the control group. This study was

conducted on 156 patients recruited from February 2008 to May 2009 and divided into 3 groups (group 1 and 2 received MSCs at kidney reperfusion and two weeks later, plus a standard dose or low dose of calcineurin inhibitors (CNIs), respectively. The control group received anti-IL-2 receptor antibody plus standard-dose CNIs.

In a pilot study, Perico *et al.*^[131] injected autologous BM-MSC in 2 living-related kidney transplant recipients at day 7 post-transplant, after induction therapy with basiliximab/low-dose thymoglobulin. The peripheral blood showed a progressive increase of the T-reg population and a strong inhibition of memory/effector CD8 T cell function/expansion, promoting a long-term tolerogenic environment compared with the control group. However, a few days after MSC infusion transient renal dysfunction was observed. A biopsy excluded graft rejection but revealed a focal inflammatory infiltrate with neutrophil and MSC recruitment as well as a complement-C3 deposition.

The same group also investigated pre-transplant infusion of autologous BM-MSCs in 2 living-related kidney transplant recipients^[132]. No renal dysfunction was observed while MSC immunomodulatory properties were preserved. In addition, it was observed that the avoidance of basiliximab in induction therapy did not facilitate further T-reg expansion.

In another recent pilot study, six patients transplanted with living-donor related kidneys received 2 donor-derived BM-MSC infusions (the first at the time of transplantation, the second one month later) in combination with sparing doses of tacrolimus^[133]. Six other patients were used as a control group and received standard doses of tacrolimus and no MSCs. The MSC-treated group had stable renal function 12 mo post-transplant despite reduced tacrolimus compared with the control group. No acute rejection occurred, except for one in the control group. Significantly increased B cell levels were observed in the MSC-treated group 3 mo after transplantation. No toxic side effects were associated with MSC infusion.

Ongoing clinical trials in liver transplantation

MSC Liege study: Taking advantage of our expertise and experience concerning the use of MSCs in the HCT context^[115], and using an already functioning good manufacturing practice (GMP)-compliant laboratory able to produce clinical-grade MSCs, we initiated a first trial in 2011 exploring the safety and tolerability of third-party MSC infusions after kidney or liver transplantation in a prospective phase I - II study (NCT01429038).

In this study, after successful transplantation, 10 liver and 10 kidney transplant recipients under standard immunosuppressive treatment (tacrolimus, mycophenolate mofetil (MMF) and steroids) receive an intravenous infusion of $1.5 \times 10^6/\text{kg}$ - $3 \times 10^6/\text{kg}$ of third-party MSCs on post-operative day 3 ± 2 . These patients are prospectively compared to the same number of liver or kidney transplant recipients who meet inclusion criteria but have not received MSC infusion. Safety is assessed by recording side effects, including opportunistic infections and

cancers. The immunosuppressive potential of MSCs will be evaluated by the rate of rejection episodes, graft/patient survivals, immunohistology of 3-mo (kidney) and 6-mo (liver) graft biopsies and *in vitro* evaluation of patient immune functions. In a second step, reduction (kidney) and progressive weaning (liver) of immunosuppression will be attempted in recipients who received MSCs. Final results are expected by the end of 2014. The next step will be to assert the immunosuppressive potential of MSCs after organ transplantation, and the opportunity to develop larger, randomised and controlled phase III trials.

“Mesenchymal stem cells in solid organ transplantation”-1 study: In a mesenchymal stem cells in solid organ transplantation phase I study (MiSOT-I) started in April 2013, the safety of MultiStem[®] infusion for immunomodulation after liver transplantation has been evaluated (NCT01841632). MultiStem is a new biological product derived from multipotent adult progenitor cells (MAPCs) which belong to the family of MSCs. Patients, divided into four cohorts, will receive 2 doses of MultiStem (first intraportal at liver transplantation, second at day 3 post-transplant) in addition to immunosuppression (calcineurin-inhibitor-free ‘bottom-up’ immunosuppressive regimen with basiliximab, mycophenolic acid, and steroids). From cohort 1 to 4, an increasing dose escalation is performed (3-6 patients in each group). The primary outcome will be infusional and acute toxicity (intraportal, pulmonary and systemic). The secondary outcomes will be biopsy-proven acute rejection, whether MultiStem promotes malignant transformation nor tumor growth, and the long-term safety of MultiStem administration (up to 6 years). Final results are expected in 2016.

The Beijing study

A third study is ongoing. This phase I study will include a total of 50 patients randomly assigned to two groups; in the first group, patients will receive conventional immunosuppressive agents plus umbilical cord (UC-) MSCs at the day of liver transplantation and then once every 4 wk, at a dose of 1×10^6 UC-MSCs/kg for 12 wk (NCT01690247). In the second group patients will receive conventional treatment plus a placebo. Both groups will be followed for 48 wk. The study will evaluate the incidence of acute rejection and early liver function recovery, as well as patient and graft survival rates, and the prevalence of adverse events as secondary outcomes.

VARIABLES TO BE CONSIDERED/ISSUES TO BE RESOLVED

At present many questions remain unanswered in the field of MSCs therapy in solid-organ transplantation. These issues could explain the conflicting data obtained in previous studies. Further *in vitro* investigations and pre-clinical studies could help to define the settings of future clinical trials through a better understanding of the mechanisms of action of MSCs.

Dosage and sources of MSCs

The ideal amount of MSCs necessary to achieve some clinical effect has not yet been studied, and additionally, the ideal source of MSCs in the setting of organ transplantation has not been determined. Usually isolated from the bone marrow, MSCs can now be isolated from other more easily accessible human tissues such as adipose tissue or cord-blood. Compared with BM-derived MSCs, adipose- and cord- derived MSCs have comparable phenotypic and immunomodulatory properties^[134]. Nevertheless, it seems that many genes are differentially expressed in MSCs depending on their tissue origin^[135]. These differences could alter the function of MSCs in clinical use.

Although not quite clear, it should be noted that MSCs derived from adipose tissue seem to be more likely to develop chromosomal abnormalities than BM-derived MSCs, after many passages in culture^[136,137]. High-passage MSCs should thus be avoided for clinical applications.

Origin of MSCs- autologous vs allogeneic

MSCs can be isolated from the organ recipient (autologous) or from the organ donor, or from a third party (allogeneic).

While some have suggested that allogeneic MSCs may be more efficient as immunosuppressors^[138], others have shown in animal models that donor-derived MSCs could be preferable^[139]. In a recent study, it has been demonstrated that both autologous and allogeneic MSCs were able to inhibit alloreactivity and had comparable efficacy^[22,127].

In terms of alloreactivity, MSCs appear to bear low immunogenicity (see above). In a clinical case of osteogenesis imperfecta, no sign of alloreactivity was observed in the recipient after infusion of fully mismatched allogeneic MSCs^[140]. Yet some papers have reported the induction of memory T cell responses and immune rejection after allogeneic MSC infusion^[18,141]. One cannot exclude that donor-derived MSCs could induce alloreactivity and accelerate graft rejection. Nevertheless, in the field of kidney transplantation, Crop *et al*^[22] have demonstrated that donor-derived MSCs are not immune-rejected and are even able to inhibit alloreactivity in kidney transplant patients when infused before transplantation.

MSC interaction with immunosuppressive drugs

In clinical transplant studies, MSCs are used concomitantly with immunosuppressive drugs. As MSCs and immunosuppressive drugs inhibit the same targets (essentially T cells), it is reasonable to consider that interactions between them can occur. Therefore, it is essential to know which drugs can (positively or negatively) affect MSC function.

In vitro, some have shown that tacrolimus (a calcineurin inhibitor) and rapamycin (a mTOR inhibitor) decrease MSCs immunosuppressive properties^[142], and conversely, that MSCs reduce the immunosuppressive capacities of tacrolimus and rapamycin. Such an effect has not been

found with mycophenolic acid (MPA). Moreover, a high dose of tacrolimus seems to be toxic for MSCs, while MPA and rapamycin at a therapeutic dose just inhibit MSC proliferation^[143]. Nevertheless, others have shown that cyclosporine A (CsA) (another calcineurin inhibitor) and MSCs exert cumulative effects against all activated lymphocytes^[138]. Furthermore, it has been demonstrated that MPA and MSCs have a synergistic immunosuppressive effect^[143].

In vivo, MPA and MSCs also synergize to promote long-term allograft tolerance in rat heart transplantation^[144]. In contrast to what is observed *in vitro*, rapamycin and MSCs synergize as immunomodulators to promote cardiac allograft long-term survival^[119]. Moreover, in a rat renal transplantation model, it has been shown that CsA antagonizes MSC efficacy, and that this combination has no advantage in terms of allograft survival rates compared with CsA alone^[122]. Nevertheless, this study has to be contrasted with other studies using various immunosuppressive drug used together with CsA in which MSC efficacy was not altered^[19,110]. The choice of concomitant immunosuppressive drugs is an important matter for debate, and more studies are needed to define which are the most effective drugs to use with MSCs.

Timing of administration of MSCs

MSCs can be injected before, during or after transplantation, and with single or repeated injection(s). Timing of administration is another important point for discussion. It has been shown *in vivo* that pre-transplant infusion could be more effective than peri-transplant infusion in preventing graft rejection in a murine heart transplantation model^[120]. On the other hand, it has been demonstrated that MSCs are effective in the treatment of steroid-resistant GVHD^[113], so at the peak of the disease. In a clinical trial, Perico *et al.*^[131] observed that early post-transplant infusion of MSCs could induce a transient renal dysfunction. This group is now investigating pre-transplant infusions^[132].

Protocols investigating timings of administration will probably have to be defined according to expected effects and drugs used concomitantly. Regarding liver transplantation, our group infuses MSCs at day 3 post-liver transplantation, while the MiSOT group performs 2 injections of MSCs at day 0 (intra operatively) and day 3 post-transplantation. In the Beijing study, an injection is performed on the day of liver transplantation and then once every 4 wk during a 12-wk period.

Administration route

In case of liver transplantation, MSCs can be injected through a peripheral vein or through intraportal infusion during surgery, or a combination of both. Intraportal infusion could be helpful in increasing the amount of MSCs homing to the liver. On the other hand, MSC homing behaviour to the inflammation site^[69] could potentially concentrate them in the liver when intravenously infused after hepatic transplantation. However, some

studies have observed that MSCs could be trapped in the lung after intravenous infusion^[86,87]. Whatever the case, it is clear that to define the best route of administration, it is necessary to better understand the homing capacity of MSCs, and whether MSCs really require close contact with the target organ in order to be effective.

MSC side effects and safety

To date, no major adverse effects have been reported in the mid-term in the significant number of clinical trials using MSC-based therapy, for example in the context of BMT^[113-117], solid-organ transplantation^[129-133] and in many completed clinical trials for various therapeutic applications^[145]. Only some studies have shown mild and transient adverse effects around the time of injection^[145]. More experience is needed in order to confirm the long-term safety of MSCs.

To reach a sufficient number of cells for MSC-based therapy, *in vitro* expansion is needed. In this context, one of the major concerns is the potential risk of a neoplastic transformation of MSCs^[122]. The occurrence of chromosomal aberrations is not uncommon after *in vitro* culture of mMSC, especially after long-term culture. It has been shown *in vivo* that these chromosomally unstable cells could transform into malignant cells with generation of tumors *in vivo*^[146-148].

Contrary to mMSCs, *in vitro* expansion of hMSC seems to be far more stable and does not seem to generate genomic instability in these cells even after long-term culture. They do not transform into malignant cells after transplantation in mice^[149,150]. Nevertheless, a French study observed the occurrence *in vitro* of transient chromosomal aberrations (aneuploidy) in twenty preparations of BM-MSCs obtained under GMP with two different culture processes. However these cells showed the same senescence as “normal” MSCs and did not lead to tumoral process after injection in immunocompromised mice^[151]. Another study has found a high rate of human MSCs spontaneously transformed in malignant cells *in vivo*^[152], but this strongly controvert suggesting a cross-contamination with cancerous cells^[153]. Moreover, in two recent reviews analysing numerous studies, no evidence was found to affirm the potential of human MSCs for malignant transformation and so far, no risk of malignant transformation has been found in clinical use of hMSCs^[149,154].

As MSCs are used as immunosuppressors, another concern is the potential emergence of opportunistic infections and induced cancers. In the case of solid organ transplantation with MSC-based immunosuppression, no increase risk of viral opportunistic infections has been observed so far-one group having even observed a decrease^[150]. Nevertheless, another group reported viral opportunistic infections in three patients^[129].

Interestingly, the MiSOT study group recently established a system to objectively score the potential emerging adverse effects related to MSC infusions (intravenous or intraportal infusion) after liver transplantation^[155]. This

score is calculated using three parameters (pulmonary toxicity, intraportal-infusional toxicity and systemic toxicity), each of them receiving a score of 0 (no adverse events) to 3 (severe adverse events). It has been retrospectively validated on a cohort of 187 liver-transplanted patients not receiving MSCs as a control population. It has been suggested that this new tool could be helpful in assessing the safety of MSC use in solid organ transplantation.

CONCLUSION

The accumulating evidence shows that MSCs have immunosuppressive and reparative capacities *in vivo* and *in vitro*, as well as a potential beneficial effect in ischemia-reperfusion injury. These three principal properties suggest that MSCs could be interesting in liver transplantation to prevent or treat IRI, allograft dysfunction and graft rejection by inducing a durable tolerogenic environment. Using MSCs, and thereby removing or reducing the need for immunosuppressive drugs could avoid the serious side effects associated with these drugs.

Currently available data in clinic show that MSCs are safe to use, at least in the medium-term, but more time is needed to evaluate their potential adverse effects on the long-term. Caution is therefore recommended. Even if encouraging, the results of MSC use *in vitro* and *in vivo* (animals and humans) are sometimes contradictory. Nevertheless, negative results do not necessarily mean that MSCs are not effective in solid-organ transplantation, but rather that a countless number of still unknown (or poorly known) parameters may influence their effectiveness. At the same time, many issues must be resolved to optimize their use. Intensive *in vitro* and pre-clinical research is certainly the key to a better understanding of the way that MSCs act, and to eventually lead to clinical success.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Causes and consequences of microsatellite instability in gastric carcinogenesis

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Core tip: This review summarizes the current knowledge on the molecular mechanisms underlying the acquisition of microsatellite instability (MSI) in gastric cancer (GC) as well as on the clinic, pathologic and molecular consequences of the MSI phenotype. Additionally, current therapeutic strategies for GC and their applicability in the MSI subset are also discussed.

Velho S, Fernandes MS, Leite M, Figueiredo C, Seruca R. Causes and consequences of microsatellite instability in gastric carcinogenesis. *World J Gastroenterol* 2014; 20(44): 16433-16442 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16433.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16433>

Abstract

Loss of DNA mismatch repair (MMR) function, due to somatic or germline epi/genetic alterations of *MMR* genes leads to the accumulation of numerous mutations across the genome, creating a molecular phenotype known as microsatellite instability (MSI). In gastric cancer (GC), MSI occurs in about 15% to 30% of the cases. This review summarizes the current knowledge on the molecular mechanisms underlying the acquisition of MSI in GC as well as on the clinic, pathologic and molecular consequences of the MSI phenotype. Additionally, current therapeutic strategies for GC and their applicability in the MSI subset are also discussed.

MICROSATELLITE INSTABILITY AND THE MISMATCH REPAIR SYSTEM

Microsatellite instability (MSI) phenotype is characterized by the accumulation of numerous mutations across the genome mainly in repetitive sequences (microsatellites) due to a defective DNA mismatch repair (MMR) system^[1].

The MMR system is composed of at least seven proteins, h-MLH1, h-MLH3, h-MSH2, h-MSH3, h-MSH6, h-PMS1 and h-PMS2, which associate with specific partners to form functional heterodimers that recognize base-pair mismatches and small nucleotide insertion/deletions (1-4 base pairs) that occur during DNA replication^[2,3]. h-MLH1 and h-MSH2 are essential components of the MMR machinery and form five

functional heterodimeric complexes: the MutS complex formed by h-MSH2/h-MSH3 (hMutS β) or h-MSH2/h-MSH6 (hMutS α) heterodimers, and the MutL complex composed by h-MLH1/h-PMS2 (hMutL α), h-MLH1/h-PMS1 (hMutL β), or h-MLH1/h-MLH3 (hMutL γ) heterodimers^[2]. DNA MMR initiates with the assembling of hMutS complex to DNA. The type of MutS heterodimer formed depends on the type of DNA alteration to be corrected. h-MSH2/h-MSH6 heterodimer is required to correct both base-base mispairs and small insertion/deletion loops whereas h-MSH2/h-MSH3 heterodimer works to repair insertion-deletion loops only^[4]. Following the initiation of DNA MMR by the MutS complex, recruitment of MutL heterodimer occurs^[5,6]. MutL proteins function to connect the mismatch recognition complex to other downstream effectors of the repair machinery such as proliferating cell nuclear antigen, DNA polymerases δ and ϵ , single-stranded DNA-binding protein and possibly helicase(s), which are needed to complete the repair process^[4,7,8]. h-MLH1/PMS2 heterodimer is the only hMutL complex shown to be linked to human MMR system and cancer. The role of the other two hMutL complexes is less well understood. *In vitro* studies showed that h-MLH1/h-MLH3 heterodimer participates in the repair of base-base mispairs and one-nucleotide insertion/deletion loops but the studies have failed to show the *in vivo* functionality of the complex^[5]. In addition, biochemical studies support the existence of h-MLH1/h-PMS1 heterodimers in human cells, unlike *in vitro* and *in vivo* studies that do not support their role in neither MMR and MSI induction nor in cancer predisposition^[5,9,10].

TYPE OF MMR SYSTEM ALTERATIONS UNDERLYING MSI IN GASTRIC CANCER

Genetic and epigenetic alterations occurring at the MMR system effectors, namely in h-MLH1 and h-MSH2, and less frequently in h-MSH6 and h-PMS2, are the main mechanism by which MMR system failure occurs in MSI gastrointestinal cancers^[4].

In stomach cancer, MSI occurs in about 15%-30% of the cases. MSI gastric cancer (GC) can occur in the context of hereditary syndromes, such as in the Lynch syndrome, but most of them arise in a sporadic form and only a small fraction show familial clustering (10%)^[11]. Lynch families are characterized by having an excess of synchronous and metachronous colorectal cancer (CRC) but frequently show extra-colonic tumours, including GC^[12,13]. Most of Lynch syndrome-associated cancers have h-MLH1, h-MSH2 germline mutations as the causal genetic event underlying MMR deficiency, and only a small fraction of them harbor alterations in h-MSH6 and h-PMS2 genes^[14,15]. In addition, loss of MMR system function may also be caused by mechanisms other than germline mutations in MMR genes. This is the case of deletions of the terminal end of the *EPCAM* gene that have been identified in a small number of families with Lynch syndrome whose tumours demonstrate loss of

h-MSH2^[16]. In these cases, a failure in transcriptional termination of *EPCAM* results in the generation of fusion transcripts with the adjacent h-MSH2 gene, giving rise to methylation of the h-MSH2 promoter, particularly in epithelial tissues where EPCAM is expressed at high levels^[16]. Constitutional epimutations of the h-MLH1 gene have also been identified in mutation-negative individuals with a clinical diagnosis of Lynch syndrome^[17-22]. This defect is characterized by soma-wide promoter methylation and transcriptional silencing of a single allele of the h-MLH1 gene^[19,20,22]. The frequencies of germline epimutations of h-MLH1 and h-MSH2 seem to be quite high in the genetically proven Lynch-syndrome cases (about 16% of all mutations) although rather infrequent in a cohort of Lynch-syndrome suspected patients (0.6% and 0.9%, respectively)^[21]. Additionally, the 944C>T germline mutation of *TGFBR2* has also been associated to Lynch-syndrome^[23].

Somatic mutations in MMR genes have also been described in sporadic MSI GC. However, in contrast to Lynch syndrome-associated cancers, these mutations were shown to constitute a molecular effect rather than a cause of the mutator phenotype^[24]. Epigenetic silencing of h-MLH1 by promoter hypermethylation is the main mechanism leading to MMR deficiency in both sporadic and familial MSI GC cases^[25-28]. In addition, *Helicobacter pylori* (*H. pylori*) infection may have a role in the impairment of nuclear MMR activity, a subject that will be further discussed in this review^[29,30].

MSI AND *H. PYLORI* INFECTION

H. pylori is the most common chronic infection worldwide and the major etiologic factor for GC^[31]. The fact that only about 1% of all infected individuals develop GC is explained by the interplay between environmental factors, host-inflammatory genetic susceptibility and variations in the pathogenicity of the bacterial strains^[32-35].

The molecular mechanisms by which *H. pylori* induces GC are not fully elucidated, but the chronic inflammation that accompanies the infection is an important trigger, since it induces cellular and DNA damage, and creates an environment rich in cytokines and growth factors that contribute to carcinogenesis^[36,37]. The persistence and combination of bacterial virulence factors and inflammatory factors acting on host gastric epithelial cells during the long-lasting *H. pylori* infection leads to epigenetic mutations, microRNA (*miRNA*) gene expression changes, and alterations in cell signaling pathways^[29,37,38]. *H. pylori* infection generates an oxidative microenvironment due to an increased production of reactive oxygen species and reactive nitrogen species, which leads to the oxidative DNA damage of the host cells and thus to mutagenesis^[39-45]. Moreover, *H. pylori* stimulates the production of pro-inflammatory mediators, either by epithelial or immune cells, such as IL-1, IL-6, IL-8, TNF- α , IFN- γ , RANTES, COX-2, 5-LOX, and growth factors such as granulocyte-macrophage colony stimulating factors

Table 1 Target genes in gastric tumours with microsatellite instability

Gene pathway	Target gene
DNA repair/chromatin structure regulation	ATR
	BLM
	CHK1
	MED1
	MRE11
	MSH2
	MSH3
	MSH6
	RAD50
	DP2
Signal transduction	IGF1R
	RIZ
	TGF- β RII
Transcriptional regulation	TCF4
	E2F4
microRNA regulation	AGO2
	TNRC6A
	APAF1
	BAX
Cell death	BCL10
	CASPASES
	FAS
	UVRAG
Other	BHD
	PAI-1

(GM-CSF) which are well-known factors involved in the different steps of tumorigenesis, such as cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis^[38,46,47].

Another mechanism through which *H. pylori* may contribute to neoplastic transformation of the gastric cells is by inducing genomic instability^[29]. It has been demonstrated that *H. pylori* induces an increased level of mutations in both the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA)^[30,43,48-50]. Genomic instability may be mediated by an impairment of the MMR pathway. In fact, it has been shown that *H. pylori* decreases the expression of *MLH1*, *PMS1*, *PMS2*, *MSH2* and *MSH6* in GC cell lines and in a mouse model of infection^[30,48,51,52], and also decreases the MMR activity^[30]. Concordantly, clinical studies have shown that *MLH1* levels are lower in *H. pylori*-infected individuals in comparison with those that do not harbor the bacteria^[53]. Furthermore, *MLH1* and *MSH2* expression increases in the gastric mucosa after *H. pylori* eradication treatment^[51]. The *H. pylori*-induced defective nDNA repair might have repercussions in mtDNA repair, due to sharing of some components of the nDNA repair that act in the mitochondria, partly explaining the increased level of mtDNA mutations in gastric cells infected by *H. pylori*^[30,49,50,54]. These data suggest that *H. pylori* impairs central DNA repair mechanisms, inducing a transient mutator phenotype, which renders gastric epithelial cells vulnerable to the accumulation of genetic instability, thus contributing to gastric carcinogenesis in infected individuals^[29].

MSI AND TARGET GENE MUTATIONS IN GC

As previously mentioned, cells with a deficient MMR system accumulate mutations throughout the genome. These mutations, typically insertions or deletions, occur mainly in microsatellite-bearing genes, and affect both coding and non-coding regions. When affecting microsatellites of coding genes, MSI-associated insertion/deletion mutations result in frameshift mutations leading to truncated proteins with impaired or no function. If these mutations affect genes that confer any tumorigenic advantage, they will likely appear at high frequency due to selection during tumour development. In contrast, when affecting non-coding intronic or intragenic regions, they are likely silent and present at low frequencies, unless they occur in gene regulatory regions (promoter regions and 3' UTR region, for example) that may control gene expression^[55-57]. Since MSI GCs show widespread somatic mutations, it is difficult to disclose which are the real target genes whose mutations drive MSI gastric carcinogenesis and which are the bystander genes whose mutations have little or no contribution to malignancy. In this regard, the frequency of mutations and their *in vitro* or *in vivo* functionality were proposed as relevant criteria to distinguish between drivers from bystander mutant genes. Additionally, inactivation of the other repeat tract by other molecular mechanism, and the involvement of the candidate MSI target gene in a *bona fide* growth suppressor pathway should also be taken into consideration^[55,58,59]. A database that gathers all mononucleotide microsatellite mutations in human MSI tumours of different organs, SelTarbase (<http://www.seltarbase.org/>), was created, allowing the identification of relevant genes for tumorigenesis based on their mutation frequency^[60]. Nevertheless, to date, several genes have been identified to be critical targets of the defective MMR and to be specifically altered in GC displaying MSI as listed in Table 1. These comprise genes involved in DNA repair, chromatin structure regulation, apoptosis, cell cycle progression, transcription regulation and signal transduction. A new class of target genes that show frameshifts mutations in MSI GC has recently been identified and include genes involved in the processing machinery of miRNA, which harbor mononucleotide repeats in their coding sequences^[61]. More recently, whole genome and exome sequencing of GC samples revealed novel genes, ARID1A and RNF43, to be mutated in 83% and 55%, of MSI cases, respectively^[62,63].

ONCOGENIC MUTATIONS IN MSI GC

In recent years, a number of studies contributed to better understand gastric tumour development demonstrating that MSI tumours are more prone to exhibit mutations in specific genes, in contrast to tumours with distinct types of genomic instability^[64-66]. Of particular relevance

are members of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways that have been found to be mutated and activated in the progression of gastric carcinogenesis. Specifically, mutations in the epithelial growth factor receptor (*EGFR*), *KRAS*, *PIK3CA* and mixed lineage kinase 3 (*MLK3*) have been described in a number of studies^[64,65].

EGFR is a transmembrane tyrosine kinase receptor that in response to extracellular stimuli leads to the activation of two major signalling cascades, the MAPK and PI3K pathways, which are critical in controlling cellular proliferation, differentiation and survival^[67]. Therefore, deregulation of this complex network of signalling pathways is known to contribute to the development of GC^[64]. *EGFR* overexpression has been reported in GC in several studies but the underlying mechanisms of aberrant expression remain poorly understood^[64,68]. *EGFR* structural alterations as amplifications and mutations have been described by many as contributing to *EGFR* overexpression. For instance, Deng *et al*^[69] reported *EGFR* amplification in about 8% in a series of primary GC samples analysed. *EGFR* increased copy number was also observed in approximately 13% of 77 primary GC, which was mainly attributed to polysomy of chromosome 7^[70]. Somatic mutations of *EGFR* have also been described in about 5% of a set of gastric adenocarcinomas^[71]. However, other studies have shown *EGFR* mutations to rarely occur in GC^[70,72]. In the MSI subset of GC, however, data is very limited. Our group has recently investigated somatic hotspot mutations of the *EGFR* gene as well as structural alterations on the A13 repeat within the 3'-untranslated region of *EGFR* (3'-UTR polyA repeat) in a cohort of 63 MSI GC. Results revealed that although no pathogenic mutations were found in the hotspot regions of *EGFR*, deletions at the 3'-UTR polyA repeat were found in a high proportion (48%) of MSI GC^[65]. Mutations in the 3'-UTR polyA repeat of *EGFR* have been found to be associated with *EGFR* overexpression in colon carcinomas through enhancement of *EGFR* mRNA stability^[73] suggesting a putative role for these mutations also in GC development. Furthermore, these *EGFR* alterations were found isolated or in concomitance with mutations in *KRAS* and/or *PIK3CA* genes suggesting a cumulative effect of both oncogenic events in MSI GC^[65].

Downstream of *EGFR*, *KRAS*, *BRAF* and *PIK3CA* have also been investigated for mutations in GC. *KRAS* mutations in codons 12 and 13 have been detected in GC in several studies and frequencies were shown to be around 4%^[74,75]. In most cases, however, *KRAS* mutations are observed in the MSI subset of GC^[65,74-76]. Indeed, our group has analysed a panel of GC samples and *KRAS* mutations were detected in about 18% of the MSI cases^[65]. Furthermore, Brennetot *et al*^[76] described *KRAS* mutations in GC samples only in the MSI subset in about 30% of the cases. A recent large international multicentre study also corroborates the idea that *KRAS* mutations are related to DNA MMR in GC^[75]. In contrast to

KRAS, *BRAF* mutations are rarely observed in GC, as demonstrated by others and our group^[74,77-80]. *PIK3CA*, a gene that encodes for the catalytic subunit p110- α of PI3K, is frequently mutated in many human cancers including GC leading to constitutive activation of the PI3K-Akt signalling pathway^[81]. More specifically, Samuels *et al*^[81] initially described a high frequency of *PIK3CA* mutations (25%) in GC, although that could be the result of a small sample size. Further studies, including those from our group, subsequently identified *PIK3CA* mutations in GC specimens that ranged from 4% to 16%^[82-87]. As for *KRAS*, *PIK3CA* mutations were also demonstrated to occur preferentially in the MSI subset of GC^[82-84]. Furthermore, *PIK3CA* and *KRAS* mutations were described as alternative oncogenic events in this subset of MSI GC^[83]. Our group also evaluated *PIK3CA* mutations in a series of MSI GC samples and identified *PIK3CA* mutations in about 14% of the samples^[65]. More recently, a meta-analysis evaluating PI3K aberrations identified *PIK3CA* mutations in 7%-15% and *PIK3CA* amplification in 46% of the GC^[88]. *PIK3CA* was also evaluated by Shi *et al*^[86] reporting that 67% of GC had amplification of the gene. In accordance with the role of PI3K pathway in MSI GC alterations in other genes besides *PIK3CA* have also been significantly associated with the MSI subset of GC^[66].

In addition to *KRAS* and *BRAF* genetic alterations, mutations in *MLK3*, a gene also involved in the MAPK pathway, were described to mainly occur in the MSI subset^[89]. Indeed, our group investigated *MLK3* mutations in gastrointestinal tumours and described these mutations to be functionally relevant^[90]. In particular, in MSI GC samples *MLK3* mutations were found in a range 3%-17%^[65,90].

Overall, the incidence of mutations in members of the *EGFR*-MAPK-PI3K signalling pathway could be proved useful for prognostic and therapeutic strategies, a subject that is discussed thereafter.

MSI IN GC - PROGNOSIS AND THERAPEUTIC APPROACHES

GC patients are often diagnosed at advanced stages of the disease mostly due to the late onset of symptoms and poor diagnostic tools. Therefore, patients diagnosed with GC are usually associated with a poor prognosis^[91]. In recent years, however, efforts have been made to identify better molecular prognostic markers as well as provide novel and more specific targeted therapies to improve overall survival of GC patients.

The different patterns of genomic instability are associated with specific subsets of GC patients having distinct clinico-pathological and molecular characteristics and subsequently have implications at the prognostic and therapeutic levels as summarized in Figure 1^[90,92]. Indeed, the overall survival of patients with GC displaying MSI phenotype is better than that of patients with MSS phenotype^[11,93]. In particular, in respect to the clinic-pathological features of the MSI GC, most are of the intes-

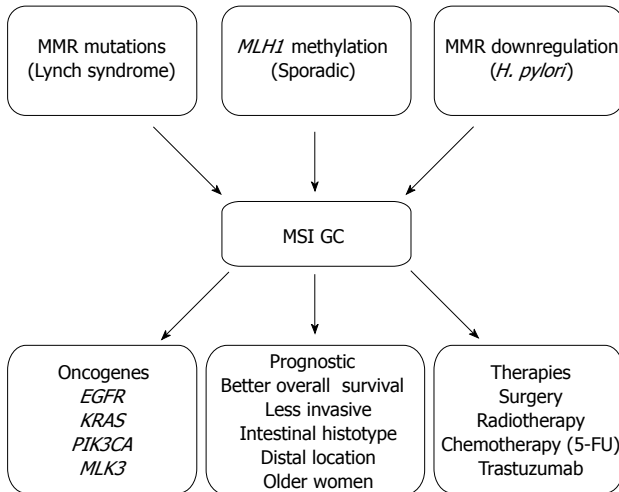


Figure 1 Summary of microsatellite instability gastric cancer associated clinico-pathologic and molecular aspects. This figure summarizes the current knowledge on the molecular mechanisms underlying the acquisition of MSI in GC as well as the clinic, pathologic and molecular consequences of the MSI phenotype. MSI: Microsatellite instability; MMR: Mismatch repair; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; 5-FU: 5-fluorouracil.

tinal histotype, located in the distal part of the stomach and occur more frequently in older women^[11,94-96]. More interestingly, MSI tumours usually have an overall long-term prognosis that is favourable even in patients with advanced disease due to the fact that these tumours have a lower ability to invade serosal layers that preferentially spread to the periphery of the stomach via the lymphatic stream to the nodes^[11,94-96]. In addition, analysis of long term survival data of patients revealed higher survival rates of patients with advanced MSI GC in comparison to patients with other types of GC even if at the same disease stage^[97]. Further, evaluation of MSI and MSS GC patients revealed a correlation of MSI at multiple loci with long term survival in advanced GC suggesting that this particular subset of MSI tumours are less aggressive and subsequently associated to a favourable prognosis^[111]. Interestingly, our group also found patients with MSI GC with familial history and patients with sporadic MSI GC to display similar clinico-pathologic characteristics^[11,26].

Molecular biomarkers have also been put forward as putative candidates with prognostic value, including EGFR, HER2 and VEGFA as recently reviewed in Durrães *et al.*^[98]. Indeed, EGFR has been throughout investigated, although its role as prognostic factor remains controversial. In several studies the expression of EGFR was shown to be related with the survival of GC patients and associated with an adverse prognostic value^[99-102]. However, recent studies found that positive EGFR expression is not prognostic of patient outcome in GC patients^[103-105]. Similarly, the prognostic value of HER2, a tyrosine kinase receptor, is also uncertain as demonstrated through the evaluation of HER2 expression by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH)^[106,107]. In contrast, VEGF-A over-expression was suggested to be associated with a poor prognosis for

overall survival and disease-free survival in patients with GC^[102,108,109]. Nonetheless, information is scarce as to the prognostic value of EGFR, HER2 or VEGFA expression in the MSI subset of GC.

In addition to the clinico-pathologic characteristics and molecular biomarkers, other inflammation-related factors have been associated with GC prognosis^[110].

Despite the many advances in the development of new lines of therapy for cancer in general, GC patients have had little benefit. The conventional therapies for GC patients include surgery, radio- and chemo-therapy regimens but the overall outcome of GC patients remains poor, in part due to the diagnosis at an advanced stage^[91]. In addition, 5-fluorouracil (5-FU) and cisplatin-based chemotherapy regimens are frequently used in patients at an advanced stage of the disease^[111]. Noteworthy, there is still controversy as to the benefits of 5-FU based adjuvant therapy in the MSI subset of GC. Early studies using CRC cells have determined that, in contrast to MSS, MSI cells were insensitive to 5-FU^[112], suggesting the same could be valid for GC cells. In fact, a recent large-scale study in GC patients with stage II and III, revealed that 5-FU-based adjuvant chemotherapy showed better disease-free survival in the MSS/MSI-low group but showed no benefits in the MSI-high group^[113]. However, conflicting data exist as other reports have shown that the survival of GC patients after the administration of 5-FU did not correlate with MSI status^[114].

In the past few years, novel targeted therapies have been tested and approved for GC patients. Regrettably, the successful rates in GC patients are not as encouraging as expected. At present, the only targeting agent approved for GC patients is trastuzumab, a recombinant humanized monoclonal antibody that targets HER2, which efficacy has been demonstrated in HER2 positive GC patients in a phase III large multicentric trial (ToGA study)^[115]. Several other targeted agents are currently being investigated or already in clinical trials, most of them focusing on the EGFR pathway or angiogenesis^[116]. More specifically, antibodies against EGFR are being evaluated in GC patients in clinical trials including cetuximab and panitumumab, though with disappointing results. Data from the phase III trial EXPAND revealed that the addition of cetuximab to capecitabine-cisplatin provided no additional benefit to chemotherapy alone in the first line treatment of advanced GC^[117]. Similarly, the addition of panitumumab to epirubicin, oxaliplatin, and capecitabine chemotherapy did not increase the overall survival of oesophagogastric adenocarcinoma in the REAL3 phase III trial^[118]. Anti-VEGF and VEGFR agents as bevacizumab, ramucirumab, apatinib, sorafenib, sunitinib and cediranib have also been evaluated in GC patients in clinical trials with variable outcomes^[116]. Furthermore, examples of other targeting agents being tested in GC include everolimus, an mTOR targeting agent; onartuzumab, an antibody against HGFR; vorinostat, an HDAC inhibitor; AZD4547, an FGFR inhibitor; and BYL719, a PIK3A inhibitor^[98,116]. Yet again, data on the effects of targeted

therapies in the MSI subset of GC is scarce and warrant further studies.

CONCLUSION

The subset of GC with MSI display specific clinic, pathologic and molecular features and therefore are associated to distinct molecular signalling pathways of tumour development^[90,92]. The available data indicates that MSI status evaluation is critical for appropriate prognosis assessment in GC patients. Despite all the recent advances, GC remains a challenging cancer. Thus, a better understanding of the molecular aspects of MSI GC is required to further develop new diagnostic and prognostic tools as well as novel therapeutic targets and strategies.

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WJG 20th Anniversary Special Issues (10): Alcoholic liver disease

Toll-like receptor-mediated signaling cascade as a regulator of the inflammation network during alcoholic liver disease

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Core tip: Alcoholic liver disease (ALD) pathogenesis is quite complex and requires the activation/inhibition of several molecular pathways. The inflammatory storm caused by alcohol abuse on the gut-liver axis and consequent activation of Toll-like receptor (TLR) signaling is topical for researchers and physicians, both for understanding ALD pathophysiology and for translating novel clues into clinical practice. Here, we focus on the current evidence of TLR involvement in inflammation during ALD in experimental models and humans, offering readers with no first-hand knowledge of this topic a valuable tool to start novel studies.

Abstract

Chronic abuse of alcohol leads to various histological abnormalities in the liver. These are conditions collectively known as alcoholic liver disease (ALD). Currently, ALD is considered to be one of the major causes of death worldwide. An impaired intestinal barrier with related endotoxemia is among the various pathogenetic factors. This is mainly characterized by circulating levels of lipopolysaccharide (LPS), considered critical for the onset of intra-hepatic inflammation. This in turn promotes hepatocellular damage and fibrosis in ALD. Elevated levels of LPS exert their effects by binding to Toll-like receptors (TLRs) which are expressed by all liver-resident cells. The activation of TLR signaling triggers an overproduction and release of some cytokines, which promote an autocatalytic cascade of other pro-inflammatory signals. In this review, we provide an overview of the mechanisms that sustain LPS-mediated activation of TLR signaling, reporting current experimental and clinical evidence of its role during inflammation in ALD.

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INTRODUCTION

Toll-like receptors (TLRs) belong to the family of pattern recognition receptors and are crucial sensors of the innate immune system committed to the recognition of both pathogen and damage-associated molecular patterns (PAMPs and DAMPs, respectively)^[1-4]. TLRs are key molecules of the innate immune system which play a major role in the control of the inflammation process, promoting the production of several circulating inflammatory molecules, including cytokines, chemokines and other molecules that may participate in tissue repair or exacerbate tissue damage in several diseases^[5]. It is noteworthy that TLRs have also been implicated in both liver physiology and in the pathophysiology of several liver diseases

as they are diffusely expressed in all types of liver cells^[6-8].

The integrity of the intestinal barrier and appropriate gut permeability are crucial for maintaining the equilibrium of commensal and pathogenic microorganisms and for avoiding their translocation from the gut. Normally, only bacterial traces can pass the intestinal mucosa and reach the liver through the portal circulation where their clearance is accomplished. A large amount of the literature in animal models and humans has reported that excessive alcohol consumption increases intestinal permeability, disrupting the intestinal barrier and leading to a strong increase of portal and systemic levels of the most studied PAMP, lipopolysaccharide (LPS)^[9-12]. However, only recently through novel metagenomic and metaproteomic approaches, the ability of acute and chronic ingestion of alcohol to alter gut microbiota composition by increased bacterial overgrowth and contributing to liver damage and inflammation has emerged^[13-15].

Over the past decade, despite numerous prevention campaigns, alcohol consumption is still at alarming levels, particularly in industrialized countries^[16]. Therefore, alcohol abuse is currently considered to be one of the major causes of chronic liver disease in Western countries, particularly in Europe, southern Europe and the United Kingdom^[17]. Prevalently, heavy drinkers are susceptible to develop alcoholic liver disease (ALD) which may be characterized by different histological abnormalities, including steatosis, steatohepatitis and fibrosis, and evolving into more severe forms of liver injury, such as cirrhosis and hepatocellular carcinoma (HCC)^[17,18].

During the last decade, the importance of research and clinical studies of the underlying molecular mechanisms that link TLRs and ALD have received increasing interest, particularly because of their therapeutic inference.

In the present review, we focus on the implication of TLRs and their role in inflammation in ALD pathogenesis and we provide an overview of their possible clinical impact in prevention and therapy.

TLR-MEDIATED SIGNALING

TLRs are regulators of innate immune response and sensors of both the pathogen signature bacteria, fungi and virus PAMPs and the endogenous components, DAMPs. They are highly conserved type I transmembrane proteins which comprise an extracellular leucine-rich ligand binding domain and an intracellular domain, Toll/interleukin (IL)-1 receptor (TIR) domain, responsible for their intracellular signal transduction^[19]. TLRs have been classified based on their ligand specificity and selectivity, accounting for more than 13 members in mammals, of which 11 are expressed in humans. However, each human TLR exhibits differential activities, depending on its tissue expression and ligand specificity^[20].

In the liver, TLRs are expressed in Kupffer cells (KCs), hepatocytes and hepatic stellate cells (HSCs) and they have been extensively studied in various chronic liver diseases^[21]. In more detail, TLR2 and TLR4 expression is shared by hepatocytes, KCs, HSCs and biliary epithelial

cells, while TLR4 is also expressed by sinusoidal endothelial cells. Moreover, KCs also express both TLR3 as biliary epithelial cells and TLR9, similar to HSCs and sinusoidal endothelial cells^[22].

LPS, a component of Gram-negative bacteria walls, composed of a carbohydrate (O-antigen), an oligosaccharide region and a lipid part (called Lipid A), is the ligand of TLR4. TLR4 cannot bind directly as the LPS molecule requires a complex assembly composed by the CD14 co-receptor which facilitates the transfer of LPS to TLR4 complex and MD-2, an adapter molecule that modulates the LPS recognition. Another cofactor is LPS-binding protein (LBP) that shuttles LPS to the CD14 molecule. The association of these auxiliary molecules triggers the signal, resulting in the homodimerization of TLR4 molecules and consequent signaling^[23,24].

Once TLRs have bound their specific ligands on the cell membrane, they transduce the downstream signal by means of the myeloid differentiation factor 88 (MyD88), a common molecule adaptor for all TLRs except TLR3^[25]. Actually, the TLR downstream signaling can be distinguished as MyD88-dependent or MyD88-independent pathways with the alternative adapter molecule as TIR-domain-containing adapter-inducing interferon- β (TRIF). As a final effect, MyD88-dependent cascade leads to the activation of nuclear factor- κ B and activating protein-1 (AP-1) by means of I κ B kinase (IKK) complex and mitogen-activated protein kinases (MAPKs) respectively. NF- κ B and AP-1, in turn, conduct the production of specific pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , IL-6 and IL-1 β ^[25]. In the liver, the MyD88 pathway also promotes the activation of the LPS-induced TNF- α factor (LITAF), another transcription factor that regulates the transcription of pro-inflammatory cytokines^[26]. Conversely, MyD88-independent cascade predominantly induces the expression of Type I interferons by the activation of IRF-3 through the critical regulators TANK-binding kinase 1 (TBK1) and IKKs^[27].

KC-dependent production and release of pro-inflammatory cytokines, such as TNF- α and IL-1 β , will further increase production and release of other pro-inflammatory cytokines (*i.e.*, IL-6 and IL-8) and chemoattractant factors in a vicious cycle, leading to the activation of other immune cells, including neutrophils, monocytes and lymphocytes. These immune cells in turn also respond to TLR activation in KCs, generating reactive oxygen species (ROS), increasing phagocytosis and secreting anti-microbial peptides and a cascade of additional pro-inflammatory molecules^[28].

In Figure 1 we have schematized the LPS-mediated TLR4 signaling pathways leading to intra-hepatic inflammation.

TLR ROLES IN ALD PATHOGENESIS

ALD pathogenesis

ALD is considered to be one of the major causes of death worldwide, accounting for half of alcohol-related fatalities^[17]. Excessive alcohol consumption may lead to

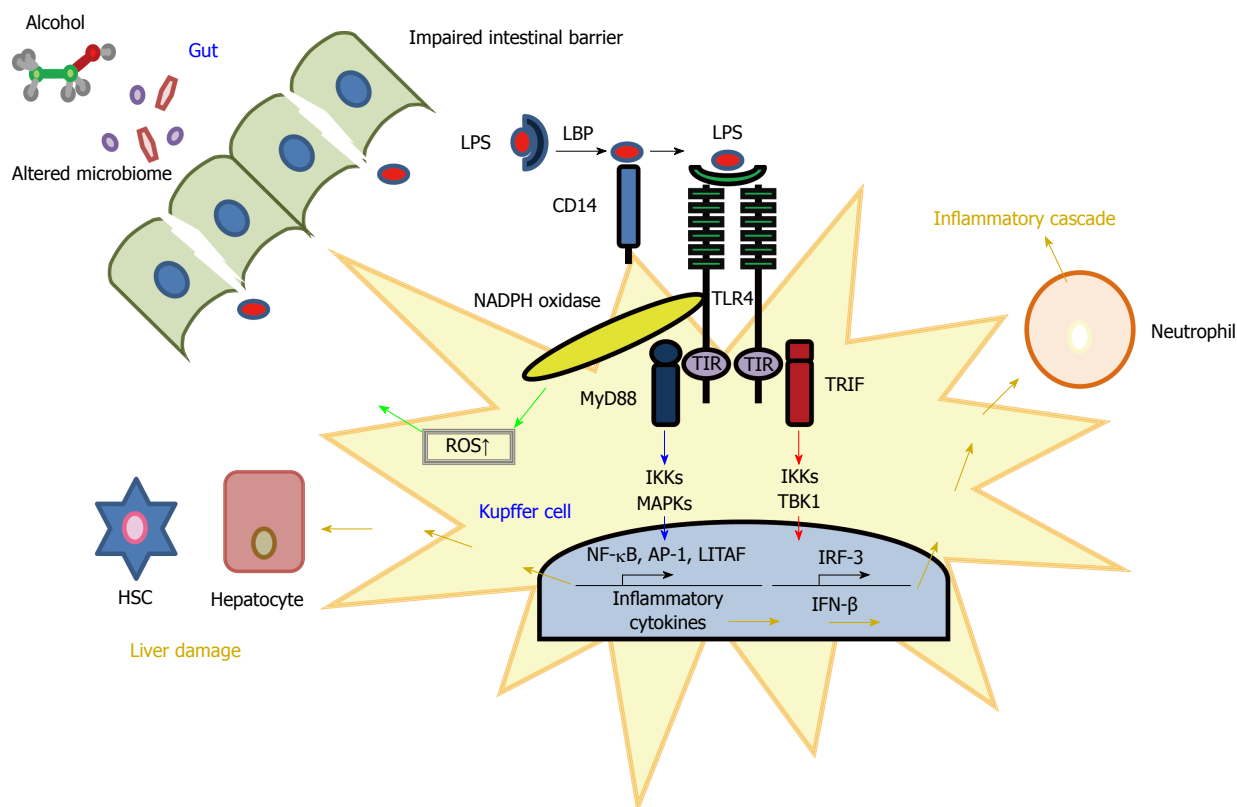


Figure 1 Schematic representation of lipopolysaccharide-mediated Toll-like receptor 4 signaling pathways. The disruption of intestinal integrity (*i.e.* caused by alcohol) results in migration of gut-derived microbial products, particularly LPS, through the portal circulation reaching the liver. LPS can prime KC-dependent production and release of pro-inflammatory cytokines (as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$), which in turn, lead to release of other pro-inflammatory cytokines and chemokines recruiting and activating immune cells, affecting HSC and hepatocyte homeostasis. In the figure, the LPS-TLR4 interaction and consequent initiation of downstream signaling cascade distinguishing both the MyD88-dependent and independent pathways in KCs and their interplay with other cells involved in liver inflammation and damage is summarized. LPS: Lipopolysaccharide; LBP: LPS-binding protein; KC: Kupffer cell; TNF: Tumor necrosis factor; IL: Interleukin; HSC: Hepatic stellate cell; TLR: Toll-like receptor; TIR: Toll/interleukin-1 receptor; TRIF: TIR-domain-containing adapter-inducing interferon- β ; $\text{NF-}\kappa\text{B}$: Nuclear factor- κB ; IKK: IKB kinase; MAPKs: Mitogen-activated protein kinases; LITAF: LPS-induced $\text{TNF-}\alpha$ factor; IFN: Interferon; ROS: Reactive oxygen species.

several liver tissue abnormalities. Therefore, histological features in ALD include steatosis, steatohepatitis and fibrosis, which may progress to cirrhosis and ultimately to HCC^[29]. Although research interests in ALD pathogenetic mechanisms have increased during the last decades, some relevant pathways have only recently been characterized^[30]. Studies on the direct effect of alcohol on intestinal permeability have shown that acetaldehyde, the most toxic metabolite of ethanol metabolism, has a major role in the increment of tight junction tyrosine phosphorylation, conducting altered localization of both tight junction (occludin and ZO1) and adherens junction (E-cadherin and β -catenin) molecules^[31,32].

Intra-hepatic lipid accumulation and steatosis in people who drink a toxic alcohol amount (40-80 g/d for men and 20-40 g/d for women for 10 years) is characterized by the impairment of the major pathways involved in alcohol metabolism, including its conversion to acetate by the activity of specific dehydrogenases which completes the conversion to acetate and mitochondrial fatty acid oxidation^[33]. Furthermore, development of the progressive severe forms of ALD-related liver damage, such as steatohepatitis and fibrosis, results from a complex interplay between the oxidative stress due to release

of ROS and the activation of several components of the innate immune system due to endotoxins such as LPS^[34]. Besides, several studies suggested that LPS increases cytokine levels by NADPH oxidase and ROS production in macrophages *via* the $\text{NF-}\kappa\text{B}$ pathway^[35]. Although the complex upstream and downstream events and molecules that may explain the LPS-mediated mechanisms in ALD have been a subject of deep investigation for decades, they are only recently becoming clear^[21]. Alcohol-induced LPS accumulation and consequent endotoxemia may occur by the alteration of gut-microbiome composition, which leads to endotoxin over-production and consequent disruption of intestinal barrier integrity. Therefore, the increased alcohol-mediated endotoxemia may reduce the detoxifying ability of KCs and the activation of the TLR4-mediated inflammatory response that promotes hepatocellular injury. Experimental and clinical evidence of this complex network of TLR4-associated upstream and downstream factors involved in ALD-associated inflammation are reported in the next paragraphs.

Evidence from animal models

The involvement of gut-derived microbial products, such as LPS, which can pass the systemic and portal circulation

due to the disruption of the intestinal barrier caused by ethanol was suggested several years ago^[36]. Furthermore, Keshavarzian *et al.*^[37] demonstrated that intestinal barrier dysfunction and endotoxemia are early events preceding ethanol-dependent hepatic injury in rats. Particularly, serum LPS has been found to be increased in animal models resembling human ALD^[38].

The relevance of TLR4-dependent KC activation followed by the initiation of the inflammatory cascade in the pathogenesis of alcohol-induced liver damage has been also proved in models of ALD^[39,40]. Furthermore, studies performed on mice that have a functional mutation in the *TLR4* gene showed an impaired response to bacterial endotoxins^[40]. Moreover, LBP knockout mice were significantly reduced in the pathological parameters characterizing ethanol-fed mice, such as endotoxin levels, steatosis, inflammation and liver injury^[41]. Further, CD14 knockout mice were protected from alcohol-caused severe liver injury and from ethanol-induced NF- κ B, transforming growth factor (TGF)- β and TNF- α increase^[42]. As previously described, TLR4 signaling encompasses two distinct cascades: the MyD88-dependent and the TRIF-dependent (MyD88-independent).

These downstream pathways have been widely studied in animal models in order to clarify their respective involvement in ALD pathogenesis. Experimental evidence established that in ALD, the TLR4 down-stream signaling is principally regulated by the MyD88-independent cascade. In fact, while TLR4-knockout (KO) mice were protected from alcohol-induced liver damage, ROS production and inflammation, MyD88-KO mice were not, both being exposed to the Lieber-De-Carli diet^[43]. Moreover, the disruption of MyD88-independent signaling, in studying TRIF-deficient mice, reported protection from alcohol-induced liver disease^[44]. Additionally, the lack of IRF-3, a transcription factor downstream to TLR4/TRIF, resulted in preserving IRF-3-KO mice from alcohol-induced liver injury, steatosis and inflammation^[45]. This evidence demonstrates the dispensable role of MyD88 adapter in TLR4-mediated liver injury.

Recently, the involvement of the protein kinase C (PKC) activity in the increase of intestinal permeability related to alcohol consumption has been described^[46]. Specifically, both *in vitro* and *in vivo* evidence showed that alcohol, in a dose-dependent manner, increases TLR4 expression, which in turn results in augmented PKC activity. The consequent reduction of occludin phosphorylation alters the intercellular junctions, leading to the intestinal permeability increment^[46].

KCs are the primary cells that respond to LPS *via* TLR4-dependent pro-inflammatory cytokines (as TNF- α , IL-1 β , IL-8, IL-6) and leading to liver inflammation. In addition, in animal models of ethanol exposure, hepatocytes can be driven to accumulate lipid and augment TLR4 levels, altering their TLR4 sensitivity and LPS hepatotoxicity^[47,48]. Moreover, damaged hepatocytes can release high-mobility group box 1 (HMGB1), an endogenous ligand that can be recognized by TLR4 and partici-

pate with a mechanism similar to that of non-alcoholic fatty liver disease in the promotion of ALD^[7]. In fact, Wang *et al.*^[49] found that HMGB1 serum levels increased in chronic alcohol feeding of mice and its balance with milk fat globule-EGF factor 8 may control macrophage efferocytosis.

Interestingly, a recent study in TLR4 deficient mice transplanted with bone marrow (BM)-derived cells (KCs) or non-BM-derived cells (HSCs included) demonstrated that in both liver cell lineages, TLR4 is essential for the progression of alcohol-induced liver steatosis, inflammation, injury and fibrosis^[50]. Besides, ROS production has been implicated in the alcohol-induced sensitization to LPS. In more detail, it has been shown that the direct interaction between TLR4 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isozyme (Nox)-4 leads to NF- κ B activation and LPS-mediated ROS generation *in vitro*^[51]. In a chronic alcohol-fed rat model, the inhibition of NADPH oxidase through diphenyleneiodonium (DPI) diminished the LPS-induced ERK1/2 phosphorylation and both ROS and TNF- α levels in KCs^[52]. Furthermore, in C57Bl6/J mice fed a modified Lieber-De Carli diet, DPI protected from steatosis and liver inflammation induced by the usage of diverse bacterial components specific for different TLRs^[48]. Previous evidence also showed that mice p47 phox -/-, depleted of the main cytosolic part of NADPH oxidase, were preserved from alcohol-induced liver injury, corroborating the crucial role of oxidative stress in TLR signaling along the ALD condition^[53].

By the way, TLR4 is an important player in the development of fatty liver, although how it can influence lipid metabolism has not been completely clarified. Experimental studies on TLR4 mutant animal models have documented that TLR4 is involved in lipid accumulation and lipid peroxidation imbalance^[40,54].

It is established that KC activation due to alcohol intake can lead to an increment in the production of prostaglandin (PG) E(2) which, interacting with prostanoid receptors and augmenting cAMP, can build up triglyceride in the liver. Furthermore, hepatic fat accumulation can be induced by the augmentation of the NADH/NAD(+) ratio, the sterol regulatory element-binding protein-1 (SREBP-1) activity and the decrease of peroxisome proliferator-activated receptor-alpha (PPAR-alpha) activity^[55,56].

Also, microRNAs (miRNAs) have been implicated in the inflammation process in ALD and its complications. MiRNAs are conserved single-strand small noncoding RNAs which have a post-transcriptional gene-expression regulation function. They act on the 3'-untranslated mRNA target with whom miRNAs pair with different specificity. This match influences RNA stability and degradation or the translation and finally leads to protein repression^[57]. Specifically, miR-155 is a ruler of inflammation and TLR signaling. It regulates TNF- α mRNA stability, as demonstrated in *in vitro* experiments performed on alcohol-treated macrophages^[58]. Such evidence has been confirmed in a mouse model of ALD in which iso-

Table 1 Experimental evidence of Toll-like receptor involvement in inflammation during alcoholic liver disease

Ref.	Experimental model	Upstream factor	Involved TLRs	Effects
Enomoto <i>et al</i> ^[39] , 2000	Ethanol-fed rats	LPS	TLR4	Kupffer cell activation and consequent inflammation
Uesugi <i>et al</i> ^[40] , 2001	TLR4 non-functional mice fed ethanol	LPS	TLR4	Decreased: Steatosis Inflammation Focal necrosis
Park <i>et al</i> ^[51] , 2004	HEK293T cells	Nox4	TLR4	NF- κ B activation LPS-mediated ROS generation
Gustot <i>et al</i> ^[48] , 2006	Mice fed a modified Lieber-DeCarli diet	LTA, PGN LPS flagellin	TLR2 TLR4 TLR5	Increased: Steatosis Liver weight -aminotransferase levels Liver mRNA expression of different TLRs.
	Addition of multiple bacterial products			Increased: TNF- α mRNA expression
Hritz <i>et al</i> ^[43] , 2008	TLR4-(KO) mice feed Lieber-De-Carli diet	Ioxoribine CpG	TLR7 TLR9 TLR4	Liver inflammatory infiltrate Protection from: Alcohol-induced liver damage ROS production Inflammation
Bala <i>et al</i> ^[58] , 2011	ALD mouse model	LPS	TLR4	MiRNA-155 increase and TNF- α levels in isolated KCs
Bala <i>et al</i> ^[59] , 2012	ALD mouse model administered with CpG + LPS	CpG	TLR9	Plasma increase of: miR-155, miR-122 and miR-146a
	TLR4-KO	LPS	TLR4	Protection from: Alcohol-induced liver disease Increase of both miR-155 and miR-122
Byun <i>et al</i> ^[61] , 2013	Mice feed high-fat diet plus binge ethanol TLR3 (KO) mice	poly I:C	TLR3	Protection from alcoholic liver damage Preservative action of poly I:C abrogated

LPS: Lipopolysaccharide; TLR: Toll-like receptor; LTA: Lipoteichoic acid; PGN: Peptidoglycan; TNF: Tumor necrosis factor; NADPH: Nicotinamide adenine dinucleotide phosphate; Nox4: NADPH oxidase 4; ROS: Reactive oxygen species; ALD: Alcoholic liver disease; KCs: Kupffer cells; poly I:C: Polyinosinic-polycytidylic acid.

lated KCs exhibited increased levels of both miRNA-155 and TNF- α . The same author showed that in alcohol-fed mice there are augmented serum/plasma miR-155 levels and an increased quantity of TNF- α in the liver^[59]. In the same model, the authors evidenced an increase of serum/plasma miR-122 which is known to exert multiple functions in hepatocytes as it is important in the regulation of cholesterol metabolism^[59,60]. Furthermore, the increase of both miR-155 and miR-122 was absent in TLR4-deficient mice that were preserved from ALD^[59]. It is also noteworthy that treatment with TLR9 ligands (that is CpG) leads to plasma increase of both miR-155 and miR-122 and increased levels of TLR9 and serum endotoxin have been reported in alcohol-fed mice^[48,59]. Actually, to date, diverse TLRs have been extensively studied and found to be involved in ALD pathogenesis. In the Lieber-DeCarli chronic alcohol feeding model, expression of TLR1, 2, 4, 6, 7, 8 and 9 liver mRNA resulted from increased enteral administration concomitantly to steatosis induction. It has been also demonstrated that the alcohol treatment sensitizes hepatic inflammation and damage since the triggering of the TLR1, 2, 4, 6, 7, 8 and 9 with their specific ligands resulted in TNF- α augmentation. Moreover, TLR expression remained unaffected after antibiotic treatment which, however, ameliorated

the alcoholic fatty liver condition^[48]. Recently, Byun *et al*^[61] studied the TLR3 involvement in alcohol liver injury both in HSCs and KCs *in vivo*. The authors demonstrated, by means of TLR3-KO and IL-10-KO mice fed a high-fat diet with added ethanol, that TLR3 is protective for alcoholic liver injury and exerts this function by stimulating IL-10 production. In more detail, polyinosinic-polycytidylic acid (poly I:C, that is TLR3 ligand) administration ameliorated alcoholic liver damage and diminished the amount of TNF- α , IL-6 and monocyte chemoattractant protein-1 (MCP-1) in control mice. The preservative action of poly I:C was abrogated in TLR3-KO and IL-10-KO murine models. Coherently, HSCs and KCs isolated from poly I:C-treated animals had higher levels of IL-10 than the controls. IL-10 was also over-expressed *in vitro* both in HSCs and KCs in the presence of poly I:C^[61].

The most relevant experimental studies on the involvement of TLRs in inflammation during ALD are summarized in Table 1.

Evidence from human studies

The role of gut dysbiosis and increased gut permeability and endotoxemia into the portal circulation has also been extensively explored in patients with ALD^[9,62]. Consequently, the critical role exerted by TLR signaling in hu-

man ALD has been investigated. Schäfer *et al.*^[63] found that circulating CD14 levels were higher in serum from patients with severe alcoholic hepatitis than in those from healthy controls, suggesting the crucial role of this TLR4 co-receptor in ALD. Furthermore, it has been reported that the CD14-159C>T polymorphism in patients with ALD may significantly correlate with the severity of disease and be associated with the risk for alcoholic cirrhosis^[64,65]. However, no correlation has been found between TLR4, TNF- α and IL-1 β gene variants and ALD in different populations to date^[66,67].

Clinical evidence of the TLR involvement in intra-hepatic inflammatory response in ALD is still scarce but reinforces the experimental data reported above. The role of TLRs on neutrophils in patients with ALD was reported by Stadlbauer *et al.*^[68]. These authors demonstrated that LPS-induced over-expression of TLR2, 4 and 9 may play a pivotal role in the neutrophil dysfunction observed in patients with alcoholic hepatitis and suggested that the use of TLR antagonists was unable to prevent this dysfunction, while the use of an endotoxin scavenger might reduce inflammatory response and improve clinical outcome^[69]. Interestingly, other authors found an impairment of TLR2 but not TLR4-mediated innate immune response in peripheral blood monocytes from patients with stable chronic ALD, explaining their susceptibility to immunodeficiency and disease worsening^[70].

Interestingly, it has been reported that TLR-dependent priming of B cells might explain the increased circulating levels of immunoglobulins in patients with alcoholic liver cirrhosis^[71].

As mentioned above, most experimental studies reported that NF- κ B transcriptional activity is crucial for the TLR signal and Stärkel *et al.*^[72] demonstrated that a persistent activation of this transcription factor, an up-regulation of TLR3 and TLR7 expression and high pro-inflammatory cytokine levels were associated with end-stage ALD and involved in disease progression in humans.

Additional proof of the activation of the TLR-dependent inflammatory network in ALD was reported by the analysis of circulating levels of cytokines and chemokines. In fact, several authors found high levels of pro-inflammatory cytokines, including TNF- α and IL-6, in the serum of actively drinking patients with ALD, predicting their outcome and long-term survival^[73,74]. Interestingly, fibroblast growth factor-inducible 14 (Fn14), a member of the TNF receptor superfamily (member 12A), was found to be over-expressed in the liver, primarily by hepatic progenitors, in patients with alcoholic hepatitis and it correlated with disease severity^[75]. In addition, it has been reported that several chemokines, including IL-8, correlated with a worse prognosis in patients with alcoholic hepatitis^[76].

Finally, a recent study demonstrated that increased serum levels of chemokine-ligand-1 (CXCL1), an inflammatory chemokine mainly expressed by mononuclear cells in response to LPS-dependent TLR activation, coupled with a polymorphism in the gene encoding for this protein are risk factors for alcoholic cirrhosis^[77].

CONCLUSION

ALD remains one of the major causes of hepatic-associated morbidity and mortality worldwide. Furthermore, the current therapeutic options for patients with ALD are alcohol abstinence and liver transplantation for end-stage liver disease. The liver inflammatory response, which is generated early in ALD, is probably triggered by bacteria and their products, particularly LPS. Due to increased intestinal permeability, they translocate into circulation and activate TLR-dependent production and secretion of pro-inflammatory cytokines and chemokines. There is a body of experimental evidence of the crucial role of TLR-mediated inflammatory network in ALD development and progression. However, to date, these findings have little translational proof in patients. More studies in humans are urgently needed to advance the field and to translate experimental/informative findings into novel therapies.

Furthermore, as qualitative and quantitative changes of the gut microbiome play a major role in the TLR-dependent liver inflammatory response and ALD pathogenesis, further metagenomic, transcriptomic and metabolomic studies may help, not only to explain the “gut-liver axis” in ALD, but also to identify novel potential therapeutic targets. In fact, according to previous studies, treatment with probiotics inhibits alcohol-induced TLR4 and TLR5 activation of TNF- α production, reducing hepatic inflammation in experimental models, and restores neutrophil phagocytic ability in patients with alcoholic cirrhosis, probably by changing TLR4 expression and IL-10 secretion^[78,79].

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WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Obesity, fatty liver disease and intestinal microbiota

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disorder that is increasing in prevalence with the worldwide epidemic of obesity. NAFLD is the hepatic manifestation of the metabolic syndrome. The term NAFLD describes a spectrum of liver pathology ranges from simple steatosis to steatosis with inflammation nonalcoholic steatohepatitis and even cirrhosis. Metabolic syndrome and NAFLD also predict hepatocellular carcinoma. Many genetic and environmental factors have been suggested to contribute to the development of obesity and NAFLD, but the exact mechanisms are not known. Intestinal ecosystem contains trillions of microorganisms including bacteria, Archaea, yeasts and viruses. Several studies support the relationship between the intestinal microbial changes and obesity and also its complications, including insulin resistance and NAFLD. Given that the gut and liver are connected by the portal venous system, it makes the liver more vulnerable to translocation of bacteria, bacterial products, endotoxins or secreted cytokines. Altered intestinal microbiota (dysbiosis) may stimulate hepatic fat deposition through several mechanisms: regulation of gut permeability, increasing low-grade inflammation, modulation of dietary choline metabolism, regulation of

bile acid metabolism and producing endogenous ethanol. Regulation of intestinal microbial ecosystem by diet modifications or by using probiotics and prebiotics as a treatment for obesity and its complications might be the issue of further investigations.

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Key words: Intestinal microbiota; Dysbiosis, Nonalcoholic fatty liver disease; Obesity

Core tip: There is increasing evidence for the relation between dietary habits, gut microbiota and obesity. Nonalcoholic fatty liver disease is a common complication of obesity. This manuscript summarizes the relationship between intestinal microbial dysregulation and fatty liver disease related with obesity, and their proposed mechanisms.

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INTRODUCTION

Human gut consists of a large number of commensal microorganisms, collectively known as “intestinal microbiota”, which are essential for the preservation of the integrity of the mucosal barrier function, for the absorption of nutrients and energy homeostasis^[1]. Recent evidence suggests that enteric microbiota may play a significant role in the development of obesity and its complications^[2].

Nonalcoholic fatty liver disease (NAFLD) describes a condition caused by a deposition of fat within the liver cells in the absence of alcohol consumption, which is linked to being obese or overweight in most cases^[3-5]. It encompasses a disease spectrum ranging from simple ste-

atosis to nonalcoholic steatohepatitis (NASH), which is histologically characterized by hepatocyte injury, inflammation and variable degrees of fibrosis. Nonalcoholic steatohepatitis progress to advanced fibrosis and cirrhosis in 37% patients^[6]. A “two hit” mechanism has been proposed; however, the complete pathogenesis remains incompletely understood. Fatty liver disease is dramatically increasing in childhood and adolescent obesity, and it has become the most common form of chronic liver disease in these age groups^[7,8].

The liver is located on the first point of the body for bacteria and microbial components, as well as other endogenous and exogenous toxins present in the portal blood and it generates the initial immunological and hormonal response to these molecules^[9]. Interactions between the gut and the liver are bidirectional; hormones, inflammatory mediators and the products of digestion and absorption all directly influence liver function. Changes prompted by specific intestinal microbiota are characterized not only by a general obesogenic and dysmetabolic framework but also by a specific *de novo* hepatic lipogenesis^[10]. This review will discuss the relationship between intestinal microbiota and obesity and also NAFLD, and their proposed mechanisms.

CHILDHOOD OBESITY AND NONALCOHOLIC FATTY LIVER DISEASE

Childhood obesity is a major health problem in all over the world because of its impact on the physical and psychological health of children, and also on the development of chronic diseases later in life such as atherosclerosis, NAFLD, hypertension, hyperlipidemia and diabetes^[3,11-13]. Global prevalence of childhood overweight/obesity varies from 5.7% to 40% in different populations^[14-17]. Several case reports, human and animal studies demonstrated that obesity is an important risk factor for carcinogenesis in many malignant neoplasms and also in hepatocellular carcinoma^[18-21]. Moreover, childhood obesity was shown to be related with increased risk of primary liver cancer in later adulthood^[22]. Obesity and related complications including fatty liver disease, cardiovascular disorders and hepatocellular carcinoma were found associated with the reduction in the life expectancy compared to general population^[23]. Several genes contribute to weight gain by controlling feeding behavior, energy expenditure and metabolism, but can only partially account for the development of obesity. Thirty two loci of the human genome had been found associated with body mass index and increased body weight, but changes of these genes affect only 2% of the population^[24]. On the other hand, exogenous obesity develops primarily due to energy intake that exceeds energy expenditure, and many environmental and host factors interact with this process in many ways.

Adipose tissue is not a passive site of energy storage. Although the major function of the adipocyte is to store and release energy in the form of triglyceride during

excess food consumption and starved periods, respectively, it is also an endocrine organ producing several proteins (adipokines like adiponectin and leptin) and cytokine mediators interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α) with many biological activities^[25]. Development and complications of obesity consist of complex mechanisms; in which numerous adipokines, hormones and cytokines take place^[26]. Although the liver participates in the systemic inflammation of obesity, the dominant controller organ is the adipose tissue. Adipokines when imbalanced, together orchestrate a proinflammatory and insulin-resistant state that further contributes to the pathogenesis of NAFLD and its progression to NASH^[27]. A large body of emerging literature seems to suggest that intestinal microbiota is also involved in the development of obesity and its complications including obesity-related liver disease.

INTESTINAL MICROBIOTA

The human intestine contains a very crowded and heterogeneous microbial system, consisting at least 100 trillion (10^{14}) microbial cells weighing about 1.5 kg and composed of more than 2000 species^[28]. Luminal microbial cells contain genes 150 times more than our own host genomes^[28]. This complex community contains taxa from bacteria, eukaryotes, viruses, and at least one archaeon, that interact with one another and with the host, involving regulation of local/systemic immunity, metabolic and trophic functions^[29].

Microbial culture studies detect only a small number of the species of intestinal bacteria. Nowadays, composition and the diversity of intestinal microbiota is revealed by culture-independent genetic and metagenomic techniques^[30]. Metagenomic analysis and 16S ribosomal RNA gene sequencing have shown that *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Spirochaetae* and *Verrucomicrobia* are the predominant bacterial phyla among the intestinal bacteria in adults^[31]. *Firmicutes* and *Bacteroidetes* constitute about 90% of all intestinal microbial cells. While the dominating phyla are relatively constant between individuals, diversity increases along the taxonomic line with each individual harboring over a hundred unique species.

Gut microbiota has evolved with humans as a mutualistic partner; however, changes in the composition of the gut microbiota (dysbiosis) have been found to be related with several clinical conditions such as obesity, diabetes, fatty liver disease, atherosclerosis, allergic diseases, gastrointestinal diseases, autoimmune diseases and cancer^[30-35] (Figure 1).

INTESTINAL MICROBIOTA AND OBESITY

There are many animal and human studies investigating the relationship between intestinal microbiota and obesity or body weight changes in the literature. In the study of Ley *et al*^[36], which investigated over five thousands of

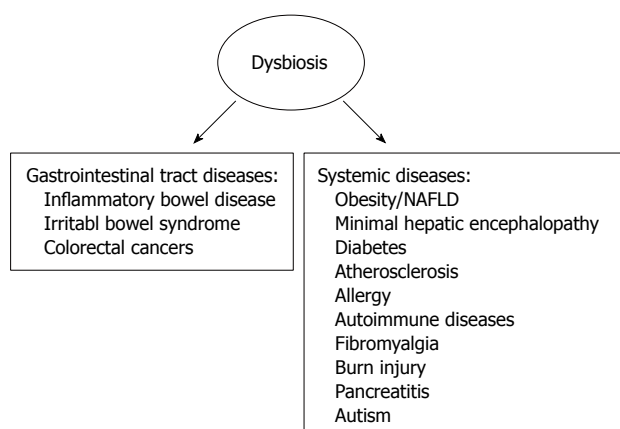


Figure 1 Some gastrointestinal and systemic conditions related with altered intestinal microbiota^[31,32,35].

bacterial gene sequences from the distal intestinal microbiota of genetically obese *ob/ob* mice, lean *ob/+* and wild-type siblings, the investigators found that genetically obese mice had a 50% reduction in abundance of *Bacteroidetes* and a proportional increase in *Firmicutes* phyla compared to lean sibling mice although they were fed with the same polysaccharide-rich diet. Since both groups of animals had been fed with the same diet, it was suggested that obesity might affect the diversity of gut microbiota^[36]. Similarly, several human studies showed that human obesity is associated with a low abundance of intestinal *Bacteroidetes* and high abundance of *Firmicutes*, and with reduced bacterial diversity^[37-39]. Obese children was also shown to have different gut microbiota compared to lean peers, and their bacterial composition have been found similar to obese adults^[40-45]. Although *Bifidobacterium* is not a predominating phylum in the gut, it seems to play an important role in host metabolism. Following a high-fat diet, reduced *Bifidobacterium* was observed, with a secondary increase of inflammatory activity, increased fat mass and insulin resistance in mice^[46]. Interestingly, these changes in the gut microbes were shown to be reversed by low-calorie diet and consequent weight loss^[34,42,47].

MECHANISMS LINKING THE INTESTINAL MICROBIOTA AND OBESITY

Western type fat- and energy-rich diet changes intestinal microbiota. On the other hand, altered microbiota also affects the host metabolism and causes inflammation and increased fat deposition of the body. Numerous animal models consistently demonstrated that gut microbiota can modulate host energy homeostasis and adiposity through different mechanisms, for example energy harvest from the diet, lipopolysaccharides (LPSs)-induced chronic inflammation, and modulation of tissue fatty acid composition, host gene expression and gut-derived peptide secretion^[48].

Intestinal microbiota and energy harvest from the diet (caloric salvage)

Mice with gut microbiota were shown to have an increased capability to harvest energy from the gut contents compared with germ-free rats^[49]. Metagenomic analyses of the microbiota performed in obese mice and humans revealed an increased capacity for the degradation (fermentation) of carbohydrates^[50,51]. This microbial fermentation increases the amount of short-chain fatty acids (SCFAs), such as acetate, propionate, butyrate, and L-lactate. These SCFAs have important roles in the reduction of intestinal pH, in the regulation of energy metabolism, immunity, and adipose tissue expansion and in modulating cancer cell development^[52]. Butyrate is used as an energy substrate for colonocytes, acetate is potentially used as a cholesterol or fatty acid precursor and propionate is used as a gluconeogenic substrate in the liver^[52-54]. In addition to these functions, SCFAs are also physiological ligands of G-protein coupled receptors GPR43 and 41 (also called free fatty acid receptor 2 and 3, respectively), which are expressed in several cell types (immune cells, endocrine cells, and adipocytes) of host tissues^[55,56] (Table 1). Activation of GPR43 by the SCFAs contributes to the inhibition of lipolysis and to adipocyte differentiation, thereby increases the adipose tissue in high-fat-diet-fed mice^[57,58]. Monosaccharides produced by microbial fermentation, absorbed and transferred to the liver via portal vein, activate the hepatic carbohydrate response element binding protein (ChREBP) that increases the transcription of several proteins involved in hepatic lipogenesis thus contributing to hepatic fat accumulation^[55]. The effect of intestinal microbiota on obtaining energy from the gut contents had also been tested in human studies. Jumpertz *et al*^[59] reported that the amount of stool energy in proportion of ingested calories was positively and negatively correlated with the abundance of phylum *Bacteroidetes* and phylum *Firmicutes* in the feces, respectively. Approximately a-150 kcal difference could be achieved with a change of 20% relative increases of *Firmicutes* and decreases of *Bacteroidetes* in the stool of lean individuals. Thus, excessive calories taken in the form of SCFAs from microbial fermentation of luminal contents may be a contributing factor in the obese state.

Fiaf (fasting-induced adipocyte factor)

In addition to extract calories from otherwise indigestible dietary polysaccharides, the presence of the intestinal microbiota also stimulates changes in the expression of genes coding for peptides in host tissues, which control energy homeostasis and nutrient availability (Table 1). Bäckhed *et al*^[60] firstly demonstrated that colonizing germ-free mice with gut microbiota had led to a decrease in the intestinal expression of angiopoietin-like factor IV [ANGPTL4, also called fasting-induced adipose factor (Fiaf)], thereby blunting the inhibition of lipoprotein lipase in the adipose tissue. In the study of Mandrad *et al*^[61] inhibition of lipoprotein lipase blocked the dissociation

Table 1 Some key host proteins and factors those their expressions were changed by intestinal microbial changes and those play role in the development of obesity

Host protein/factor	Function
Fiaf (fasting-induced adipocyte factor)	A protein that inhibits lipoprotein lipase activity
ChREBP (carbohydrate response element-binding protein)	A transcription factor that recognizes monosaccharides in the portal vein and plays a key role in the hepatic carbohydrate metabolism
SREBP-1 (liver sterol response element-binding protein type-1)	A transcription factor family that controls the lipid synthesis in the liver and other tissues
G-protein coupled receptors (GPR43 and GPR41)	Proteins expressed in enteroendocrine L-cells those recognize luminal SCFAs and mediate SCFA-induced GLP-1 release. They also present in adipocytes and promote adipogenesis by increasing lipid accumulation and inhibiting lipolysis and stimulate leptin production in response to SCFAs
Toll like receptors	Transmembrane molecules those recognize bacterial breakdown products
GLP 1 (Glucagon-like peptide 1)	A protein produced by intestinal epithelial endocrine L-cells that stimulates insulin secretion, inhibits gastrointestinal motility, regulates appetite and food intake
Peptide YY	A peptide hormone produced by intestinal epithelial endocrine L-cells that inhibits intestinal motility
Farnesoid X receptor	A receptor expressed in liver and intestine that regulates bile acid synthesis, transport and detoxification

GLP-1: Glucagon-like peptide 1; SCFAs: Short-chain fatty acids.

of fatty acids from triglycerides for uptake into tissues and upregulated fatty acid oxidation and uncoupling proteins, and potentially reduced the amount of fat storage. These results may explain why conventionalized mice are more sensitive than germ-free mice to fat storage when fed with a high-fat diet and supports the role of the gut microbiota in the development of obesity^[50,52,62].

Increased intestinal permeability and inflammation

Obesity, diabetes and insulin resistance are associated with a low grade systemic inflammation^[63-65]. Several studies have been conducted on the effect of systemic inflammation on glucose and lipid metabolism, but little is known about its triggers.

Intestinal microorganisms have highly conserved microbial molecules, called “pathogen-associated molecular patterns” (PAMPs) and endogenous products called “damage-associated molecular patterns” (DAMPs), which are recognized by pattern recognition receptors. These receptors include membranous toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs). TLRs recognise the potential pathogens in the intestinal lumen and induce the immune response. Microbial products such as LPSs, lipopeptides, DNA and RNA have potentially hepatotoxic effects as they are potent inducers of inflammation. Among 13 known TLRs, TLR2, TLR4 and TLR9 have been shown to play a role in the development of NAFLD. Stimulation of TLRs results in the activation of several different intracellular signaling cascades including stress-activated and mitogen activated protein (MAP) kinases, Jun N-terminal kinases, p38 MAP kinase, interferon regulatory factor 3 and nuclear factor kappa B (NF- κ B) pathways^[9,66]. NF- κ B is an important transcription factor in the cell, translocates to the nucleus and induces the transcription of a variety of inflammatory cytokines and chemokines such as TNF- α and IL-1 β ^[67,68]. de la Serre *et al*^[69] showed that rats prone to weight gain exhibited an increase in TLR4 activation associated with ileal inflammation, decreased intestinal alkaline phosphatase activity, a luminal enzyme that detoxifies the bacterial

component (LPS), known to cause inflammation, and increased innate immune system activation in the luminal wall when compared to the rats resistant to obesity. In another study, Cani *et al*^[46] demonstrated knockout rats of an immunoprotein (CD14), which was necessary to cause an inflammatory reaction to LPS, were resistant to weight gain. Vijay-Kumar *et al*^[70] showed that TLR5 knockout mice exhibited hyperphagia and developed hallmark features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased visceral fat deposition. These metabolic changes correlated with changes in the composition of the gut microbiota, and transfer of the gut microbiota from TLR5-deficient mice to wild-type germ-free mice conferred many features of metabolic syndrome to the recipients^[70]. Lam *et al*^[71] showed that mice fed with high-fat diet had reduced zona occludens-1 mRNA expression (40%) and increased permeability in proximal colon, and increased levels of TNF- α and IL-1 in mesenteric fat compared to mice fed with the control diet. In human studies, low grade endotoxemia (metabolic endotoxemia) was found associated with high-fat meal, obesity, NAFLD and diabetes^[72-75]. All these data shows that the inflammatory milieu is the key component of the development of obesity and its complications.

Releasing of gut hormones

Intestinal microbial system regulates entero-endocrine cells and promotes the release of several gut hormones. Peptide YY (PYY) is an enteroendocrine cell-derived hormone normally inhibits gut motility. Samuel *et al*^[76] showed that PYY expression was lower in both *Gpr41* -/- germ-free and conventionalized mice compared to *Gpr41* +/+ mice. Reduced PYY expression resulted in increased intestinal transit time and reduced harvest of dietary energy^[72-76]. In other studies, dietary fructo-oligo-saccharides increased the abundance of *Bifidobacterium* in the distal intestine, which led to increased colonic fermentation and glucagon-like peptide 1 (GLP-1) levels, decreased serum orexigenic peptide ghrelin and decreased food intake, fat

mass, and hepatic steatosis in rats^[77,78]. Based on these results, it can be concluded that dietary inulin-type fructans could play a role in the management of obesity and diabetes through their capacity to promote secretion of endogenous gastrointestinal peptides involved in appetite regulation^[77,78].

INTESTINAL MICROBIOTA AND FATTY LIVER DISEASE

Nonalcoholic fatty liver disease is a multifactorial disease and the underlying mechanisms are incompletely understood. Various genetic, metabolic, inflammatory, nutritional and environmental factors are thought to contribute to its pathogenesis^[9]. Nutrition is the most important environmental factor; its role may be more complex than inducing fat accumulation in the liver and may involve interactions with the microbiota^[9]. The potential role of the intestinal microbiota on the liver diseases had been known since 1921^[79]. Several animal and human studies have investigated possible relationships between the intestinal microbiota and NAFLD^[46,60,75,79-85]. Bäckhed *et al.*^[60] firstly showed that microbiota stimulated monosaccharide absorption from the intestinal lumen, promoted *de novo* fatty acid synthesis and triglyceride production, as confirmed by increased activity of acetyl-CoA carboxylase and fatty acid synthase. The relation between altered microbiota and liver was shown even in pediatric age group studies. Karlsson *et al.*^[45] showed that obese/overweight preschool children had an increased amount of the gram-negative family *Enterobacteriaceae* and had inverse correlation between *Bifidobacterium* concentration and alanine aminotransferase levels.

MECHANISMS LINKING THE INTESTINAL MICROBIOTA AND NAFLD

Studies suggest that intestinal microbiota may stimulate liver steatosis through several mechanisms^[86]: (1) induction of obesity by harvesting energy from otherwise indigestible dietary polysaccharides (above mentioned); (2) regulation of gut permeability and stimulation of low grade inflammation; (3) modulation of dietary choline metabolism; (4) regulation of bile acid metabolism; and (5) stimulation of endogenous ethanol production by enteric bacteria.

Small intestinal bacterial overgrowth and increased intestinal permeability

Intestinal epithelial cells separate the intestinal microbial environment from the host immune system. Epithelial cells are linked to each other with tight junctions, which play a central role in maintaining intestinal barrier integrity^[87]. In human studies NAFLD has been associated with increased LPS plasma levels, through mechanisms involving increased intestinal permeability, small intestinal bacterial overgrowth (SIBO), tight junction alteration and

bacterial translocation^[83,84,88-90].

SIBO is defined as an increase in the number and/or alteration in the composition of bacteria in the proximal gastrointestinal tract. Association of increased intestinal permeability and fatty liver was first demonstrated by Miele *et al.*^[84]. They reported that patients with fatty liver disease had increased gut permeability related to SIBO and disrupted tight junctions compared to healthy adults^[84]. They also showed that gut permeability and SIBO were correlated with the severity of the liver steatosis. In the study of Gäbele *et al.*^[91], mice were fed with high-fat diet to stimulate NASH and then exposed to dextran sulphate sodium, an agent that causes intestinal epithelial injury. They showed that combined administration of high-fat diet and dextran sulphate sodium induced fibrosis in the liver^[91]. All these data support the hypothesis that altered homeostasis between host and intestinal microbial system at the intestinal epithelial barrier level promotes bacterial translocation from the gut into the portal circulation and induces the liver damage^[86,92,93].

Low grade inflammation and fatty liver

Several studies demonstrated that the altered microbiota caused low-grade inflammation, which had a pivotal role for the development of obesity and its complications including NAFLD. Association of inflammation and fatty liver was firstly demonstrated by Cani *et al.*^[46]. They showed that continuous LPS infusion in mice increased the insulin resistance, liver triglyceride content and adipose tissue inflammation. After this study, other animal studies conducted in TLR4 knockout mice confirmed that TLR4 was essential for hepatic steatosis and NASH development^[94-96]. Kupffer cells, which express the highest levels of TLR4 in the liver, are the primary cells in liver inflammation that respond to LPSs in order to produce inflammatory cytokines, chemokines and reactive oxygen species (ROS)^[97,98]. In genetically obese mice, the administration of LPS induces changes in Kupffer cells function and increases liver parenchymal sensitivity to TNF- α ^[99]. TNF- α , which is the most important of LPS-TLR4 induced cytokines in these cells, is recognized as a mediator of hepatotoxicity, inflammation and NASH development in mice^[100]. Besides, hepatic stellate cells might also have a substantial role in constituting the inflammatory cascade of the liver consequently associated with metabolic endotoxemia. Indeed, these cells are the major fibrogenic cell type in injured liver were shown to be the target through which TLR4 promoted fibrogenesis via enhancement of transforming growth factor- β (TGF- β) signalling^[101-103].

The “second hit” mechanism of the NAFLD/NASH pathogenesis include enhanced lipid peroxidation and increased generation of ROS^[104]. Inflammasomes, major contributors of inflammation, are cytoplasmic multiprotein complexes, which include nucleotide-binding domain (NLRPs). NLRPs are sensors of the bacterial PAMPs and DAMPs^[105,106]. They manipulate the cleavage of pro-inflammatory cytokines such as pro-IL-1 β and pro-IL-18.

Most DAMPs induce the production of ROS, which is known to activate NLRP3 inflammasome^[107,108]. Henao-Mejia *et al*^[108] reported that inflammasome alterations or IL-18 deficiency cause intestinal microbial changes by enhancing portal influx of TLR4 and TLR9 ligands, which in turn increase hepatic TNF- α production in mice. Human studies also demonstrated that endotoxin levels were increased in both adult and pediatric obese patients with fatty liver disease^[74,109,110]. Moreover, endotoxin levels were found to be correlated with the severity of the disease^[75,111]. All these data support that chronic low-grade inflammation caused by obesogenic microbial ecosystem is a real “hepatotoxin” and has a key role in the pathogenesis of obesity related fatty liver disease.

Altered choline metabolism

Choline is a water-soluble essential nutrient. It is an important phospholipid component of the cell membrane and is the precursor molecule for the neurotransmitter acetylcholine. Choline has important roles in fat metabolism in the liver and a very-low-lipoprotein assembly, and also it promotes lipid transport from the liver^[86,112]. Exogenous sources of choline are meat, dairy products, fish, soybeans, nuts and whole grains. Endogenous sources of choline, in the form of phosphatidylcholine, are biliary lipids, exfoliated epithelial cells and intestinal bacteria^[113]. Buchman *et al*^[114] showed that choline-deficient nutrition stimulated the liver steatosis. Furthermore, they demonstrated that a 6-wk choline supplementation reversed this pathology in patients. Gut microbiota secrete enzymes that cleave the dietary choline to its toxic metabolites (dimethylamine and trimethylamine). Liver uptakes these toxic methylamines and converts them to trimethylamine-N-oxide which induce inflammation in the liver^[115,116]. Spencer *et al*^[117] showed that the compositions of the gastrointestinal microbial communities changed with dietary choline content and especially *Gammaproteobacteria* and *Erysipelotrichi* levels were directly associated with changes liver steatosis in each subject during choline depletion. The role of dietary choline in NAFLD can be explained by the bioavailability of free choline to for lipoproteins in the liver (especially very- low-density-lipoprotein-VLDL), which allows the export of free fatty acids from this organ^[113]. If the gut microbiota converts excessive amounts of dietary choline into trimethylamine, this leads to reduced choline bioavailability and consequent fatty liver disease^[118].

Altered bile acid metabolism

Bile acids modulate lipid absorption and cholesterol homeostasis. The nuclear bile acid receptor, called farnesoid X receptor (FXR), is strongly expressed at bile acid excretion (liver) and absorption (intestine) regions. Bile acids also act as signaling molecules and activate FXR and the G-protein coupled receptor TGR5. Through activation of downstream signaling pathways of these key receptors, bile acids regulate not only their own synthesis and enterohepatic circulation, but also impact on hepatic lipid, glucose, and energy homeostasis^[119]. FXR plays a key

role in the control of hepatic *de novo* lipogenesis, VLDL triglyceride export and plasma triglyceride turnover^[120]. TGR5 binds secondary bile acids and promotes glucose homeostasis, by stimulating secretion of GLP-1^[121]. Besides, bile acids have a bacteriostatic activity. Gut microbiota can modulate bile acid metabolism. Swann *et al*^[82] showed that gut microbiota can indirectly promote hepatic steatosis and lipid peroxidation through FXR stimulation changes in bile acid secretion. On the other hand, high-fat diet changes the bile acid composition, which influences the conditions for gut microbial environment and causes dysbiosis^[122,123].

Stimulation of endogenous ethanol production by enteric bacteria

Intestinal microbiota produces a number of potentially hepatotoxic compounds such as ethanol, phenols, ammonia and they are transported to liver by portal system. These toxins stimulate hepatic Kupffer cells for production of nitric oxide and cytokines such as TNF- α ^[9,124]. Acetaldehyde and acetate are two major metabolites of ethanol. Ethanol can increase acetate production via inhibition of the Krebs cycle. Acetate is a substrate for fatty acid synthesis. On the other hand, acetaldehyde and its metabolites may lead to the formation of reactive oxygen species. ROS production could be involved in liver injury by contributing to the disruption of intestinal barrier function and to the two hit mechanisms of steatohepatitis^[80,86,125]. Ethanol and LPS also stimulate the production of ROS by parenchymal and nonparenchymal liver cells. Gustot *et al*^[124] showed that enteral ethanol exposure induced steatosis and increased liver weight, aminotransferase levels, and TLR1, 2, 4, 6, 7, 8, and 9 liver mRNA expressions in mice. They concluded that ethanol-fed mice exhibited an oxidative stress dependent on upregulation of multiple TLRs in the liver and were sensitive to liver inflammation induced by multiple bacterial products recognized by TLRs^[124].

In a human study, Nair *et al*^[126] demonstrated that obese women with NASH had higher breath ethanol concentrations than healthy controls detected by gas chromatography. Similarly, Zhu *et al*^[127] showed in their pediatric age group study that *Proteobacteria*, *Enterobacteriaceae*, and *Escherichia* (is a well-known ethanol producer bacteria) were the only phylum, family and genus types exhibiting significant difference between the patients with and without NASH microbiomes. Similar blood-ethanol concentrations were observed between healthy subjects and obese non-NASH patients; however, NASH patients exhibited significantly elevated blood ethanol levels^[127]. Ethanol contributes to iNOS-mediated intestinal hyperpermeability, and therefore enhances the passage of endotoxins from the intestinal lumen into the portal system^[128].

CONCLUSION

High energy diets alter intestinal microbiota, induce gut dysfunction, which subsequently result in visceral fat

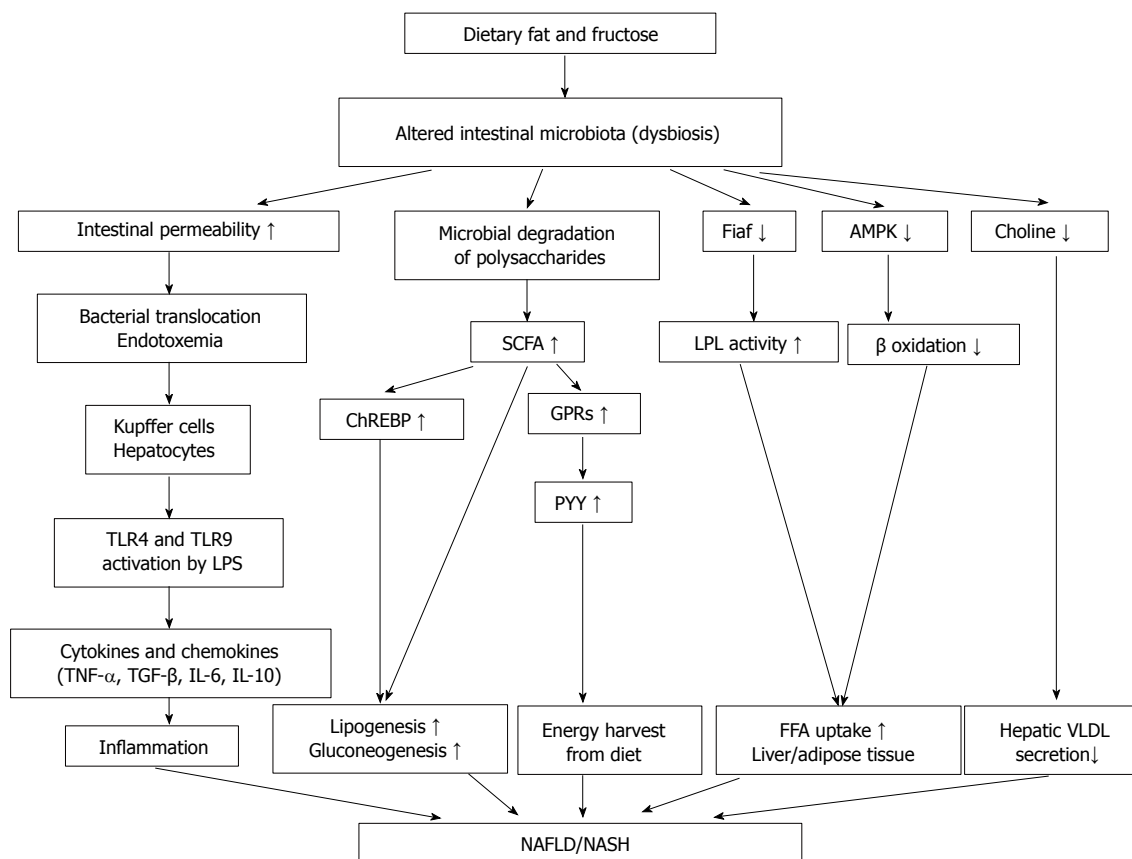


Figure 2 Effects of gut microbiota on the development of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis through the gut-liver axis. Altered intestinal bacterial composition (dysbiosis) results in degradation of carbohydrates in the intestinal lumen and produces short-chain fatty acids (SCFAs), which are substrates for hepatic lipogenesis and gluconeogenesis. The interaction of SCFAs with G-protein coupled receptors (GPRs) releases the peptide YY (PYY), which modulates gut motility and nutrient absorption. SCFAs stimulate hepatic carbohydrate response element binding protein (ChREBP) and increase lipogenesis. Bacterial translocation to the portal circulation causes interaction of bacterial endotoxins (lipopolysaccharides, LPS) with hepatic toll-like receptors (TLR4 and TLR9) and results in the release of cytokines and chemokines. Decreased Fiaf (fasting-induced adipocyte factor) levels enhance lipoprotein lipase (LPL) activity and cause fat accumulation. Choline deficiency causes liver steatosis via decreased secretion of very-low-density-lipoprotein-(VLDL) from the liver.

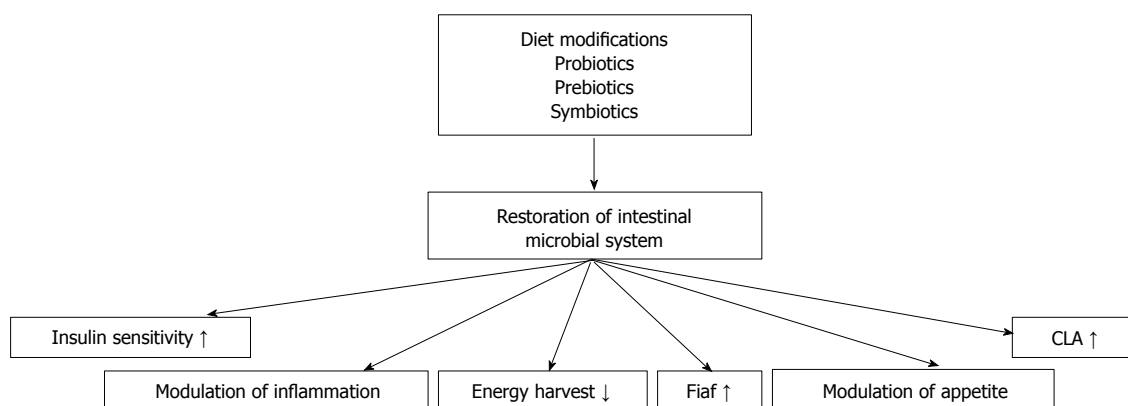


Figure 3 Probable mechanisms of action of the antiobesity effects of modulated intestinal microbiota. CLA: Conjugated linoleic acid; Fiaf: Fasting-induced adipocyte factor.

inflammation and systemic metabolic dysregulation. An obesogenic microbiota can alternate liver function by stimulating hepatic triglyceride and by modulating systemic lipid metabolism that indirectly impact the storage of fatty acids in the liver (Figure 2). Several studies

suggested that intestinal microbiota might also play an important part in progression of NAFLD to NASH. Modulation of gut microbiota by diet modifications or by using probiotics, prebiotics and synbiotics as a treatment for obesity and fatty liver disease might be the issue of

further investigations (Figure 3).

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WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Non alcoholic steatohepatitis a precursor for hepatocellular carcinoma development

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been associated with NASH and subsequent HCC progression. We will focus our discussion on inflammation and gut derived inflammation and how they contribute to NASH driven HCC.

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Key words: Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Inflammation; Microbiome; Bile acids

Core tip: Non alcoholic steatohepatitis (NASH) is a metabolic inflammatory disease the can often advance to liver cancer. Previously, it was assumed that obesity, hepatocyte cellular death and insulin resistance were the dominant drivers of NASH progression to hepatocellular carcinoma. Herein, we discuss the latest concepts concerning the gut microbiome and bile acids, which have now been shown to have a role in promoting hepatic inflammation, and subsequent liver tumor growth.

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Abstract

Hepatocellular carcinoma (HCC) is increasing in prevalence and is one of the most common cancers in the world. Chief amongst the risks of attaining HCC are hepatitis B and C infection, aflatoxin B1 ingestion, alcoholism and obesity. The later has been shown to promote non alcoholic fatty liver disease, which can lead to the inflammatory form non alcoholic steatohepatitis (NASH). NASH is a complex metabolic disorder that can impact greatly on hepatic function. The mechanisms by which NASH promotes HCC are only beginning to be characterized. Here in this review, we give an overview of the recent novel mechanisms published that have

INTRODUCTION

Hepatocellular carcinoma (HCC) is now the fifth cancer of greatest incidence worldwide. Hepatitis B and C leading to cirrhosis are the dominant risk factors and the most common causes of HCC^[1]. But in recent years studies have found that non-alcoholic steatohepatitis (NASH) can promote liver fibrosis, end-stage liver failure, cirrhosis, and ultimately progression to HCC. NASH is a clinical and pathological syndrome that is not associ-

ated with increased alcoholic intake, but has histological features similar to alcoholic hepatitis, with prominent fatty deposition and fat storage in the liver parenchymal cells, that can promote inflammation and necrosis^[2,3]. Day *et al*^[4] proposed an initial theory for the pathogenesis of NASH, known as the “two-hit hypothesis”. Here it was suggested that the “first hit” of hepatic triglyceride accumulation or steatosis, increased the vulnerability of the liver to the “second hit” of injury due changes in inflammatory cytokines and/or adipokines, mitochondrial dysfunction and elevated oxidative stress, that can together promote steatohepatitis and fibrosis. However, data from many sources has now attributed other “hits” that include insulin resistance and the metabolic syndrome. These include changes in serum cytokines, which have been extensively reviewed elsewhere^[5-10], and more recently and of relevance to this review, inflammation, altered gut microflora and bile acids that can contribute to the generation of NASH and HCC.

NASH: A COMPLEX BIOLOGICAL ENTITY

Numerous clinical studies have shown strong links between NASH and consequent cirrhosis, which naturally increases the risk of progression to HCC^[11-13]. Studies have also shown that HCC is now a major cause of mortality in NASH patients^[14]. Importantly, work has illustrated in comparison to diabetes and hepatitis C virus that non alcoholic fatty liver disease (NAFLD)/NASH is a growing underlying etiological risk for HCC^[15]. Significantly, research has shown that HCC is now occurring more frequently from non-cirrhotic NASH^[16,17] (and reviewed in^[18]). Taken together, these data suggest that NASH in the presence of cirrhosis or non-cirrhosis, can promote HCC through diverse pathways. However, an underlying theme that is now emerging from the literature is the role of inflammation in cancer and in particular HCC. Moreover, these pathways have activity in the presence or absence of cirrhosis and we believe that unbridled inflammation is one of the principle factors that can drive the progression from NASH to HCC, and enhance HCC growth. The targeting of these pathways offers a potential avenue of therapeutically restricting HCC growth. Thus, in this review we will focus on clarifying some of the principal and novel inflammatory mechanisms that have been recently described for promoting HCC in the presence of NASH.

FACTORS LINKING INFLAMMATION TO NASH AND HCC PROGRESSION

Cytokines

Cytokines represent a family of small bioactive proteins and peptides that have signaling qualities in mediating intercellular communication signals, cellular interactions, growth and differentiation in cells. Therefore, in disease states when imbalances in cytokine levels occur they have important effects in promoting aberrant signaling, and in

particular modulating inflammatory responses^[19-21]. Studies have shown that cytokines such as tumor necrosis factor (TNF)- α , leptin, adiponectin and interleukin-6 (IL-6) occupy important roles in hepatic pathology. Thus, we will now examine their relationship and role in progression from NASH to HCC.

TNF- α

Studies have shown that TNF- α has an important role in liver cancer progression and growth^[22]. The binding of TNF- α to TNFR1 receptor activates the transcriptional regulator nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)^[23]. NF- κ B consists of five different family members: RelA (p65), RelB, c-Rel, p50/p105, and p100/p52 that are localized to the cytoplasm by inhibitors of κ B (I κ B)^[24]. In response to proinflammatory stimuli, the I κ B kinase complex of IKK α and IKK β subunits and the regulatory protein, NF- κ B essential modulator (NEMO) or inhibitor of NF- κ B kinase subunit gamma (IKK γ), phosphorylates I κ B, leading to its degradation and the subsequent movement of NF- κ B into the cell nucleus^[25]. Genetic removal of components of this pathway, have illustrated the importance of NF- κ B in liver inflammation and HCC.

In mice, the ablation of IKK β from hepatocytes to foster NF- κ B inactivation, and subsequent treatment with the mutagen diethylnitrosamine (DEN), resulted in a greater incidence of HCC^[26]. To explain this phenotype it was found that on exposure to excess TNF- α , prolonged c-Jun N-terminal kinase (JNK) activation was stimulated, and resulted in increased hepatocyte apoptosis with compensatory hepatocyte proliferation. These events facilitated the accumulation of genetic errors to enhance HCC growth. Accordingly, increased JNK activity can also promote insulin resistance as JNK^{-/-} mice have improved insulin sensitivity and less hepatic inflammation and fibrosis^[27,28]. Alternatively, the removal of IKK β from hepatocytes and Kupffer cells reduced the number and size of HCCs, after DEN treatment^[26]. Mechanistically, it was found that the proliferation of hepatocytes after exposure to DEN, were dependent on the IKK β induced production of the hepatic mitogens TNF- α , IL-6 and HGF from Kupffer cells.

Further studies showed that the deletion of NEMO from hepatocytes, which modulates the phosphorylation and degradation of I κ Bs in the TNF- α pathway, spontaneously promoted HCC development. It was found that NEMO absence completely blocked NF- κ B activation and sensitized the NEMO-null hepatocytes to lipopolysaccharide (LPS) treatment, suggesting the involvement of the innate immune response and microbiome in HCC development^[29].

To convey the importance of obesity in driving steatosis and inflammation, it has been shown that TNF- α and IL-6 are important in obesity driven HCC. Here liver cancer was promoted by DEN treatment of IL-6 or TNFR1 null mice and subsequent high-fat feeding. It was observed that compared to wild-type controls that IL-6

or TNFR1 null mice had significantly reduced steatosis, HCC number and size^[30]. Collectively, these studies show the key role of the TNF- α pathway in hepatic inflammation, and suggest that imbalances in this pathway are important in the transition from NASH to HCC.

IL-6

IL-6 has been shown to have defined and important roles in HCC pathology. IL-6 is largely secreted by inflammatory cells and can bind to IL-6 receptors on hepatocytes and liver non-parenchymal cells to promote the binding of the signal-transducing receptor gp130 to the IL-6R complex, to activate Janus Kinase1 (JAK1). Subsequent activation and phosphorylation of the transcription factor signal transducer and activator of transcription-3 (STAT3) factor, promotes a transcriptional program to prevent apoptosis and initiate cell growth and differentiation. Studies have shown that phosphorylated STAT3 is expressed in up to 60% of human HCCs^[31], and that increased IL-6 protein levels is associated with NASH as compared to steatosis in patients^[32]. Moreover, IL-6 has been shown to be upregulated in two established models murine models of NAFLD^[33], and mice overexpressing IL-6 and soluble IL-6R, have more extensive liver cancer than wild-type mice^[34]. In agreement with the concept that IL-6 is a promoter of HCC, it has been shown that male IL-6 deficient mice generate less HCC than wild-type males after DEN treatment^[35]. Finally, it has been demonstrated that IL-6 serum levels are increased in the majority of hepatic acute and chronic events^[36]. Thus, these data suggest that IL-6 is associated with NASH and is a promoter of HCC formation and growth.

Leptin

Leptin (Ob) is a multifunctional adipocytokine secreted by fat cells and has a variety of biological effects mediated by its binding to a specific leptin receptor (ObR). It plays an important role in hepatic stellate cell (HSC) activation and hepatic fibrosis. It was found that the inhibition of endogenous leptin activity can retard HSC function and have powerful anti-fibrotic effects^[37]. Moreover, leptin can stimulate tissue inhibitor of metalloproteinase 1 (TIMP-1) production *via* the JAK/STAT pathway to promote fibrogenesis^[38,39]. The injection of leptin into carbon tetrachloride-treated mice increased the synthesis and secretion of pro-fibrotic genes in the liver including procollagen and TGF β 1. Leclercq *et al*^[40] utilised leptin-deficient mice and found that the injection of leptin increased TGF- β 1 levels and completely restored fibrosis. These data suggest that leptin plays an important role in liver fibrosis.

The relationship of leptin with NASH is less well determined. In patients Chittur *et al*^[41] found that serum leptin levels were higher in NASH patients than in the normal group. Studies from another group, failed to find an association between leptin and NASH^[42]. Leptin can regulate liver cancer development by promoting tumor cell proliferation and angiogenesis. Leptin acts on endo-

thelial cells to promote tube formation and migration, while limiting leptin impairs angiogenesis^[43]. In the presence of vascular endothelial growth factor, leptin-mediated neovascularization in the liver increased in line with NASH progression, indicating a pro-angiogenic role^[44]. In HCC patients both leptin and ObR are expressed at higher levels in livers. Interestingly, poorly differentiated HCCs have greater blood vessel density and ObR expression, again suggesting an angiogenic role^[45]. Furthermore, leptin has been shown to promote HCC proliferation, migration, and invasiveness through activation of the JAK/STAT pathway^[46]. Taken together, these observations suggest that elevated serum leptin levels through increased adipose tissue mass, may promote progression by enhancing hepatic fibrogenesis, angiogenesis, and cancer cell division and behavior. Thus, it is plausible that increased leptin levels could promote progression from NASH to HCC. However, as studies in humans have not revealed a clear correlation it is possible that leptin is associated only with a subset of HCCs that originate in the background of NASH.

Adiponectin

Adiponectin is an anti-inflammatory cytokine produced by the adipose tissue. It has roles in regulating glucose and fatty acid metabolism, with decreased plasma concentrations correlating to increased BMI, insulin resistance, type 2 diabetes and atherosclerosis. Adiponectin circulates in the serum in different molecular forms: a low molecular weight trimer, a middle molecular weight hexamer, and high molecular weight multimers, that is considered to be the most biologically active. There are three known APN receptors: AdipoR1, AdipoR2 and T-cadherin that have distinct affinities for the various circulating forms of APN^[47].

Given the different adiponectin forms and receptors, adiponectin has a plethora of activities. In the liver adiponectin activates AMPK to reduce hepatic gluconeogenesis, stimulate fatty acid oxidation, and limit hepatic *de novo* lipogenesis through inhibition of sterol-regulatory element binding protein-1c (SREBP-1c), a dominant regulator of triglyceride and fatty acid synthesis^[48,49]. Adiponectin can also activate peroxisome proliferator-activated receptor α (PPAR α) to promote fatty acid oxidation. Importantly, in the context of liver diseases adiponectin can limit inflammation by inhibiting the NF- κ B activation to suppress TNF- α release^[50,51]. Adiponectin can also further suppress macrophage function and the proliferation and migration of vascular smooth muscle cells^[52].

In NASH, hypoadiponectinemia is an early feature, and it has been shown that low serum adiponectin levels are associated with increased hepatic steatosis and with necroinflammation^[53]. Likewise, adiponectin null mice have more steatosis and fibrosis after high fat feeding, and develop more fibrosis on carbon tetrachloride treatment^[54-56]. Moreover, adiponectin has strong hepatoprotective properties and can diminish steatosis and hepatic damage, in endotoxin and alcohol injury models by limit-

ing hepatic production of TNF- α ^[57,58].

Nevertheless, studies have showed that in cirrhotic and HCC patients that impaired hepatic function is associated with increases in serum adiponectin levels^[59-61]. It has been recently shown that in advanced NASH there is a significant correlation between increased serum bile acids, circulating adiponectin and reduced liver fat^[62]. It is probable that similar mechanisms are operating in HCC patients. It remains open whether adiponectin can influence HCC once developed. In HCC cell lines adiponectin increased JNK activation and subsequent apoptosis in tumors, and promoted increased AMPK phosphorylation and the inhibition of the mammalian target of rapamycin (mTOR) phosphorylation to limit tumor growth in nude mice^[63]. In a separate study, it was shown that adiponectin treatment decreased HCC tumor growth and macrophage infiltration, and secondary lung metastasis^[64]. In summary, these data suggest that lower adiponectin levels associated with obesity are a driver of NASH and HCC formation.

EMERGING INFLAMMATORY PATHWAYS FOR PROMOTING NASH HCC DEVELOPMENT

The above illustrate that NASH driven HCC is associated with a robust inflammatory response. For some time the standard model assumed that the increased adipose mass associated with insulin resistance enhanced liver injury. Additionally, evidence also suggested that the accumulation of hepatic cellular fat could promote the release of reactive oxygen species to interfere with cellular functions such as cellular respiration to cause the release of toxic lipids species, to result in hepatocyte dysfunction and apoptosis (as reviewed by us^[65]). However, recent studies now include another layer of factors that are responsible for contributing and perhaps enhancing the effect of inflammation and damage to the liver. Specifically, research has found that diet affects the constitution of the gut bacteria (microbiome) to subsequently influence inflammation. Furthermore, it has been demonstrated that the microbiome can modulate bile acids levels, which are now known to have both systemic and hepatic specific signaling. Together, these findings suggest a novel pathogenic pathway between the gut and liver driven by dietary changes that has the potential to generate hepatic inflammation and ultimately influence HCC.

Microbiome and NASH

Studies have linked NASH with dysbiosis of the gut. In NASH patients Wigg *et al.*^[66] observed small intestinal bacterial overgrowth and increased TNF- α levels. Similarly, in another study NASH patients had greater bacterial overgrowth, elevated expression of Toll-like receptor (TLR) 4 and increased levels of serum IL-8^[67]. Commensurate with these changes qPCR for the major gut bacteria species has shown that NASH patients have less

gut Gram-negative Bacteroidetes, than that observed in patients with simple steatosis^[68]. Likewise, in NASH patients an increase in the abundance of alcohol producing bacteria has been observed, suggesting that these strains may be involved in NASH pathogenesis^[69].

In view of these findings, interventional studies have been undertaken in rodents and patients. Treatment of mice fed the methionine-choline-deficient diet with the probiotic VSL#3 reduced liver fibrosis but had no effect on inflammation and steatosis^[70]. However, in a genetic model, treatment of ApoE knock-out mice with VSL#3 had a more pronounced effect and reversed insulin resistance and steatohepatitis^[71]. In choline-deficient/L-amino acid define (CDAA) fed rats a butyrate-producing probiotic reduced hepatic lipid deposition and significantly improved insulin resistance, serum endotoxin levels, and hepatic inflammatory indexes^[72]. In patients there has been limited studies undertaken. A small Chinese study of 20 NASH patients has shown that probiotics can reduce liver fat and AST levels^[73]. Similarly, in an Italian study of 66 NASH patients, the 33 that received probiotics had reduced TNF- α , C-reactive protein, AST, HOMO-IR, serum endotoxin levels, steatosis and NASH activity^[74]. Together, these data suggest a role for the microbiome in mediating NASH and that the correction of gut dysbiosis to a more healthy phenotype can possibly be used as a therapy to limit NASH progression.

How does the microbiota induce hepatic inflammation and metabolism?

Numerous studies over the past decade have shed light on the role of the microbiota in modulating hepatic metabolism and inflammation. A focus point has been lipopolysaccharide (LPS), a large molecule present on and released by gram negative bacteria. LPS binds to the cluster of differentiation 14/TLR2/lymphocyte antigen 96 (CD14/TLR4/MD2) receptor complex on immune cells, which represents part of the innate immune system to promote a pro-inflammatory immune response. It has been shown that the consumption of a high fat diet can loosen the intestinal tight junctions, leading to the increased delivery of bacterial products like LPS *via* the portal vein to the liver. Moreover, as constituents of this signaling cascade have links with regulating metabolism, data has shown that LPS can also regulate insulin resistance. For example, obese *ob/ob* mice have intestinal overgrowth, increased intestinal permeability, more TNF- α , hepatic stellate cell activation and an enhanced LPS inflammatory response^[75]. The treatment of *ob/ob* mice or HFD mice with antibiotics to alter the gut microbiota reduced glucose intolerance, body weight gain, fat mass development, lowered inflammation and oxidative stress. Moreover, the authors showed that the absence of CD14 in *ob/ob* CD14^{-/-} mutant mice mimicked the metabolic and inflammatory effects of antibiotics, suggesting a key role for LPS^[76]. In a separate study, leptin whose levels are induced by obesity has been shown to upregulate CD14, to promote hyper responsiveness to LPS and

enhance progression to NASH^[77]. These studies show a critical role for the TLR4 signaling complex in promoting inflammation and NASH.

Another arm of the innate immune system are the inflammasomes, which are a signaling complex consisting of caspase 1, apoptosis-associated speck-like protein containing (ASC) and nucleotide-binding oligomerization domain receptors (NOD-like receptors: NLRs). The complex can be stimulated by TLRs *via* LPS, but can also be activated by other bacterial ligands, depending on the NLR family member. A principle downstream action of the complex is to activate the caspase-1 cascade, leading to the production of pro-inflammatory cytokines IL-1 β and IL-18. In an elegant study by Henao-Mejia *et al.*^[78], they showed that inflammasome-deficient mice (Casp1^{-/-}, Asc^{-/-}, Nlrp3^{-/-} or IL-18^{-/-}), fed a methionine and choline-deficient (MCD) diet had increased severity of NASH and decreased glucose tolerance. Critically, the authors showed that co-habitation of these mice with wild-type could result in the transfer of the NASH phenotype to the wild-type animals. Furthermore, they showed that the microbiota from these animals mediated the inflammatory response through TLR4, TLR9 and TNF- α . These data show that inflammatory cross-talk between the gut and liver has an important role in protecting the organism against metabolic disease, and that changes in the microbiome or that alternatively the decreased responsiveness of the innate immune system can lead to metabolic diseases such as diabetes and NASH.

Evidence of microbiota links to NASH driven HCC

The above evidence suggests that the microbiota can promote NASH a risk factor for HCC development. To test if the microbiota can influence HCC promotion, the group of Schwabe in a refined study, initiated tumorigenesis with DEN and followed with subsequent carbon tetrachloride treatment to promote fibrosis driven HCC^[79]. They used this experimental protocol on TLR4 null mice and found that TLR4 absence limited HCC growth. Antibiotic treatment of wild-type mice subjected to the DEN/CCl₄ treatment reduced tumor growth, suggesting that the microbiota and LPS through TLR4 promoted HCC. Further, the authors identified that the mitogen epiregulin, a ligand for the epidermal growth factor receptor (EGFR), was expressed by activated stellate cells, suggesting a mechanism by which activated HSCs could drive tumor cell proliferation. Moreover, they observed that the microbiota and TLR4 supported the expression of survival signals to promote tumor growth.

The gut microbiota apart from stimulating an immune response has other important functions. Specifically, they catalyse the generation of secondary bile acids such as deoxycholic acid (DCA), which is known to induce DNA damage^[80]. Further to this concept, Yoshimoto *et al.*^[81] found that DCA can promote the activation of a senescence-associated secretory phenotype in HSCs, reflected by the secretion of IL-1 β . They observed that the absence of IL-1 β limited obesity-induced HCC development and similarly that antibiotic treatment

could alleviate HCC development. Furthermore, the lowering of DCA or feeding of DCA to HFD-fed mice, respectfully limited or enhanced HCC growth. These data suggest in sum that bacterial metabolites can instigate hepatic inflammation either directly or indirectly *via* the generation of metabolites such as DCA that can enhance HCC growth and progression. These observations suggest a potential role for bile acids in NASH HCC progression.

Bile acids and NASH HCC

Primary bile acids are derived from cholesterol, the most abundant being chenodeoxycholic acid (CDCA) and cholic acid (CA). They are secreted into the intestine where modification by intestinal bacteria leads, as explained above, to the generation of the secondary bile acids, such as DCA and lithocholic acid (LCA). To maintain bile acids at physiological levels they are efficiently reabsorbed in the ileum and transported by the enterohepatic circulation back to the liver. For many years it was considered that bile acids were mere detergents, important for absorbing, transporting, and distributing dietary lipids, vitamins and steroids. However, it is now appreciated that they represent a distinct class of hormones that bind to highly specific receptors, the best described being the nuclear hormone receptor farnesoid X receptor (FXR) and the G-protein-coupled cell surface receptor TGR5.

FXR regulates bile acid synthesis by inhibiting the transcription of cholesterol 7 α -hydroxylase (CYP7A1), the rate-limiting enzyme in the conversion of cholesterol to bile acids. FXR can also repress the SREBP-1c transcription to reduce triglyceride synthesis, and promote the β -oxidation of fatty acids through augmented PPAR α signalling^[82]. In agreement FXR null mice have elevated serum triglycerides and cholesterol and are prone to develop steatohepatitis^[83]. Moreover, FXR agonists can antagonize NF- κ B activity and limit hepatic inflammation *in vivo*^[84]. In this light, studies have illustrated the potential of FXR agonists to treat NASH. In the mouse MCD model, treatment with WAY-362450, reduced liver injury and inflammation^[85]. In a recent phase II clinical trial a study was undertaken to evaluate the effects of Obeticholic acid (OCA; INT-747, 6 α -ethyl-chenodeoxycholic acid) on insulin sensitivity in patients with nonalcoholic fatty liver disease and type 2 diabetes mellitus. It was found that within 6 wk OCA increased insulin sensitivity and reduced the markers of liver inflammation treatment and fibrosis^[86]. Given FXR's important role in liver function, it has also been shown that FXR null mice can spontaneously develop liver tumors as they age, and treatment with CA further potentiated DEN-initiated liver cancer. Mechanistically, it was found that the null mice have increased levels of the proinflammatory cytokine IL-1 β , activation of the Wnt/ β -catenin pathway activation, and target gene c-myc^[87,88].

TGR5 is expressed in the gall bladder, cholangiocytes, ileum, colon, brown and white adipose tissue and to a lesser extent in skeletal muscle, liver and immune cells^[89]. TGR5 activation in muscle and brown adipose increases

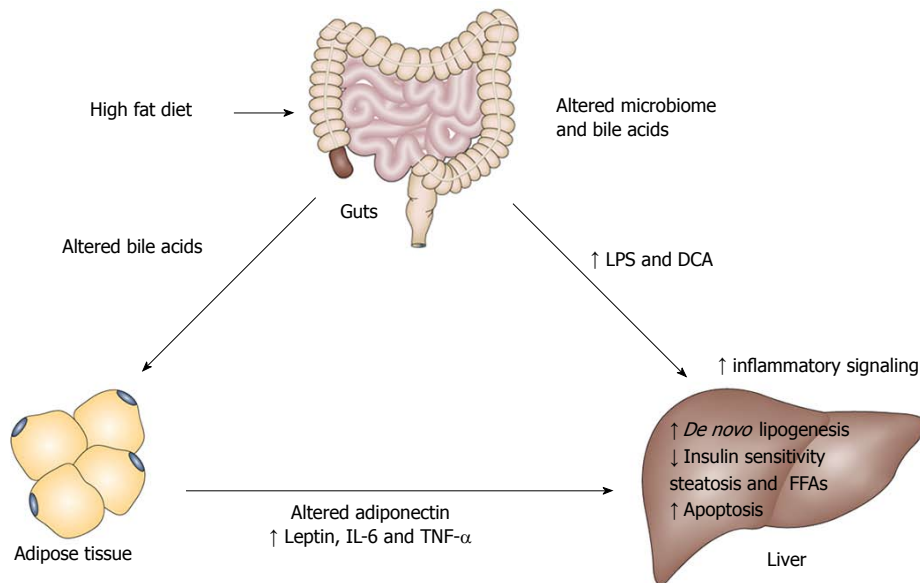


Figure 1 Model illustrating the role of inflammation in non alcoholic steatohepatitis driven hepatocellular carcinoma. The current literature supports the concept that hepatic inflammation can in part come from dietary changes that promote an altered microbiome to release inflammatory factors such as lipopolysaccharide (LPS) and the inflammatory bile acid deoxycholic acid (DCA). They impact on the liver to activate the innate immune system and a senescence - associated secretory phenotype in hepatic stellate cells. Bile acids can also modulate hepatic inflammation through farnesoid X receptor and TGR5 receptors. The increased adipose mass can produce less adiponectin and more leptin, interleukin-6 (IL-6) and tumor necrosis factor (TNF)- α that can further impact on the liver. The build up of fat and elevated FFAs, induce hepatocyte apoptosis, further amplifying the inflammatory effects. The net effect of the systemic and hepatic inflammation is to support neoplastic growth in the liver. It remains to be determined in appropriate mouse models whether bile acids can influence adipocyte function, non alcoholic steatohepatitis and hepatocellular carcinoma progression.

the intracellular secondary messenger cyclic adenosine monophosphate (cAMP), which in turn increases transcription of the Type II iodothyronine *deiodinase* gene (*Dio2*) and the prerequisite protein Type II iodothyronine *deiodinase* (D2), which converts thyroxine (T4) to triiodothyronine (T3). The net effect is augmented energy and oxygen consumption. TGR5 promotes hormone secretion in the gut, of intestinal glucagon-like peptide-1 (GLP-1), which in turn stimulates insulin release. The concept that TGR5 is involved in energy modulation, is supported by TRG5 null mice which accumulate fat faster than wild-type. Moreover, treatment of wild-type mice with the TGR5 specific agonist INT-777 increases brown adipose tissue D2 and energy metabolism, and improves obesity and steatosis^[90]. In the liver, TGR5 is expressed in Kupffer cells, sinusoidal endothelial cells and cholangiocytes. In keeping with this expression pattern, TGR5 inhibits liver inflammation by suppressing NF- κ B in macrophages and is protective, as TGR5 null mice have more liver damage after bile-duct ligation^[91-93]. In sum, these observations show that TGR5 has been shown to repress hepatic inflammation, but as yet no definitive study has linked it with HCC progression *in vivo*. In sum, these data illustrate that elevated bile acids or inhibited bile acid signaling could be a deciding step in the progression from NASH to HCC.

CONCLUSION

Taken together we have illustrated here the critical impor-

tance of adipokines, inflammatory factors, the generation of inflammation through gut dysbiosis and bile acids in supporting and enhancing NASH driven liver tumor growth. Obviously, inflammation from other sources such as the adipose and liver generated injury due to steatosis and cholesterol are also consequential factors in NASH and HCC^[65]. Jointly, these observations show that cross-talk between the fat and liver and gut and liver can contribute to NASH HCC. We illustrate these concepts in Figure 1.

In light of the fact that NASH originates from being overweight and obese, the correction of the microbiome to a lean phenotype and utilization of bile acid agonists are attractive future therapeutic options to limit hepatic inflammation and NASH progression, and possible HCC formation. Nevertheless, it must be mentioned that few of these studies have demonstrated a genetic mechanism through which NASH HCC tumor initiation is influenced. Therefore, this suggests that in the context of NASH driven HCC there are clearly genetic and epigenetic factors that can promote the initiation of HCC. A review of the literature reveals that research into the association of NASH HCC with alterations in microRNA, methylation, chromatin remodeling and chromosomal changes is only in its infancy. Thus, even through the suppression of inflammation is an attractive target for limiting and restraining HCC growth, conceptually further research is urgently needed to elucidate the identity of genetic and epigenetic factors that can initiate HCC in the presence of NASH.

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WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Pathology of alcoholic liver disease, can it be differentiated from nonalcoholic steatohepatitis?

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pathologist should recognize.

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Abstract

The liver involvement in alcoholic liver disease (ALD) classically ranges from alcoholic steatosis, alcoholic hepatitis or steatohepatitis, alcoholic cirrhosis and even hepatocellular carcinoma. The more commonly seen histologic features include macrovesicular steatosis, neutrophilic lobular inflammation, ballooning degeneration, Mallory-Denk bodies, portal and pericellular fibrosis. Nonalcoholic steatohepatitis (NASH) is a condition with similar histology in the absence of a history of alcohol intake. Although the distinction is essentially based on presence or absence of a history of significant alcohol intake, certain histologic features favour one or the other diagnosis. This review aims at describing the histologic spectrum of alcoholic liver disease and at highlighting the histologic differences between ALD and NASH.

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Key words: Alcoholic liver disease; Steatosis; Steatohepatitis; Fibrosis; Cirrhosis; Hepatocellular carcinoma; Non-alcoholic steatohepatitis

Core tip: Alcoholic steatohepatitis (ASH) is well described. Absence of steatosis should not rule out ASH. Nonalcoholic steatohepatitis though histologically similar to ASH, does have important differences, which a

INTRODUCTION

The effect of alcohol on the liver has been known since centuries. A PubMed search reveals descriptions of the pathology and ultra-structure in the 1950-1960s^[1,2]. Non-alcoholic fatty liver disease (NAFLD) was first described by Ludwig in 1980 for a set of histological features similar to those of alcoholic hepatitis, which he noted in liver biopsies of patients without a significant history of alcohol intake or any clinical evidence of alcohol abuse^[3]. Thus the close similarity of histologic findings in the two entities is obvious, however the differences in histology if any, are not well described. This review intends to describe in detail the histology of alcoholic liver disease (ALD) while highlighting the differences from NAFLD.

ALCOHOLIC LIVER DISEASE

Diagnosis of alcoholic liver disease can be made in persons with excessive alcohol intake (20-40 g/d for men and 20 g/d for women) and evidence of liver injury^[4]. Histologically the spectrum of liver injury in both ALD and NAFLD can vary from simple steatosis to cirrhosis.

HISTOLOGY OF ALCOHOLIC LIVER DISEASE

The liver involvement in ALD classically ranges from

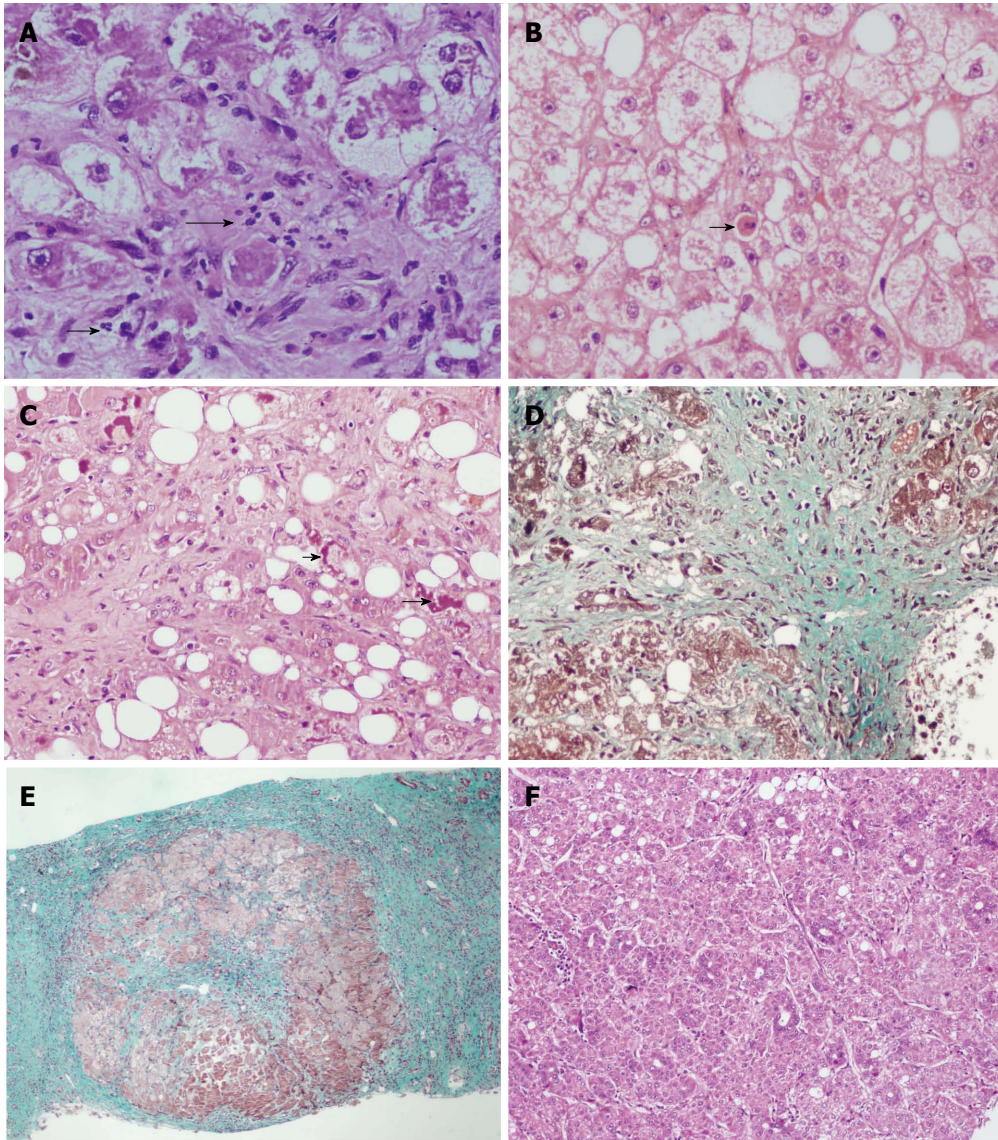


Figure 1 Histological image. A: Neutrophilic inflammation (arrow) and ballooning of hepatocytes in alcoholic liver disease (ALD) (HE staining, $\times 400$); B: Apoptotic cell death (arrow) in ALD (HE staining, $\times 400$); C: Prominent Mallory hyaline (arrow) in ALD (HE staining, $\times 400$); D: Sclerosing hyaline necrosis (masson trichrome staining, $\times 200$); E: Cirrhosis with associated pericellular fibrosis also seen in ALD (masson trichrome staining, $\times 40$); F: Hepatocellular carcinoma with focal steatosis in a case of known ALD (HE staining, $\times 100$).

alcoholic steatosis, alcoholic hepatitis or steatohepatitis (ASH), and alcoholic cirrhosis.

Alcoholic steatosis

Steatosis is defined as the accumulation of lipid droplets in the hepatocyte cytoplasm. The term “fatty degeneration” has been used when $> 5\%$ of hepatocytes show steatosis while fatty liver is the term described when $> 50\%$ of hepatocytes show steatosis^[5]. Presence of steatosis however is not essential for the diagnosis of ALD as it may even decrease despite continuing alcohol intake and progression of disease^[6].

Steatosis can be macrovesicular or microvesicular, depending not only on the size of the lipid droplet accumulated, but also on the pathogenesis and etiology of disease. In ALD the steatosis is usually macrovesicular

or mixed microvesicular and macrovesicular. The steatosis begins in the centrilobular Zone 3 and progresses towards the periportal Zone 1. It may begin with small droplets of fat in the cytoplasm (microvesicular), which later enlarge to large fat droplets (macrovesicular), which push the nucleus to the periphery. Macrovesicular fat droplets can coalesce to form fat cysts (large irregular extracellular fat vacuole). Continuing accumulation of fat may lead to rupture of fat cyst with a histiocytic reaction or lipogranuloma. The macrovesicular fat droplets have a high surface/volume ratio and are less susceptible to action of lipases. This allows macrovesicular fat to persist for a few months even after alcohol intake is stopped^[7]. Microvesicular steatosis is seen as multiple fat droplets with a central nucleus. Pure microvesicular steatosis may be seen in Alcoholic foamy degeneration. This has not

been described in non-ASH (NASH).

Alcoholic steatohepatitis

Steatohepatitis indicates evidence of hepatic injury accompanying the steatosis. The injury may be seen in the form of hepatocyte ballooning, neutrophil rich inflammation in the lobular parenchyma (Figure 1), apoptosis or Mallory Denk bodies. Ballooning degeneration of hepatocytes is the predominant mode of cellular injury in alcoholic hepatitis. The hepatocytes are markedly swollen with rarified cytoplasm, clumping of intermediate filaments and loss of staining for cytokeratins 8 and 18. The oncotic swelling as a result of severe ATP depletion and increase in intracellular calcium results in loss of plasma membrane volume control, disruption of intermediate filament network, cell swelling and oncotic necrosis^[7]. Lytic necrosis following ballooning degeneration is the commoner form of injury in ASH, however apoptosis can also be seen and indicates either ongoing or current injury. Apoptosis may be triggered by oxidative damage to the mitochondrial inner membrane. The apoptotic hepatocytes (Figure 1), also known as councilman bodies or acidophil bodies, show cell shrinkage, chromatin condensation and nuclear and cellular fragmentation. These are more frequent in NASH than in ASH^[8]. Mallory-Denk bodies (MDB) are clumps or skeins of eosinophilic ropy material in the hepatocyte cytoplasm usually in a perinuclear location. Misfolded and aggregated keratin filaments accumulate to form MDB^[9]. These are degraded by ubiquitination-proteasome pathway to give ubiquitinated keratin, ubiquitinated protein p62, Heat shock proteins 70 and 25. Thus, MDB demonstrate immunoreactivity with antibodies to ubiquitin, keratins 8 and 18, and p62. The formation of MDB could either be a result of toxic damage to the hepatocyte or they may actually contribute to continuing inflammatory injury^[7]. Lobular inflammation in ALD is often neutrophil rich and the neutrophils may surround ballooned hepatocytes - "satellitosis"^[10]. Portal Inflammation is usually milder than is seen in other forms of chronic hepatitis such as viral hepatitis. Besides lymphocytes and plasma cells, neutrophils, eosinophils and even mast cells can be seen in the inflammatory infiltrate. Portal inflammation is more common and of higher grade in ALD than in NAFLD. It may be accompanied by ductular reaction and periportal fibrosis and may be associated with underlying chronic pancreatitis^[11].

Other histologic features described in ALD include glycogenated nuclei, megamitochondria, hemosiderin deposition, cholestasis and ductular reaction^[12]. Glycogenated nuclei are nuclear vacuolations in the hepatocyte nuclei. They are more frequent in NAFLD where they are seen in a periportal location.

Fibrosis: Alcoholic cirrhosis

Fibrosis in alcoholic liver disease begins in Zone 3, peri-venular region and extends in a pericellular/perisinusoidal pattern, giving rise to the classic "chicken-wire fibrosis".

A Masson trichrome or Sirius red stain better identifies this. Later the fibrosis progresses to extend to the portal tracts and central-portal or portal-portal bridging fibrosis are seen. Finally, if the alcoholic injury continues, the simultaneous fibrosis and hepatocyte regeneration results in nodule formation and finally cirrhosis^[13]. An orcein stain is helpful in later stages to differentiate broad bands of fibrosis (orcein positive) from areas of collapse due to superadded acute alcoholic hepatitis.

Areas of parenchymal extinction are seen as a result of involvement of central and portal veins by the fibrotic process, while ductular reaction is a result of expansion of stem cell compartment^[13].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) can develop in the background of alcoholic liver disease in up to 5%-15% cirrhotics. Alcohol is likely the commonest cause of HCC in the west. Autopsy studies reveal the presence of dysplastic nodules in large cirrhotic regenerative nodules greater than 5 mm in size. These may be precursor lesions to the development of HCC.

The tumor cells may show presence of Mallory hyaline, steatosis and even focal microvesicular foamy degeneration.

NAFLD

Non-alcoholic fatty liver disease and Alcoholic fatty liver disease, as the name implies, are diseases with differing etiologies and similar morphologic spectrum. The term NAFLD indicates the entire spectrum of fatty liver diseases not related to alcohol intake. Non-alcoholic fatty liver is the term used when there is simple steatosis in the liver not accompanied by inflammation, cell injury or fibrosis. NASH is the term used when steatosis is accompanied by inflammation and cell injury/ballooning degeneration with or without fibrosis. Matteoni *et al*^[14] in 1999 have divided NAFLD into four types or classes based on the clinical spectrum and pathological severity. Class 1 is simple steatosis, class 2 represents steatosis with lobular inflammation, class 3 shows the additional presence of ballooned hepatocytes and class 4 requires the presence of either Mallory's hyaline or fibrosis. These were correlated with increasing severity of disease and likelihood of progression to cirrhosis.

Diagnosis

NAFLD is essentially a clinicopathological diagnosis, wherein clinically, besides ruling out significant alcohol consumption, other causes such as viral hepatitis, autoimmune, A1AT and cholestatic etiologies should be excluded, and evidence of metabolic syndrome^[15] should be sought. Minimal histological criteria for diagnosis of NASH were defined at the American Association for the Study of Liver Diseases Single Topic Conference on NASH held in September 2002^[4] (Table 1). These recommendations though helpful, most pathologists use varying combinations of histologic features to diagnose

Table 1 Histopathologic abnormalities in nonalcoholic steatohepatitis^[4]

Necessary components	
Steatosis, macro > micro; accentuated in Zone 3	
Lobular inflammation, mild; scattered polymorphonuclear leukocytes as well as mononuclear cells	
Ballooning of hepatocytes; most apparent near steatotic liver cells, in Zone 3	
Usually present but not necessary	
Zone 3 perisinusoidal fibrosis	
Zone 1 hepatocellular glycogenated nuclei	
Lipogranulomas in the lobules; usually small	
Occasional acidophil bodies or periodic acid Schiff-stained Kupffer cells	
Fat cysts	
May be present, not necessary	
Mallory's hyaline in ballooned hepatocytes - usually Zone 3; typically poorly formed, may require immunostaining for ubiquitin, p62 or CK 7, 18, 19 to confirm	
Hepatocellular iron, usually grade 1	
Megamitochondria in hepatocytes	

Table 2 Grading system for alcoholic liver disease^[6]

Grade 0-11	
Steatosis	
0-3 as in NIDDK NASH CRN	
Lobular inflammation	
0-3 as in NIDDK NASH CRN	
Cell death	
0 none	
1 focal apoptosis	
2 many acidophil bodies	
3 confluent necrosis	
Ballooning	
0-2 as in NIDDK NASH CRN	
Fibrosis stages 0-6	
Stage 1 mild Zone 3 pericellular and/or perivenular	
Stage 2 marked Zone 3 fibrosis in most zones 3	
Stage 3 fibrous linkage HV to septa	
Stage 4 fibrous linkage HV, PT and septa	
Stage 5 incomplete or probable cirrhosis	
Stage 6 definite cirrhosis	

NASH CRN: Nonalcoholic Steatohepatitis Research Network; NIDDK: National Institute of Diabetes and Digestive and Kidney Diseases; HV: Hepatic venule; PT: Portal tracts.

NASH, but the four most important features are steatosis, ballooning, lobular inflammation and perisinusoidal fibrosis^[16].

GRADING AND STAGING

Scoring systems for ALD are few and not used frequently. In early stage of disease if clinical history and evaluation point to the diagnosis, a biopsy is often not done. Biopsy is evaluated when advanced diseases is suspected or if diagnosis is in doubt, and thus grading systems are not routinely referred to. Yip and Burt^[6] in 2006 proposed a system which may be useful in assessing prognosis, or comparing biopsies in clinical trials (Table 2).

Brunt *et al*^[17] in 1999 proposed a grading and staging system for NASH using steatosis, ballooning, lobular

Table 3 Non-alcoholic fatty liver disease activity score as defined by nonalcoholic steatohepatitis clinical research network^[16]

Item	Definition	Score
Steatosis (grade)	Low to medium power evaluation of parenchymal involvement by steatosis	
	< 5%	0
	5%-33%	1
	> 33%-66%	2
Lobular Inflammation	> 66%	3
	Overall assessment of all inflammatory foci	
	No foci	0
	< 2 foci per 200 × field	1
Ballooning	2-4 foci per 200 × field	2
	> 4 foci per 200 × field	3
	None	0
	Few balloon cells	1
Fibrosis stage	Many cells/prominent ballooning	2
	None	0
	Perisinusoidal or periportal	1
	Mild, Zone 3, perisinusoidal	1A
	Moderate, Zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4

and portal inflammation for the activity grading. Subsequently the pathology subcommittee of the Clinical Research Network for NASH designed and validated a histologic feature scoring system for the full spectrum of lesions of NAFLD. This group evaluated 14 histologic features and after analysis proposed a NAFLD activity score (NAS)^[16] (Table 3). Routine hematoxylin and eosin and Masson Trichrome stains are recommended for evaluation. The NAS score specifically included only those features that were related to active injury and were potentially reversible. It has been defined as the un-weighted sum of scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2). The sum is thus 0-8. Fibrosis has been scored separately as in chronic hepatitis scoring systems and is elaborated in greater detail than the previous staging system. The NAS has been defined for the purpose of evaluating histologic changes after therapeutic trials and to assess overall histologic change and not to replace diagnostic criteria or assess severity of NAFLD.

Inter and intra-observer variation may be present as in other grading systems. The degree of steatosis may be over or underestimated based on the power at which observations are made. For example small drop-let steatosis may be missed on low power examination and may lead to underestimation of grade of steatosis. Estimation at × 20 objective magnification is recommended. Hall *et al*^[18] have shown that hepatopathologists show “excellent” inter-observer agreement in estimated fat proportionate area, however measured fat proportionate area using digital image analysis is more

Table 4 Differences in histologic features of nonalcoholic steatohepatitis and alcoholic liver disease

NAFLD (NASH)	ALD
Usually mild disease	Varying severity
Bridging necrosis rare	Bridging necrosis common
Poorly formed MH	Well formed MH
-/rare	Sclerosing hyaline necrosis
-/rare	Phleboscclerosis
-/rare	Canalicular cholestasis
Not described	Foamy degeneration
Nuclear vacuolation - more common	Nuclear vacuolation - less common
Presence of Iron/hemosiderin is less frequent	Presence of Iron/hemosiderin is more frequent
Ductular reaction is less frequent/prominent	Ductular reaction is more frequent/prominent
Fibrosis/cirrhosis - less common	Fibrosis/cirrhosis - more common

NAFLD: Non-alcoholic fatty liver disease; ALD: Alcoholic liver disease; NASH: Nonalcoholic steatohepatitis; MH: Mallory hyaline.

accurate. Degree of ballooning may be difficult to grade when steatosis is extensive.

ASH vs NASH

Historically, NASH was described as a result of its morphologic similarity to Alcoholic Steatohepatitis. Thus the overlap in histologic features is prominent; however alcoholic liver disease shows more severe disease histology at the time of biopsy. Whether this is a function of greater toxic injury due to repeated bouts of alcohol abuse, or because liver biopsy is rarely performed in the early stage of disease when a clinical diagnosis is obvious, is debatable. Despite the commonality in the histologic features in ASH and NASH, some features are rarely seen or not reported in NASH (Table 4). Sclerosing hyaline necrosis is characterized by perivenular liver cell necrosis with fibrosis in the same region. This may result in occlusion of the terminal hepatic venules and precirrhotic portal hypertension^[19]. Alcoholic foamy degeneration was described by Uchida *et al*^[20] in a set of 20 patients who recovered rapidly once the alcohol was withdrawn. The hepatocytes show a diffuse prominent microvesicular fatty change (may be more in perivenular zone), with minimal inflammation and MDB are either minimal or absent. Perivenular hepatocytic and canalicular cholestasis may be associated. Goodman and Ishak^[21] have described three types of vascular lesions in an autopsy series of ALD. These include lymphocytic phlebitis, phleboscrosis (narrowing of the hepatic vein lumen) and veno-occlusive lesions. Phleboscrosis is seen in all cases of ASH. Cholestasis is seen in severe fatty liver, alcoholic foamy degeneration and can be seen in alcoholic hepatitis and decompensated alcoholic cirrhosis. Liver histology may show cholestasis, marginal ductular proliferation, cholangiolitis and portal tract edema. Adaptive changes such as oncotic or groundglass change in cytoplasm of hepatocytes can be seen in advanced ALD. These may reflect increased numbers of mitochondria or smooth

endoplasmic reticulum respectively^[6].

Pinto *et al*^[22] studied the histologic features of liver biopsy in 32 non-alcoholics, 21 asymptomatic ambulatory and 52 hospitalized patients of alcoholic hepatitis. Histologic findings in the ambulatory alcoholic group were intermediate between the other two groups with nonalcoholic group having least degree of severity of hepatocellular damage, inflammation, MDB and fibrosis. The obese, diabetic non-alcoholics had significant fibrosis in 47% and cirrhosis in 8% while 38% of ambulatory and 89% of hospitalized alcoholic hepatitis patients had cirrhosis.

Singh *et al*^[23] also compared the liver histology in alcoholic versus non-alcoholic fatty liver disease. They reported more severe ballooning degeneration of hepatocytes, portal inflammation, prominent MDB, neutrophil-rich inflammation, and fibrosis in ASH as compared to NASH. Cholestasis and bile ductular proliferation were observed only in ASH.

The differentiation between the two conditions becomes even more difficult in overlapping clinical scenarios such as obese alcoholics, alcoholics with diabetes and obese or diabetic individuals with a borderline alcohol intake. The degree of injury caused by alcohol varies greatly in different individuals due to various genetic factors.

Role of liver biopsy in ALD

Although liver biopsy is not essential in a clinical setting of ALD wherein the history of alcohol abuse is forthcoming, it may be useful in the following settings: (1) To look for or rule out the presence of a coexisting etiology for liver injury which may be seen in up to 20% of patients^[24]; (2) To assess the severity of liver injury and the stage of fibrosis, especially in patients who do not have decompensated liver disease; (3) The liver biopsy may also give helpful prognostic information. The severity of inflammation, presence of neutrophils and presence of cholestatic changes may indicate poorer prognosis while presence of megamitochondria indicates a better prognosis, long-term survival with fewer complications and slower progression to cirrhosis^[25]; (4) In patients where the history of alcohol use is suspected but not forthcoming or there may be a possibility of both ALD and NAFLD; (5) In severe forms of ALD where specific treatment modality is to be given (such as corticosteroids or pentoxifylline); (6) Patients who are to be enrolled in a clinical trial; and (7) Where acute-on-chronic liver injury is suspected.

However, the decision to biopsy has to be taken for each individual case based on a balance between the information it can provide, the impact on treatment and the risk of complications.

Reporting on a biopsy

Before designating a biopsy as alcoholic steatohepatitis, in a liver biopsy showing histology of steatohepatitis, a clinical history of significant alcohol intake must be sought. For a pathologist where details such as quantum

and frequency of alcohol intake may not be clearly mentioned on the requisition form, this diagnosis is based only on a brief input from the clinician. Furthermore, there is a distinct possibility of the patient having additional clinical features of metabolic syndrome. Thus a report stating that the histology is “consistent” with alcoholic steatohepatitis is more accurate. In cases where history of alcohol intake or features related to metabolic syndrome and other causes of steatohepatitis are not clearly stated, a morphologic diagnosis alone should be given with clinical possibilities stated in order of likelihood based on the morphology. For example, a liver biopsy clearly showing about 40% macrovesicular steatosis, associated with ballooning degeneration of hepatocytes, well defined Mallory hyaline and a neutrophil-rich lobular inflammation with a mild portal inflammation, may be reported as “Steatohepatitis - possibly alcohol related” - clinical correlation required.

Additional information such as the degree of steatosis and grading and staging of the biopsy may be helpful. Prussian blue stain for iron overload may also be helpful especially in alcoholic liver disease.

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High intensity focused ultrasound: A noninvasive therapy for locally advanced pancreatic cancer

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Abstract

The noninvasive ablation of pancreatic cancer with high intensity focused ultrasound (HIFU) energy is received increasingly widespread interest. With rapidly temperature rise to cytotoxic levels within the focal volume of ultrasound beams, HIFU can selectively ablate a targeted lesion of the pancreas without any damage to surrounding or overlying tissues. Preliminary studies suggest that this approach is technical safe and feasible, and can be used alone or in combination with systemic chemotherapy for the treatment of patients with locally advanced pancreatic cancer. It can effectively alleviate cancer-related abdominal pain, and may confer an additional survival benefit with few significant complications. This review provides a brief overview of HIFU, describes current clinical applications, summarizes characteristics of continuous and pulsed HIFU, and discusses future applications and challenges in the treatment of pancreatic cancer.

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Key words: Pancreatic cancer; High intensity focused ultrasound; Focused ultrasound surgery; Thermal ablation; Hyperthermia; Therapeutic ultrasound

Core tip: Prognosis in unresectable locally advanced

pancreatic cancer is extremely poor. Standard treatments are currently limited to chemotherapy, radiotherapy, or a combination of the two. Though few regimens may offer a limited survival benefit, novel treatment strategies are urgently needed. As a non-invasive approach, high intensity focused ultrasound therapy can selectively ablate a targeted lesion of the pancreas. Preliminary studies indicate that this approach is safe and feasible, and can be used alone or in combination with chemotherapy. It can effectively alleviate cancer-related abdominal pain, and may confer an additional survival benefit with few significant complications.

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INTRODUCTION

Carcinoma of the exocrine pancreas is the fourth leading cause of cancer-related death in the United States and the Western world. In 2013, 45220 estimated new cases were diagnosed for the United States, with 38460 associated deaths^[1,2]. Because of the frequent delay in diagnosis, more than 80% of patients have locally advanced or metastatic disease at presentation, and are unsuitable for curative surgical resection^[1,2]. Prognosis in pancreatic cancer is generally dismal. Median survival for locally advanced disease is just 6-10 mo, but this falls to 3-6 mo in patients with metastatic disease; overall 5-year survival rate is about 5%^[1,2].

Standard options available for treating patients with unresectable pancreatic cancer are limited to chemothera-

py, radiotherapy, or a combination of the two. Gemcitabine is the most commonly used chemotherapeutic agent in pancreatic cancer, and recent studies have shown that a combination of gemcitabine with other chemotherapy agents may offer a limited survival benefit in patients with locally advanced pancreatic cancer^[3,4].

As so few patients with pancreatic cancer are suitable for curative surgery and most have only a limited response to chemotherapy, high intensity focused ultrasound (HIFU) has been recently investigated as a potential additional therapy with the intention of tumor debulking and symptom control. Using an extracorporeal approach, it employs focused ultrasound energy to raise the temperature between 56 °C and 100 °C in a targeted tumor while ultrasound beam is transmitted into a pancreatic lesion, leading to a complete destruction of all the targeted pancreatic cancer cells, instead of local tumor removal^[5]. The main advantages of HIFU therapy are less invasive with no incision, no scarring, cheap, less pain and short recovery time. These result in an associated reduction in mortality, morbidity, hospital stay, cost and improved quality of life for cancer patients. The purpose of this article is to review recent developments in the use of HIFU therapy for pancreatic cancer, and to discuss its potential in this application.

DEFINITION OF HIFU ABLATION

Ultrasound is a form of vibrational wave. It can be brought to a tight focus at a distance from its source while an ultrasound beam propagates harmlessly through living tissues. Just as energy in the sun can be concentrated to a point, and used to set fire to combustible material using a magnifying glass, the power of an ultrasound beam can be focused. If the concentrated energy is sufficient, there may be tissue destruction solely within the focal volume, while cells lying elsewhere remain unharmed.

Ultrasound energy absorption by living tissue can result in measurable temperature rises. For HIFU, the energy is greatest within the focal volume, and thus the temperature is maximal there. The mechanism for cell killing is primarily thermal. The temperature rises rapidly, and is held in excess of 56 °C for 1 s or longer. This causes immediate coagulation necrosis of the targeted volume. The extent of cellular thermal damage is determined both by the temperature achieved, and the length of time for which it is maintained, the higher the temperature, the shorter the time required to produce identical effects. The boundary of the thermally necrosed region, referred to in HIFU as the “lesion” represents the “56 °C for 1 s or longer” contour. Higher temperatures will have been reached at its centre, and in reality, the temperature within the focal volume may rise rapidly above 80 °C during HIFU treatments^[6,7]. A steep temperature gradient exists at the lesion boundary, and therefore a sharp demarcation between the treated and normal extra-focal tissue is only less than the size of 10 cells, which is histologically observed under light microscope^[8,9].

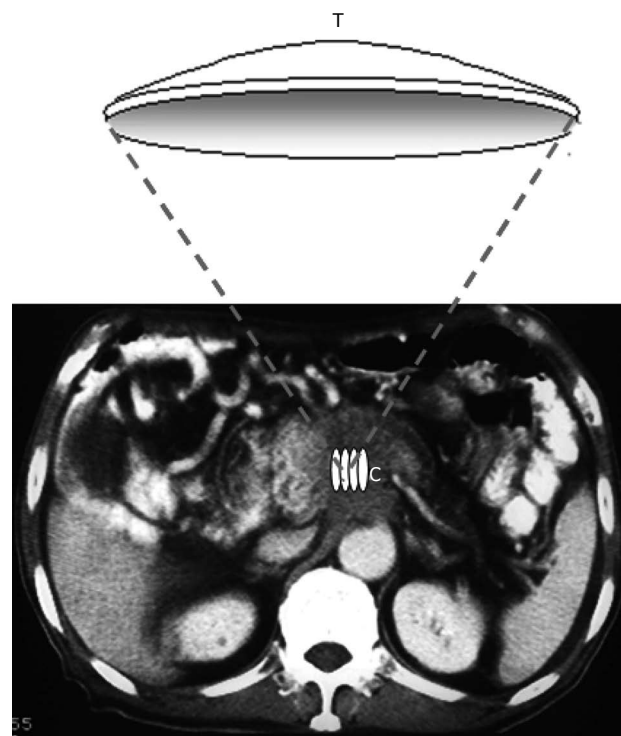


Figure 1 Schematic diagram demonstrating the principle of high intensity focused ultrasound treatment for pancreatic cancer. Ultrasound beam is focused into a small volume in which ultrasound energy is converted into heat to induce the required coagulation necrosis of a targeted pancreatic tumor. T: HIFU transducer; C: The targeted pancreatic cancer.

At high ultrasound intensity levels, not only thermal effects, but those resulting from mechanical mechanisms become important^[10]. The most important non-thermal mechanism for tissue disruption in HIFU fields is acoustic cavitation, which leads to the local destruction of the tissue due to cavitation-induced high pressures and temperatures^[9].

The intention of a HIFU treatment is to deliver ultrasound energy to a well-defined targeted volume at depth, and to induce complete coagulation necrosis of the tumor. A single (1-3 s) HIFU exposure usually produces a very small cigar-shaped lesion of dimensions of 10-20 mm along the beam axis and 1-2 mm in the transverse direction. However, by placing lesions side by side, conformal confluent volumes of ablation of clinically relevant size can be achieved, as shown in Figure 1. It is important that individual lesions overlap in order that no viable tumor cells remains between them. Due to the nature of using a small lesion to cover the large volume of tumor, theoretically there should be no limitation of tumor size, but it will take long and costly treatment times when attempting to ablate a large tumor. For safety reasons, in weaken and old patients HIFU procedure may be divided into two sessions when tumor is too large, and each session ablates the separated part of the targeted tumor. However, as HIFU is guided by either US or MRI, it is unsuitable to treat small tumors (less than 0.5 mm), if they are not clearly detected by both images.

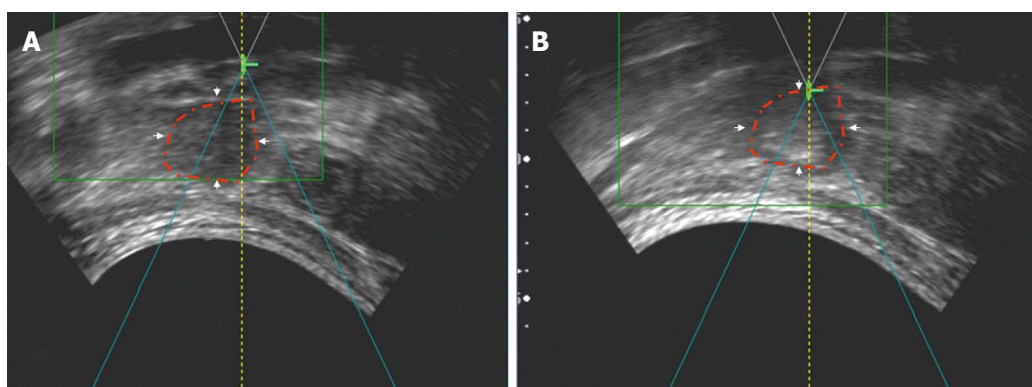


Figure 2 Grey-scale changes in a treated pancreatic cancer on real-time ultrasound images during high intensity focused ultrasound exposure. A: Ultrasound (US) image obtained before high intensity focused ultrasound (HIFU) shows a pancreatic cancer lesion present in the body of the pancreas (arrowheads); B: US image obtained immediately after the one-slice HIFU treatment shows the obvious hyperechogenicity of the treated pancreatic tumor (arrowheads).

DESTRUCTIVE EFFECTS OF HIFU ABLATION

Direct thermal and non-thermal effects

The effects of thermal ablation on a targeted tumor are determined by increased temperatures due to thermal energy deposition, rate of removal of heat, and the specific thermal sensitivity of the tissue. As the tissue temperature rises, the time required to achieve irreversible cellular damage decreases exponentially. At temperatures between 50 °C and 55 °C, cellular death occurs instantaneously in cell culture^[11]. Protein denaturation, membrane rupture, cell shrinkage, pyknosis, and hyperchromasia occur *ex vivo* between 60 °C and 100 °C, leading to almost immediate coagulation necrosis^[12]. In addition, acoustic cavitation, one of the mechanical effects induced by HIFU ablation, is the most important non-thermal mechanism for tissue disruption^[10]. Small gaseous nuclei existing in subcellular organelles and fluid in tissue are the sources of cavitation, which can expand and contract under the influence of the acoustic pressure. During the collapse of bubbles, the acoustic pressure, shear stress, and subsequently high temperature can induce the local destruction of a targeted tissue^[13].

Thermal effects on tumor blood vessels

Structural and functional changes are directly observed in tumor blood vessels after thermal ablation^[14-17]. These changes are not as well described as thermal effects on the tissues, but they rely on varying temperatures. At temperatures between 40 °C and 42 °C, there is no significant change in tumor blood flow after 30-60 min exposure^[18]. Beyond 42 °C to 44 °C, there is an irreversible decrease in tumor blood flow, with vascular stasis and thrombosis, resulting in heat trapping and progressive tissue damage^[19]. When temperatures exceed 60 °C, immediate destruction of tumor microvasculature occurs^[20]. It cuts the blood supply to the tumor directly through the cauterization of the tumor feeder vessels, leading to deprivation of nutrition and oxygen. Thus, tissue destruction can be

enhanced by the damage caused by thermal ablation to tumor blood vessels.

CLINICAL OUTCOMES

Up to now, HIFU has been largely reported as a palliation option to treat patients with locally advanced pancreatic cancer. There are mainly two HIFU commercial devices available to clinical application, and the HIFU-treated patients are almost from Asia. Both devices incorporate B-mode ultrasonography to target and monitor the therapeutic procedure. One is Chongqing HIFU system (Model-JC and JC200 HIFU system, Haifu Medical Technology, Chongqing, China). It is an extracorporeal ultrasound-guided HIFU device, and employs continuous HIFU wave with high intensity (5-20 kW/cm²). The therapeutic regime is a typically thermal ablation, and each patient receives HIFU treatment only once. Treatment time is dependent on the size of a targeted tumor, which ranges from 45 min to 3.2 h. During the procedure, acoustic intensity should gradually increase in the focus until a hyperechogenic change is clearly observed within the targeted lesion on ultrasound imaging (Figure 2). This tissue response is not only a good real-time imaging assessment to determine whether coagulation necrosis could occur during each HIFU shot in the targeted tumor, but also a imaging feedback to control energy delivery of HIFU exposures. Chongqing HIFU device got CE approval in 2005 for the treatment of pancreatic cancer, and now it has been increasingly used for clinical applications in Europe. The other is a FEB-BY Serial HIFU System (China Medical Technologies, Beijing, China). It is also an extracorporeal ultrasound-guided HIFU device, but uses pulsed-wave HIFU with low intensity (< 3 kW/cm²). The therapeutic regime is similar to focused ultrasound hyperthermia treatment. Each patient has separately undergone 4-7 sessions over the course of 10-14 d, and every session lasts about 1-1.5 h. During the procedure, acoustic intensity should drop down if a patient feels abdominal pain or discomfort. The clinical outcomes of the both HIFU devices are summarized in Tables 1 and 2.

Table 1 Studies of continuous-wave high intensity focused ultrasound treatment for patients with advanced pancreatic cancer

Study	n	Patients	Treatment method	HIFU Device	Outcome and survival	Complications
Wu <i>et al</i> ^[21]	8	A phase I - II study of HIFU for advanced pancreatic cancer, unresectable. Average tumor size 5.89 cm (4.5-8 cm)	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 8/8 (100%); Median survival: 11.25 mo (2-17 mo)	None
Orsi <i>et al</i> ^[22]	6	Late-stage pancreatic cancer, unresectable. Average tumor size 4.6 ± 1.4 cm	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 6/6 (100%); Median survival: 7 mo; Overall survival: 42.9% at 12 mo and 21.4% at 24 mo	Portal vein thrombosis: 1/6 (16%)
Wang <i>et al</i> ^[24]	40	Advanced pancreatic cancer, unresectable. Average tumor size 4.3 cm (2-10 cm)	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 35/40 (87.5%); Median survival: 8 mo (stage III: 10 mo; stage IV: 6 mo); Overall survival: 58.8% at 6 mo and 30.1% at 12 mo	None
Sung <i>et al</i> ^[25]	46	Advanced pancreatic cancer, unresectable. Average tumor size 4.2 ± 1.4 cm (1.6-9.3 cm)	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	A significant reduction of pain score ($P < 0.001$); Median survival: 12.4 mo; Overall survival: 52.2% at 6 mo, 30.4% at 12 mo, and 21.79% at 18 mo	Mild abdominal pain: 16/46 (34%); severe abdominal pain with vomiting: 2/46 (4%); transient fever: 3/46 (6%); 2 nd -3 rd skin burns: 2/46 (4%); pancreaticoduodenal fistula: 1/46 (2%); gastric bleeding due to ulcer: 1/46 (2%)
Wang <i>et al</i> ^[26]	224	Advanced Pancreatic cancer	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief and survival data not reported	Abdominal distension, anorexia and nausea: 10/224 (4%); asymptomatic vertebral injury: 2/224 (1%); obstructive jaundice: 1/224 (1%)
Gao <i>et al</i> ^[27]	39	Locally advanced pancreatic cancer, unresectable. Tumor size unclear	One-session HIFU alone: 14 pts; HIFU + gemcitabine: 25 pts	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 31/39 (79.5%); Median survival: 11 mo; Overall survival: 82.1% at 6 mo, and 39.5% at 12 mo	None
Zhao <i>et al</i> ^[28]	37	A phase II study of HIFU + gemcitabine for locally advanced pancreatic cancer, average tumor size 3.4 cm (1.7-8.5 cm).	Gemcitabine on days 1, 8 and 15, and multiple HIFU sessions on days 1, 3 and 5. The combined treatment repeated every 28 d	Continuous HIFU irradiation, HIFUNIT-9000 HIFU System	Overall survival: 12.6 mo (95%CI: 10.2-15.0); Pain relief: 29/37 (78%)	Fever: 26/37(70%); neutropenia: 6/37 (16%); thrombocytopenia 2/37 (5%); nausea and vomiting 3/37 (8%); diarrhea 2/37 (5%)

HIFU: High intensity focused ultrasound; pts: Patients.

Continuous-wave HIFU treatment

The first success of HIFU ablation for advanced pancreatic cancer was conducted in Chongqing China in 2000^[21]. It was a phase I - II prospective clinical trial, and both survival benefit and pain control were observed during follow-up period. Eight patients with locally advanced pancreatic cancer were treated only once with continuous-wave HIFU alone for palliation. The tumor ranged from 4.5 to 8 cm in diameter (mean 5.89 cm), and was mainly located in the body and tail of the pancreas. The results showed that HIFU treatment was safe and feasible, and no complications were recorded. After HIFU, pre-existing severe back pain of presumed malignant origin disappeared in each patient. Follow-up images showed reduction or absence of tumor blood supply in the treated region with significant shrinkage of the ablated tumor, as shown in Figure 3. Of them, 4 patients died (median survival time 11.25 mo, range 2-17 mo), and the remaining 4 patients were still alive with median

follow-up time of 11.5 mo (range 9-16 mo). The authors concluded that HIFU could be safe, effective and feasible in the treatment of patients with advanced pancreatic cancer.

Subsequently, several clinical studies were performed to investigate the safety and feasibility of HIFU for the treatment of patients with advanced-stage pancreatic cancer^[22-25]. They were one-arm phase I - II trials, and clinical results were very encouraging, as shown in Table 1. Orsi *et al*^[22] reported a preliminary experience of using HIFU for 6 patients with unresectable pancreatic cancer. After treatment, either PET/CT or contrast-enhanced MR images showed complete ablation in 5 of 6 patients, and pain relief was observed in all patients. Median survival was 7 mo, and 1- and 2-year survival rates were 42.9% and 21.4% respectively. Local skin burn was not observed, but portal vein thrombosis was detected as a major complication in one patient after treatment. The same group also treated 2 inoperable patients with pancreatic

Table 2 Studies of pulsed-wave high intensity focused ultrasound treatment for patients with advanced pancreatic cancer

Study	n	Patients	Treatment Method	HIFU Device	Outcome and Survival	Complications
Wang <i>et al</i> ^[29]	15	Late-stage pancreatic cancer, unresectable, average tumor size 5.6 cm (2.2-8 cm)	Multiple-session HIFU monotherapy, average sessions 8.1 (2-12)	Pulsed HIFU irradiation, FEB-BY HIFU System	Pain relief: 13/13 (100%) No survival data available	Mild abdominal pain: 2/15 (13%)
Li <i>et al</i> ^[30]	25	Advanced pancreatic cancer, unresectable, average tumor size unclear	One-session HIFU: 19 pts; 2-session HIFU: 6 pts; average sessions 1.2	Pulsed HIFU irradiation, FEB-BY HIFU System	Performance status and pain improvement: 23/25 (92%); median overall survival: 10 mo; 1-year survival: 42%	First-degree skin burn: 3/25 (12%)
Ge <i>et al</i> ^[31]	20	A retrospective study for unresectable pancreatic cancer, average tumor size (4.5 ± 1.2) × (3.5 ± 1.0) cm	Multiple-session HIFU monotherapy; average HIFU session 9.3 ± 4.1	Pulsed HIFU irradiation, FEB-BY HIFU System	Pain relief and survival data not reported	Mild abdominal pain: 5/25 (25%); subcutaneous fat callus: 4/25 (20%); 2nd-degree skin burn: 1/25 (5%); pancreatic effusion: 1/25 (5%)
Xiong <i>et al</i> ^[32]	89	A retrospective study for unresectable pancreatic cancer. Tumor size not reported	Multiple-session HIFU monotherapy: 84 pts; HIFU + gemcitabine: 5 pts; HIFU sessions ranging 4-10	Pulsed HIFU irradiation, FEB-BY HIFU System	Pain relief: 54/67(80%), median survival: 26.0 mo (stage II), 11.2 mo (stage III) and 5.4 mo (stage IV)	Superficial skin burns: 3/89 (3%); subcutaneous fat sclerosis: 8/89 (6%); asymptomatic pseudocyst: 1/89 (1%)
Lee <i>et al</i> ^[33]	12	Advanced pancreatic cancer, unresectable, average tumor size 3.5 cm (2.3-5.3 cm)	Multiple-session HIFU monotherapy: 9 pts; HIFU + gemcitabine: 3 pts; average HIFU sessions: 4.2 (1-18)	Pulsed HIFU irradiation, FEB-BY HIFU System	Median survival for those receiving HIFU alone: 10.3 mo; Overall survival for 3 patients receiving the combined treatment: 26.0, 21.6 and 10.8 mo, respectively	Pancreatitis: 1/12 (8%); skin burn: 5/12 (41%); subcutaneous fat sclerosis: 2/12 (16%)

HIFU: High intensity focused ultrasound; pts: Patients.

neuroendocrine tumor (insulinomas)^[23]. Both patients suffered from episodes of severe nightly hypoglycemia, which was not efficiently controlled by medication. During 9-mo follow-up, local disease control and symptom relief were achieved in them without any complications. Wang *et al*^[24] followed up HIFU-treated 40 patients with advanced pancreatic cancer (stage III, 13 patients; stage IV, 27 patients). Average tumor size was 4.3 cm (range 2-10 cm). After HIFU, pain relief was achieved in 87.5% of the patients. The median overall survival was 8 mo for all patients, including 10 mo in stage II and 6 mo in stage III patients. Six-month and 1-year survival rates were 58.8% and 30.1% respectively. No severe complications were observed during follow-up period.

Sung *et al*^[25] treated 46 patients with advanced pancreatic cancer, including 18 in stage III and 28 in stage IV disease. Average tumor size was 4.2 ± 1.4 cm (range: 1.6-9.3 cm). After HIFU treatment, contrast-enhanced MR images showed 90%-100% ablation in 38 lesions, 50%-90% in 8 and within 50% in 3 lesions. Pain score (visual analog scale) was significantly reduced from 4.9 ± 1.1 to 2.1 ± 1.1 ($P < 0.001$). Overall median survival from initial diagnosis was 12.4 mo. Overall survival rates at 6, 12, and 18 mo from HIFU were 52.2%, 30.4%, and 21.79%, respectively, with a median survival of 7.0 mo. Minor complications (abdominal pain, fever and nausea) was observed in 28 (57.1%) of 49 HIFU treatment. Major complications were detected in 5 (10.2%) of 49 treatment, including 2-3 degree skin burn in 2, pancreaticoduodenal fistula in 2 and gastrointestinal tract

bleeding due to gastric ulcer in one patient. The authors concluded that HIFU was safe and effective, and it could induce excellent local tumor control in most patients with advanced pancreatic cancer.

The largest clinical experience of using HIFU treatment for advanced pancreatic cancer was reported by Wang *et al*^[26]. A total of 224 patients were enrolled in this study for safety analysis of HIFU treatment. Gastrointestinal dysfunction such as abdominal distension and anorexia with slight nausea was observed in 10 cases (4.5%) after HIFU treatment. One case with pancreatic head cancer developed obstructive jaundice 2 wk after HIFU treatment. Vertebral injury, identified by MRI, occurred in 2 cases, although no symptoms were seen. No severe complications were observed in all enrolled patients. These results indicated that HIFU was a safe, non-invasive treatment. However, no long-term follow-up and survival data were reported in this study.

HIFU combined with chemotherapy was also used to treat advanced pancreatic cancer. Gao *et al*^[27] reported an initial use of HIFU alone or HIFU plus gemcitabine for the treatment of 39 patients with locally advanced pancreatic cancer. Among them, 14 patients received one-session HIFU monotherapy, and the remaining 25 patients underwent HIFU combined gemcitabine therapy. After treatment, no severe complications were observed, and pain relief was achieved in 31 (79.5%) of 39 patients who had previous pain. Median overall survival was 11.0 mo, and 6- and 12-mo survival rates for all patients were 82.1% and 39.5% respectively. However, median survival

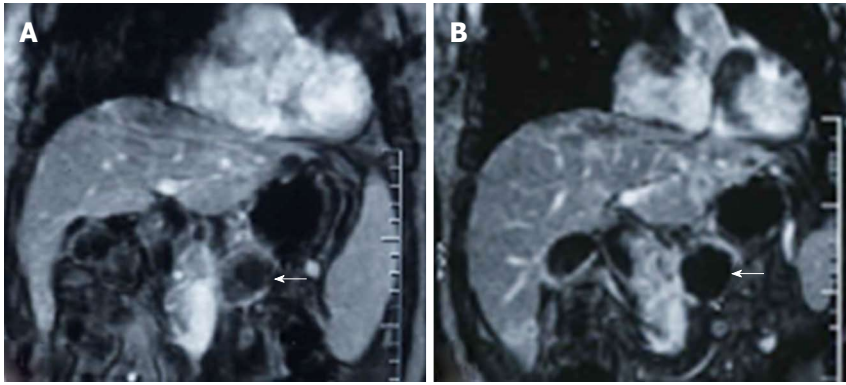


Figure 3 Contrast-enhanced T-weighted MR images obtained in a patient treated with high intensity focused ultrasound for advanced pancreatic cancer. The tumor was 4.5 cm in diameter and located in the body of the pancreas. A: Image obtained before high intensity focused ultrasound (HIFU) shows blood supply in the pancreatic lesion (arrows); B: Image obtained 2 wk after HIFU shows no evidence of contrast enhancement in the treated lesion (arrows), which is indicative of complete coagulation necrosis in the treated pancreatic cancer.

and 1-year survival were significantly higher in patients treated with HIFU plus chemotherapy while compared with those in patients treated with HIFU alone. There were statistical differences between two groups ($P < 0.01$). Zhao *et al*^[28] also reported a phase II trial investigating the safety and efficacy of concurrent gemcitabine and HIFU for the treatment of 37 patients with locally advanced pancreatic cancer. The average tumor size was 3.4 cm (range 1.7-8.5 cm). All patients received gemcitabine 1000 mg/m² on days 1, 8, and 15, and concurrent HIFU treatment (HIFUNIT-9000, AS Sci-Tech, Shanghai, China) on days 1, 3, and 5. The combined treatment regime was repeated every 28 d and continued until disease progression, patient refusal, or an unacceptable toxicity. The results showed that overall survival was 12.6 mo (95%CI: 10.2-15.0), and the estimates of overall survival at 12 and 24 mo were 50.6% (95%CI: 36.7%-64.5%) and 17.1% (95%CI: 5.9%-28.3%), respectively. Pain was relieved in 22 (78.6%) of 28 patients who had complained of abdominal pain consistent with tumor-related pain. After treatment, grade 1 or 2 fever was detected in 70.3% of patients. Six patients (16.2%) experienced grade 3/4 neutropenia, and 2 (5.4%) had grade 3 thrombocytopenia was documented. Grade 3 nausea/vomiting and diarrhea were observed in 3 (8.1%), and 2 (5.4%) patients respectively. The authors concluded that concurrent gemcitabine and HIFU was a tolerated treatment modality with promising activity in patients with previously untreated locally advanced pancreatic cancer.

Pulsed-wave HIFU treatment

Compared to continuous-wave HIFU treatment, pulsed HIFU usually uses low energy with a multiple-session treatment regime. The first study of pulsed HIFU for advanced pancreatic cancer was reported by Wang *et al*^[29] in 2002, and 15 patients received multiple-session pulsed HIFU treatment for the purpose of palliation. HIFU session ranged from 2 to 12 (average 8.1). The average tumor size was 5.6 cm (range 2.2-8 cm). Seven patients had a lesion located in the head of the pancreas, including 4 who had previously received gallbladder-intestine bypass

operation. The remaining 8 patients had carcinoma of the body and tail of the pancreas. After HIFU, pain relief was observed in 13 (100%) of 13 patients who had previously cancer-related pain. Tumor size shrank in 3 patients while the other 12 patients had no change. Unfortunately, there were no survival benefit data available in this study. Mild abdominal pain was recorded as a complication in 2 of 15 patients.

Li *et al*^[30] reported a clinical result of pulsed HIFU for the treatment of 25 patients with unresectable pancreatic cancer. Of them, 19 patient received one-session HIFU, and the remaining 6 had two session treatments. The treatment time was less than 60 min in each session. After HIFU treatment, 3 patients had first degree skin burn, but they recovered without any medication. Performance statue and pain improvement were observed in 23 (92%) of 25 patients during follow-up period. Overall average survival time was 10 mo, and 1-year survival rate was 42% for all patients. Ge *et al*^[31] analyzed clinical results of HIFU treatment for advanced pancreatic cancer in a retrospective study. Twenty patients received multiple-session HIFU treatment, and the average number of HIFU sessions was 9.3 ± 4.1 for each patient. After treatment, mild abdominal pain was observed in 5 (25%) patients, and subcutaneous fat callus was found in 4 (20%) of 25 patients. One patient experienced 2nd-degree skin burn, and pancreatic effusion was also detected in 1 patient. However, no pain relief and survival data were reported in this study.

Xiong *et al*^[32] reported the largest retrospective study of using pulsed HIFU treatment for advanced pancreatic cancer. Eighty-nine patients with pancreatic cancer were analyzed after HIFU, including 4 in stage II, 39 in stage III, and 46 in stage IV disease. Tumors were located in the pancreatic head in 34 patients (38.2%), and in the body and/or tail of the pancreas in 55 patients (61.8%), although tumor size was unclear. In order to treat an entire volume of the tumor, 4-10 HIFU sessions were needed for each patient. After treatment, pain relief was achieved in 54 (80.6%) of 67 patients who had pain prior to HIFU. The median survival was 26.0 mo in stage II patients,

11.2 mo in stage III, and 5.4 mo in stage IV patients. Complications included superficial skin burns (3.4%), subcutaneous fat sclerosis (6.7%), and an asymptomatic pancreatic pseudocyst (1.1%). The authors concluded that although this retrospective study had significant limitations, preliminary results suggested that the clinical application of HIFU for pancreatic cancer appeared to be safe and was a promising modality of treatment for palliation of pain related to pancreatic cancer.

Similar to continuous HIFU treatment, pulsed HIFU combined with chemotherapy were also used to treat advanced pancreatic cancer. Lee *et al*^[33] reported initial experience of using pulsed HIFU for the treatment of 12 patients with unresectable pancreatic cancer, including 9 treated with HIFU alone, and 3 treated with pulsed HIFU combined with gemcitabine. Median tumor size was 3.5 cm (range: 2.3-5.3 cm), and HIFU sessions ranged from 1 to 18 (average 4.8 sessions). After HIFU treatment, skin burn was observed in 5 patients including 1 in 2nd-degree and 4 in 1st-degree skin burn. Subcutaneous fat sclerosis caused by thermal injury was detected in 2 patients, and one patient developed acute pancreatitis with a large pseudocyst after treatment. The median survival for those receiving HIFU treatment alone was 10.3 mo. However, the overall survival of three patients treated by HIFU combined with gemcitabine was 26.0, 21.6 and 10.8 mo, respectively, suggesting that concurrent pulsed HIFU and chemotherapy could be potentially more effective in the treatment of unresectable pancreatic cancer.

DISCUSSION AND CONCLUSION

HIFU is an attractive emerging therapy for unresectable pancreatic cancer. It has been offered as a palliation option for improving the quality of life in patients with advanced-stage pancreatic cancer. Almost all studies have been conducted for the assessment of technical safety and feasibility, and clinical outcome have showed that HIFU therapy is safe and reproducible.

Many of early concerns that surrounded the safety of HIFU treatment for pancreatic cancer have been addressed in the pilot studies. As shown in the Tables 1 and 2, the incidence of complications directly caused by HIFU is relatively lower while compared with radiation therapy and minimally-invasive thermal ablation approaches. Mild complications include abdominal pain, nausea and vomiting, skin burn, and subcutaneous fat sclerosis. They usually occur in 3%-20% patients, and recover in a short time after HIFU treatment, without any medication. Severe complications are observed in 3 patients, including 1 case with portal vein thrombosis, 1 with pancreaticoduodenal fistula, and 1 with obstructive jaundice. Two patients experience pancreatitis with a large pseudocyst around the inflammation site, and 1 patient has gastrointestinal bleeding due to gastric ulcer after treatment. These demonstrate that HIFU is a promising approach with a few adverse effects for the treatment of unresectable pancreatic cancer. However, contraindications

should be considered if a targeted lesion is too close to the duodenum and bile duct. It can extremely increase the risk of bowel perforation and bile leakage because of HIFU damage on these normal structures. Unfortunately, there is no exact safe distance between the tumor and adjacent vital structures available to HIFU treatment currently, and further studies are needed in animal models to define it.

Most clinical results to date are obtained in retrospective studies, and there are a few phase II prospective clinical trials performed in research settings for assessment of HIFU efficacy. These studies have shown that HIFU can significantly improve the quality of life in patients with advanced-stage pancreatic cancer. Pain relief is obviously observed in 78%-100% patients after treatment. Median survival time ranges from 7 to 12 mo, which is dependent on the TNM stage of disease. Case reports reveal that while HIFU is combined with chemotherapy (gemcitabine), median survival and overall survival rate seem better than HIFU alone, but this claim needs to be confirmed in randomized, two-arm clinical trials. In addition, almost all studies uses symptom relief, survival and MRI/CT changes as evidences of assessing treatment effects on pancreatic cancer, instead of histomorphological examination following HIFU treatment. Further studies are needed to investigate the characteristics of histological changes in pancreatic cancer after HIFU treatment.

Two various regimes of therapeutic strategy have been noticed in HIFU treatment. One is continuous HIFU, and the other is pulsed HIFU treatment. They are totally different in both technical parameter and therapeutic strategy, as shown in Table 3. Using high intensities ranging from 5 to 20 kW/cm², each continuous HIFU shot can induce coagulation necrosis of a targeted tumor. It is a one-session treatment, and can be used alone for the treatment of unresectable pancreatic cancer. There is no need to be repeated if the tumor is significantly ablated. In addition, the appearance of a hyperechoic region of in the focus is clearly observed on ultrasound imaging immediately after each shot, as shown in Figure 2. Either sedation or general anesthesia is required for patients during treatment procedure due to discomfort and pain. After treatment, the patients require hospitalization for several days.

In contrast, pulsed HIFU uses lower ultrasound intensities, which is usually less than 3 kW/cm². It is a multiple-session treatment, and needs to be repeated for many times ranging from 5 to 10 sessions if the patients are suitable. Some patients require sedation during treatment procedure, but most of them don't need it if there is no pain or discomfort. It is a one-day procedure, and there is no need for patients to stay in hospital after treatment. Recent studies have indicated that pulsed HIFU can significantly enhance chemotherapeutic agents against tumor cells^[33-36], suggesting that pulsed HIFU may be a treatment approach using focused ultrasound for hyperthermia, instead of HIFU for inducing coagulation necrosis. Actually, focused ultrasound hyperthermia has

Table 3 Technical and therapeutic differences in continuous high intensity focused ultrasound and pulsed focused ultrasound therapy

	US wave	US intensity	Treatment session	Change in US image during procedure	Treatment mechanism	Treatment use	Required anesthesia	Appearances in follow-up images
Continuous HIFU therapy	Continuous	5-20kW/cm ²	One session	Real-time hyperechoic change in the focus during each shot	Thermal ablation for coagulation necrosis	Monotherapy, used alone	Sedation or general anesthesia	No contrast enhancement in tumor on MRI/CT; negative uptake on PET/CT
Pulsed focused ultrasound therapy	Pulsed	< 3 kW/cm ²	Multiple sessions	No real-time US image change during each shot	Hyperthermia for enhancing sensitive to chemotherapeutic agents	Need to be combined with chemotherapy	Sedation or none	Tumor shrinkage on MRI/CT; negative uptake on PET/CT

HIFU: High intensity focused ultrasound; US: Ultrasound.

been used as adjuvant to radiotherapy and chemotherapy for cancer treatment in the 1990s^[37,38]. It can raise the temperature of the tumor from 37 °C to 42-45 °C for 60 min. This may make some cancer cells more sensitive to radiation and chemotherapy, or harm other cancer cells that both therapies cannot damage^[39,40]. It is obvious that focused ultrasound hyperthermia uses lower acoustic energy to heat tumor, and there is no coagulation necrosis that occurs in the treated tumor while compared with HIFU treatment. However, HIFU is a therapeutic approach to locally heat and destroy diseased tissues through thermal ablation. In order avoid any confusion related to the definition of HIFU and hyperthermia, it is highly recommended to use pulsed focused ultrasound hyperthermia rather than pulsed HIFU treatment in the future.

In conclusion, HIFU ablation has been shown a promising approach for the palliative treatment of advanced pancreatic cancer. The nature of non-invasiveness and highly treatment precision has made HIFU become more attractive emerging therapy. It has much potential for further clinical investigation and technical improvements. Currently, preliminary studies suggest that this approach is technical safe and feasible, and can be used alone or in combination with systemic chemotherapy. It can effectively alleviate cancer-related abdominal pain, and may confer an additional survival benefit with few significant complications. However, large, prospective, multi-center randomized clinical trials will be needed to assess the long-term efficacy, and determine the future role of this technique for the treatment of locally advanced pancreatic cancer. Once oncologic efficacy data from those trials are available, HIFU ablation will become an attractive treatment option for patients with pancreatic cancer.

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WJG 20th Anniversary Special Issues (17): Intestinal microbiota

Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs

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Abstract

The intestinal microbiota is the collection of the living microorganisms (bacteria, fungi, protozoa, and viruses) inhabiting the gastrointestinal tract. Novel bacterial identification approaches have revealed that the gastrointestinal microbiota of dogs and cats is, similarly to humans, a highly complex ecosystem. Studies in dogs and cats have demonstrated that acute and chronic gastrointestinal diseases, including inflammatory bowel disease (IBD), are associated with alterations in the small intestinal and fecal microbial communities. Of interest is that these alterations are generally similar to the dysbiosis observed in humans with IBD or animal models of intestinal inflammation, suggesting that microbial responses to inflammatory conditions of the gut are conserved across mammalian host types. Studies have also revealed possible underlying susceptibilities in the innate immune system of dogs and cats with IBD, which further demonstrate the intricate relationship between gut microbiota and host health. Commonly identified microbiome changes in IBD are decreases in bacterial groups within the phyla *Firmicutes* and *Bacte-*

roidetes, and increases within *Proteobacteria*. Furthermore, a reduction in the diversity of *Clostridium* clusters XIVa and IV (*i.e.*, *Lachnospiraceae* and *Clostridium coccooides* subgroups) are associated with IBD, suggesting that these bacterial groups may play an important role in maintenance of gastrointestinal health. Future studies are warranted to evaluate the functional changes associated with intestinal dysbiosis in dogs and cats.

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Key words: Microbiome; 16S rRNA; Inflammatory bowel disease; Probiotic; Dog; Cat

Core tip: Several studies in dogs and cats have demonstrated that acute and chronic gastrointestinal diseases, including inflammatory bowel disease (IBD), are associated with alterations in the small intestinal and fecal microbial communities. Of interest is that these alterations are generally similar to the dysbiosis observed in humans with IBD or animal models of intestinal inflammation, suggesting that microbial responses in inflammatory conditions of the gut are conserved across mammalian host types, and dogs and cats may serve as models to study therapeutic approaches to spontaneous inflammatory conditions of the gastrointestinal tract.

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INTRODUCTION

The intestinal microbiota is the collection of the living

microorganisms (bacteria, fungi, protozoa, and viruses) inhabiting the gastrointestinal (GI) tract. Novel bacterial identification approaches have revealed that the gastrointestinal microbiota of dogs and cats is, similarly to humans, a highly complex ecosystem, comprising at least several hundred different bacterial phylotypes^[1-3]. It has been suggested that the intestine of mammals is home to a total of 10^{10} - 10^{14} microbial cells, which is approximately 10 times more than the number of host cells. This complex microbial ecosystem and its interplay with eukaryotic host cells have a significant impact on health and disease of dogs and cats. The stimulation of the host immune system and the microbial metabolites produced by the resident microbiome are thought to be one of the most important driving forces behind the coevolution of gastrointestinal microbiota with their host. Gut microbes aid the host by acting as a defending barrier against enteropathogens. They also aid in digestion of complex fiber sources and produce various short-chain fatty acids and other metabolites that provide nutritional support for enterocytes, and which play an important role in the development and regulation of the host immune system^[4,5].

Several studies in dogs and cats have demonstrated that acute and chronic gastrointestinal diseases, including inflammatory bowel disease (IBD), are associated with alterations in small intestinal and fecal microbial communities^[6-14]. Of interest is that these alterations are generally similar to the dysbiosis observed in humans with IBD or animal models of intestinal inflammation^[15-20], suggesting that microbial responses in inflammatory conditions of the gut are conserved across mammalian host types, and dogs and cats may serve as models to study therapeutic approaches to spontaneous inflammatory conditions of the gastrointestinal tract. Recent data support this model, as it has been shown that for example probiotic products (*i.e.*, VSL#3 strains) show similar clinical benefits in dogs with IBD as have been previously demonstrated in humans^[21].

Studies have also revealed possible underlying susceptibilities in the innate immune system of dogs and cats with IBD, which further demonstrates the intricate relationship between gut microbiota and host health^[10,22-25]. Currently, a major hurdle for a more detailed understanding of host-microbe interactions in dogs and cats is the fact that to date most studies evaluating microbiota in GI diseases have examined only a single time point or have evaluated only a small number of diseased animals.

Yet the possibility to alter the microbiome holds promise as a therapeutic mean in veterinary medicine, and recent studies would confirm that direct or indirect manipulations of the intestinal microbiome *via* antibiotics, diet and/or probiotics may have beneficial effects in gastrointestinal diseases of dogs and cats^[21,26-29].

INTESTINAL MICROBIOTA IN HEALTHY DOGS AND CATS

Various studies have evaluated the bacterial communities

in healthy dogs and cats using either traditional bacterial culture or novel next-generation sequencing approaches. Based on traditional bacterial culture, the small intestine of dogs and cats harbors generally low bacterial counts, ranging between 10^2 to 10^5 cfu/g of small intestinal content; however, some studies have identified much higher counts in healthy dogs and cats with up to 10^9 cfu/g^[30,31]. Cats appear to have higher counts of anaerobic bacteria compared to dogs in the proximal small intestine^[31]. The total bacterial count in the colon ranges between approximately 10^9 and 10^{11} cfu/g and the most abundant cultivable groups are *Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, and *Enterobacteriaceae*^[32,33]. Next-generation sequencing studies of the 16S rRNA gene have described the canine and feline microbiome, which on higher phylogenetic levels resembles the microbiome of humans and other mammals. On average, 10 different bacterial phyla have been identified in the feline and canine gut, with *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* making up the vast majority of all gut microbes^[1,3,11,34-36]. Minor abundant members are the phyla *Tenericutes*, *Verrucomicrobia*, *Cyanobacteria*, and *Chloroflexi*. The *Firmicutes* contain various sequences affiliated with *Clostridium* cluster IV and *Clostridium* cluster XIVa and these are together with *Bacteroides* or *Prevotella* the predominant bacterial groups in fecal samples^[3,35,37]. *Helicobacter* are the predominant group in the stomach (> 90% of sequencing reads)^[38], while the duodenum is home to *Enterobacteriaceae*, *Clostridiales*, *Bacteroidales*, and *Lactobacillales*^[36].

The canine^[39] and feline^[40,41] fecal metagenomes (*i.e.*, shotgun sequencing of genomic DNA) have also been studied. This approach yields information regarding microbial genes present in a sample, and allows assessment of the functional capabilities of the microbiota, summarized in Figure 1. Despite variation in the microbial populations of cats and dogs, the functional capabilities are noted to be highly conserved.

More detailed overviews about the canine and feline microbiota in healthy animals have been reported previously^[42-45].

MICROBIOME IN GASTROINTESTINAL DISEASES OF DOGS AND CATS

In recent years, the GI microbiota has garnered strong interest due to the potential etiopathologic role in host health and disease. Many studies in humans and animal models have suggested that various GI disorders are associated with alterations of the GI microbiota. While specific enteropathogens have been recognized in cats and dogs (*i.e.*, *Campylobacter jejuni*, *Clostridium difficile*, *Clostridium perfringens*, and *Salmonella*), most of them are found in similar frequency across healthy animals. Therefore, their cause-effect relations remain unclear^[46,47]. It is now well recognized that more broad changes in the intestinal microbiome are associated with acute and chronic GI disease. Examples of recent studies in companion ani-

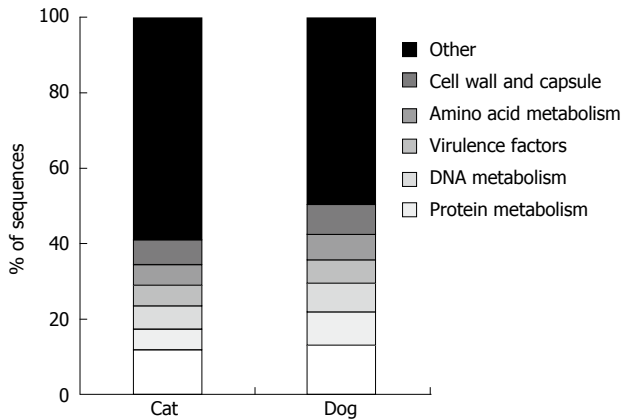


Figure 1 Relative proportion of major microbial gene functions in cats and dogs. Metagenomic data adapted from Swanson *et al.*^[39], Barry *et al.*^[40], and Tun *et al.*^[41].

imals and their findings are summarized in Table 1. The cause-effect relationships of the alterations are still being elucidated, but especially in chronic enteropathies such as IBD there is now strong evidence that the gut microbiota plays an important part in the pathogenesis of the disease. Studies in humans have shown an association between IBD and microbial dysbiosis in the intestine. In these studies, a decrease in the bacterial phyla *Firmicutes* and *Bacteroidetes*, and an increase in *Proteobacteria* and *Actinobacteria* were associated with IBD^[16]. Furthermore, a reduction in the diversity of *Clostridium* clusters XIVa and IV (*i.e.*, *Lachnospiraceae* and *C. coccoides* subgroups) are associated with IBD, suggesting that this bacterial group may play an important role in maintenance of gastrointestinal health, possibly due to production of short chain fatty acids (SCFA). Similar studies have now been reported in dogs and cats with IBD, and a comparison of the observed microbial shifts for humans, dogs, and cats with IBD is provided in Table 2.

Microbiota alterations in canine and feline IBD

In veterinary medicine, chronic enteropathies with intestinal inflammation are commonly seen in dogs and cats. The response to treatment is used to allow for distinction of different types of enteropathies, such as food-responsive diarrhea, antibiotic-responsive diarrhea, and steroid-responsive diarrhea. Idiopathic IBD is a subgroup of enteropathies and it is defined as an inflammation of the GI tract with persistent or recurrent GI signs due to unknown cause^[48]. To diagnose IBD, known causes for GI inflammation need to be excluded. Therefore, empirical treatments are applied sequentially, starting with a dietary trial, followed by antibiotic therapy if there is a lack of response to diet, and finally, treatment with anti-inflammatory drugs, if response to previous treatments was inadequate. Similarly to human IBD, the exact pathogenesis of canine/feline IBD is unknown, but is suspected to be the result of an abnormal interplay between an altered intestinal microbiota, an underlying genetic susceptibility of the host, and dietary and/or en-

vironmental factors^[48]. Consequently, several studies have revealed possible underlying susceptibilities in the innate immune system of dogs and cats with chronic GI inflammation. These include altered differential expression of Toll-like receptors (TLR)-2 and 4^[25,49], single nucleotide polymorphisms that lead to hyper-responsiveness of TLR-5 to flagellin in German Shepherd dogs (GSDs)^[22], and decreased expression of CD11c(+) cells in dogs with IBD^[50]. There is also well known anecdotal evidence that certain breeds are more prone to chronic GI inflammation. In addition to GSDs, which have been shown to possess polymorphisms in the TLR-4 and TLR-5 genes that are significantly associated with IBD^[51], other dog breeds such as Rottweiler, Border Collie, Boxer dog, and Weimaraner have been shown to possess increased risks for developing IBD^[23]. Of those breeds, breed specific studies evaluating the association between mucosa-adherent microbiota and intestinal inflammation were performed only in GSDs and Boxer dogs. In GSDs with chronic intestinal inflammation, the mucosa-adherent microbiota were analyzed in small intestinal brush samples and showed a significant over-representation of *Bacilli* and *Erysipelotrichi* when compared to healthy Greyhound dogs^[6]. Interestingly, this is somewhat different to the results observed in other studies where more diverse populations of dogs with chronic intestinal inflammation were evaluated. In these studies, the most frequently observed changes in the mucosa-adherent microbiota in the small intestine were increases in members of the *Proteobacteria*, especially *Escherichia coli*-like organisms^[9] or *Pseudomonas*^[8], with concurrent decreases of members of *Firmicutes* and *Bacteroidetes*. In a more recent study evaluating mucosa-adherent microbiota in the duodenum of dogs with IBD by next-generation sequencing, the proportions of *Fusobacteria*, *Bacteroidaceae*, *Prevotellaceae*, and *Clostridiales* were significantly increased in healthy dogs. In contrast, specific bacterial genera within *Proteobacteria*, including *Diaphorobacter* and *Acinetobacter*, were either more abundant or more frequently identified in dogs with IBD^[7]. One study evaluated specifically the presence of *Mycobacterium avium* subspecies *paratuberculosis* in duodenal biopsies of dogs with IBD or intestinal neoplasia by qPCR and reported that 19% of diseased dogs were PCR positive for this organism^[52]. Less published information is available about the mucosa-adherent microbiota of cats with IBD. While sequencing methods have not yet been reported for the characterization of feline IBD, a study using fluorescent *in situ* hybridization (FISH) has revealed an increase in *Enterobacteriaceae* in duodenal biopsies of cats with IBD^[10]. Furthermore, a relationship between increased bacterial numbers and the severity of histological inflammation was observed^[10].

Several studies have evaluated the fecal microbiota in dogs and cats with chronic GI disease. In one study, cats with IBD had lower FISH counts for total bacteria, *Bacteroides* spp., and *Bifidobacterium* spp., but higher counts of *Desulfovibrio* spp. compared to healthy cats^[53]. *Desulfovibrio* spp. are a sulfate-reducing bacterial group and able to

Table 1 Reported microbial shifts in dogs and cats with gastrointestinal disease

Ref.	Species	Sampling location	Animal (sample size)	Method	Microbial changes in diseased animals
Suchodolski <i>et al</i> ^[1] , 2012	Dog	Duodenal biopsies	IBD (<i>n</i> = 14) HC (<i>n</i> = 6)	454-pyrosequencing (16S rRNA gene)	Increase in <i>Proteobacteria</i> (<i>Diaphorobacter</i> , <i>Acinetobacter</i>) Reduction in <i>Fusobacteria</i> , <i>Bacteroidaceae</i> , <i>Prevotellaceae</i> , <i>Clostridiales</i>
Suchodolski <i>et al</i> ^[1] , 2010	Dog	Duodenal biopsies	IBD (<i>n</i> = 7) HC (<i>n</i> = 7)	Gene clone libraries (16S rRNA gene)	Increase in <i>Proteobacteria</i> Decrease in <i>Clostridia</i>
Allenspach <i>et al</i> ^[6] , 2010	Dog	Duodenal brushings	Chronic enteropathies (<i>n</i> = 13) HC (<i>n</i> = 8)	Gene clone libraries (16S rRNA gene)	Increase in <i>Actinobacteria</i> , <i>Lactobacillales</i> , <i>Erysipelotrichales</i>
Xenoulis <i>et al</i> ^[9] , 2008	Dog	Duodenal brushings	IBD (<i>n</i> = 10) HC (<i>n</i> = 9)	Gene clone libraries (16S rRNA gene)	Increase in <i>Enterobacteriaceae</i> (<i>E. coli</i>); Reduction in biodiversity
Suchodolski <i>et al</i> ^[36] , 2008	Dog	Duodenal brushings	Chronic enteropathies (<i>n</i> = 71) HC (<i>n</i> = 64)	Gene clone libraries (fungal ITS gene)	No significant differences in fungal communities
Glanemann <i>et al</i> ^[52] , 2008	Dog	Stomach, duodenum, Colon biopsies	Chronic GI disease (<i>n</i> = 42) HC (<i>n</i> = 14)	PCR	Presence of <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> Detected in 8/42 (19%) of dogs with chronic GI disease
Manchester <i>et al</i> ^[59] , 2013	Dog	Colon biopsies	Granulomatous colitis (<i>n</i> = 6)	FISH	Presence of invasive <i>E. coli</i>
Simpson <i>et al</i> ^[58] , 2006	Dog	Colon biopsies	Granulomatous colitis (<i>n</i> = 13) HC (<i>n</i> = 38)	FISH	Intracellular translocation of adherent and invasive <i>E. coli</i>
Rossi <i>et al</i> ^[21] , 2014	Dog	Fecal samples	IBD (<i>n</i> = 20) HC (<i>n</i> = 10)	qPCR (16S rRNA gene)	Decreased in <i>Faecalibacterium</i> spp. And <i>Turicibacter</i> spp.
Foster <i>et al</i> ^[62] , 2013	Dog	Fecal samples	Acute diarrhea (<i>n</i> = 7) HC (<i>n</i> = 12)	454-pyrosequencing (18S rRNA gene)	No significant differences in fungal communities
Suchodolski <i>et al</i> ^[14] , 2012	Dog	Fecal samples	IBD (<i>n</i> = 19) AHD (<i>n</i> = 13) NHD (<i>n</i> = 12) HC (<i>n</i> = 32)	454-pyrosequencing (16S rRNA gene) qPCR (16S rRNA gene)	AHD: most profound alterations in their microbiome Increase in <i>Sutterella</i> , <i>Clostridium perfringens</i> Decrease in <i>Blautia</i> , <i>Ruminococcaceae</i> , <i>Turicibacter</i> IBD: Decrease in <i>Faecalibacterium</i> spp., <i>Fusobacteria</i>
Markel <i>et al</i> ^[55] , 2012	Dog	Fecal samples	Chronic enteropathies (<i>n</i> = 87) AHD (<i>n</i> = 48) HC (<i>n</i> = 180)	qPCR (16S rRNA gene)	Decrease in <i>Faecalibacterium</i> spp., <i>Turicibacter</i> spp., <i>Ruminococcaceae</i> Increase in <i>C. perfringens</i> and <i>E. coli</i>
Jia <i>et al</i> ^[65] , 2010	Dog	Fecal samples	Chronic diarrhea (<i>n</i> = 9) HC (<i>n</i> = 8)	FISH	Increase in <i>Bacteroides</i>
Glanemann <i>et al</i> ^[52] , 2008	Dog	Fecal samples	Diarrhea (<i>n</i> = 4) HC (<i>n</i> = 9)	T-RFLP	Increase in <i>C. perfringens</i> , <i>E. faecalis</i> , and <i>E. faecium</i>
Ghosh <i>et al</i> ^[13] , 2013	Cat	Ileum full-thick biopsies	Severe systemic ill (<i>n</i> = 50) HC (<i>n</i> = 50)	FISH PCR	Increase in <i>E. faecalis</i> Attachment of <i>E. coli</i> to intestinal epithelial cell
Janeczko <i>et al</i> ^[10] , 2008	Cat	Small intestine biopsies	IBD (<i>n</i> = 17) HC (<i>n</i> = 10)	FISH	Increase in <i>Enterobacteriaceae</i>
Abecia <i>et al</i> ^[54] , 2010	Cat	Fecal samples	IBD (<i>n</i> = 8) HC (<i>n</i> = 10)	FISH	No significant differences in specific bacterial population
Inness <i>et al</i> ^[53] , 2007	Cat	Fecal samples	IBD (<i>n</i> = 11) HC (<i>n</i> = 34)	FISH	Decreased total bacteria, <i>Bifidobacterium</i> spp. and <i>Bacteroides</i> Increase in <i>Desulfovibrio</i>

IBD: Inflammatory bowel disease; HC: Healthy control; AHD: Acute hemorrhagic diarrhea; NHD: Non-hemorrhagic diarrhea; FISH: Fluorescence *in situ* hybridization; T-RFLP: Terminal restriction fragment polymorphism; qPCR: Quantitative polymerase chain reaction.

produce hydrogen sulfides, which may be associated with the pathogenesis of feline IBD. However, another study did not identify significant differences in FISH counts

between cats with IBD and controls, although the same bacterial groups were targeted^[54]. A recent study utilized 454-pyrosequencing of 16S rRNA genes to describe

Table 2 Comparison of reported microbial shifts in inflammatory bowel disease relative to healthy subjects across species

Organism	Human (Crohn's disease)	Human (ulcerative colitis)	Dog	Cat
Firmicutes	Decreased ^{[15,18,56]1,2,4,5}	Decreased ^{[15,18]1,2,4}	Decreased ^{[7]1,6}	
Class Clostridia			Decreased ^{[7,8,14]1,2,4,6,7}	
Family Ruminococcaceae (Clostridial cluster IV)	Decreased ^{[15]2,4}	Decreased ^{[15]2,4}	Decreased ^{[7,8,14]1,2,4,6,7}	
Family Lachnospiraceae (Clostridial cluster XIVa)	Decreased ^{[15,18]1,2,4,5}	Decreased ^{[15,18]1,2,4,5}	Decreased ^{[7,8]1,6,7}	
Bacteroidetes	Decreased ^{[18]1,4}	Decreased ^{[18]1,4}	Decreased ^{[7,9]1,6,7} ; increased ^{[8]1,7}	
Genus Bacteroides			Decreased ^{[7]1,6}	Decreased ^{[53]1,5}
Fusobacteria			Decreased ^{[7,8]1,6,7}	
Proteobacteria	Increased ^{[18]1,4}	Increased ^{[18]1,4}	Increased ^{[7-9]1,6,7}	Increased ^{[10]1,5}
Family Enterobacteriaceae	Unchanged ^{[18]1,4} ; increased ^{[20]2,5}	Unchanged ^{[18]1,4} ; decreased ^{[20]2,5}	Increased ^{[7-9]1,6,7}	Increased ^{[10]1,5}
<i>E. coli</i>	Unchanged ^{[15]2,4} ; Increased ^{[19]1,4,5}		Increased ^{[8]1,7}	
Adherent-Invasive <i>E. coli</i>	Increased ^{[19]1,5}		Increased ^{[58]1,3,5}	
Actinobacteria	Increased ^{[18]1,4}	Increased ^{[18]1,4}	Increased ^{[7]1,6}	
<i>Mycobacterium avium</i> subspecies <i>pseudotuberculosis</i>	Controversial ^{[18]1}		Increased ^{[52]1,4}	
Genus Bifidobacterium	Decreased ^{[15]2,4}	Decreased ^{[15]2,4}		Decreased ^{[53]1,5}

¹Based on mucosal samples; ²Based on fecal samples; ³Granulomatous colitis in Boxer dogs and French bulldogs; ⁴Quantitative polymerase chain reaction;

⁵Fluorescent *in situ* hybridization; ⁶454-Pyrosequencing; ⁷Gene clone libraries. *E. coli*: *Escherichia coli*.

changes in fecal microbiota in cats with chronic diarrhea and their response to dietary modifications^[29]. Several bacterial groups correlated with improved fecal scores after therapeutic response to diet. Those included *Slackia* spp., *Campylobacter upsaliensis*, *Enterobacteriaceae* *Raoultella* spp., *Collinsella* spp., and unidentified genera within *Clostridiales* and *Lachnospiraceae*^[29].

More data about the fecal microbiota are available in dogs. In one study, fecal samples from healthy dogs, dogs with acute non-hemorrhagic diarrhea, dogs with acute hemorrhagic diarrhea, and dogs with active or therapeutically controlled idiopathic IBD were analyzed by sequencing of the 16S rRNA gene^[14]. Dogs with acute diarrhea, especially those with acute hemorrhagic diarrhea, had the most profound changes in bacterial groups in their microbiome. Dogs with acute hemorrhagic diarrhea had significant decreases in *Blautia*, *Ruminococcaceae* including *Faecalibacterium*, and *Turicibacter* spp., and significant increases in genus *Sutterella* and *C. perfringens* compared to healthy dogs. In another recent study, the fecal microbiome of healthy dogs, dogs with chronic enteropathies, and dogs with acute hemorrhagic diarrhea was evaluated by qPCR assays for selected bacterial groups^[55]. The most pronounced changes were decreases in *Faecalibacterium* spp., *Turicibacter* spp., and *Ruminococcaceae* in CE and AHD. *E. coli* and *C. perfringens* were significantly increased in CE and AHD^[55]. Especially *Faecalibacterium* spp. is an important group that frequently appears depleted in canine GI disease. This has been confirmed in another study evaluating the fecal microbiota of dogs with idiopathic IBD, in which *Faecalibacterium* spp. was the major bacterial group decreased in diseased dogs^[21]. Noteworthy, *Faecalibacterium* spp. correlated with improvement in clinical activity index, suggesting that *Faecalibacterium* spp. may be important for canine GI health, and also may be useful as a monitoring marker for improvement of fecal dysbiosis^[14,21].

While the above discussed studies have reported

changes in microbial groups in GI disease of dogs and cats, only limited information is available about the metabolic consequences that are associated with this dysbiosis, as currently no comprehensive functional studies have been reported in dogs or cats. Alterations in the composition of intestinal microbiota are thought to be an important factor in the pathogenesis of chronic GI diseases. It can be hypothesized that the observed microbiome changes may lead to altered intestinal barrier function, damage to the intestinal brush border and enterocytes, an increased competition for nutrients and vitamins, and to an increased deconjugation of bile acids. Of interest is that commonly depleted groups in GI disease are *Lachnospiraceae*, *Ruminococcaceae*, and *Faecalibacterium*. These bacterial groups, important producers of SCFA, may play an important role in maintenance of gastrointestinal health, as their depletion leads to decreased production of SCFA (e.g., butyrate, acetate), which may impair the capability of the host to down-regulate aberrant intestinal immune response. The importance of some of these bacterial groups that are depleted in IBD have recently been demonstrated in humans. For example, *Faecalibacterium prausnitzii* is consistently reduced in human IBD and this bacterium has been shown to secrete metabolites with anti-inflammatory properties, thereby down-regulating interleukin (IL)-12 and interferon gamma and increasing IL-10 secretions^[56]. Disturbances may result in a dysregulation of adaptive immune responses, and lead to inflammation and/or reduced activity against infection. Also, some bacteria produce various toxic agents such as ammonia, D-lactate, endotoxin (LPS), or exotoxin (enterotoxin), and compete for vitamins or other nutrients. Consequently, depletions in serum vitamin B12 concentrations and also increases in serum concentrations of D-lactate are potential consequences of intestinal dysbiosis in cats^[57]. However, more comprehensive metabolomics studies are needed in companion animals to elucidate the consequences of

the dysbiosis observed in GI disease.

Invasive and adherent bacteria

A specific form of colitis occurs in Boxer dogs^[58] and occasionally also in French Bulldogs^[59]. This disease is termed granulomatous colitis. Microbiota analysis based on sequencing of 16S rRNA genes in combination with FISH has revealed invasive bacteria in the colonic mucosa of Boxer dogs with granulomatous colitis. Based on comparative 16S rRNA gene analysis, these bacteria have high phylogenetic similarity to *Escherichia coli* (*E. coli*) and *Shigella*. *In situ* analysis with 16S rRNA gene based FISH probes against *E. coli* showed multifocal clusters of invasive bacteria within macrophages in the colonic mucosa^[58]. The eradication of these invasive *E. coli* in Boxer dogs and French Bulldogs with granulomatous colitis correlates with clinical remission, inferring a causal relationship between these bacteria and the disease^[59]. Of interest is that these observed phylotypes of *E. coli* isolated from Boxer dogs have high phylogenetic resemblance to *E. coli* associated with Crohn's disease in humans^[16,59]. The breed specific predisposition of Boxer dogs and French bulldogs to *E. coli* associated granulomatous colitis highly suggests the presence of a genetic susceptibility that impairs their ability to fend off adherent and invasive *E. coli*.

Bacteria invading the intestinal mucosa may also be part of neutrophilic IBD in other dog breeds. Due to the recognized association of granulomatous and neutrophilic IBD with invasive bacteria, specialized testing based on FISH has been developed that allows localizing the bacteria in intestinal biopsies for better guidance of treatment decisions^[59].

ALTERATIONS IN FUNGAL MICROBIOTA

While bacteria are by far the most abundant constituents of the mammalian GI tract, it is now recognized that the gut harbors a highly diverse population of fungal organisms. FISH and shotgun sequencing studies of human and canine fecal DNA have estimated the abundance of fungal organisms and archaea as < 2% of total microbiota^[39,60]. A recent metagenomic approach estimated that the feline GI microbiota constitutes 0.02% fungi, 0.09% archaea, and 0.09% viruses^[41]. Fungi were described using pyrosequencing of the fungal 18S rRNA gene in pooled fecal samples of cats^[3], with *Aspergillus* and *Saccharomyces* being the most abundant fungal genera. A study reported the prevalence and identification of fungal organisms in the small intestine of healthy dogs and dogs with chronic enteropathies^[61]. The results indicated a high prevalence (up to 76.1% of dogs) and high diversity of fungal organisms in the canine duodenum. Furthermore, dogs with gastrointestinal disease harbored opportunistic fungal pathogens. A total of 51 different phylotypes were identified, with the most frequently observed phylotypes being *Pichia* spp., *Cryptococcus* spp., *Candida* spp., and *Trichosporon* spp.^[61].

A recent study has characterized the fungal micro-

biome (mycobiome) of 19 dogs (12 healthy dogs and 7 dogs with acute diarrhea) using fungal tag-encoded FLX-Titanium amplicon pyrosequencing^[62]. Five distinct fungal phyla were identified, with *Ascomycota* (median: 97.9% of obtained sequences) and *Basidiomycota* (median 1.0%) being the most abundant. A total of 219 fungal genera were identified across all 19 dogs with a median (range) of 28 (4-69) genera per sample. *Candida* was the most abundant genus found in dogs. However, no significant differences were observed in the relative proportions of fungal communities between healthy and diseased dogs. Therefore, additional studies are needed to elucidate the importance of fungi on intestinal health and disease of animals.

CONCLUSION

Studies using molecular approaches have provided clear evidence for alterations in microbial communities in the small and large intestine of dogs and cats with GI disorders. However, currently there is a lack of comprehensive studies evaluating the functional consequences of these alterations. A better understanding of these mechanisms will allow for the development of treatment modalities (*e.g.*, prebiotics, probiotics, metabolites) aiming at modulating microbial communities and their produced metabolites. Anecdotal case reports have reported some success using fecal transplantation in dogs with chronic diarrhea. Results of initial studies suggest that the administration of probiotic strains can be useful in dogs with GI disease. For example, probiotic strains have shown benefits in dogs with IBD^[21], puppies with acute parvoviral enteritis^[63], and adult dogs with non-specific diarrhea^[26,27]. In cats, probiotics strains have been shown to be beneficial in cats with chronic diarrhea^[28] and stress-related diarrhea in a shelter environment^[64]. However, future studies will need to evaluate how these microbial changes impact the immune and metabolic status of dogs and cats.

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WJG 20th Anniversary Special Issues (17): Intestinal microbiota

Mechanistic links between gut microbial community dynamics, microbial functions and metabolic health

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factors have contributed to discrepancies between studies. These include the high level of functional redundancy in host-microbiome interactions combined with individual variation in microbiome composition; differences in study design, diet composition and host system between studies; and inherent limitations to the resolution of rRNA-based community profiling. Accounting for these factors allows for recognition of the common microbial and host factors driving community composition and development of dysbiosis on high fat diets. New therapeutic intervention options are now emerging.

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Key words: Microbiome; Dysbiosis; High fat diet; Bile; Intestinal mucosa; Microbe-associated molecular patterns; Short chain fatty acids; Immunomodulation; Enteroendocrine cells

Abstract

Gut microbes comprise a high density, biologically active community that lies at the interface of an animal with its nutritional environment. Consequently their activity profoundly influences many aspects of the physiology and metabolism of the host animal. A range of microbial structural components and metabolites directly interact with host intestinal cells and tissues to influence nutrient uptake and epithelial health. Endocrine, neuronal and lymphoid cells in the gut also integrate signals from these microbial factors to influence systemic responses. Dysregulation of these host-microbe interactions is now recognised as a major risk factor in the development of metabolic dysfunction. This is a two-way process and understanding the factors that tip host-microbiome homeostasis over to dysbiosis requires greater appreciation of the host feedbacks that contribute to regulation of microbial community composition. To date, numerous studies have employed taxonomic profiling approaches to explore the links between microbial composition and host outcomes (especially obesity and its comorbidities), but inconsistent host-microbe associations have been reported. Available data indicates multiple

Core tip: The development of dysbiosis is driven by multiple factors. These include selective pressures imposed on the microbial community by the diet composition and feedback effects that involve either diet-host interaction or diet-microbiome-host interaction. The role of microbial signals in dysbiosis is well established but the involvement of host feedback mechanisms in aberrant host-microbial interactions is an under-appreciated part of disease progression. New opportunities to intervene in diseases of dysbiosis can result from targeting these distinct processes. These include stimulation of the host ability to self-regulate and blocking of deleterious host responses.

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INTRODUCTION

The gastrointestinal tract of animals typically harbours a large resident community of microorganisms that we will term the microbiome. The main function of the gut is to enable harvesting of nutrients from the external environment, however, animals live in a dynamic environment where their energy demands, exposure to foreign microorganisms and their access to nutrients are continually changing. Consequently gut functions also include containment of microbial activity to the intestinal lumen and integration of sensory perception of the intestinal environment with behavioural and physiological responses. Put simply, the gut is a major site for endocrine, immune and neural signalling in addition to digestion and nutrient absorption.

Many aspects of host physiology are strongly shaped by the presence and activities of the gut microbiome. The primary axis of host-microbiome interaction is in the intestinal tissues where microbial growth in the lumen contributes to the digestion of ingested food and directly shapes the chemical milieu of the gut. Host cells in the intestines are highly exposed to microbial activity, and microbial influence ranges from stimulation of receptors on those cells, to supply of energy sources to epithelial cells and triggering of developmental pathways in intestinal tissues^[1,2] (Figure 1). Although the primary interaction with microbes is at the intestinal epithelium, their influence is projected beyond the gut through secondary host-microbiome interactions, which occur externally to the epithelium. Some of these influences such as nutrient uptake and systemic inflammation, result from translocation of or “escape” of microbial products^[3,4]. Others such as appetite regulation, gut motility, energy balance and immune tone, result from the integration of multiple signals from the gut environment and bidirectional communication along the gut-brain axis^[5,6]. Accordingly, it is now widely recognised that differences in microbial composition and activity result in effects of fundamental importance to health.

The breadth of potential influence of the microbiome means mechanisms that serve to regulate the microbial interface with host systems are critical for health. This view gives rise to the concept of dysbiosis: Disease states that result from dysregulated host-microbe interactions. Dysbiosis contributes to the underlying pathophysiology of a wide range of diseases, including obesity^[7], diabetes^[4,8], inflammatory bowel diseases^[9], non-alcoholic fatty liver diseases^[10,11] and cardiovascular diseases^[12,13]. With awareness of the importance of dysbiosis in multiple diseases, attention has focused on how to define the microbe involvement in different diseases. The objectives here encompass the following: Identification of microbiota signatures (or biomarkers) that help define different dysbiosis states, ideally at the pre-clinical stage. Identifica-

tion of the triggers of dysregulated host-microbe interactions that ultimately lead to disease. Development of intervention strategies based around restoration of normal host-microbiome interactions. Underpinning all these objectives is the need to understand the dynamics of gut microbial community composition. This review focuses on mechanisms that drive the changes in microbial community composition that ultimately lead to shifts in host-microbiome interactions.

EVIDENCE FOR, AND LIMITS OF, MICROBIOME INFLUENCE ON HEALTH

Comparative studies on germ-free (GF) and conventionally raised (CONV) animals have been instrumental in establishing that the gut microbiome has influence on the physiological, immunological and nutritional state of its host. Such studies have consistently shown that GF animals are characterised by reduced intestinal vasculature^[1], undeveloped gut-associated lymphoid tissue^[14] and alterations in nutrition and energy metabolism^[15], all of which are largely restored by reintroduction of gut bacteria. Collectively there is compelling evidence that the gut microbiome can influence postnatal development of gut tissues and the physiological state of animals.

The effects of microbes are interdependent with effects of diet or the host genotype. For instance, GF and CONV comparisons are not precisely recapitulated in different animal models^[16], and there are also characteristic variations in microbiome composition between species^[17]. Some of these variations almost certainly reflect genetically encoded differences in life history (carnivores *vs* herbivores) or gut structure (ruminants *vs* monogastrics). Others will reflect more subtle tissue specific differences, for example, the organisation of gut-associated lymphoid tissue in dogs and rodents are distinct^[18]. Collectively these points serve to illustrate a broader issue. Host-microbiome interaction involves effects of the microbiome on the host, as well as effects of the host on the microbiome and these both occur within the context of environmental effects on the system (especially the nutritional environment). Studies that have addressed the influence of microbiome on differences between GF and CONV against defined genetic and diet differences in animals highlight the importance of this tripartite interaction^[9,19].

The importance of variation in host diet and genotype has been observed through GF-CONV comparisons across different strains and species of inbred rodents. In a seminal paper Bäckhed, Gordon *et al.*^[15] raised the prospect that gut microbiota represent an environmental factor in obesity. They showed that GF C57BL/6 mice had less fat deposition than CONV counterparts despite higher food consumption. Moreover, the faecal caloric content of GF mice was significantly higher than that of CONV counterparts. These findings led to the conclusion that gut microbiota promote energy harvesting and fat storage, and the hypothesis that GF

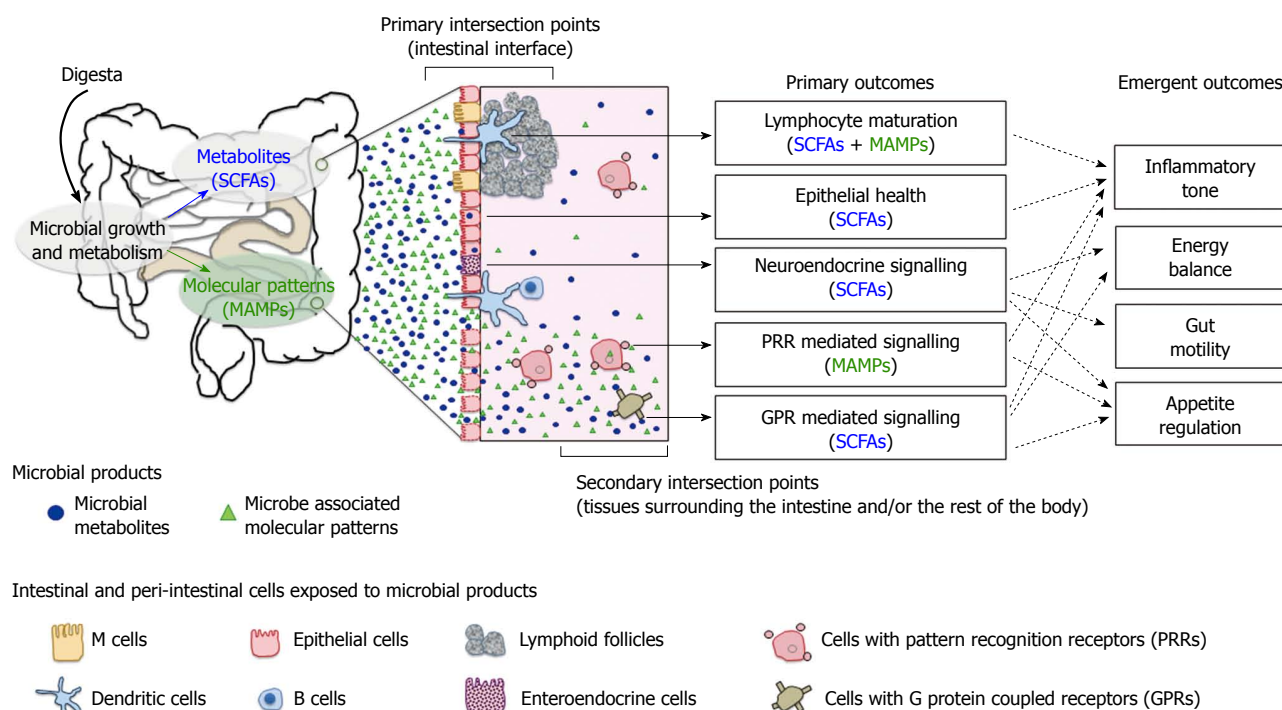


Figure 1 Axes of host-microbiome interaction that influence health. Short chain fatty acids (SCFAs) and microbe-associated molecular patterns (MAMPs) are the key microbial signals detected by the host. Outcomes of host-microbiome interactions are contingent on the microbial product involved, the type of host cells exposed to microbial signals and the location of contact. The primary intersection points occur at the intestinal epithelial interface. Sampling of luminal MAMPs and uptake of SCFAs have a direct impact on gut epithelium, lymphoid and neuroendocrine systems. The secondary intersection points occur externally to the intestinal tissues. Translocated or “escaped” microbial products can activate pattern recognition receptors (PRRs) and specific G protein coupled receptors (GPRs) on a wide range of host cells beyond the epithelium. A compromised gut barrier amplifies host-microbiome interactions in the secondary intersection points and the downstream effects of PRR and GPR signalling cascades. Host outcome is an emergent property of all axes of interactions.

animals are protected from obesity^[15,20]. In contrast to this mouse model, GF Fischer 344 rats displayed similar body weight and adiposity relative to CONV in two out of three experimental cohorts, and differences in daily food intake between the GF and CONV groups were insignificant^[21]. Although this suggests different animal species may respond differently, it is important to note that these studies used standard rodent chow from different suppliers and almost certainly the diets were compositionally distinct^[15,21].

Intersection between diet and genotype can also influence the phenotype of GF and CONV animals. The significance of this issue is highlighted in a report comparing the effect of three different diets on GF and CONV C3H mice^[22]. There was no difference in weight gain between GF and CONV groups under low fat diet, but GF C3H mice actually showed significantly higher weight gain on a high fat diet (HFD) compared to CONV. Previous reports of obesity resistance on HFD in GF C57BL/6 mice had used a formulation with similar macronutrient balance but distinct sources of carbohydrates and fat^[20]. When the two versions of high fat formulation were directly compared, GF and CONV C3H had comparable body fat content on the HFD with low sugar formulation but GF C3H mice was obesity resistant on the HFD with high sugar^[22]. In summary, GF-CONV comparisons in different animal/diet models consistently show differences in energy harvest (faecal

caloric content), energy storage (weight and body fat) and energy expenditure. Typically the effect of microbial presence is to increase adiposity, however, this does vary between experimental models and even between cohorts in the same model system. The major identifiable variables are animal species/strain and diet composition which differ between experimental cohorts.

Further exploration of the importance of microbiome composition has provided robust evidence supporting a causal link between gut microbiome composition and host outcomes. Specifically, some phenotypic traits of CONV animals can be recapitulated by conventionalisation of GF animals through microbiome transplantation^[11,23-25]. When GF mouse models are conventionalised with gut microbiota from either obese or lean mice, metabolic profiles and physiological attributes of the recipients reflect their donors^[23,24]. Evidently emergent properties of the total microbial community can drive differences in metabolic and physiological phenotypes. Precisely which microbes or how many are needed is unclear. For example, monocolonisation of GF mice with *Enterobacter cloacae* (a member of *Proteobacteria* isolated from an obese human) induced obesity and systemic insulin resistance in mice on HFD, while GF mice on HFD did not exhibit the same disease phenotypes^[26].

In conclusion, host metabolic health is strongly influenced by the gut microbiome. The influence of gut microbes is dependent on microbiome composition and

is interactive with the effects of diet and host genotype. The mechanisms of microbial influence stem from microbial activity in the intestinal tract, but are projected to the body system *via* multiple integrated pathways. The complexities of these interactions mean that although variations in microbial community composition can lead to different outcomes, associations may be diet or system-specific.

IDENTIFYING MICROBIAL MARKERS FOR METABOLIC DISEASES

Gut microbial community in health and disease-taxonomic insights

Broadly speaking microbiome association studies have two objectives: (1) To identify links with specific disease states^[27], and (2) To identify features of a healthy microbiome that may be a target in the restoration of health^[28]. Although there have been many reports of microbiome associations with obesity or metabolic health indicators in cross-sectional studies^[29,30], experimentally controlled treatments in humans^[31,32] and animal models (Table 1), consistent patterns across studies are hard to discern. As discussed above the influence of the microbiome on host health is interdependent with diet and the host system. As such the apparent lack of consistent associations is likely to reflect the confounding effects of diet, host genotype and host epigenetic state. Since HFDs in Table 1 are not of the same formulation, some of the discrepancies observed almost certainly reflect variations in diet. Differences will also reflect some inherent limitations of taxon-based description of the gut microbial community.

Community profiling has two key requirements. These are the ability to recognise biologically distinct units and the capacity to effectively sample all such units in a community. The size and diversity of microbial communities mean that it is essential to meet these requirements with high throughput approaches. The limitations of the species concept in bacteriology, combined with poor cultivability of bacteria meant that historically this has been impossible. Advances in sequencing technologies and analysis programs over the past decade have made effective sampling possible for the first time. However, recognition of biologically meaningful taxonomic units is still limited.

The most widely used marker for community profiling is the 16S ribosomal RNA (rRNA) gene. Sample sizes of thousands to even millions of sequence reads are now readily obtained. A feature of the 16S rRNA is that it is a very flexible phylogenetic marker and taxonomic units can be readily made at a variety of scales. Generally defining taxonomic units at coarse scale (*e.g.*, phylum; about 80% 16S rRNA identity) simplifies the analytical task of comparing units but at the expense of explanatory power. Variation in the gut microbiome is readily observable at this scale^[48]. Many studies have reported an association between the ratio of the two dominant gut phyla, *Bacteroidetes* and *Firmicutes*, with obesity in cross-sectional studies and in experimental treatments^[24,29,49]. However

numerous exceptions have also been reported^[50-52], and a recent exhaustive meta-analysis of human microbiome project data found no consistent relationship between the *Bacteroidetes:Firmicutes* ratio and obesity^[53]. An almost certain contributing factor is that such coarse taxonomic units are less biologically meaningful than fine scale units.

There are some attributes of the gut microbiome that one can reasonably predict from the taxonomic profiles at phylum scale. For instance, *Firmicutes* and *Bacteroidetes* have fundamental differences in cell envelope composition, and polysaccharide foraging strategy^[54]. However, detailed predictions of microbial functions and/or properties based on phylum classification alone are unrealistic. At finer scales of classification the biological homogeneity of taxa increases and more consistent patterns are observable. For example, it has been proposed that human gut microbiome variation occurs in three predominant variants termed enterotypes, which are recognisable through co-occurrence patterns defined by the genera *Bacteroides*, *Prevotella* and *Ruminococcus*^[52]. Recently this concept has been intensively explored, highlighting that observation of specific patterns of association is subject to analytical and classification approaches^[55], particularly how sequences are clustered into operational taxonomic units (OTUs) and how OTU-based distances between communities are calculated. This effect of analytical approach is likely to exist wherever community profiling does not (or cannot) classify into ecologically homogeneous units (ecotypes).

The inability to recognise ecotypes is an inherent limitation of 16S rRNA sequencing based approaches. Closely related species can have differential responses to specific nutrient sources and have divergent ecological roles^[42,56,57]. Perhaps the most striking illustration of this issue derives from a study conducted by Li *et al.*^[58], where they used community fingerprinting and metabolomics to test for associations between *Clostridia* and urinary metabolites in humans. Distinct populations in the fingerprinting analysis that had mutually exclusive associations to different sets of urinary metabolites were classified to *Faecalibacterium prausnitzii* (*F. prausnitzii*). This indicates that strains of *F. prausnitzii* inseparable by rRNA-based classification had distinct metabolic impacts in the gut system. Hence, it is not surprising that even microbiome associations reported at the finest scales possible with rRNA-based classification are often contradictory between different studies. For instance, *F. prausnitzii* was found to be over-represented in obese subjects in comparison to the lean counterparts^[59], which suggests high proportion of *F. prausnitzii* within the gut community is an indicator of poor health outcomes. Yet, other investigations have reported that healthy individuals carry more *F. prausnitzii* than patients with type 2 diabetes^[30] or chronic inflammation^[60]. Another example is the association of *Akkermansia muciniphila* (*A. muciniphila*) with health in some animal studies^[61], other studies have noted an increased proportion of *A. muciniphila* in obesity^[33] and type 2 diabetes^[30], or a role in

Table 1 Murine gut microbiome and host outcomes after exposure to high fat diets

Type of high fat diet and duration	Detection method	F:B	Key microbial features ¹				Other	Observation and proposed mechanism for microbial outcomes	Reported host phenotype	Observation and proposed mechanism for host outcomes	Ref.
			Firmicutes	Bacteroidetes	Proteobacteria	Actinobacteria					
HF/HIS ² for 8 wk	Fecal 454 [V4]	↑ F:B	↑ unclassified Lachnospiraceae, unclassified Ruminococcaceae, Turicibacter, Dorea, Roseburia	↓ Barnesiella, unclassified Porphyromonadaceae		↑ Bifidobacterium	↑ Akkermansia	Host genotype influences gut microbiota plasticity in response to diet.	↑ Body fat percent		[33]
HF/HIS ³ for 8 wk	Cecal full length 16S sequencing, shotgun sequencing and transcriptomics	↑ F:B	↓ Oscillibacter ↑ Molllicutes/ Erysipelotrichaceae				↓ Microbial diversity ↑ Genes for PTS system ↑ SCFAs concentration	Altered substrate availability ↑ microbes with the capacity to import and degrade sugars found in diet and/or host mucosa.	Weight gain, ↑ Body fat percent	Increased energy harvest. Specific microbes facilitate the transfer of calories from the diet to the host in the form of SCFAs.	[24]
HF/HIS ⁴ for 12 wk	Colonic tissue 454 [V1-2], qPCR and DGGE [V3-5]	↓ F:B	↑ mucin-degrading Ruminococcus torques	↑ Bacteroides-Prevotella spp	↑ Proteobacteria		↓ 16S rRNA gene copies	Diet type and host genotype ↑ bacteria with the ability to bind to glycosylated proteins and colonise mucosal surfaces.	Leaky gut	Diet-induced microbial changes at gut mucosa may aggravate inflammation in genetically susceptible host.	[34]
HFD ⁵ for 8 wk	Fecal 454 [V4] at baseline, Week 4 and Week 8	Progressive ↑ F:B			Progressive ↓ Proteobacteria			Dietary factors determine microbial composition. Microbial community may adapt to HFD overtime	Weight gain and ↑ fat mass	Microbes may promote obesity via LPS or SCFA modulation of host gene expression rather than energy harvesting.	[35]
HFD ⁶ for 20 wk	Fecal 454 [V4] and qPCR	↑ F:B	↑ Lactobacillus	↓ Bacteroides					Weight gain, IR, fatty liver, adipose, and systemic inflammation	Antibiotic improves metabolic abnormalities. Gut microbiota modulates inflammatory responses.	[36]
HFD ⁷ for 21 wk	Fecal 454 [V1-2] and shotgun sequencing	↑ F:B	↑ Clostridiaceae	↓ Bacteroidaceae, Prevotellaceae and Rikenellaceae	↑ Desulfovibrionaceae		↑ genes for ABC transporters, two-component system and cell motility ↓ metabolic genes	Altered substrate availability ↑ microbes with the capacity to enhance nutrient uptake in an environment of limiting substrates	Weight gain		[37]
HFD ⁸ for 8 to 12 wk	Fecal 454 [V6-8]	↑ F:B	↑ Oscillibacter, Blautia ↓ Lactobacillus	↓ Barnesiella, Parabacteroides					Weight gain, leaky gut, IR, adipose, gut and liver inflammation	Gut bacteria modulate gut barrier integrity. Leaky gut coupled with aberrant microbiota drive metabolic dysfunction.	[38]

HFD ⁷ for 12 wk	Cecal MiSeq [V4], metaproteome, metabolomics	↓ Ruminococcaceae ↑ Erysipelotrichales	↑ Rikenellaceae	↑ proteins for amino acid metabolism and transport and cell motility No difference in microbial richness No difference in microbial diversity	Altered substrate availability shifts the composition and/or activity of microbiota, which favours amino acid metabolism	Weight gain, hyperglycemia	[39]
HFD ⁸ for 8 wk	Fecal 454 [V1-3], culture	↑ F:B ↑ Ruminococcaceae ↓ Clostridiales	↑ Rikenellaceae ↓ Bacteroidaceae	↑ LPS No difference in microbial diversity		Weight gain, hyperglycemia, adipose, systemic and gut inflammation	[40]
HFD ⁸ for 12 wk	Fecal 454 [V3] at every 2-4 wk	Progressive ↑ F:B ↑ Lachnospiraceae, Ruminococcaceae, Lactococcus	↑ selected OTUs in Bacteroides, Alistipes ↓ Barnesiella	↓ microbial diversity ↑ LPS binding protein	Age-related effects and/or altered substrate availability	Weight gain, ↑ fat mass, IGT	[41]
HFD ⁸ for 25 wk	Fecal 454 [V3], DGGE [V3] and T-RFLP	Lineages in Mollicutes/Erysipelotrichaceae responded differentially			Altered substrate availability and host genetics have differential impact on gut microbial profile	Weight gain, IGT	[42]
HFD ⁸ for more than 35 wk	Cecal 454 [V1-2]	↓ F:B ↑ unclassified Lachnospiraceae, Lactococcus, Unclassified Ruminococcaceae, Roseburia	↑ Bacteroides	↑ Mucispirillum	Leptin may affect microbial composition by modulating mucin production in the intestine	Weight gain, ↑ leptin, adipose inflammation	[43]
HFD ⁸ for life, gut microbiota at week 62 is described here	Fecal 454 [V3]	↓ Allobaculum ↑ selected OTUs in Allobaculum, Ruminococcaceae, Papillibacter, Lactococcus ↓ selected OTUs in Allobaculum	↑ Bilophila	↓ Akkermansia ↑ Mucispirillum ↑ LPS binding protein	Altered substrate availability. Low plant polysaccharides may alter the balance of gut barrier protecting bacteria, butyrate producers and pathobionts	Weight gain, IGT, fatty liver, ↓ liver function, abnormalities	[44,45]
HFD ⁹ for 4 wk	Cecal FISH	↓ Eubacterium rectale/ Clostridium coccoides	↓ Bifidobacterium	↑ LPS		Weight gain, IR, fatty liver, systemic and adipose inflammation	[4]
HFDs ¹⁰ with different sources of fat (safflower oil, milk fat or lard) for 24 d	Cecal 454 [V2-4]	↑ F:B in lard HFD ↓ F:B in other HFDs	↑ Bilophila in milk fat HFD	↓ microbial diversity in milk fat and safflower oil HFDs	Altered substrate availability. Milk-derived saturated fat ↑ the pool of sulphated bile acid, an antimicrobial but a growth substrate for Bilophila	Gut inflammation in genetically susceptible host	[9]

HFDs ¹⁰ with different sources of fat (safflower oil, milk fat or lard) for 4 wk	Fecal Illumina [V3-4]	↑ F:B in all HFDs	↑ Proteobacteria in milk fat and safflower oil HFDs	↑ Actinobacteria	↑ Tenericutes in lard HFD	Altered substrate availability. Dietary fat source modulates gut microbial profile	Weight gain (highest in milk fat), adipose inflammation (highest in safflower)	Diet induced alterations in the gut microbiota influence localised inflammation	[46]
HFDs ¹¹ with different sources of fat (palm, olive or safflower oil) for 8 wk	Fecal MITChip (microarray)	↑ F:B in palm oil HFD only	↑ Bacilli, Clostridium cluster XI, XVII, and XV III in palm oil HFD only	↓ microbial diversity in palm oil HFD only	Saturated fat diet leads to an overflow of dietary fat in the gut which may have an antimicrobial effect on microbiota	Weight gain (highest in palm oil), IR, fatty liver			[47]

¹⁰This table features the microbial shifts in wild type mice after dietary interventions, patterns for knockout models are excluded. Sampling site and technique used to monitor the hypervariable region of 16S rDNA are noted; ²31.8% and 51.4% calories from fat (corn oil and butter fat) and carbohydrates, respectively, Research Diets, New Brunswick; ³40.6% and 40.7% calories from fat (beef tallow, vegetable shortening) and carbohydrates, respectively, Harlan-Teklad, United States; ⁴60.6% and 26.3% calories from fat (lard) and carbohydrates, respectively, SAFE, France; ⁵45% and 35% calories from fat (lard and soybean oil) and carbohydrates, respectively, Research Diets, New Brunswick; ⁶60% and 20% calories from fat (lard and sunflower oil) and carbohydrates, respectively, in house; ⁷60% and 21% calories from fat (beef tallow and soybean oil) and carbohydrates, respectively, Sniff GmbH, Germany; ⁸60% and 20% calories from fat (lard and soybean oil) and carbohydrates, respectively, Research Diets, New Brunswick; ⁹72% and < 1% calories from fat (corn oil and lard) and carbohydrates, respectively, SAFE, France; ¹⁰37.5% and 47% calories from fat and carbohydrates, respectively, Harlan-Teklad, United States; ¹¹45% and 35% calories from fat and carbohydrates, respectively, The Netherlands. F:B: Firmicutes to Bacteroidetes ratio; HF/Hs: High fat and high sugar diet; HFD: High fat diet; PTS: Phosphotransferase system; SCFAs: Short chain fatty acids; ABC transporters: ATP-binding cassette transporters; DGGE: Denaturing gradient gel electrophoresis; T-RFLP: Terminal restriction fragment length polymorphism; IR: Insulin resistance; IGT: Impaired glucose tolerance; LPS: Lipopolysaccharides; OTUs: Operational taxonomic units; Hs: Hydrogen sulphide; FISH: Fluorescent in situ hybridisation.

exacerbating gut inflammation^[62].

In summary, consideration of diet, host system and great care in methodological approaches to community profiling is necessary to identify consistent associations between microbes and metabolic health. The main limitation from a methodological perspective is linkage of relevant ecological properties of the microbial group to the taxonomic marker. An alternate approach to this is to profile the gut system and its resident bacteria from a functional perspective.

Gut microbial community in health and disease-functional insights

In effect functional profiling is delineation of taxonomic units based on a biochemical property. It is generally accepted that there is a high level of functional redundancy in the gut microbiome. This means bacteria from different taxonomic groups may contribute to the same ecological process (belong to the same guild) and they can substitute for one another. For instance, many gut bacteria can produce butyrate, a short chain fatty acid (SCFA) with widespread health implications, but the bacteria that carry out this function are phylogenetically diverse^[63]. Associations between rRNA-based taxa and host outcomes that are critically dependent on butyrate availability are likely to be inconsistent because different members of the butyrate-producer guild may be dominant under different diets or host systems. Thus functional redundancy is almost certainly a contributor to the wide variation in associations of microbiome response and host outcomes to HFDs summarised in Table 1.

If the diet-microbiome-host outcomes listed in Table 1 are cross-examined from the perspective of microbial metabolites or microbe-associated molecular patterns (MAMPs) that are likely to be common features of ecological guilds, a more encouraging picture of associations between microbiome and metabolic health starts to emerge. Inferred or measured changes of microbial metabolite such as elevated total SCFA, elevated serum lipopolysaccharides (LPS) and hydrogen sulphide (H₂S) production are recurrently observed. In the case of LPS and H₂S these are also associated to taxa that are recognisable by rRNA-based classification, such as *Enterobacteriaceae* and *Desulfovibrionaceae* from the phylum *Proteobacteria*.

Metagenomic analysis provides a global dataset for functional profiling whereby multiple guilds can be looked at simultaneously. Such analyses have reported differences in the total level of carbohydrate degradation genes in the metagenomes of obese *vs* lean microbiomes raising the prospect that energy harvesting may be predictable from

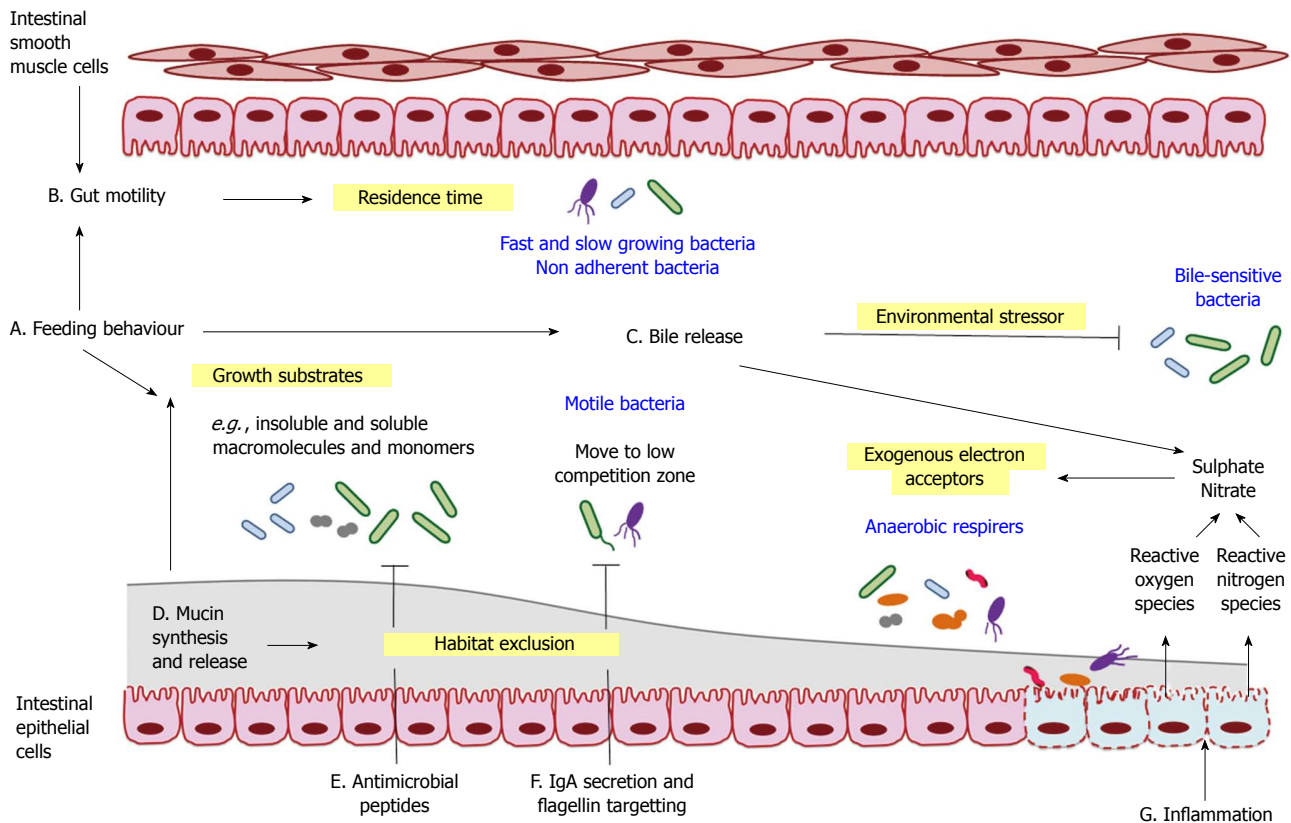


Figure 2 Multiple host-mediated mechanisms regulate bacterial growth and their activities. These pathways may act against the microbiota in a generalised manner or influence bacteria with distinct properties (blue). A: Substrates from diet are key energy sources for bacterial growth. Changes in feeding pattern will shape the microbiome structure and associated products; B: Ingestion of dietary fibre and osmotically active compounds promotes gut motility. Faster transit rate flushes out slow growing organisms and those without the ability to adhere to the intestines; C: Release of bile in response to dietary fat selects against bile-sensitive bacteria but promotes those with the capacity to obtain energy via anaerobic respiration; D: Mucin secreted by goblet cells physically prevents the penetration of bacteria into gut epithelium, and it also promotes bacteria that utilise mucin as growth substrates; E: Paneth cells in the gut epithelium secrete effector molecules with broad-spectrum antimicrobial activity, e.g. defensins, lysozyme and Reg III γ , which contribute to the innate barrier against microbial colonisation; F: Migration of flagellated bacteria is inhibited by secretory immunoglobulin A (IgA), which facilitates the exclusion of bacteria at the epithelium; G: When mucin synthesis and release is impaired, pathobionts may penetrate the mucosal epithelium and trigger the inflammatory cascade. Byproducts of inflammation confer a growth advantage for organisms that obtain energy through anaerobic respiration.

metagenome signatures^[64], but more specific signatures have also been reported. Aside from microbial metabolites, MAMPs also stimulate host responses. Consistent with this, metagenome studies have found enrichment of microbial genes that encode cell motility^[37] as well as an increase in flagellin proteins^[65] associated with the obese state.

In summary, small scale single-cohort, rRNA-based studies of diet-microbiome-host interactions in response to HFD typically identify associations. cursory comparisons of such studies reveal a confusing picture, however more detailed consideration of common ecological or physiological features reveals common patterns. Microbial structural motifs and metabolites with robust associations to HFD formulations and disease states have been seen and are regarded as the mechanistic links between gut microbiome and systemic complications. It is noteworthy that these MAMPs and microbial metabolites are present in the intestinal lumen but their systemic loads are known to increase during a HFD challenge^[4,66-68] and in various aspects of metabolic disorders^[4,51,69]. This raises the question of feedback processes that may further shape

microbial community structure and the progression into dysbiosis.

FACTORS THAT SHAPE GUT COMMUNITY DYNAMICS AND FUNCTION

Intrinsic factors

Multiple host mechanisms are involved in restricting microbial growth and activity to the intestinal lumen (Figure 2). These processes may act against the gut microbiome in a generalised manner or target specific bacteria with distinct properties. Host secretions in the gut can function as environmental stressors that regulate bacterial growth. The primary role of bile acids is to facilitate dietary fat absorption but their amphipathic properties also disrupt bacterial membrane integrity and result in antibacterial activity^[70]. When rats are fed with diet supplemented with bile acids, their gut communities are characterised by a reduction in *Bacteroidetes* and enrichment in *Clostridia* and *Erysipelotrichi*^[71]. Intriguingly, this

compositional change mirrors the patterns reported in HFD studies^[24,37,38]. Higher amounts of bile acids are also linked to lower caecal concentrations of butyrate^[71], a metabolite produced by subsets of gut bacteria. This finding suggests bile acids either select against the proliferation of butyrate producing bacteria or inhibit the metabolic pathways leading to butyrate synthesis. Collectively, bile acids have a contributing role in determining microbial composition and the products released by the gut microbiome.

At the intestinal interface, host-derived molecules work in synergy to exclude microbial colonisation along the gut epithelium and modulate the microbial composition in the vicinity. Secretory immunoglobulin A (IgA) is known to control bacterial migration patterns by sequestering the movement of motile organisms, thereby preventing their penetration across the gut epithelium^[72]. Antimicrobial peptides such as defensins and RegIII γ also influence microbial composition^[73,74]. Mice expressing human α -defensin genes had marked depletion of segmented filamentous bacteria and less interleukin 17-producing T cells in the lamina propria than those with α -defensin deficiency^[75]. RegIII γ , on the other hand, generally selects against Gram positive bacteria, as LPS on Gram negative bacteria inhibit RegIII γ activity^[74,76]. Host secretions can also shape the gut microbiome by providing an ecological niche for specific bacteria. For instance, mucin, a glycosylated protein covering the intestinal epithelium, is a specific growth substrate for many commensal gut microbes, including *Ruminococcus*^[77], *Bacteroides*^[78] and *Akkermansia*^[79]. In the event of gut inflammation, byproducts of immune responses may alter the gut microbiome by favouring the growth of selected organisms. For instance, host cells release reactive oxygen and nitrogen species into the lumen, which react to form nitrate^[80-82]. It has been shown that *Escherichia coli* uses exogenous nitrate as electron acceptors for anaerobic respiration, giving it a competitive advantage over fermentative organisms^[83].

Host feeding behaviour

While host secretions play an important role in determining the gut community structure, external factors such as host feeding behaviour are equally influential (Figure 2). A main driver of microbial change is the macronutrient intake of the host, in particular the type of carbohydrate ingested^[57,84]. Changes in intake are likely to influence the gut microbiota composition or their nutrient acquisition strategies^[85]. For instance, experiments in monocolonised mice have found that *Bacteroides thetaiotaomicron* responded to depletion of dietary polysaccharides by upregulating a set of genes adapted to degradation of host mucus glycans^[78]. Similarly, *Ruminococcus gnavus* switches on different sets of carbohydrate-utilising enzymes in response to the availability of carbon sources (monosaccharides *vs* mucin) in the environment^[86]. *Escherichia coli* can also adapt to nutrient changes in the environment by altering porin-mediated outer membrane permeability, broadening nutritional acquisition capacity^[87], but at the expense of

reduced resistance against bile^[88]. Increase in the amount of fermentable polysaccharides changes intestinal transit rate, which modulates the membership of the gut community^[89]. Faster transit rate may flush out slow growing organisms and those without the ability to adhere to the mucosal lining of epithelial cells. Altered microbial composition and associated metabolites, in turn, feedback to gut motility^[89,90], which strongly influences nutrient absorption in the gut^[91,92]. Additionally, high consumption of dietary saturated fat enhances the secretion and taurine conjugation of bile acids^[9,93,94], which provides a strong selection pressure on the gut commensals due to its antibacterial activity. However, influx of taurocholic acid presents an additional source of sulphated compounds for bile tolerant, sulphate/sulphite-reducing bacteria (SRBs) to utilise in anaerobic respiration^[9], thereby promoting their expansion in the gut community. Changes in diet can alter microbial composition in the matter of days^[95,96]. If the altered state persists over time, it will result in a different repertoire of microbial products accumulating in the gut system^[97].

HOST-MICROBIOME FEEDBACKS IN METABOLIC DYSFUNCTION AND INFLAMMATION

MAMPs as mechanistic links between gut community and host outcome

A number of pattern recognition receptors (PRRs) on host cells, such as toll-like receptors (TLR4 and TLR5) and nucleotide-binding oligomerisation domain receptors (NOD1 and NOD2) are specialised for detection of MAMPs such as LPS, peptidoglycan (PGN) and flagellin. The structure and/or the extent to which MAMPs are released from bacterial cells can vary between species. Thus modification in community composition, or MAMPs expression, can promote changes in the host system. However MAMPs profile alone cannot determine host outcomes, specific host receptors and loss of gut barrier function are required to potentiate metabolic dysfunction. Localisation and expression of PRRs differ between cell types^[98], this may explain the divergent outcomes of each MAMP/PRR interaction.

Flagellin

A wide range of gut bacteria have the capacity to produce flagella, including members of the phyla *Firmicutes*^[99] and *Proteobacteria*^[72]. Flagellin proteins derived from motile organisms are detected by TLR5, which is selectively expressed at a higher level in the cecum and proximal colon^[100]. TLR5 are present on the basolateral surface of intestinal epithelial cells, apical surface of epithelial cells associated lymphoid follicles and mucosal dendritic cells^[98,100]. TLR5 detection of flagellin is known to induce the secretion of anti-flagellin IgA, which quenches the motility of various *Proteobacteria* and *Firmicutes* species^[72]. This restriction of microbial migration is a normal host

response. When flagellin gains access into the intestinal mucosa, it triggers pro-inflammatory responses and increases the risk of chronic inflammation^[101].

Aside from localised responses in the gut, flagellin activation is linked to regulation of physiological processes beyond the gut system. Mice lacking TLR5 had higher food consumption, and developed obesity, dyslipidemia, insulin resistance and hypertension in comparison to wild type (WT)^[102]. While some of these phenotypes can be explained by increased dietary intake, food restriction in TLR5 knockout (KO) mice was only effective in preventing obesity but not insulin resistance. Remarkably, antibiotic treatment of TLR5 KO mice normalised food intake and ameliorated metabolic defects, while transplantation of TLR5 KO gut microbiota into WT recipients recapitulated metabolic dysfunction^[102]. These results suggest that appropriate flagellin/TLR5 signalling cascade have a beneficial role in host feeding behaviour and thus, promote metabolic health.

Lipopolysaccharides

LPS is a component of the outer membrane of most Gram negative bacteria, including *Bacteroidetes* and *Proteobacteria*. Chemical properties of LPS vary between species, which lead to differential capacity in activating the TLR4 signalling cascade^[103]. It is thought that species from *Proteobacteria* exert a stronger immunostimulatory effect than *Bacteroides*^[104]. In comparison to TLR5, TLR4 expression in intestinal epithelial cells is relatively low^[105] and they are localised in the basolateral compartment^[98]. Under normal circumstances, only small amounts of LPS pass through the gut epithelium and reach the bloodstream^[4]. Consumption of HFD, however, is associated with reduced expression of tight junction proteins in the gut epithelium^[106]. Loss of tight junction integrity increases the paracellular space in the epithelium and facilitates the leakage of luminal contents, including LPS, into adjacent tissues and the circulatory system^[106]. Dietary fat is also believed to enhance chylomicron absorption of LPS from the intestinal lumen or enterocytes, which are then exported into the circulatory system^[107,108]. Once LPS escapes from the intestinal lumen it can be recognised by cells with TLR4 in the peri-intestinal region or in insulin-targeting tissues, such as adipose tissue, liver, skeletal muscle and pancreas^[109]. Activation of TLR4 induces the release of pro-inflammatory cytokines, which drives helper T cell (T_H helper) expansion and impairs insulin signalling^[109,110]. In summary, LPS is an immunostimulatory agent but its exposure to TLR4 expressing cells and the capacity to drive dysbiosis is dependent on physiological properties of the host system such as intestinal permeability.

Physiological consequences of LPS/TLR4 signalling are demonstrated in mice with CD14 or TLR4 deficiencies. During HFD treatment or LPS infusion, both KO mouse models are protected from the hallmark features of metabolic dysfunction observed in the WT counterparts, including obesity, insulin resistance and inflamma-

tion^[4,111]. These results indicate that TLR4 agonists, such as LPS, can influence health. Yet, TLR4 is also stimulated by non microbial structures, such as saturated fatty acids^[112]. Systemic lipid infusion can trigger the TLR4 inflammatory cascade in adipose tissue and give rise to insulin resistance^[113]. One might argue the activation of TLR4 cascade and associated metabolic defects is due to an excess of dietary lipid from HFD, rather than a consequence driven by a microbiota-derived compound. However, detoxification of LPS by intestinal alkaline phosphatase^[114], reduced microbial load after antibiotic administration^[106,115] or altered microbial profile after prebiotics treatment^[61,116] can all lower plasma LPS. All these are thought to be concomitant with improved gut barrier function and/or restoration of metabolic health^[106,114-116]. Since broad (antibiotics) and selective (prebiotics) alterations in the gut microbiota lead to improvements of metabolic parameters during HFD, these findings are in agreement that the availability of LPS has a fundamental role in driving metabolic outcomes.

Peptidoglycan

NOD1 and NOD2 are sensors of PGN, but each receptor has a different substrate preference. NOD1 preferentially binds to a structural variant commonly found in Gram negative bacteria^[117], while NOD2 detects a common motif of gram positive and gram negative organisms^[118]. Similar to TLR4, NOD1 activation is implicated in the development of insulin resistance. Administration of NOD1 agonist to adipocytes upregulates the expression of pro-inflammatory cytokine TNF- α and chemokine MCP-1 in a dose dependent manner, which affects insulin signalling and decreases insulin-mediated glucose uptake^[119]. Mice lacking NOD1 are protected from HFD-induced glucose intolerance and translocation of intact Gram negative bacteria from the gut lumen to mesenteric adipose tissue (MAT) and blood, compared to the WT^[120]. The authors also demonstrated that bacterial translocation to MAT and the associated inflammation preceded glucose intolerance, suggesting NOD1 interaction with Gram negative gut bacteria drives the pathophysiology associated with HFD.

Apart from NOD1 signalling, NOD2 activation in the skeletal muscle also influences insulin action and glucose homeostasis. Tamrakar *et al.*^[121] have shown that a NOD2 agonist significantly reduced insulin-stimulated glucose uptake in rat skeletal muscle cell line, whereas NOD1 activation had minimal effect. However, interference with the NOD2 cascade does not necessarily protect the host from dysbiosis. Malfunctions in NOD2 signalling in patients with Crohn's disease or in NOD2 KO mice, are linked to dysregulation of microbial containment, resulting in bacterial translocation to intestinal surface and aberrant stimulation of mucosal immune system^[122,123]. Taken together, these findings demonstrate the diverse outcomes of host-microbial immune signalling. The net response is strongly dependent on the target site and is possibly linked to the ratio of Gram negative to

Gram positive organisms as different PGN ligands lead to divergent downstream response.

SCFAs as mechanistic links between gut community and host outcome

SCFAs, such as acetate, propionate and butyrate, are arguably the most influential microbial metabolites in the context of health and disease. Both community composition and the available fermentable substrates influence the net SCFA profile^[54,124,125]. As a consequence SCFA profile is an emergent property of the community and it is difficult to predict from taxon-based analysis. The majority of SCFA production is utilised locally by the gut epithelial cells but significant amounts are also transported across the epithelium to distant tissues *via* the circulatory system. Butyrate is metabolised in the gut epithelium and is the key energy source for colonocytes^[126]. Propionate and acetate are metabolised as substrates for energy metabolism and lipid synthesis in the liver and other peripheral tissues^[127]. Absorption of SCFAs accounts for 6%-9% of the total energy intake for humans and can contribute up to 44% in other animals^[128,129]. In addition to their role as an energy substrate, SCFAs are signalling molecules in modulating neuroendocrine and anti-inflammatory responses at various sites.

SCFA signalling: neuroendocrine function and energy regulation

G protein coupled receptors, GPR41 and GPR43, are the primary mediators of SCFA signalling. Butyrate and propionate have high stimulatory effect towards GPR41, while butyrate, propionate and acetate all show similar activity towards GPR43^[130]. Evidence from KO models has led to the proposal that SCFA signalling *via* GPRs modulates energy balance, with WT mice having higher fat deposition than GPR41 KO^[131]. The GPR41 KO is also characterised by a reduced expression of intestinal peptide YY (PYY), an enteroendocrine L cell hormone that in WT animals inhibits gut motility, potentially increasing the time for energy harvest and absorption^[131]. Similarly, GPR43 KO mice are resistant to HFD-induced obesity, insulin insensitivity, and dyslipidemia^[132], and there is supporting evidence that acetate and propionate promote adipogenesis through GPR43^[133].

Other gut hormones are also influenced by SCFA signals. Glucagon-like peptide 1 (GLP-1) secreted by enteroendocrine cells has a range of effects that encompass promotion of satiety and glucose homeostasis^[134], and its release can be stimulated by oral administration of butyrate^[135]. Supplementation of butyrate to HFD fed mice reduced food intake and improved glucose control compared to HFD mice without the treatment^[135], these phenotypic differences might be driven by differential secretion of GLP-1. Consistent with this observation, mice with impaired GPR43 signalling had reduced GLP-1 secretion, concomitant with glucose intolerance^[136]. In adipocytes, SCFA activation of GPR41 induce the expression and

production of leptin^[137], a hormone that regulates feeding behaviour, metabolic rate and immune response.

Interactions *via* the gut-brain axis are also involved in the coordination of metabolic homeostasis. Propionate produced in the gut can activate GPR41 in the nerve fibres of the portal vein, which resulted in upregulation of genes required in intestinal synthesis of glucose, or intestinal gluconeogenesis (IGN)^[138]. The IGN-derived glucose contributes to reduced appetite, improved glucose control and decreased hepatic glucose production, concomitant with lower body weight^[138,139]. These emergent outcomes of propionate-induced IGN are mediated by the portal nervous system as denervation can abolish these effects^[138,139].

It is evident that SCFA interactions with GPRs and subsequent neuroendocrine signalling affect a wide range of physiological functions, and the emergent outcomes are contingent on the type and location of the receptors as well as the agonists. As a consequence variation in microbial community composition that alters the SCFA profile can drive host responses *via* signalling pathways. The range of pathways triggered is influenced by other factors such as gut barrier function and SCFA translocation that impact which tissues are exposed to SCFA. The host responses, including appetite and intestinal motility, have potential to feedback to gut community composition.

SCFAs and immune regulation

The actions of SCFAs extend beyond energy balance and endocrine function, they are also involved in shaping immune regulation and possibly the progression of autoimmune diseases. In models of colitis, arthritis and asthma, GF mice and CONV GPR43 KO mice showed increased production of inflammatory mediators and enhanced recruitment of immune cells. Notably, exacerbated inflammation in GF mice was attenuated by acetate supplementation, supporting SCFA/GPR43 signalling resolves inflammatory responses^[140]. However, other studies have proposed that SCFA mediated GPR43 signalling also has a role in potentiating tissue destruction^[141,142].

Despite the competing views on the role of SCFAs/GPR signalling in inflammatory outcomes, SCFAs have emerged as the key microbial signal in modulating the balance of pro-inflammatory T_HHelper and anti-inflammatory T regulatory cells (T_{Reg}). Atarashi *et al.*^[143] have shown that SCFA-producing species from *Clostridium* clusters IV and XIVa had greater capacity in expanding the population of colonic T_{Reg} than *Bacteroides fragilis*, which releases polysaccharide A (PSA) to promote immune homeostasis. More importantly, SCFAs on their own can modulate T_{Reg} responses and increase the expression of anti-inflammatory cytokine interleukin-10, which dampens pro-inflammatory responses and reduces the proliferation of effector CD4⁺ T cells^[144]. Diets which promote SCFA production or administration of butyrate alone are able to recapitulate these effects^[145,146]. Butyrate can also down regulate the expression of pro-inflammatory mediators in

intestinal macrophages, such as nitric oxide, interleukin-6, and interleukin-12 by histone deacetylase inhibition, a mechanism independent of GPR activation^[147].

These host-microbial immune feedbacks in the gut are proposed to have a role in the pathophysiology of autoimmune diseases in genetically susceptible individuals, such as type 1 diabetes (T1D). T1D is characterised by T cell mediated destruction of pancreatic β cells and deficiencies in T_{reg} numbers or function^[148,149]. Given the link between butyrate and T cell homeostasis, gut microbiota might be an environmental risk factor in T1D. High throughput sequencing studies have shown that the T1D gut is depleted in butyrate producing bacteria and a key gene involved in butyrate synthesis^[8]. Butyrate depletion is linked to increased intestinal permeability, which precedes the clinical onset of T1D^[150,151]. In individuals who are genetically susceptible to T1D, an aberrant gut microbiota with reduced butyrate production is predicted to increase the risk of the following events: increased intestinal permeability, leakage of MAMPs, subclinical intestinal inflammation, homeostatic imbalance of T cells and ultimately autoimmunity in pancreas^[152,153].

In conclusion the widespread effects of SCFAs mean that factors altering their concentration and profile have multiple interacting consequences for the host and microbiome. SCFA are primary metabolites of microbial growth. Consequently the SCFA profile of the gut will be especially responsive to diet as changes in microbial nutrient supply can alter both community composition and their metabolic activity. These SCFA changes can lead to changes in gut barrier integrity, energy metabolism and inflammatory responses. All these may impact on host health, but also can feedback to impact microbial community structure. SCFAs are key factors in the interaction between gut microbiome and the host.

Hydrogen sulphide and gut epithelial function

While butyrate fortifies the structural integrity of gut epithelium, other microbial metabolites, such as H₂S, are implicated in impaired epithelial function. H₂S is produced when sulphated compounds are utilised as terminal electron acceptor in anaerobic respiration. Most gut bacteria with this capability belong to the *Desulfovibrionaceae* family^[154]. H₂S is known to interfere with energy metabolism in the gut epithelium^[155], ultimately leading to cell death, concomitant with gut inflammation^[156]. *In vitro* studies of intestinal epithelial cells have demonstrated that H₂S influences the expression of genes linked to cell cycle progression and stimulates both inflammatory and DNA repair responses^[157,158]. Collectively, there is robust evidence that H₂S has deleterious effects on the gut epithelium. A recurrent feature of HFD studies, especially those in which diet formulations have a high proportion of saturated fat, is an increase in *Desulfovibrionaceae* and gut inflammation (Table 1). Again the inferred loss of gut barrier function and associated changes in host-microbiome interaction have the potential to drive feedback responses in the microbial community.

DIET, PATHOBIONT EXPANSION AND DYSBIOSIS-A MODEL REVISITED

The interplay between diet, gut microbiome and host health has been the subject of numerous studies, and mechanisms that tip homeostasis to dysbiosis are starting to emerge. Nutrient competition is a major driver of community dynamics. Available evidence indicates that access to inorganic electron acceptors such as nitrate and sulphate occupies a special place in determining the outcome of nutrient competition between pathobionts and commensals at the epithelial interface^[9,82]. The availability of these is tightly linked to inflammation and cell damage^[9,82]. We postulate that microbes whose competitive advantage is dependent on anaerobic respiration adopt a pro-inflammatory life history strategy (which results in increased nitrate) and that their competitors promote mucosal homeostasis (which limits nitrate). Obesity and diet can skew the outcome of these opposing strategies by altering the “tipping point” at which inflammatory processes lead to elevated gut nitrate (Figure 3).

The effect of obesity, or more specifically MAT, is due to their potential to amplify the host response to metabolites that escape the intestine. Adipose tissue macrophages stimulated by MAMPs such as LPS switch to a pro-inflammatory state and increase the production of pro-inflammatory cytokines^[159]. Pro-inflammatory cytokines can “escape” from the adipose tissue and promote inflammation and insulin resistance in other tissues^[160].

The effects of diet are multiple but can be summarised as driving microbial changes that alter gut barrier function and immune tone. Diets that are depleted in fermentable polysaccharides are associated with lower levels of SCFA production. This state increases the risk of epithelial cell starvation (due to low butyrate levels) and reduces the numbers of T_{reg} cells. Both host responses have the effect of increasing the potential for inflammation. Epithelial cell starvation and/or inflammation can both increase the availability of inorganic electron acceptors in the lumen that supports expansion of pro-inflammatory pathobionts, many of which are *Proteobacteria*. At this point the potential for positive feedback exists since the LPS of *Proteobacteria* is strongly pro-inflammatory. Diets that are also high in saturated fat exacerbate this basic model. Dietary fat results in increased bile secretion which has been observed to select against key groups of fermentative bacteria. Fat types that specifically promote taurocholate may exacerbate the inflammatory processes since they are strongly linked to expansion of SRBs and production of H₂S. Collectively these two aspects of diet composition, levels of fermentable polysaccharide and saturated fat, can operate in synergy to reduce the fitness of bacteria that promote mucosal function *via* butyrate production and enhance the competitiveness of bacteria that drive inflammation *via* LPS.

In this conceptual framework there are two independent host feedback pathways, bile secretion and nitrate production, that facilitate the enrichment of pathobionts

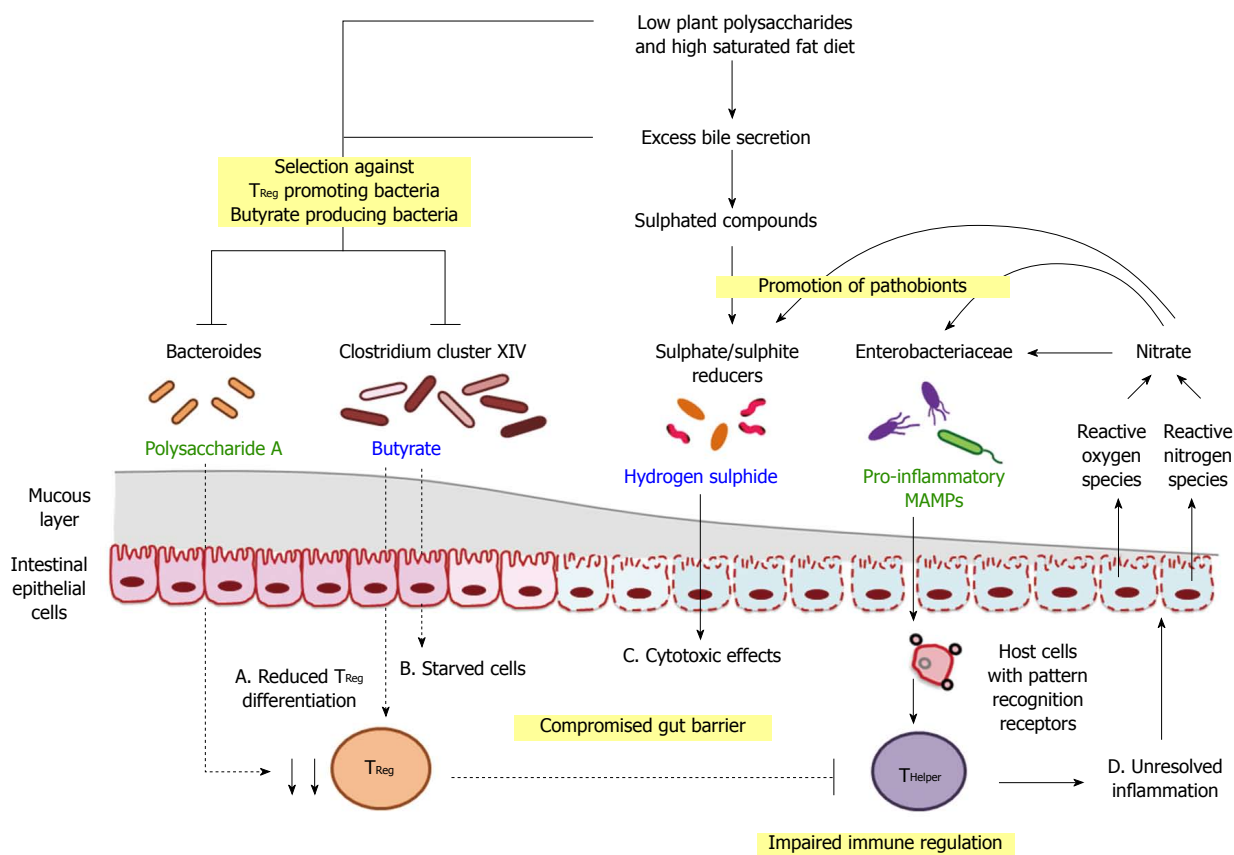


Figure 3 Hypothesised triggers and drivers in diet-induced dysbiosis. Progression from homeostasis to clinical manifestation of metabolic dysfunction may emerge from shifts in microbe-associated molecular patterns (MAMPs; green) and metabolites (blue), initiated by long-term consumption of diets with reduced amount of dietary fibre but high saturated fat. A: Reduction in the availability of fermenting substrates in conjunction with excess secretion of anti-bacterial bile acids can alter the competition dynamics of commensal organisms and pathobionts. Consequent depletion of polysaccharide A and butyrate promotes immune dysfunction by altering the balance of regulatory T cells (T_{Reg}) and helper T cells (T_{Helper}); B and C: Shifts in microbial products contribute to the impairment of gut barrier function and the leakage of MAMPs; D: Dietary factors, microbial signals and host responses act in concert to drive inflammation, which provides a positive feedback pathway in favour of chronic disease development.

and drive pro-inflammatory responses. Host feedbacks to the gut microbiome may be an important determinant in disease progression, which warrants further investigation. Furthermore, there may be more than one type of commensal or pathobiont that influence disease states, especially when alternate microbial groups fulfil similar ecological functions within the gut community. Although *Bilophila* was the leading SRB pathobiont in the initial saturated fat/taurocholic acid/inflammation model^[9], the above mechanism is applicable to other SRBs that produce H_2S , such as *Desulfovibrio* in the *Desulfovibrionaceae* family and other representatives within the *Clostridia* class^[154,161]. Similarly, several SRBs in the *Desulfovibrionaceae* family and other *Proteobacteria* have the capacity to utilise nitrate^[162] and thus, *Enterobacteriaceae* such as *E. coli* may not be the only organisms with increased fitness during inflammation.

FUTURE DIRECTIONS AND CONCLUSION

With many mechanistic links between gut community dynamics and host health are now established, microbiome-based applications for preventing and attenuating the progression of gut-related diseases are emerging. Poten-

tial therapeutic strategies may be in the form of restoring function or blocking feedback at specific nodes of the host-microbial network. If pro-inflammatory tone at the intestinal interface is the predominant driver of disease states, improving T_{Reg} ability to suppress T_{Helper} actions may ameliorate local and systemic complications associated with aberrant immune responses. Prebiotics with fermentable dietary carbohydrates are known to promote the proliferation of organisms that produce butyrate and PSA^[163,164]. Stimulation of T_{Reg} differentiation by these beneficial microbial signals may help resolve inflammation.

Aside from rational modifications in diet composition, a change in feeding cycle, *e.g.*, intermittent fasting, has been shown to have metabolic benefits^[165]. Since periodic fasting will change nutrient availability to gut microbes and potentially interrupt host feedbacks to the gut microbiome, this may also help reverse dysbiosis. However, these postulated links require further investigations for validation. In conclusion, integration of metagenomics, metabolomics and taxonomic profiling has provided important insights into the functions of gut microbiome and the role of host-microbial crosstalk in dysbiosis. Our emerging understanding of interplay between nutrition,

gut microbial dynamics and host responses will further the development of effective interventions on pathophysiology of lifestyle diseases.

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WJG 20th Anniversary Special Issues (17): Intestinal microbiota

Effect of probiotic administration on the intestinal microbiota, current knowledge and potential applications

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Abstract

Although it is now known that the human body is colonized by a wide variety of microbial populations in different parts (such as the mouth, pharynx and respiratory system, the skin, the gastro- and urogenital tracts), many effects of the complex interactions between the human host and microbial symbionts are still not completely understood. The dysbiosis of the gastrointestinal tract microbiota is considered to be one of the most important contributing factors in the development of many gastrointestinal diseases such as inflammatory bowel disease, irritable bowel syndrome and colorectal cancer, as well as systemic diseases like obesity, diabetes, atherosclerosis and non-alcoholic fatty liver disease. Fecal microbial transplantations appear to be promising therapies for dysbiosis-associated diseases; however, probiotic microorganisms have been growing in popularity due to increasing numbers of studies proving that certain strains present health promoting properties, among them the beneficial balance of the intestinal microbiota. Inflammatory bowel diseases and

obesity are the pathologies in which there are more studies showing this beneficial association using animal models and even in human clinical trials. In this review, the association of the human gut microbiota and human health will be discussed along with the benefits that probiotics can confer on this symbiotic activity and on the prevention or treatment of associated diseases.

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Key words: Probiotics; Dysbiosis; Gut microbiota; Symbiosis; Treatment

Core tip: The human body is colonized by a wide variety of microorganisms that constantly interact with the host. The dysbiosis of the gut microbiota is considered to be one of the most important contributing factors in the development of gastrointestinal as well as systemic diseases. Many studies relate the health promoting properties of probiotic microorganisms with a beneficial balance of the host intestinal microbiota. In this review, the association of the human gut microbiota and human health will be discussed along with the benefits that probiotics can confer on this symbiotic activity and on the prevention or treatment of associated diseases.

de Moreno de LeBlanc A, LeBlanc JG. Effect of probiotic administration on the intestinal microbiota, current knowledge and potential applications. *World J Gastroenterol* 2014; 20(44): 16518-16528 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16518.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16518>

INTRODUCTION

In February 2014, if one performed a MEDLINE search using the keywords “probiotics” crossed with “microbi-

ota” or “microbiome”, the total hits would be over 1294 articles. From these, almost 75% (962) were published in the last 5 years (between 2009 and the beginning of 2014) showing that the association between probiotics and microbiota is not only recent but also is gaining the attention of scientists from around the worlds. The objective of this review is to give an overview of the most recent studies that have shown that the use of probiotics can modify the human microbiota and in turn can help in the prevention or treatment of a growing number of diseases that can be caused by a dysbiosis in the microbiota composition.

DEFINITIONS

Before going any further, it is important to clearly define the 2 terms that are going to be described in this review, probiotics and microbiota. The most commonly accepted definition of probiotics was published by the World Health Organization/Food and Agricultural Organization in 2001 that stated that probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit to the host”^[1]. However, according to the International Scientific Association for Probiotics and Prebiotics (ISAPP), a non-profit scientific organization dedicated to advancing the science of probiotics and prebiotics, the term probiotic is commonly misused both commercially, when the term is featured on products with no substantiation of human health benefits, and scientifically, where the term has been used to describe bacterial components, dead bacteria or bacteria with uncharacterized health effects in humans (<http://www.isapp.net/Portals/0/docs/ProbioticDefinitionClarification.pdf>). The ISAPP does not provide a new definition for probiotics, it simply points out the important elements that are contained in the FAO/WHO definition. This being said, they clarify that a probiotic must: (1) be alive when administered; (2) have undergone controlled evaluation to document health benefits in the target host; (3) be a taxonomically defined microbe or combination of microbes (genus, species and strain level); and (4) be safe for its intended use.

Although the terms are sometimes used synonymously, “microbiome” and “microbiota” are terms that describe either the collective genomes of the microorganisms that reside in an environmental niche or the microorganisms themselves, respectively^[2-4]. The term “microflora” is an equivalent term for “microbiota” that was used in the past and still appears in recent articles. The term “microbiota” is thus “the microscopic living organisms of a region” or “the microorganisms of a particular site, habitat, or geological period” according to the Dorland’s Medical Dictionary for Health Consumers (2007) and the Oxford Dictionary, respectively.

A keyword that has gained a lot of attention is “the human holobiont”. In this theory, humans did not evolve as a single species instead they evolved with a complex-associated microbiota, building a kind of “superorgan-

ism” or holobiont^[5]. The human superorganism is a conglomerate of mammalian and microbial cells, with the latter estimated to outnumber the former by ten to one and the microbial genetic repertoire (microbiome) to be approximately 100-times greater than that of the human host^[6]. The association between the host and its microbiota (also referred to as symbiote) provides a mutual beneficial relationship. It has recently been shown that the symbiote not only protects the host from pathogens but also decreases immune disorders by immunomodulation; while the host provides shelter and nutrients to the symbiote, the symbiote in turn also improve various body functions such as digestion to provide essential nutrients to the host^[7].

Although it is now known that the human body is colonized by a wide variety of microbial populations in different parts of the human body (such as the mouth, pharynx and respiratory system, the skin, the gastro- and urogenital tracts), many effects of these complex interactions are still not completely understood. In this review, the association of the human gut microbiota and human health will be discussed along with the benefits that probiotics can confer on this symbiotic activity and on the prevention or treatment of associated diseases.

EFFECT OF THE HUMAN INTESTINAL MICROBIOTA ON HEALTH AND DISEASES

The human body has over 10^{14} microorganisms in the gastro-intestinal tract (GIT), literally 10 times more than the cells of the entire human body itself. In the past, it was thought that this microbiota was useful for the host because they could contribute nutrients and energy *via* the fermentation of non-digestible dietary components in the large intestine. Now, it is recognized that the microbiota is also extremely important to human health due to the emergence of studies that have shown that a dysbiosis of the GIT microbiota can cause diseases or that in certain diseases there is an observable change in the composition of this microbiota.

According to a recent review, a healthy microbiota is defined by high diversity and an ability to resist change under physiological stress; in contrast, microbiota associated with disease is defined by lower species diversity, fewer beneficial microbes and/or the presence of pathobionts^[8]. In this review, diet-induced dysbiosis was described to be a contributing factor in the development of gastrointestinal diseases like inflammatory bowel disease, irritable bowel syndrome and colorectal cancer (CRC), as well as systemic diseases like obesity, diabetes, atherosclerosis and non-alcoholic fatty liver disease (NAFLD) (Figure 1).

The close proximity of the GIT microbiota with the mucosa and gut lymphoid tissue helps explain why a balanced microbiota is likely to preserve mucosal health, whereas an unbalanced composition, as seen in dysbio-

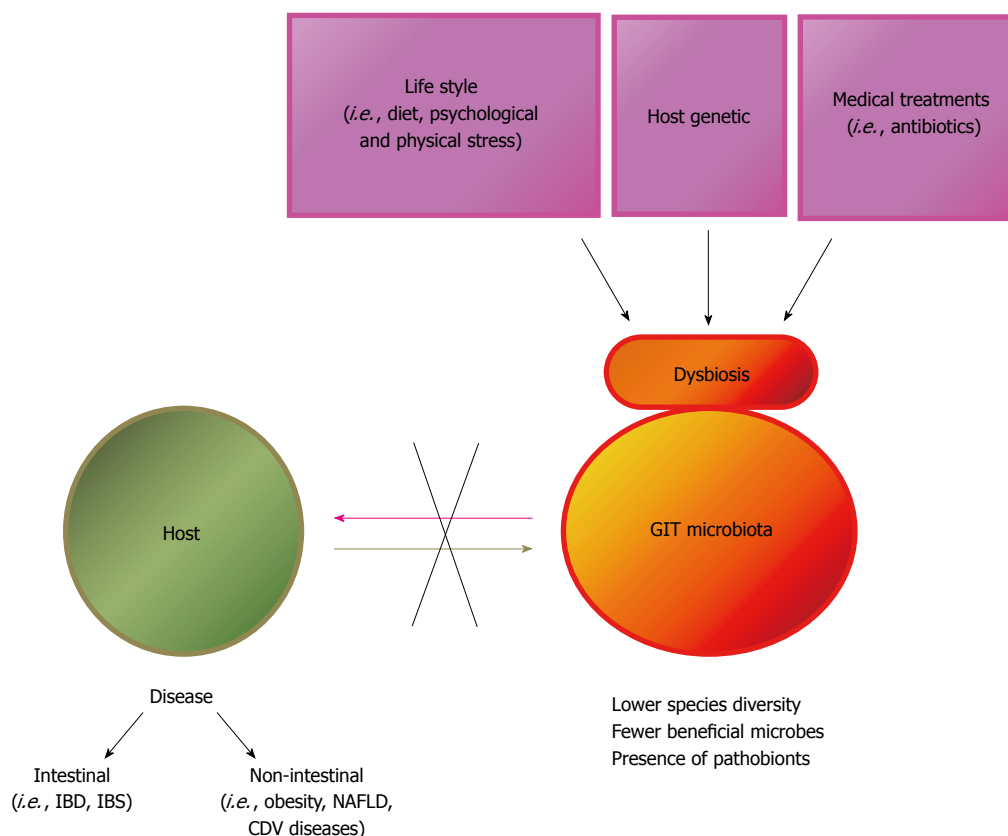


Figure 1 Causes of gastrointestinal tract microbiota dysbiosis and effect on host health. GIT: Gastrointestinal tract; NAFLD: Non-alcoholic fatty liver disease; IBD: Inflammatory bowel diseases; IBS: Irritable bowel syndrome; CDV: Canine distemper virus.

sis, may increase the prevalence of diseases not only of the mucosa but also within the body due to the strong interactions with the gut immune system, the largest immune organ of the body^[9]. Such abnormalities have been pinpointed as etiological factors in a wide range of diseases, including autoimmune disorders, allergy, irritable bowel syndrome, inflammatory bowel disease, obesity, and colon cancer. The intestinal mucosa is the body's first line of defense against pathogenic and toxic invasions from food. After ingestion, orally administered antigens encounter the GALT (Gut Associated Lymphoid Tissue), which is a well-organized immune network that protects the host from pathogens and prevents ingested proteins from hyperstimulating the immune response through a mechanism called oral tolerance. The main mechanism of protection given by the GALT is humoral immune response mediated by secretory IgA (s-IgA) which prevents the entry of potentially harmful antigens, while also interacting with mucosal pathogens without potentiating damage. The stimulation of this immune response could thus be used to prevent certain infectious diseases that enter the host through the oral route. Numerous studies have shown that certain probiotic strains can increase s-IgA and modulate the production of cytokines (mediators produced by immune cells) that are involved in the regulation, activation, growth, and differentiation of immune cells and have recently been reviewed^[10].

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD), ulcerative colitis (UC) or irritable bowel

syndrome (IBS) can arise from the disruption of immune tolerance to the gut commensal microbiota, leading to chronic intestinal inflammation and mucosal damage in genetically predisposed hosts^[11,12]. The gut microbiota composition and activity of IBD patients are abnormal, with a decreased prevalence of dominant members of the human commensal microbiota (*i.e.*, *Clostridium* IXa and IV groups, *Bacteroides*, *Bifidobacteria*) and a concomitant increase in detrimental bacteria (*i.e.*, *Sulphate-reducing bacteria*, *Escherichia coli*)^[13]. *Enterobacteria* and *Bacteroides* species have been implicated as important factors in the observed dysbiosis and in the development and recurrence of IBD^[14]. The observed dysbiosis is concomitant with defective innate immunity and bacterial killing (*i.e.*, reduced mucosal defensins and IgA, malfunctioning phagocytosis) and overaggressive adaptive immune response (due to ineffective regulatory T cells and antigen presenting cells), which are considered the basis of IBD pathogenesis.

Changes in the equilibrium of the intestinal microbiota were also associated to the presence of CRC. A comparative study of the stool microbioma of healthy individuals and CRC patients showed that butyrate-producing bacterial species were under-represented in the CRC samples and this finding was correlated with proportionately lower amounts of butyrate and higher concentrations of acetate in stools of CRC patients, compared to the healthy individuals^[15]. These results agree with the conception that butyrate is a microbial me-

tabolite reported to have anti-tumorigenic effects, which were associated to the decrease of colonic inflammation, the reinforcement of the colonic barrier and the decreasing of oxidative stress^[16]. Similar results were recently observed using a 1,2-dimethylhydrazine (DMH)-induced colon cancer model in rats. The animals from tumour group showed reduction of butyrate-producing bacteria such as *Roseburia* and *Eubacterium* in the gut microbiota. This experimental work also showed that DMH-induced carcinogenesis was associated to decrease of other beneficial species such as *Ruminococcus* and *Lactobacillus* in the gut microbiota of the rats^[17]. New studies continue to show the differences in the intestinal microbiota between healthy individual and CRC patients. In this sense, it was described that a reduction of biodiversity and richness of microbial community, with increases of bacteroides was associated with colon cancer^[18]. The analysis of the exact mechanisms by which these changes in the intestinal microbiota can be related to colon carcinogenesis are largely unknown. It was demonstrated that in CRC patients, in addition to the modification of intestinal metabolites, changes in the intestinal microbiota influence the host's immune response. In this sense, it was demonstrated that IL-17C has an important role in microbiota-mediated tumorigenesis^[19]. IL-17C was upregulated in human CRC samples and also in mouse models of CRC. IL-17C was induced in the intestinal epithelial cells by the dysregulated microbiota and promoted the survival of these cells, contributing to the tumorigenesis.

A detailed microbiota analysis of a well-characterized cohort of infants with food allergy (FA) showed that dysbiosis of fecal microbiota with several FA-associated key phylotypes, but not the overall microbiota diversity, may play a pathogenic role in FA^[20]. In this study, the proportion of abundant *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* phyla were significantly reduced, while the *Firmicutes* phylum was highly enriched in the FA group.

Recent studies have suggested that an imbalance of the intestinal microbiota may be involved in the development of obesity and type 2 diabetes mellitus (T2DM). In a recent review it was stated that a high-fat diet may induce dysbiosis, which can result in a low-grade inflammatory state, obesity and other metabolic disorders and that modifying this diet can play a role in T2DM management due to positive intestinal microbiota modulation^[21]. Also, a metagenome-wide association study analysis showed that patients with type 2 diabetes were characterized by a moderate degree of gut microbial dysbiosis, a decrease in the abundance of some universal butyrate-producing bacteria and an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring sulphate reduction and oxidative stress resistance^[22].

In another study, it was suggested that the obesity epidemic in the United States may be partly driven by the mass exposure of Americans to foods containing low-residue antimicrobial agents that can alter the composition of the gut microbiota^[23]. Studies that link microbiota

ome modifying early life events to subsequent obesity risk provide some indirect evidence to support a causal role for gut microbiota in the pathogenesis of obesity^[24]. Published data have proposed that dysbiosis of gut microbiota (at phyla, genus, or species level) affects host metabolism and energy storage stating that among the mechanisms involved, metabolic endotoxemia (higher plasma LPS levels), gut permeability and the modulation of gut peptides (GLP-1 and GLP-2) have been proposed as putative targets^[25]. The mechanisms by which the gut microbiota affects metabolic disorders such as obesity, diabetes, and cardiovascular diseases have been proposed to be by two major routes: (1) the innate immune response to the structural components of bacteria [*e.g.*, lipopolysaccharide (LPS)] resulting in inflammation; and (2) bacterial metabolites of dietary compounds (*e.g.*, SCFA from fiber), which have biological activities that regulate host functions^[26]. The concept of crosstalk, the biochemical exchange between host and microbiota, is also important to understand obesity since it maintains the metabolic health of the superorganism and whose dysregulation is a hallmark of the obese state^[27].

Since the GIT and liver are connected by the portal venous system, the liver is thus more vulnerable to translocation of bacteria, bacterial products, endotoxin or secreted cytokines present in the GIT^[8]. An obesogenic microbiota can alternate liver function by stimulating hepatic triglyceride and modulating systemic lipid metabolism that indirectly impact the storage of fatty acids in the liver^[28]. A recent systematic database search was conducted and demonstrated that common mechanisms are involved in many of the local and systemic manifestations of NAFLD that can lead to an increased cardiovascular risk, and IBS, leading to microbial dysbiosis, impaired intestinal barrier and altered intestinal motility^[29].

Studies in patients and animal disease models are shedding new light on the critical roles of the microbiota, metabolome and host responses in primary and recurrent *Clostridium difficile* (*C. difficile*) infection (CDI), which is the leading cause of antibiotic-associated diarrhea and pseudomembranous colitis in the healthcare setting^[30]. In a recent study, culture-independent pyrosequencing was used to compare the distal gut microbiota for individuals with CDI, subjects with *C. difficile*-negative nosocomial diarrhea (CDN), and healthy control subjects^[31]. This genomic analysis revealed significant alterations of organism lineages in both the CDI and CDN groups, which were accompanied by marked decreases in microbial diversity and species richness driven primarily by a paucity of phylotypes within the *Firmicutes* phylum. Normally abundant gut commensal organisms, including the *Ruminococcaceae* and *Lachnospiraceae* families and butyrate-producing C2 to C4 anaerobic fermenters, were significantly depleted in the CDI and CDN groups.

These examples of the effects of microbiota dysbiosis are just a few of the most recent studies published on the subject and show the immense lack of knowledge of the effect of the holobiont on human health. Correcting

this dysbiosis is now the aim of many groups due to the diverse diseases that are directly or indirectly associated with this imbalance of the symbiotic microbiota.

PROS AND CONS OF FECAL

TRANSPLANTATION ON THE INTESTINAL MICROBIOTA AND DISEASE

Fecal transplantation and synthetic microbiome transplants are being considered as promising therapies for dysbiosis-associated diseases.

Fecal microbial transplantation (FMT) is the process of transplantation of fecal bacteria from a healthy donor into a host with disease. Clinical criteria for inclusion and exclusion of both donor and recipient should be performed to limit the risk associated to this procedure and increase the chances of success^[32]. Fecal transplantation represents a therapy with a high potential of success, and has been mostly studied in the treatment of chronic gastrointestinal infections^[33]. The effectiveness of FMT was remarkable for recurrent *C. difficile* infection. Recently, it was reported that FMT was effective to improve clinical symptoms and eliminated fecal *C. difficile* toxins in a study of 27 patients with recurrent *C. difficile* infection who were given a single session of FMT^[34]. This effect was associated to increased microbial diversity in all the patients and the effectiveness was also associated to the correction of the metabolism of bile salts that is disrupted in patients with recurrent *C. difficile* infection^[35].

Considering that microbial dysbiosis is associated to many intestinal and non-intestinal diseases, FMT was considered for treatment of different disorders, including IBS, IBD, insulin resistance, multiple sclerosis, obesity, and heart diseases^[36]. However, its use remains controversial in patients with IBD^[37]. There is a study showing the safety and positive clinical response after FMT in children and young adults with UC^[38]. A totally different response was also reported where the case of a patient with UC (quiescent for more than 20 years) who was treated with FMT for a *C. difficile* infection and developed a flare of UC, indicating the need to be cautious in the use of this procedure in patients with IBD^[39]. It was also suggested the value of characterizing not only the composition but also the temporal dynamics of the microbiota for a better understanding of FMT efficacy in the treatment of UC^[40].

The current knowledge shows that FMT has a high potential to be used^[41], but controlled trials of FMT in specific disorders and complemented by animal models of fecal transplantation, in which variables can be controlled and manipulated, are needed before FMT can be more accepted and applied clinically. Concerns over donor-derived infections (especially viral infection that are not normally detected) also exist, and it is difficult to quantify the true risk. The possibility to modify the transplantation of whole microbial communities from a healthy donor stool by another methodology has also re-

cently been suggested in which specific fecal microorganisms grown in vitro could afterwards be transplanted^[42]. The discovery of these commensal microorganisms will lead to the development of new probiotics that can replace FMT as applied today.

EFFECT OF PROBIOTIC ADMINISTRATION ON THE INTESTINAL MICROBIOTA AND DISEASE

Probiotic microorganisms have been growing in popularity due to increasing numbers of studies proving that certain strains present health promoting properties, among them the beneficial balance of the intestinal microbiota that can be also associated to other benefits to the host (Figure 2A). The most commonly used strains as probiotics are members of *Lactobacilli*, *Enterococci* and *Bifidobacteria* groups^[43]. Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms that are present in the normal diet of many people and also in the gastrointestinal and urogenital tract of animals, and some of these claimed to be probiotics. Although most of the studies about probiotic have been mainly focused on bacteria, there are also many reports showing the potential of probiotic yeasts. In this context, Ianiro *et al.*^[44] reviewed the role of the “gut mycome”, and demonstrated that intestinal yeasts fulfill an important role in health maintenance. Selected yeast strains, especially from *Saccharomyces boulardii* were reported as probiotic, and their beneficial effects against different types of diarrhea were demonstrated using experimental animal models^[45] and also in human trials^[46,47]. Currently, many products containing LAB or other probiotic microorganisms are available on retail shelves throughout the world because of the increase consumer demand for healthier natural foods that can improve their overall well-being.

EFFECTS OF PROBIOTICS ON INTESTINAL DISEASES

It has been shown that LAB and other probiotic microorganisms can counteract inflammatory processes in the gut by stabilizing the microbial environment and the permeability of the intestinal barrier, and by enhancing the degradation of enteral antigens and altering their immunogenicity^[48]. *Lactobacillus reuteri* (*L. reuteri*) was used to prevent colitis in IL-10 knock-out (KO) mice and to increase the number of lactobacilli in the gastrointestinal tract^[49]. The normalization of *Lactobacillus* levels was obtained by oral administration of a prebiotic and rectal swabbing with *L. reuteri* to neonatal IL-10 KO mice. In a placebo-controlled trial, orally administered *L. salivarius* UCC118 reduced prevalence of colon cancer and mucosal inflammatory activity in IL-10 KO mice by modifying the intestinal microbiota in these animals with reduction in *C. perfringens*, coliforms, and enterococcus levels in the probiotic fed group^[50]. The administration of yoghurt,

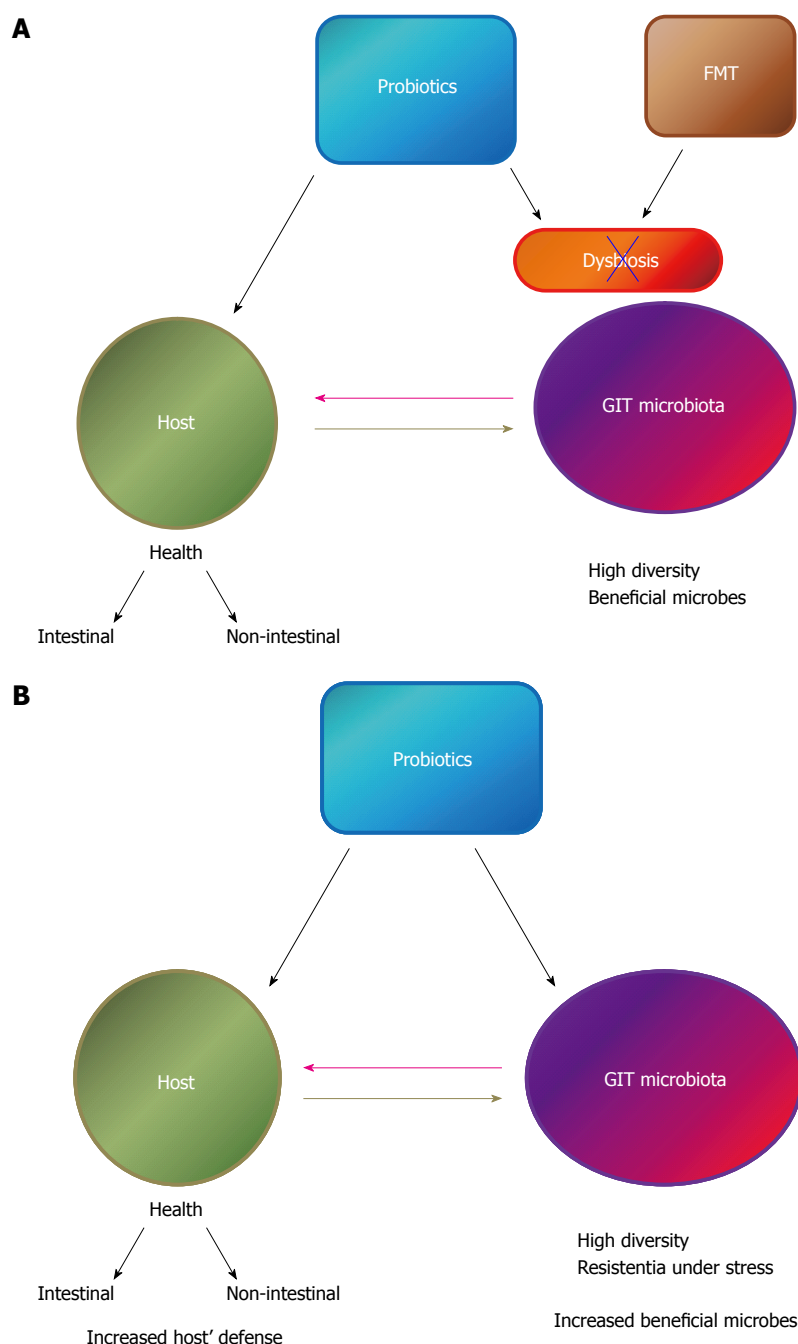


Figure 2 Effect of probiotic administration on gastrointestinal tract dysbiosis (A) or healthy individuals (B) and the interaction of the gastrointestinal tract microbiota with the host. GIT: Gastrointestinal tract; FMT: Fecal microbial transplantation.

with potential probiotic strains, decreased the inflammation by modulation of the host immune response in a trinitrobenzene sulphonic-induced mouse model of IBD. This effect was related to beneficial changes in the large intestine microbiota of the mice, with increases of bifidobacteria population^[51].

The translation of the potential use of probiotics for IBD patients remains uncertain^[52], and even when some authors reported their effectiveness against specific pathologies and the modification of GIT microbiota is one of the benefits attributed to them, there are only few reports where the fecal microbial composition of the patients was evaluated. A randomized, double-blind,

placebo-controlled trial evaluated the effect of a probiotic mixture containing *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, *Bifidobacterium breve* (*B. breve*), *B. lactis*, *B. longum*, and *Streptococcus thermophilus* in patients with IBS^[53]. The fecal flora composition was analyzed by polymerase chain reaction denaturing gradient gel electrophoresis (DGGE) and it was reported that the therapeutic effect of this probiotic mixture was associated with the stabilization of intestinal microbiota. Another study showed that probiotic supplementation (Ecologic 825, Winclove, Amsterdam, the Netherlands) to patients with UC and severe pouchitis restored the mucosal barrier, which was correlated with the bacterial diversity of mucosal pouch

microbiota^[54].

Regarding the use of probiotic yeasts, the influence of the administration of *Saccharomyces boulardii* on the composition of the fecal microbiota was evaluated in a human microbiota-associated mouse model. The animals received antibiotic treatment that induced modifications in the intestinal microbiota. The administration of probiotic yeast was related with quicker return to the initial level for the *Clostridium coccoides*-*Eubacterium rectale* and *Bacteroides*-*Porphyromonas*-*Prevotella* groups, compared to the control animals without any special administration, and this effect was suggested as a possible mechanism by which *S. boulardii* affect beneficially human with antibiotic-associated diarrhea^[55].

The use of probiotic *S. boulardii* was also examined in humans, but as was explained for probiotic bacteria, not many studies in human analyzed the modification of the intestinal microbiota. Regarding IBD patients, it was reported that *S. boulardii* was effective to reduce symptoms of disease and this was related to the improvement of intestinal microbiota composition^[56]. *S. boulardii* was also evaluated for the treatment of diarrhea-predominant IBS and its effect was compared to mesalazine^[57]. It was reported that all the treatments improved the symptoms of the patients; however, mesalazine alone or its combination with *S. boulardii* was more effective than the treatment with the probiotic yeast alone. A recent work demonstrated that probiotic *S. boulardii*, associated to conventional treatment improved the quality of life of patients with diarrhea-dominant IBS^[58]. This effect was associated to an anti-inflammatory profile of cytokines in blood and tissues of patients that receive the probiotic compared to the placebo group.

EFFECTS OF PROBIOTICS ON NON-INTESTINAL DISEASES

The use of probiotics to beneficially affect the GIT microbiota was also evaluated in non-intestinal diseases (Figure 2B). Recent studies suggested that GIT microbiota might play a critical role in the development of obesity and LAB were pointed as candidate for an anti-obesity effect^[59]. A review from 61 original articles showed that the main effect observed at the microbiota level (usually accompanied by weight loss) after probiotic or prebiotic administration in obese hosts was associated to increases in bifidobacteria populations^[60].

Studies in diet induced obese mice showed that the supplementation of *L. curvatus* HY7601 and *L. plantarum* KY1032 reduced the obesity and modulated pro-inflammatory and fatty acid oxidation-related genes in the liver and adipose tissue; and this effect was associated to modulation of gut microbiota^[61]. The relative abundance of 4 species belonging to the Ruminococcaceae and Lachnospiraceae families of the order Clostridiales and phylum Firmicutes were decreased by high fat diet and increased in mice receiving probiotic treatment. It was also observed that other GIT microbial species not asso-

ciated with changes caused by high fat diet were affected in mice that received probiotics, standing out the relative abundance of endogenous *Bifidobacterium pseudolongum*.

VSL#3 is a mixture containing eight different strains of probiotic bacteria that was evaluated against different diseases, including the prevention and treatment of obesity and diabetes in several mouse models. This effect was associated to the modulation of the gut microbiota-short chain fatty acid (SCFA)-hormone axis^[62]. VSL#3 supplementation induced changes in the microbiota that were associated with an increase in the levels of butyrate, and it was demonstrated *in vitro* that this SCFA stimulated the release of GLP-1 from intestinal cells. The hormone GLP-1 reduces food intake and improves glucose tolerance.

Recently, the beneficial effect of *L. coryniformis* CECT5711 was demonstrated in a high fat diet induced mouse model. Probiotic administration to obese mice induced marked changes in microbiota composition and reduced the metabolic endotoxemia by decrease of the LPS plasma level^[63].

The effect of probiotics in humans was also observed; however, as was explained for other pathologies, there are not many articles that evaluate the intestinal microbiota. A clinical trial with the probiotic bacterium *L. salivarius* Ls-33 was conducted in obese adolescents to investigate the impact on fecal microbiota^[64]. Ratios of *Bacteroides*-*Prevotella*-*Porphyromonas* group to Firmicutes belonging bacteria were significantly increased after administration of Ls-33; however, these changes were not related to effects on their metabolic syndrome.

A randomized, double-blind, placebo controlled study was conducted in order to evaluate the effects of probiotic capsule when combined with herbal medicine in treatment of obesity^[65]. In this trial, each probiotic capsule contained viable cells *Streptococcus thermophilus*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus*, *B. lactis*, *B. longum*, and *B. breve*. It was reported that probiotic administration prevented endotoxin production, which can lead to GIT microbiota dysbiosis associated with obesity. Gut *B. breve* population showed negative correlation with endotoxin level.

NAFLD is a disease linked to obesity and the beneficial role of probiotics was also reported^[66]. Recently, it was shown that *L. rhamnosus* GG protected against NAFLD in a mice model^[67]. The effect was associated to increase total bacterial numbers including the phyla Firmicutes and Bacteroidetes in the distal small intestine. This result was in concordance to the previous one that reported modulation of the microbiota in the small intestine with a concomitant anti-obesity effect in mice that received *L. rhamnosus* GG and *L. sakei* NR28^[68].

The human GIT microbiota has also been related with a possible cardiovascular risk. GIT microbiota profiles were not only associated with metabolic diseases, but also the flux of metabolites derived from microbial metabolism of choline, phosphatidylcholine and l-carnitine that contribute directly to cardiovascular disease. In this sense, probiotics were reported among dietary strategies to modulate the GIT microbiota or their metabolic ac-

tivities^[69-71]. The improvement of disease biomarkers, especially plasma cholesterol levels, appears to be possible after probiotic administration to lower cardiovascular risk. In this sense, it was shown that the administration of a probiotic soy product containing *Enterococcus faecium* CRL 183 and *L. helveticus* 416 supplemented or not with isoflavones was associated with an improved cholesterol profile and inhibition of atherosclerotic lesion development in a rabbit model^[72]. The authors reported that of *Enterococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. were negatively correlated with total cholesterol, non-HDL-cholesterol, and lesion size. The intake of the probiotic soy product increased significantly these bacterial species in the fecal microbiota.

EFFECTS OF PROBIOTICS IN HEALTHY HOSTS

There are also reports that showed the potential of probiotics in healthy hosts, maintaining a balanced microbiota, which, as was explained above, is an important key for health. The consumption of a probiotic product containing *L. coryniformis* CECT5711 and *L. gasseri* CECT5714 was analyzed in 30 children with no gastrointestinal pathology^[73]. An increase in faecal lactobacilli counts was shown at the end of the experimental protocol, and these findings were associated to enhancing the defence against gastrointestinal aggressions and infections and enhancing the immune function with increase IgA concentration in faeces and saliva. A recent work reported a clinical trial that included 40 participants with no known digestive diseases. *Laminaria japonica*, a widely used ingredient in seaweed kimchi, and LAB of traditional fermented Korean food were given to volunteers and was related to increases in the number of some administered LAB species in their GIT microbiota^[74].

CONCLUSION

The dysbiosis of the gastrointestinal tract microbiota is considered to be one of the contributing factors in the development of certain gastrointestinal and non-gastrointestinal diseases. Fecal transplantations appear to be promising therapies for dysbiosis-associated diseases; however, controlled trials of FMT in specific disorders are needed before FMT can be more accepted and applied clinically. The possibility to modify the traditional FMT by specific probiotic fecal microorganisms was also reported and would be a better alternative from a safety and therapeutic point of views.

Recent reports showed the potential of the administration of specific probiotic strains to improve the balance of the GIT microbiota that is altered in different diseases, being IBD and obesity the pathologies in which there are more studies showing this association using animal models and even in human clinical trials. The importance of probiotic consumption in healthy hosts was also demonstrated because its relationship with beneficial

balance in GIT microbial populations, which is also associated to improved defense against gastrointestinal aggressions and infections and the enhancing of the host's immune function.

However, as was explained for FMT, there are not enough human trials where the application of probiotics as biotherapeutic agents was evaluated in double-blinded large scale clinical trials. These assays are very important before the medical community will accept the addition of probiotic as supplements for specific patients with diseases associated to gut microbial dysbiosis as a viable alternative to FMT.

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WJG 20th Anniversary Special Issues (18): Pancreatitis

Drug induced acute pancreatitis: Does it exist?

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Core tip: While the literature has reported over 130 drugs as causing acute pancreatitis, the evidence that these drugs have a true causal role is lacking in the vast majority of drugs. While idiopathic pancreatitis is common, accounting for almost a third of patients with acute pancreatitis, drug induced acute pancreatitis is probably an uncommon, perhaps a rare disease. Before a clinician blames a drug as causing acute pancreatitis, a thorough evaluation for more common causes should be made, even a consideration that the disease is merely idiopathic.

Abstract

As the incidence of acute pancreatitis continues to rise, establishing the etiology in order to prevent recurrence is important. Although the etiology of acute pancreatitis is not difficult in the majority of patients, almost a quarter of patients are initially labeled as having idiopathic acute pancreatitis. When confronted with a patient with acute pancreatitis and no clear etiology defined as an absence alcoholism, gallstones (ultrasound and/or MRI), a normal triglyceride level, and absence of tumor, it often appears reasonable to consider a drug as the cause of acute pancreatitis. Over 100 drugs have been implicated by case reports as causing acute pancreatitis. While some of these case reports are well written, many case reports represent poorly written experiences of the clinician simply implicating a drug without a careful evaluation. Over-reliance on case reports while ignoring randomized clinical trials and large pharmaco-epidemiologic surveys has led to confusion about drug induced acute pancreatitis. This review will explain that drug induced acute pancreatitis does occur, but it is rare, and over diagnosis leads to misconceptions about the disease resulting in inappropriate patient care, increased litigation and a failure to address the true entity: idiopathic acute pancreatitis.

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PROBLEM OF IDIOPATHIC PANCREATITIS

Idiopathic Acute Pancreatitis accounts for 20-40 percent of patients with acute pancreatitis^[1,2]. That is, normally, approximately a third of patients who present with acute pancreatitis defy the clinician's ability to determine what caused the disease. Idiopathic acute pancreatitis is defined as acute pancreatitis with no etiology established after initial laboratory (including lipid and calcium level) and imaging tests (trans-abdominal ultrasound, MRI and CT in the appropriate patient)^[3]. These patients do not have gallstones, a significant history of alcohol use, hypertriglyceridemia and a tumor. Anatomic and physiologic anomalies of the pancreas occur in 10%-15% of the population, including pancreas divisum and sphincter of Oddi dysfunction^[4]. However, it remains controversial if these disorders alone cause acute pancreatitis.

There may be a combination of factors, including anatomic and genetic, that predispose to the development of acute pancreatitis in susceptible individuals^[5]. The influence of genetic defects, such as cationic trypsinogen mutation, SPINK, or CFTR mutations, in causing acute pancreatitis is being increasingly recognized. These defects, furthermore, may also increase the risk of acute pancreatitis in patients with anatomic anomalies, such as pancreas divisum^[6]. The idea that acute pancreatitis may result from a combination of factors working together should not be a surprise when one considers that most patients with gallstones, hypertriglyceridemia, alcoholism and pancreas divisum will never develop acute pancreatitis.

Clinician's caring for a patient with acute pancreatitis yearn to find a diagnosis to prevent a recurrent attack. This is compounded by the patient's desire to understand what has happened to them to cause so much pain and suffering. In addition to endoscopic interventions, clinicians search the literature for possible causes. The profession demands it, the patient's deserve it, and the literature provides a plethora of possibilities.

DRUGS AS A CAUSE OF ACUTE PANCREATITIS

Most patients who are admitted to a hospital are already taking a medication. Nearly 240 million Americans take at least one prescription drug weekly, and pharmacies fill over ten million prescriptions each day^[7]. Over 100 drugs have been reported to cause acute pancreatitis in the scientific literature. Most reviews claim that drug induced acute pancreatitis accounts for 3%-5% of all cases of acute pancreatitis^[8]. The diagnosis of drug induced acute pancreatitis is difficult to establish since drug-induced pancreatitis rarely is accompanied by clinical or laboratory evidence of a drug reaction, such as rash, lymphadenopathy, and/or eosinophilia, few ancillary data are available to help with the diagnosis.

While a few medications have been reported to cause acute pancreatitis based on a large body of evidence, most of the drugs implicated are based on case reports that suffer from a combination of inadequate criteria for the diagnosis of acute pancreatitis, failure to rule out more common etiologies, and/or lack of a rechallenge with the medication^[9]. A rechallenge is a case in which a patient who develops acute pancreatitis has the medication suspected as causing acute pancreatitis withheld. After the acute pancreatitis resolves, the medication is restarted (typically as the medication was not originally suspected of causing the acute pancreatitis). Within a period of time (typically shorter), after the medication is restarted, the patient has another attack of acute pancreatitis. A valid rechallenge case report should be considered when evaluating whether a particular drug causes acute pancreatitis; however, it is not proof of causation. For example, it is clear that many patients with idiopathic pancreatitis or microlithiasis have recurrent attacks of acute pan-

Table 1 Classification of drug induced pancreatitis

Class I a drugs
At least 1 case report with positive rechallenge, excluding all other causes, such as alcohol, hypertriglyceridemia, gallstones, and other drugs
Class I b drugs
At least 1 case report with positive rechallenge; however, other causes, such as alcohol, hypertriglyceridemia, gallstones, and other drugs were not ruled out
Class II drugs
At least 4 cases in the literature
Consistent latency (75% of cases)
Class III drugs
At least 2 cases in the literature
No consistent latency among cases
No rechallenge
Class IV drugs
Drugs not fitting into the earlier-described classes, single case report published in medical literature, without rechallenge

creatitis. Therefore, stopping and restarting a drug with recurrence of pancreatitis may be a coincidence and not cause and effect^[10].

Badalov *et al*^[9] published an extensive review of published case reports in the peer reviewed literature. Using criteria based on the presence of a rechallenge, latency, and the number of case reports (Table 1), a classification system "based on the evidence" was provided. Table 2 shows the medications from the published case reports with the "most evidence" of causing acute pancreatitis. At the time the authors published the paper, none of them were aware that the United States Food and Drug Administration ("FDA") and trial lawyers would use the classification as a partial basis for assigning blame to drugs as causing acute pancreatitis^[11].

FDA ADVERSE EVENT REPORTING SYSTEM

Through the Federal Food, Drug, and Cosmetic Act ("FDCA"), the FDA is empowered to verify the safety of drugs on the market^[12]. Although the FDA employs a rigorous review process to ascertain the safety and efficacy of drugs prior to approval, reports have consistently warned that pre-market research often fails to provide an accurate risk-benefit profile for marketed products^[13]. Many drugs come to the market and subsequently are found to have significant side effects that pre-market trials did not reveal^[14]. To rectify this problem, the FDA had developed the Adverse Event Reporting System (FAERS)^[15].

Based on "MedWatch Reports"^[16] filed by interested clinicians, the FDA's reporting programs generate a "deluge of information. Annually the agency has received more than 200000 adverse event reports regarding drugs or biologic products. It is not surprising that the agency describes its analysis of this flood of data as triage^[17]. The reports are typically incomplete and often, biased. Although more work on the database and system is need-

Table 2 Summary of drug-induced acute pancreatitis

Class 1a
Azodisalicylate; Bezafibrate; Cannabis; Carbimazole; Codeine; Cytosine; Arabinoside; Dapsone; Enalapril; Furosemide; Isoniazid; Mesalamine; Metronidazole; Pentamidine; Pravastatin; Procainamide; Pyritinol; Simvastatin; Stibogluconate; Sulfamethoxazole; Sulindac; Tetracycline; Valproic acid
Class 1b
All trans-retinoic acid; Amiodarone; Azathioprine; Clomiphene; Dexamethasone; Ifosfamide; Lamivudine; Losartan; Lynesterol/methoxyethinylestradiol; 6-MP; Meglumine; Methimazole; Nelfinavir; Norethindronate/mestranol; Omeprazole; Premarin; Sulfamethazole; Trimethoprim-sulfamethazole
Class 2
Acetaminophen; Chlorthiazide; Clozapine; DDI; Erythromycin; Estrogen; L-asparaginase; Pegaspargase; Propofol; Tamoxifen

ed to distinguish reliable findings from “variability and noise”, more resources are necessary and lacking^[18].

Despite incomplete data, the FDA often relying on the FAERS will issue warnings and require manufacturers to add “black box warnings” intended to alert physicians to the importance of the adverse information learned. However, with premature data causing unsubstantiated fears, the FDA has added, modified, and often removed black box warnings from the drugs in question. The addition of these black box warnings has fueled litigation^[17].

AN ILLUSTRATION OF THE FALLACY OF DRUG INDUCED ACUTE PANCREATITIS: EXENATIDE (BYETTA®)

The claim that Byetta (exenatide and other GLP-1 agonists) cause acute pancreatitis exemplifies the problem with drug induced acute pancreatitis. Based on case reports, especially following the criteria set forth in the paper by Badalov *et al*^[9] MedWatch reports, the FAERS, resultant black box warnings, and poor science, confusion and litigation resulted as “experts” claimed the exenatide caused acute pancreatitis.

Exenatide, an incretin mimetic, was approved as Byetta by the FDA on April 25, 2005. The drug is an adjunctive therapy to improve glycemic control in patients with type II diabetes mellitus. The first published case reports of acute pancreatitis thought to be caused by exenatide appeared shortly after the drug was approved^[19]. Additionally, by December 31, 2006, according to the FAERS database, there were 48 documented domestic cases of acute pancreatitis in patients taking exenatide^[20]. Noting slightly more cases of acute pancreatitis than expected in the general population, the FDA asked the manufacturer, Lilly and Amylin Pharmaceuticals to strengthen the labeling of acute pancreatitis from the Adverse Reactions section to the Warnings and Precautions section of the exenatide label.

While the FDA was comparing the incidence of acute pancreatitis in the exenatide using diabetic population to the general population, it is not clear that they were

Table 3 Methods of causal inference

Randomized controlled trials
Controlled trials without randomization
Cohort studies
Case-control studies
Ecologic studies
Case reports and case series

aware that diabetic persons were at a significant increased risk of developing acute pancreatitis. For a variety of reasons, including increased incidence of gallstones and hypertriglyceridemia, the incidence of acute pancreatitis in patients with diabetes is higher than the general population^[21]. Therefore, regardless of the drug used, if one simply compared the normal population incidence of acute pancreatitis with the diabetic population, one would find a higher incidence in the diabetics. This is a classic confounding variable rather than a drug effect.

The limitations to Medwatch reports cannot be overstated. In many reports the diagnosis of acute pancreatitis is not clearly established. Thus, there is no reason to proceed with considering the case as the adverse event suspected may be another pathology in the abdomen. Misdiagnosis of acute pancreatitis often occurs by clinicians who search for a reason for abdominal pain and merely rely on mild elevations in the amylase and lipase to reach a diagnosis. This is not appropriate, however, as any inflammatory process in the abdomen can cause a mild 2-3 fold elevation of the amylase and/or lipase^[22]. Additionally, many patients with diabetes have been shown to have mild elevations, greater than three times the upper limit of normal, of amylase and/or lipase^[23]. Thus, many patients with abdominal pain from other sources are falsely labeled as having acute pancreatitis. In the patients who truly have acute pancreatitis, many of the reports fail to identify if the patient has more likely causes of acute pancreatitis, such as gallstones, a history of alcoholism, hypertriglyceridemia^[9].

Despite the limitations to the reports and the FDA's position that the FAERS is for hypothesis testing, Elashoff and colleagues^[24] examined the FAERS database from 2004-2009 for reported adverse events for exenatide and other medications (which served as controls) in order to determine if patients were at an increased risk of developing pancreatitis. The authors found that the risk of developing pancreatitis from exenatide was higher compared to from other therapies, but importantly the issue of reporting bias could not be entirely ruled out.

Although the FDA agreed to study the issue further, in the meantime it required Amylin and Lilly to alert health care professionals in several ways - including *via* industry letters, published articles, and reports of these cases in the FDA Newsletter^[25]. The result was a surge of FAERS cases involving exenatide as a cause of acute pancreatitis immediately followed the FDA notification requirement. Despite the obvious reporting bias induced by the FDA notification, and the failure of the FDA to

Table 4 Bradford Hill criteria for causation

Temporality - causal factor must precede effect
Strength of association - magnitude of the relative risk estimates observed
Consistency of the association - extent to which scientific results are similar across the entire body of evidence
Biologic gradient (dose-response) - the extent to which the relative risk estimates increase in magnitude as the dose of the exposure increases
Biologic plausibility - the extent to which a mechanism of action has been proposed, studied and demonstrated in toxicological or other laboratory based studies
Specificity - refers to the precision with which the exposure and the outcome can be defined
Coherence - the extent to which the evidence and hypotheses for the results fit together into a reasonable and well-tested explanation
Experimentation - the extent to which a randomized clinical trials or cohort studies are available
Analogy - the extent that the purported exposure-disease relationship under consideration is similar to other relationships

note that the population using exenatide-diabetics-inherently had a predisposition for acute pancreatitis, the FDA subsequently added a black box warning to the drug's labeling. The black box warning stated that exenatide could cause acute pancreatitis^[26].

Immediately thereafter, thousands of persons who had developed acute pancreatitis while taking exenatide initiated multiparty litigation suits. They relied on the FAERS database and resultant black box warning. The plaintiffs, diabetics already at risk for developing acute pancreatitis, claimed that the defendants Lilly and Amylin Corporations knew or should have known of the hazards associated with exenatide in causing acute pancreatitis. In addition, by claiming that the defendants actively concealed information that demonstrated the dangers of their drug and thus misled the public and prescribing physicians, the plaintiffs were granted broad access to company documents during discovery^[27]. The costs of litigation skyrocketed.

Despite the persistent litigation occurring, over the last year, the FDA independently evaluated the post marketing reports that exenatide was a cause of acute pancreatitis. After an exhaustive evaluation of more than 250 toxicology studies conducted in nearly 18000 live animals, no evidence of pancreatic disease was found^[28]. In addition to the laboratory data, the FDA reviewed data from 200 trials (including other GLP-1 agonists), involving 41000 patients, and found no evidence of an increased risk of pancreatic disease. The FDA has promulgated that "assertions concerning a causal relationship between incretin drugs are inconsistent with the scientific literature. Simply, despite case reports and MedWatch reports, exenatide does not cause acute pancreatitis.

RETHINKING CAUSATION

It is important to use the general scientific method in making causal claims about human health and disease^[29]. The basic structure of the scientific method to determine causation includes: hypothesis generation, observable predictions, alternatives, and tests to distinguish between the causal hypothesis of interest and its alternatives. There could be competing explanations for any scientific observation. Epidemiologic methods involving human subjects are the most important means for identifying and testing hypotheses involving human disease causation. Random-

ized controlled trials are the strongest means and case reports are the lowest means^[30] (Table 3). The use of the scientific method avoids falsely claiming causation when the truth is mere chance. Chance is not the only alternative to causation, but must be considered strongly.

The criteria of causation is best understood by the Hill criteria^[31]. An "association" in this methodology is not satisfied by the existence of individuals with exposure to the putative cause and the disease of concern. Rather, an "association" from a causal perspective would only exist if a statistically-significant relationship (*e.g.*, between the rate of acute pancreatitis in patients with diabetes mellitus patients exposed to exenatide and the rate of acute pancreatitis in similar diabetic patients not exposed to exenatide) was demonstrated in analytical epidemiological studies. Those studies should be well-designed, with careful attention to diagnostic criteria, adherence to medication, control of confounders, and avoidance (or correction) of important sources of bias. Case reports would never meet this level of evidentiary need to determine causation.

Hill's 9 criteria evaluate the totality of evidence for causation evaluating for temporality, strength of association, consistency of the association, the presence of a biologic gradient (dose-response), biologic plausibility, specificity, coherence, experimentation and analogy (Table 4). In applying the 9 criteria to a drug like exenatide, the evidence shows no causal association. There is no temporality as the latency for exenatide causing acute pancreatitis varies among the reports. As to strength, large epidemiologic studies show no causal relationship of exenatide to acute pancreatitis. There is no consistency of the data. Results from clinical trials, epidemiology, case reports, and animal studies are inconsistent. Based on animal and clinical trial data there is no biologic plausibility (no established mechanism) or gradient. There is no evidence that increase in dosage and/or increase in time results in a linear increase in episodes of acute pancreatitis. Experimental data, in both animals and humans, do not establish that exenatide is a cause of acute pancreatitis. There is also a coherence that exenatide does not cause acute pancreatitis from laboratory, clinical, case report, and epidemiologic studies. Analogy to other anti-diabetic drugs does not strengthen the causal hypothesis as other GLP-1 agonists have also been shown not to cause acute pancreatitis from clinical trials.

Making reliable causal claims in pharmacovigilance is difficult if not impossible when case reports and case series are used as the primary evidentiary source^[15]. While the case reports and series generate hypothesis testing, as was shown for exenatide, it is irresponsible to assign causation based on causal hypothesis^[32].

DRUG INDUCED ACUTE PANCREATITIS AND IDIOPATHIC ACUTE PANCREATITIS

Although the vast majority of drugs that have been purported to cause acute pancreatitis probably do not, drug induced acute pancreatitis does exist! When evaluating drugs for causation on the basis of the evidence as described by Hill, two drugs meet the evidence of causation: Azathioprine (and its metabolite 6-mercaptopurine) and 2'3'-dideozinosine (DDI). The strong evidence comes not from case reports but a consideration of the totality of the evidence, including randomized prospective trials, cohort trials, case reports and a molecular basis^[33,34]. For example, in the National Cooperative Crohn's Disease study, almost 6% of the 116 patients treated with 6-MP developed acute pancreatitis^[35]. Similarly, Haber *et al*^[36] treated 400 patients with inflammatory bowel disease with 6-MP and 3.25% developed acute pancreatitis. There are many more randomized trials that support the simplistic case reports.

More recently, Floyd *et al*^[34] performed a large population based study including 1388 patients taking azathioprine and 13836 controls in a single county. The incidence rate for acute pancreatitis among all users of azathioprine was one per 659 treatment year. The crude odds ratio (OR) of having redeemed prescriptions for azathioprine within 90 d before admission for acute pancreatitis was 7.5 (95%CI: 2.6-21.6). After adjustment for gallstone disease, alcohol-related diseases, inflammatory bowel disease, and use of glucocorticoids, the OR increased to 8.4 (95%CI: 2.4-29.4). Although there was a significant risk of persons on azathioprine in developing acute pancreatitis, the population-attributable risk, which measures the proportion of all cases of pancreatitis that are attributable to the use of azathioprine in this study population, was 0.4%.

This finding of less than a half percent attributable risk of azathioprine as a cause of acute pancreatitis is extremely important when considering the claims that drug induced acute pancreatitis accounts for 3%-5% of all cases^[37-39]. In the absence of data from controlled clinical trials and large pharmacoepidemiologic trials, there is little to no evidence that other drugs cause acute pancreatitis. Although similar data exists for DDI, the drug is not widely used at this time^[40,41]. Therefore, drug induced acute pancreatitis probably accounts for less than 1% of cases, and maybe extremely rare in patients who are not taking obvious drugs.

Premarket approval and post-marketing surveillance has become sensitive to determining complications of drugs such as acute pancreatitis. Randomized controlled

trials that evaluate for other complications, such as cardiac complications, would detect significant risks of drugs causing acute pancreatitis^[42]. In addition, large pharmacoepidemiologic databasis and meta-analyses are often searched for signals to determine whether drugs cause acute pancreatitis^[43].

Azathioprine (and 6-MP) and exenatide represent the two extremes of the data demonstrating a causal association for a drug and acute pancreatitis. While there are case reports in the literature and Medwatch reports on the FARS that both drugs cause acute pancreatitis, only for azathioprine (and 6-MP) have multiple randomized controlled trials and large pharmacoepidemiologic studies showing a statistically significant association. For exenatide (and the other GLP-1 agonists), the opposite is true. Multiple controlled trials, pharmacoepidemiologic databases fail to show any causal association with acute pancreatitis.

While clinicians continue to publish case reports blaming drugs as causing acute pancreatitis, it is important to consider the ideas discussed in this paper. Be critical, cynical and remember that idiopathic pancreatitis is common. Clinicians should perform a thorough workup as described to verify the absence of gallstones, alcoholism, hypertriglyceridemia, tumors. However, the struggle to identify a cause, especially in assigning blame to a drug should be done with extreme caution. When a patient asks "what caused my acute pancreatitis?" Clinicians must remember that almost a third of cases will not be clear and are labeled as idiopathic. As clinicians do not have trouble explaining to patients that "bad luck" is the cause of appendicitis, diverticulitis, cholecystitis, telling a patient that it appears simply "idiopathic" may be correct.

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Evidence for a role of mitogen-activated protein kinases in the treatment of experimental acute pancreatitis

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termine the onset and course of the disease have been explained. Aim of this article is to review the role of mitogen-activated protein kinases in pathogenesis of acute pancreatitis.

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Key words: Experimental acute pancreatitis; Mitogen-activated protein kinases; Mitogen-activated protein kinases inhibitors; Cytokines; Cholecystokinin; Cerulein

Core tip: The review focuses on the role of mitogen-activated protein kinases (MAPKs) in the treatment of acute pancreatitis. In fact, acute pancreatitis is a disease characterized by a marked inflammatory reaction and it is usually associated with severe upper abdominal pain, organ failure and also mortality. The activation of MAPKs is an early event in AP and exerts a central role in the onset and development of acute pancreatitis. Thanks to the pivotal function played by MAPKs in acute pancreatitis, the use of specific inhibitors may represent a potential therapeutic target for the treatment of this inflammatory disease.

Abstract

Acute pancreatitis (AP) is an inflammatory disease characterized by acute inflammation and necrosis of the pancreatic parenchyma. AP is often associated with organ failure, sepsis, and high mortality. The pathogenesis of AP is still not well understood. In recent years several papers have highlighted the cellular and molecular events of acute pancreatitis. Pancreatitis is initiated by activation of digestive enzymes within the acinar cells that are involved in autodigestion of the gland, followed by a massive infiltration of neutrophils and macrophages and release of inflammatory mediators, responsible for the local and systemic inflammatory response. The hallmark of AP is parenchymal cell necrosis that represents the cause of the high morbidity and mortality, so that new potential therapeutic approaches are indispensable for the treatment of patients at high risk of complications. However, not all factors that de-

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease characterized by acute inflammation and necrosis of the pancreatic parenchyma^[1]. AP is often associated with organ failure, sepsis and high mortality. Approximately 20% of patients may develop a more severe form of the disease

with evidence of organ dysfunction^[2]. 80% of cases of acute pancreatitis are associated with alcohol excess or gallstones; 10% are idiopathic and a further 10% are related to trauma, biliary interventions and drugs such as antibiotics, diuretics, immunosuppressants and antiretroviral agents. Pancreatitis is associated with parenchymal oedema and apoptosis^[3]. The pathogenesis of acute pancreatitis (AP) is still not well understood. In recent years several papers have highlighted the cellular and molecular events of acute pancreatitis. It is now generally known that pancreatitis is initiated by premature activation of digestive enzymes within the acinar cells leading to autodigestion of the gland, followed by a massive infiltration of neutrophils and macrophages and production of inflammatory mediators released from the infiltrated pancreatic connective stroma, such as cytokines, adhesive molecules, platelet activating factors, nitric oxide, oxygen reactive species and lysosomal enzymes that represent the cause of the local and systemic inflammatory response. The hallmark of AP is parenchymal cell necrosis that is responsible for the high morbidity and mortality, so that new potential therapeutic approaches are essential for the treatment of patients at high risk of complications^[1,4]. However, not all factors that determine the onset and course of the disease have been explained. Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with several cellular activities as cell proliferation, differentiation, survival, death, and transformation^[5]. It has been hypothesized that activation of mitogen-activated protein kinases (MAPKs) is an early event in AP and seems to exert a central role in development and onset of AP^[6]. Aim of this article is to review the role of MAPKs in pathogenesis of acute pancreatitis and the potential of MAPKs as therapeutic targets.

MAPKS SIGNALING PATHWAY AND ACUTE PANCREATITIS: THE ROLE OF ERK AND JNK

One of the most important cascades involved in several cellular processes is the mitogen-activated protein kinases (MAPKs) pathway. MAPKs play key roles in signal transduction pathways and are involved in directing cellular response to a variety of stimuli and regulate processes as gene expression, differentiation, mitosis, cell survival, and apoptosis^[7-9]. There are three major classes of MAPKs in mammals, the extracellular signal-regulated kinases (ERKs) and the two stress-activated protein kinase (SAPKs) families, c-jun N-terminal kinase (JNK) and p38. MAPKs are activated *via* a signalling cascade that is conserved from yeast to mammals^[10,11]. ERK1/2 is mainly activated by mitogens stimuli through the Ras/Raf pathway but can also be activated, independently of Ras, by proinflammatory stimuli including cytokines. JNK and p38 are mainly activated by a variety of stresses and proinflammatory stimuli. Once activated, MAPKs path-

ways orchestrate the recruitment of gene transcription leading to activation of cellular mechanisms such as proliferation, cell differentiation, and inflammation regulated by the release of others growth factors and hormones. In recent years much interest has focused on inhibitors of the mitogen-activated protein kinases (MAPKs) primarily because they have been implicated as key regulators of inflammatory diseases as acute pancreatitis. It has been demonstrated that activation of MAPKs signaling cascades is an early event in AP contributing to the progression of acute pancreatitis^[12,13]. Indeed, MAPKs pathways participate to the release of inflammatory mediators highly involved in the development of inflammatory reaction from local to the systemic level^[14,15]. At cellular level, time course of MAPKs activation showed that the p38 MAP kinase increases in pancreatic acinar cells most rapidly, with the peak of activity after three hours. JUN kinase activity is the highest after 12 h and after 24 h its activity becomes undetectable^[16,17]. Involvement of MAPKs cascade in the pathogenesis of AP is also demonstrated by the fact that hyperstimulation with cholecystokinin (CCK) activates the two isoforms of ERK, p42 and p44, and JNK/SAPK (slowly activated compared with ERK) in pancreatic acini^[12,18]. Moreover, CCK activation of JNK/SAPKs results slower than ERK's activation, so that CCK's concentrations for the activation of JNK/SAPKs are higher than the concentrations required for the activation of ERK. Cerulein (CER) is a cholecystokinin-pancreozymin analogue used for experimental acute pancreatitis models in rats and mice, leading to proteolytic enzyme secretion that causes pancreatic acinar autolysis with progressive interstitial oedema just one hour after injection^[19]. The stimulation with a low dose of caerulein causes physiological activation both ERKs and JNK/SAPKs. Hyperstimulation both *in vitro* and *in vivo* determines an increase of JNK/SAPKs as a consequence of cellular stress. So, it has been demonstrated that after CCK or CER stimulation *in vitro* as well as *in vivo*, the activation of ERK occurs early than JNK's activation^[20]. JNK and ERK1/2 were proposed as important early mediators during caerulein-induced pancreatitis due to their pattern and activation time course^[21]. Activation of ERK1/2 and JNK occurs within 5 min, peaks within 30-40 min and decreases, generally, within 1 h following caerulein hyperstimulation. The two MAP kinases cannot be detected anymore 2 h after caerulein injection, thus confirming the very early involvement of this signalling pathway in the inflammatory cascade^[22]. Active MAPKs are responsible for the phosphorylation of a variety of effector proteins including several transcription factors that trigger an inflammatory cascade^[11,23]. Furthermore, in experimental models it has been shown that also reactive oxygen species (ROS) are responsible for the activation of ERK and JNK in pancreatic acinar cells^[24]. In fact, the administration of caerulein *in vivo* stimulates the release of ROS, demonstrating a relationship between increased ROS concentrations and activation of both ERK and JNK^[25]. The incubation of pancreatic acini with H₂O₂ causes a dose-

Table 1 Summary of the actions of the mitogen-activated protein kinases inhibitors

Inhibitor	Mechanism of action	Effects
SP600125	Selective and reversible inhibitor of JNK	Dose dependent inhibition of JNK Inhibition of inflammatory genes (COX-2, IFN, IL-2, TNF- α) <i>in vivo</i> Reduction of pancreatic inflammatory mediators (TNF- α , IL-1 β) <i>in vivo</i>
CEP1347	Potent and selective inhibitor of JNK	Dose dependent inhibition of JNK both <i>in vivo</i> than <i>in vitro</i> Reduction of inflammatory cytokines
PD98059	Inhibitor of ERK 1/2, prevents phosphorylation binding MEK	Protection against inflammatory process in the pancreas <i>in vivo</i> Protective effects probably related to the inhibition of COX-2
UO126	Selective inhibitor of MEK1 and MEK2; it prevents the activation of ERK1/2	Protection against inflammatory process in the pancreas <i>in vivo</i>
SB203580	Selective inhibitor of p38. Inhibition of p38 catalytic activity	Downregulation of the expression of proinflammatory mediators (TNF- α and IL-1 β) <i>in vivo</i>

JNK: c-jun N-terminal kinase; COX-2: Cyclooxygenase 2; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; ERKs: Extracellular signal-regulated kinases.

dependent, rapid and strong activation of MAPKs: ERK, JNK and p38. These findings underline the potential role of ROS in the pathogenesis of acute pancreatitis, in fact large amounts of ROS are produced near to pancreatic acinar cells^[26,27]. Reports describe as ERK can also be activated by exogenous ROS through EGF receptor^[28,29]. High concentrations of ROS may cause cytoskeleton disruption in pancreatic acini cells directly and can modify its function *via* activation of MAPKs and p38, so long as these molecules play an important role in the regulation of cytoskeleton function^[30]. As described, both inflammatory response and oxidative stress play essential roles on the development of acute pancreatitis, and are correlated with the severity of the disease^[31,32]. Pretreatment with an antibody against tumor necrosis factor (TNF)- α or blockade of TNF- α production with pentoxifylline ameliorates experimental AP^[33]. The role of oxidative stress in AP has been demonstrated by the beneficial effects of antioxidants^[34]. It has been demonstrated that combined treatment by simultaneous blocking of inflammation and oxidative stress pathways has positive effects as therapy in the AP. Blockade of TNF- α production with pentoxifylline partially prevented glutathione depletion and pancreatic inflammation in cerulein-induced AP^[35]. Simultaneous inhibition of xanthine oxidase (XO) and TNF- α with oxypurinol and pentoxifylline significantly reduced inflammation in taurocholate-induced pancreatitis^[36]. In addition, oxidative stress, as reported, causes activation of MAPKs^[37], which activation leads to TNF- α production. In fact, it has been demonstrated that oxypurinol reduces p38 phosphorylation and pentoxifylline reduces ERK and JNK phosphorylation. The combination of the two treatments decreases activation of MAP kinases, and this reduction has been observed in other tissues, such as lung and liver, that are involved in systemic inflammatory process^[37]. So, the p38 pathway is related to oxidative stress; ERK and JNK may be associated to inflammatory process and release of pro-inflammatory cytokines. The blockade of these two processes and the concomitant inhibition of MAP kinases can represent a potential therapy to reduce the local and systemic effects in AP, as well as decrease inflammation and production of reactive species

which are involved in development and progression of acute pancreatitis.

PHARMACOLOGICAL MAPKS MODULATION IN AP

Given the role of MAPKs signaling pathway in the development of AP, interest in protein kinases as drug targets has exploded in the past few years, and MAPKs pathways inhibition represents an alternative target in the treatment of AP. Pharmacological inhibitors have been identified which impact on the MAPKs ERK1/2, p38 and JNK/stress activated protein kinases and have been tested in different studies^[38], as resumed in Table 1. It has been shown that selective JNK inhibition leads to amelioration of AP. Different JNK inhibitors have been used, among these, SP600125 is one of the most promising inhibitors for treatment of inflammatory diseases involving MAPKs signalling, as acute pancreatitis^[39]. SP600125 is a potent, selective and reversible inhibitor of the three JNK enzymes over 300-fold more selective for JNK as compared to ERK1 and p38 MAP kinases, acting through a competitive inhibition with respect to ATP and having an IC₅₀ of 40 nmol/L for JNK1 and JNK2, and 90 nmol/L for JNK3^[40]. SP600125 was shown to cause a dose-dependent inhibition of the phosphorylation of c-Jun, and thereby the expression of inflammatory genes cyclooxygenase 2 (COX-2), IFN- γ , interleukin (IL)-2, TNF- α ^[39]. Minutoli *et al.*^[41] showed that treatment with SP600125 blunted caerulein-induced pancreatic JNK activation (90%) and partially ERK1/2 activation (45%). The observed greater effect on JNK activity obtained with SP600125 is in agreement with previous “*in vitro*” data showing that this compound exhibits a greater selectivity for JNK as compared to ERK1/2 MAP kinase^[39]. In the same study SP600125 reduced the pancreatic content of proinflammatory mediators as TNF- α and adhesion molecules as ICAM-1 with a significant reduction in the oedema and in the inflammatory cell infiltrates, thus confirming the positive effect of MAPKs inhibition on the cell survival during AP^[41]. Samuel *et al.*^[44] provided new evidence that MAP kinases (ERK, JNK, and p38)

are involved in caerulein-stimulated exocrine pancreatic production of cytokines. The group used pancreatic fragments stimulated with caerulein. As awaited, the stimulation wreaked a significant increase of phospho-ERK and phospho-p38. Specific inhibitors of these MAPKs significantly reduced IL-1 β and TNF- α production. Using this specific inhibitor of JNK, SP600125, they observed an attenuation of levels of both JNK and IL-1 β . Therefore, there is also a connection between the activation of MAPKs and the production of cytokines, responsible for inflammatory events.

Within the MAPKs signaling cascades inhibitors, CEP-1347 is a potent and selective inhibitor of the JNK but not the p38 or the extracellular signal-regulated kinase signalling cascades, studied principally for its neuroprotective effects^[42]. The correlation between inhibition of the JNK signaling cascade and pancreatitis amelioration by CEP-1347 is showed in *in vitro* and *in vivo* studies^[19,43]. *In vitro* studies demonstrated that CEP-1347 (2 μ M) inhibited caerulein-induced JNK activation in a dose dependent manner. Pretreatment of rats with CEP-1347 strongly reduced caerulein-induced pancreatic JNK activation without p38 or ERK inhibition leading to a consequent reduction of pancreatic damage as demonstrated by reduced pancreatic oedema formation and reduced histological severity of pancreatitis. CEP-1347 inhibits JNK activation *in vivo* and ameliorates caerulein-induced pancreatitis. Furthermore, PD98059 and U0126, both inhibitors of ERK1/2, afford significant protection against inflammatory sequelae following experimental acute pancreatitis^[44].

Since AP is a condition associated with an inflammatory response, an important role is played by the cytokines TNF- α and IL-1 β , which initiate and propagate acute pancreatic inflammation^[45]. In fact, patients affected by acute pancreatitis show elevated serum IL-6 levels^[46]. IL-6-blocking antibody attenuates experimental pancreatitis and associated pulmonary injury^[47].

PD98059 mediates its inhibitory properties by binding to the ERK-specific MAP kinase MEK, therefore preventing phosphorylation of ERK1/2 (p44/p42 MAPK) by MEK1/2, with an IC₅₀ values of 4 μ M/L and 50 μ M/L for MEK1 and MEK2. PD98059 binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf^[48]. Similar to PD98059, also U0126 is a selective inhibitor of MAP kinase kinases, MEK1 and MEK2, acting by inhibiting the kinase activity of MEK1/2 thus preventing the activation of MAP kinases p42 and p44. Inhibition of pancreatic ERK1/2 with PD98059 or U0126 *in vivo* protects against the inflammatory sequelae characteristic of the cerulein model of AP^[44] confirming the role of ERK1/2 activation in the progression of AP. Moreover, the protective effects of PD98059 might be related to the inhibition of COX-2, although this mechanism has not been well investigated^[49].

Evidences have shown that the local pancreatic renin-angiotensin system (RAS) is involved in AP^[50]. Angioten-

sin II, *via* ROS activation, leads to activation of ERK. Leung *et al.*^[51] demonstrated in their study the involvement of ERK in regulating angiotensin II-induced IL-6 expression in pancreatic acinar cells during pancreatic inflammation. The administration of angiotensin II augmented the expression of IL-6, and angiotensin II led to ERK activation. The effect of ERK activation has been confirmed using its inhibitor, PD98059. In this model, it has been observed that the activation of ERK is mediated by the release of ROS; in fact, pretreatment with antioxidants reduced ERK activation. Blockade of AT₁ receptors can represent a potential therapeutic approach to the treatment of AP, ROS mediated, too. Using two different inhibitors, SP600125 and PD98059, it has been demonstrated that they completely inhibited the activation of CER-induced pancreatic JNK and ERK^[52].

CONTROVERSIAL ROLE OF P38 IN ACUTE PANCREATITIS

Despite the MAPKs have been largely involved in acute pancreatitis, the p38 has an unclear role in the development of the disease. As a matter of fact, studies have suggested that p38 MAP kinase activation could worsen acute pancreatic inflammation or protect against it^[43,53]. It has been suggested that the inhibition of p38 exacerbates cerulein-induced pancreatitis in rats^[53]. Others experimental evidences demonstrate that the activation, and not the inhibition, of p38 may exacerbate the progression of AP. This kinase regulates activation of nuclear factor (NF)- κ B in isolated pancreatic acinar cells, but it is unclear the effective role of p38 MAP kinase in acute pancreatitis.

Moreover, p38 signaling pathway is involved in cytokine-mediated pancreatic beta-cell injury. The activation of p38 MAPK occurs through two different upstream kinases, mitogen-activated protein kinase kinase 3 (MKK3) and MKK6. When activated, it is involved in a lot of responses, such as apoptosis, inflammation and fibrosis^[54]. Several studies showed positive effects of systemic p38 inhibitor drugs in a lot of models^[55]; other studies demonstrated that systemic p38 blockade could have negative effects^[56]. It has been studied the role of MKK3-p38 signaling in a model of cytokine-dependent pancreatic injury induced by multiple low doses of streptozotocin, using mice deficient for the *MKK3* gene^[57]. In this study, the group demonstrated that *MKK3* gene deletion has a protective effect, probably due to the suppression of islet inflammation. These findings suggest that MKK3 signaling plays an essential role in the development of pancreatic injury, leading to destruction of beta-cells and hyperglycemia. p38 is activated by CCK in a time and dose dependent manner, with a peak at 5 and 10 minutes, respectively. Twait *et al.*^[58] expressed a dominant negative form of the p38 MAP kinase (DNp38) and evaluated its effect on NF- κ B pathway activation in an exocrine pancreatic cell line (AR42J cells). They observed that DNp38 reduced nuclear translocation of NF- κ B and decreased NF- κ B-dependent gene transcription after CCK or

TNF- α stimulation in AR42J cells. These results support the hypothesis that p38 regulates transcription factors such as NF- κ B in pancreatic exocrine cells^[59]. In a recent paper, Wang *et al.*^[60] investigated the effect of SB203580 which is the inhibitor of p38 mitogen-activated protein kinase on pathologic change of pancreatic tissue and expression of TNF- α and IL-1 β in rats with severe acute pancreatitis. This compound is a pyridinyl imidazole inhibitor widely used to elucidate the roles of p38 mitogen-activated protein kinase acting through the blocking of the activation of MAPKAPK-2 by p38 MAPK and subsequent phosphorylation of HSP27^[61]. SB203580 inhibits p38 MAPK catalytic activity by binding to the ATP-binding pocket, but does not inhibit phosphorylation of p38 MAPK by upstream kinases. Wang *et al.*^[60] showed that treatment with SB203580, inhibiting p38 MAPK signaling pathway led to a down regulation of the expression of pro-inflammatory mediators such as TNF- α and IL-1 β . All these studies highlighted the central role of MAPKs activation in acute pancreatitis pathogenesis and the real possibility to use pharmacological inhibition of these pathways for treatment of this disease.

OTHER MOLECULAR MECHANISMS INVOLVING MAPKS ACTIVATION IN AP

A number of other molecules participate to the complex network of events triggering the MAPKs activation and the inflammatory response associated with the progression and the onset of AP. In this context, recent advances showed an interaction between p38 and JNK activation and cannabinoid receptor 1 (CB₁) and 2 (CB₂) in pancreas, where non selective CB₁/CB₂ agonist HU210 ameliorated experimental pancreatitis^[62]. However, the real role of CB₁ and CB₂ in acute pancreatitis has not been totally investigated. The agonist HU210 carries out a protective effect in pancreatitis also in CB₁ deficient mice, and the selective CB₂ antagonist, AM630, activates JNK and increases apoptosis in acute pancreatitis. The administration of cerulein in CB₁ deficient mice is not responsible for a more severe pancreatitis, if compared to wild type animals, excluding a prominent role of CB₁ receptor in the development of the disease^[63]. On the other hand the protective effect of CB₂ receptor seems to be due to the inhibition of cytokines involved in inflammatory processes, for example, IL-6, which is an activator of JNK^[64]. MK2 is a downstream target of p38; the genetic disruption of the *MK2* gene protects against cerulein-induced pancreatitis^[65]. Several experiments with MK2 deficient mice have suggested a connection between MK2 and JNK activation: the presence of MK2 determines the activation of CB₂ that causes consequently the inhibition of JNK and therefore the attenuation of acute pancreatitis. In MK2 deficient mice, the absence of MK2 creates opposite effects when CB₂ receptor is activated, and leads to activation of JNK and increase of IL-6 levels. So, the activation of CB₂ receptor has probably protective effects through inhibition of MAPKs cascade in experimental

acute pancreatitis and the use of CB₂ agonist can represent an interesting therapeutic target for humans.

In the complex molecular network involved in the regulation of inflammation during AP seems to have a role also protease-activated receptor 2 (PAR2)^[66], a member of the G protein coupled receptor superfamily, that plays important roles not only stimulating pro-inflammatory response but also mediates anti-inflammatory effects^[67]. PAR2 is activated by activated trypsin in acute pancreatic inflammation; it has pro-inflammatory effects since activates immune and endothelial cells^[68]. The protective effects of PAR-2 in acute pancreatitis were investigated in the cerulein-induced pancreatitis model. It has been demonstrated that PAR-2 can activate MAPKs^[69]. In contrast, it has been shown that of PAR-2 activation decreases the cerulein-induced activation both ERK and JNK by accelerating their dephosphorylation, activating MAP kinase phosphatases (MKPs), in rat's pancreas. The expression of MKPs provides a negative feedback mechanism for MAP kinases, and the induction of MKP's expression may be activated both by PAR2 and by cerulein. It has been demonstrated that the protective effect obtained by using ERK's and JNK's inhibitors is similar to the effect observed with PAR2 activation, and ameliorates the course of acute pancreatitis^[68].

An additional molecule involved in the progression of acute pancreatitis is pancreatitis-associated protein (PAP1). PAP1 is not expressed under physiological conditions whereas is overexpressed during acute pancreatitis^[69]. Its activation is linked to a large number of diseases such as inflammatory bowel disease, Alzheimer's disease, and cancer^[70-72]. The peak of expression of PAP1 in pancreatic tissue or juice has been observed 24 h after the induction of acute pancreatitis by cerulein^[73]. In pancreatic acinar cells the augmented expression of PAP1 led to an increase of resistance to apoptosis^[74,75]. Ferrés-Masó *et al.*^[76] demonstrated an anti-inflammatory role of PAP1, since its induction occurs during inflammatory diseases (pancreatitis, Crohn's disease, ulcerative colitis). *In vivo* studies showed that the administration of anti-PAP1 antibodies worsened the inflammatory response. Treatment with PAP1 prevented TNF- α -induced NF- κ B activation in macrophages. Gironella *et al.*^[70] furthermore demonstrated the anti-inflammatory role of PAP1 in a PAP1-deficient mice model. The anti-inflammatory mechanism of the protein is related to the activation of JAK/STAT3 pathway. PAP1 increases the transactivation activity of the nuclear transcriptional factors associated with MAPKs family. *In vitro* experiments on AR42J pancreatic acinar cell line showed a time-dependent induction of PAP1 gene expression after addition of PAP1 to the culture cells. It has been shown that this cellular line presented basal levels of expression of the proteins members of the MAPKs family: ERK, JNK and p38. Treatment with PAP1 enhanced the phosphorylation of MAP kinases, underlining that PAP1 signal transduction involves MAPKs family^[76]. Treatment with MAPK specific inhibitors, such as SB203580 (p38 MAPK inhibitor), PD98059 (ERK inhibitor) and JNK inhibitor,

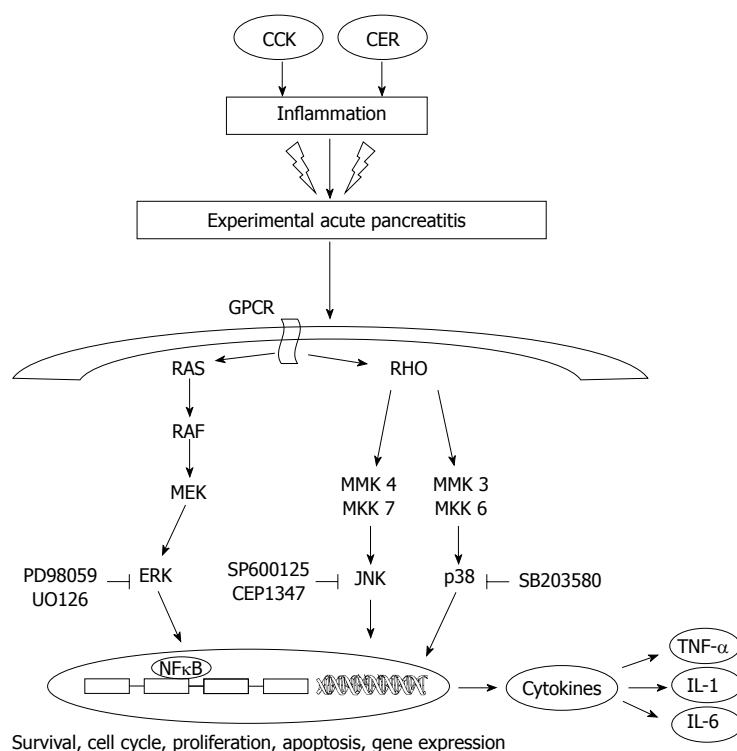


Figure 1 Involvement of mitogen-activated protein kinases and their inhibitors in pancreatic damage. CCK: Cholecystokinin; CER: Cerulein; GPCR: G protein coupled receptor; JNK: c-jun N-terminal kinase; TNF: Tumor necrosis factor; ERKs: Extracellular signal-regulated kinases; NF: Nuclear factor; IL: Interleukin; NF: Nuclear factor.

caused the inhibition of the activation of PAP1. This result demonstrates that the involvement of MAPKs family is essential for the synthesis of PAP1. Some reports indicate that ERK mediates STAT3 phosphorylation both *in vivo* and *in vitro*^[77]. Probably a linkage exists between MAPK and JAK/STAT3 pathway upon activation by PAP1.

Also Substance P (SP)^[78,79], a neuropeptide released from nerve endings in many tissues, plays an important role in inflammatory processes. SP binds to a G protein-coupled receptor, neurokinin-1 receptor (NK1R). Pancreatic acinar cells express NK1R, SP has been found in pancreas^[80], and levels of SP and NK1R are increased in AP^[81]. It has been demonstrated that genetic deletion of NK1R reduces the severity of pancreatitis and pancreatitis-associated lung injury. Knockout mice deficient in the preprotachykinin-A gene, which encodes for SP, are protected against AP^[82]. These evidences suggest an important interaction between SP and NK1R in development of acute pancreatitis and lung injury. Studies have shown that SP induces an increase of cytosolic calcium, and probably elevated concentration of calcium is one of the causes of AP^[83]. Pancreatic acinar cells treated with SP showed an upregulation of phosphorylation of both ERK and JNK. The inhibitor U73122, a PLC inhibitor, decreased phosphorylation of ERK and JNK, as well as inhibited the activation of NF-κB^[84]. These findings are important to demonstrate that drugs targeting SP could represent a therapeutic approach for the treatment of AP.

CONCLUSION

Acute pancreatitis is an autodigestive disease resulting in

acute inflammation of the pancreas and MAPKs have been demonstrated to play a pivotal role in the development of the disease (Figure 1). As a consequence of the above reported observations, it is possible to speculate that the blockade of MAPKs may represent a strategic target for future treatment of acute pancreatitis.

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Imaging tests for accurate diagnosis of acute biliary pancreatitis

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Abstract

Gallstones represent the most frequent aetiology of acute pancreatitis in many statistics all over the world, estimated between 40%-60%. Accurate diagnosis of acute biliary pancreatitis (ABP) is of outmost importance because clearance of lithiasis [gallbladder and common bile duct (CBD)] rules out recurrences. Confirmation of biliary lithiasis is done by imaging. The sensitivity of the ultrasonography (US) in the detection of gallstones is over 95% in uncomplicated cases, but in ABP, sensitivity for gallstone detection is lower, being less than 80% due to the ileus and bowel distension. Sensitivity of transabdominal ultrasonography (TUS) for choledocholithiasis varies between 50%-80%, but the specificity is high, reaching 95%. Diameter of the bile duct may be orientative for diagnosis. Endoscopic ultrasonography (EUS) seems to be a more effective

tool to diagnose ABP rather than endoscopic retrograde cholangiopancreatography (ERCP), which should be performed only for therapeutic purposes. As the sensitivity and specificity of computerized tomography are lower as compared to state-of-the-art magnetic resonance cholangiopancreatography (MRCP) or EUS, especially for small stones and small diameter of CBD, the later techniques are nowadays preferred for the evaluation of ABP patients. ERCP has the highest accuracy for the diagnosis of choledocholithiasis and is used as a reference standard in many studies, especially after sphincterotomy and balloon extraction of CBD stones. Laparoscopic ultrasonography is a useful tool for the intraoperative diagnosis of choledocholithiasis. Routine exploration of the CBD in cases of patients scheduled for cholecystectomy after an attack of ABP was not proven useful. A significant rate of the so-called idiopathic pancreatitis is actually caused by microlithiasis and/or biliary sludge. In conclusion, the general algorithm for CBD stone detection starts with anamnesis, serum biochemistry and then TUS, followed by EUS or MRCP. In the end, bile duct microscopic analysis may be performed by bile harvested during ERCP in case of recurrent attacks of ABP and these should be followed by laparoscopic cholecystectomy.

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Key words: Biliary; Pancreatitis; Lithiasis; Endoscopic ultrasonography; Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography

Core tip: Gallstones represent the most frequent aetiology of acute pancreatitis estimated between 40%-60%. Clearance of lithiasis (gallbladder and common bile duct, CBD) rules out recurrences. Confirmation of biliary lithiasis is done by imaging. Endoscopic ultrasonography (EUS) seems to be a more effective tool to diagnose acute biliary pancreatitis rather than endoscopic

retrograde cholangiopancreatography, which should be performed only for therapeutic purposes. As the sensitivity and specificity of computerized tomography are lower as compared to state-of-the-art magnetic resonance cholangiopancreatography or EUS, especially for small stones and small diameter of CBD, the later techniques are preferred nowadays.

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INTRODUCTION

Gallstones represent the most frequent aetiology of acute pancreatitis in many statistics all over the world. The proportion from the total number of acute pancreatitis cases is estimated between 40%-60%, with variations due especially to diagnostic efforts and availability of imaging tests^[1]. Accurate diagnosis of acute biliary pancreatitis (ABP) is of outmost importance because clearance of lithiasis (gallbladder and common bile duct, CBD) rules out recurrences, very frequent otherwise, with 30% to 50% of the patients developing recurrent acute pancreatitis relatively soon after discharge (average time 108 d), some of them maybe more severe than the previous episode^[2].

Once the diagnosis of acute pancreatitis is made, grounded on generally acknowledged criteria of abdominal pain and three times more than normal hyperamylasemia/hyperlipidemia and/or intravenous (*iv*) contrast-enhanced helical computerized tomography (CT) scan/magnetic resonance imaging (MRI)/ transabdominal ultrasonography (TUS), the biliary aetiology is suspected if jaundice, elevated alanine aminotransferase (ALT) (three times more than normal) or a dilated CBD are present^[3]. To those criteria we might add statistical data of a higher incidence in women, between 50 and 70 years of age^[1].

Confirmation of biliary lithiasis is done by imaging. Clearance of biliary lithiasis implies a cholecystectomy and the removal of CBD stones. The minimal invasive approach is preferred nowadays, either by combined approach of laparoscopic cholecystectomy and endoscopic extraction of CBD stones, or total laparoscopic approach (cholecystectomy and CBD exploration and calculi extraction). Thus, once a diagnosis of gallbladder lithiasis is made, especially for microlithiasis, the most important thing is to establish whether there is also a CBD stone. Over 90% of the CBD stones come from the gallbladder through the cystic duct. Primary stones arising in the CBD are rarer and usually due to conditions that alter the normal flow of the bile and create conditions for bile stasis. "Silent stones" in the CBD may be present in up to 15% in patients younger than 60 years undergoing cho-

lecystectomy, and even more frequent in older patients^[4]. However, the incidence of ABP in choledocholithiasis is only 3%-8%^[1]. Even more important, after triggering the acute pancreatitis, most of stones pass through the papilla into the duodenum^[5]. Thus, the percentage of CBD stones in ABP decreases from 28.6% in the first 4 h to 8% at 1 wk^[6,7].

IMAGING TESTS

Transabdominal US

The first, and the most available and commonly performed is TUS. It seeks for lithiasis in the gallbladder, CBD or indirect signs of biliary obstruction, *e.g.*, dilation of the CBD. The sensitivity of the US in the detection of gallstones is over 95% in uncomplicated cases, but in ABP, sensitivity for gallstone detection is lower, being only 67%-78% due to the ileus and bowel distension^[8]. Sensitivity of TUS for choledocholithiasis varies between 50%-80%, but the specificity is high, reaching 95%^[9].

Diameter of the bile duct may be orientative for diagnosis. In a prospective study, the diameter of the CBD was measured before cholecystectomy and it was compared afterwards with finding stones at the surgical intervention. There were no stones in the CBD if the diameter was less or equal to 3 mm, while 7.7% of patients with the ducts measuring 4 mm or more had stones. If the size increased, the probability of having stones also increased, nearly all ducts of 9 mm or more had stones^[10] (Figure 1).

Endoscopic US

Endoscopic US is more accurate than transcutaneous US, with a sensitivity of over 90% and an even higher specificity^[11,12]. Nevertheless, the technique is more expensive and it requires a longer learning curve. EUS seems to be a more effective tool to diagnose ABP rather than ERCP, which should be performed only for therapeutic purposes. In a systematic review of clinical trials from 1994 to 2010, comparing EUS and ERCP in ABP, it was found that EUS avoided ERCP in 71.2% of cases, had no related complication, while ERCP was complicated in over 20% of cases. The clinical course of ABP was not influenced by either of those explorations^[13]. A meta-analysis performed on 36 studies with 3532 patients revealed a sensitivity of 89% and a specificity of 94% for choledocholithiasis^[14], with another meta-analysis performed on 2673 patients showing even higher numbers of 94% sensitivity and 95% specificity^[15]. Consequently, EUS is an important diagnostic tool for the presence of CBD stones, as it accurately visualizes the CBD without the need of instrumentation^[16]. There is now enough evidence to support the use of EUS before ERCP, even for smaller stones (less than 4 mm), as it can spare at least two thirds of ERCPs^[17]. Moreover, as compared to MRCP, EUS has the same sensitivity, specificity and accuracy, although the sensitivity of MRCP seems to diminish in small (less than 6 mm) CBD stones. Thus,

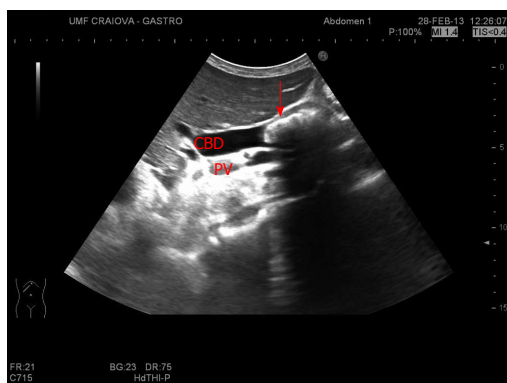


Figure 1 Large, conglomerated stones into a dilated common bile duct (over 12 mm).

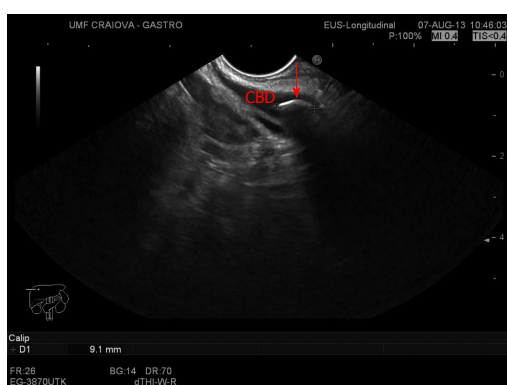


Figure 2 A 9 mm stone, within a slightly dilated, elongated common bile duct.

EUS has a significant impact for surgical decision making, especially in the patients with suspected ABP^[18] (Figure 2).

CT

Unenhanced helical CT scan has a variable accuracy for the detection of choledocholithiasis, with a sensitivity of 60%-87% and a specificity of 97%-100%^[19,20]. CT-choangiography has a higher performance for the diagnosis of choledocholithiasis with a sensitivity of 85%-96% and a specificity of 88%-98%^[19,21]. As the sensitivity and specificity of CT are lower as compared to state-of-the-art MRCP or EUS, especially for small stones and small diameter of CBD, the later techniques are nowadays preferred for the evaluation of ABP patients.

MRCP

MRCP has a high reported accuracy in the diagnosis of choledocholithiasis. Meta-analyses report pooled sensitivities of 92%-94%^[7,22] and a specificity of 99%. There are still controversies regarding the optimal imaging method in the preoperative assessment of patients with ABP, but MRCP has the advantage of a non-invasive method that could properly detect CBD lithiasis. The efficacy of MRCP in detecting CBD stones and to assess the time of choledochal passage of calculi was also compared to

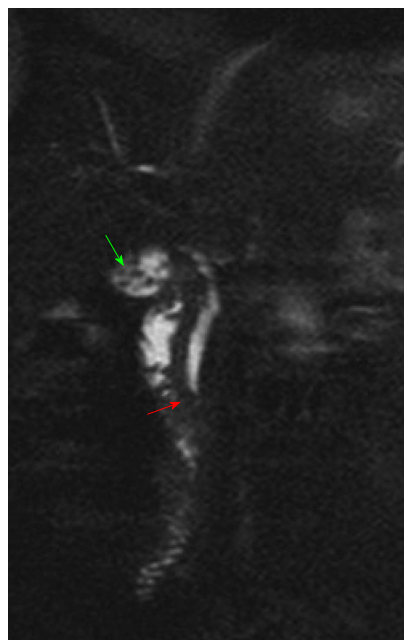


Figure 3 Multiple gallstones in T2 hyposignal less than 5 mm diameter (green arrow), diameter of the common bile duct 6 mm, with a 4 mm migrated stone (red arrow).

ERCP. Overall, MRCP had a positive predictive value 90.5%, negative predictive value 95.2%, sensitivity 82.6%, specificity 97.5% and overall accuracy 94.2%. Moreover, MRCP diagnoses anatomical variants of cystic duct and acute cholecystitis^[6,7]. A prospective study compared the efficacy of EUS compared to MRCP and ERCP in the same patients with suspected extrahepatic biliary disease, taking into account also the economic aspect. Results regarding choledocholithiasis were that EUS was more sensitive than MRCP in the detection of choledocholithiasis (80% *vs* 40%), with similar specificity. Rate of acute pancreatitis after ERCP was 6.6%. EUS strategy had the greatest cost-utility by avoiding unnecessary ERCP examinations^[23]. Nevertheless, a systematic review showed a similar diagnostic value for prospective studies that compared MRCP and EUS for the detection of CBD stones^[24] (Figure 3).

ERCP

ERCP has the highest accuracy for the diagnosis of choledocholithiasis and is used as a reference standard in many studies, especially after sphincterotomy and balloon extraction of CBD stones. Diagnostic ERCP does not, however, detect all stones and in one study its sensitivity was 89% in comparison with EUS, especially for small stones hidden by contrast injection^[12]. EUS has been compared to ERCP in a prospective randomized fashion in cases of acute pancreatitis suspected to have a biliary cause. The patients had EUS or ERCP examinations within 24 h from admission. If EUS detected choledocholithiasis, therapeutic ERCP was performed immediately. EUS was successful in all patients, but ERCP failed in 10%, the difference being significant. Also ERCP

failed to identify stones in 8.5%. Morbidity, hospital stays and mortality was similar in both groups^[25]. The preferred approach for concomitant gallbladder and CBD stones in the laparoscopic era is sequential preoperative ERCP followed by laparoscopic cholecystectomy, although this has been found to have similar efficacy, maybe with a shorter hospital stay with laparoscopic CBD exploration during cholecystectomy^[26]. The same conclusion was also reached by a Cochrane systemic review comparing the endoscopic versus surgical treatment of CBD stones, with laparoscopic CBD clearance being as effective as pre- or post-operative ERCP^[27].

Laparoscopic ultrasonography

Laparoscopic ultrasonography (LUS) is a useful tool for the intraoperative diagnosis of choledocholithiasis. Thus, LUS was compared to laparoscopic cholangiography with the same specificity (100%) and positive predictive value (100%), and a sensitivity of 93%^[28]. Nevertheless, laparoscopic exploration of the bile duct is as safe and effective as postoperative ERCP in clearing stones from the common duct^[29]. The benefit of routine intraoperative cholangiography at the time of cholecystectomy in patients with ABP submitted to laparoscopic cholecystectomy was also questioned. Thus, patients with ABP submitted to cholecystectomy with or without intraoperative cholangiography and CBD exploration were compared in terms of outcome. At 3.8 years of follow up there was no significant difference regarding the rate of recurrent pancreatitis or biliary complications, suggesting that intraoperative cholangiography does not improve outcome after cholecystectomy for gallstone pancreatitis^[30]. Another study showed that laparoscopic cholecystectomy (LC) can be performed safely without intraoperative cholangiography (IOC). Thus, from the patients with symptomatic gallstone disease, about 9.2% were selected for preoperative ERCP based upon preoperative clinical, laboratory and ultrasound criteria. In those patients, 58% were found with choledocholithiasis, and stone clearance was achieved in all cases. The other patients were submitted to laparoscopic cholecystectomy with no injury of CBD, no mortality and a rate of retained CBD stones of 1.5% at 2 years follow-up^[31].

The necessity of routine exploration of the CBD in cases of patients scheduled for cholecystectomy after an attack of ABP was submitted to question. Ito *et al*^[32] investigated this in cases of low risk for choledocholithiasis. The authors included 148 patients without preoperative ERCP, normal and decreasing liver function tests, and normal CBD diameter. They were divided into 2 groups - with or without intraoperative cholangiography. Follow-up didn't find any significant differences between the 2 groups regarding postoperative episodes of acute pancreatitis, cholangitis or changes in liver function tests. Authors concluded that direct CBD exploration could be safely avoided in selected cases of ABP, with low-risk for choledocholithiasis.

ETIOLOGY

Some of the acute pancreatitis cases remain idiopathic even after complete serum biochemistry, ultrasound and CT evaluations. Nevertheless, the aetiology of acute pancreatitis should be determined in at least 80% of cases and no more than 20% should be classified as idiopathic (recommendation grade B)^[33]. These represent between 10% and 30% in different series. Some studies suggested that more accurate imaging tests for biliary lithiasis detection may reveal the biliary cause in those cases. In our experience, it also happened that once we introduced in our hospital EUS and ERCP there was a shift between the leading causes for acute pancreatitis between the alcoholic and biliary causes, many of idiopathic pancreatitis being actually biliary ones. Recently, some studies showed that a significant rate of the so-called idiopathic pancreatitis are actually caused by microlithiasis and/or biliary sludge, identified by the presence of cholesterol monohydrate and/or calcium bilirubinate microcrystals in the biliary sediment.

Microlithiasis

Microlithiasis is a viscous precipitate containing mucin, cholesterol and calcium bilirubinate which can obstruct the pancreatic duct. US has a sensitivity of only about 55% in detecting microlithiasis and does not allow for analysis of the chemical composition of bile^[34]. This is an important cause of recurrent acute pancreatitis. Though a EUS procedure is diagnostic, with a high sensitivity and specificity^[35] a duodenal aspirate or a bile duct aspirate for the microliths^[36] at ERCP is confirmatory. In a series of 86 patients^[37] with acute pancreatitis, 21 patients had microlithiasis. Six patients were subjected to cholecystectomy and 4 patients to endoscopic sphincterotomy. Fewer recurrences were noted in patients receiving either of the two treatment modalities compared to the group managed conservatively. The treatment protocol would warrant a cholecystectomy in all patients unless contraindicated. In those with a high operative risk, endoscopic biliary sphincterotomy is a safe and viable option^[38]. Ursodeoxycholic acid is an alternative in those with bleeding tendencies^[39]. Thus, microlithiasis or biliary sludge as a causative aetiology for acute pancreatitis remains controversial and not well understood. Several studies have demonstrated the presence of biliary sludge in as many as 75% of patients with unexplained acute pancreatitis^[37]. Bile analysis with microscopic examination is considered the gold standard for diagnosis. Bile can be obtained directly while cannulating the bile duct during ERCP or following CCK stimulation on EGD. ERCP with bile aspiration from the CBD has a reported sensitivity of 83% in detecting microlithiasis^[40].

In patients considered to have idiopathic acute pancreatitis, after negative routine work-up for biliary etiology, EUS is recommended as the first step to assess for occult microlithiasis, neoplasms and chronic pancreatitis.

If EUS is negative, rare and uncommon causes should be looked for. MRCP (secretin-stimulated) is advised to identify or rule out rare morphologic abnormalities. If aetiology still remains unidentified, genetic counselling (not necessarily genetic testing) should be considered in order to search for hereditary or other genetic causes^[3].

In conclusion, the general algorithm for CBD stone detection starts with anamnesis, serum biochemistry and then TUS, followed by EUS or MRCP. In the end, bile duct microscopic analysis may be performed by bile harvested during ERCP in case of recurrent attacks of ABP and these should be followed by laparoscopic cholecystectomy.

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Retroperitoneal disorders associated with IgG4-related autoimmune pancreatitis

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Abstract

IgG4-related autoimmune pancreatitis is frequently accompanied by relevant lesions in the genitourinary tract and retroperitoneal organs, which cause various clinical problems, ranging from non-specific back pain or bladder outlet obstruction to renal failure. The diagnosis of IgG4-related retroperitoneal fibrosis requires a multidisciplinary approach, including serological tests, histological examination, imaging analysis, and susceptibility to steroid therapy. Radiological examinations are helpful to diagnose this condition, but surgical resection is occasionally unavoidable to exclude malignancy, particularly for patients with isolated retroperitoneal involvement. Steroid therapy is the treatment of choice for this condition, the same as for other manifestations

of IgG4-related disease. For patients with severe ureteral obstruction, additional ureteral stenting needs to be considered prior to steroid therapy to preserve the renal function. Some papers have suggested that IgG4-related disease can affect male reproductive organs including the prostate and testis. IgG4-related prostatitis usually causes lower urinary tract symptoms, such as dysuria and pollakisuria. Patients sometimes state that corticosteroids given for IgG4-related disease at other sites relieve their lower urinary tract symptoms, which leads us to suspect prostatic involvement in this condition. Because of the limited number of publications available, further studies are warranted to better characterize IgG4-related disease in male reproductive organs.

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Key words: IgG4; Autoimmune pancreatitis; Retroperitoneum; Genitourinary tract; Management

Core tip: Patients with IgG4-related autoimmune pancreatitis frequently have associated conditions involving genitourinary organs. Since clinical presentations and imaging findings vary among patients, the differential diagnoses are broad. Serum IgG4 elevation is highly sensitive but not entirely specific for this condition, which is one reason why the diagnosis should be established in a multidisciplinary way. Although recent radiological advances have facilitated the effective characterization of IgG4-related retroperitoneal fibrosis, surgical resection is occasionally necessary to exclude malignancies. In addition to steroid therapy, ureteral stenting is required for patients with severe ureteral obstruction. A new concept of IgG4-related prostatitis is being increasingly recognized.

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INTRODUCTION

IgG4-related disease has been widely recognized over the last decade^[1]. Type 1 autoimmune pancreatitis is a prototypic manifestation of this systemic condition, and investigations of autoimmune pancreatitis led to the establishment of a novel entity, "IgG4-related disease"^[2-4]. IgG4-related autoimmune pancreatitis is sometimes accompanied by synchronous or metachronous lesions at other anatomical sites. Previous studies suggested that retroperitoneal fibrosis is the most commonly associated condition outside the pancreatobiliary system in patients with IgG4-related autoimmune pancreatitis^[5,6]. Tubulointerstitial nephritis is another well-known manifestation of IgG4-related disease. Other urinary tract organs that can be affected by IgG4-related disease include the renal pelvis and ureter. Interestingly, reproductive-organ involvements such as IgG4-related prostatitis have been also confirmed in male patients^[7-12].

In this paper, we review features of retroperitoneal and reproductive-organ manifestations related to IgG4-related autoimmune pancreatitis to promote their better understanding and management. We did not include IgG4-related tubulointerstitial nephritis, as it has already been well described^[13,14].

RESEARCH

A PubMed search was performed for articles published until November 2013 using the keywords of IgG4, pancreatitis, and retroperitoneal fibrosis or testis or prostate. We also referred to studies published in Japanese, as many studies on this entity have been conducted in Japan. Written informed consent was obtained from all the patients for case presentation.

IgG4-RELATED RETROPERITONEAL FIBROSIS: GENERAL ASPECTS

An association between serum IgG4 elevation and autoimmune pancreatitis was first reported by Hamano *et al.*^[15] in 2001. The same group also described a case of IgG4-related pancreatitis complicated by retroperitoneal fibrosis, where abundant IgG4-positive plasma cells were histologically identified^[16]. This is the first proven case of IgG4-related retroperitoneal fibrosis in the literature. In 2006, Kamisawa *et al.*^[17] suggested that IgG4-related pancreatitis and retroperitoneal fibrosis belong to a systemic condition, which is now recognized as IgG4-related disease. Since then, many papers have described IgG4-related retroperitoneal fibrosis, but most of them are case reports.

It is worth emphasizing several aspects of IgG4-related retroperitoneal fibrosis. Firstly, some patients present with isolated IgG4-related retroperitoneal fibrosis with no identifiable extra-retroperitoneal lesions. Secondly, retroperitoneal fibrosis is not always IgG4-related. Only approximately 60% of retroperitoneal fibrosis is IgG4-related. Due to marked overlap in clinical features between IgG4-related and non-related cases, this discrimination is not straightforward without histological analysis^[18,19]. Yet, if a patient is younger than 40 years, non-IgG4-related retroperitoneal fibrosis is more likely.

The diagnosis of IgG4-related disease thus requires a multidisciplinary approach, where serological tests, tissue diagnosis, and imaging examination need to be considered. Serum IgG4 elevation is highly sensitive, but not entirely specific for this condition. IgG4 elevations up to twice the upper limit of the normal range (280 mg/dL) in the serum can be seen in a variety of diseases, including both inflammatory and neoplastic conditions. IgG4 elevations of more than 280 mg/dL are highly specific for this condition.

IgG4-RELATED RETROPERITONEAL FIBROSIS: PATHOLOGY

IgG4-related retroperitoneal fibrosis is histologically characterized by massive lymphoplasmacytic infiltration, storiform fibrosis, and obliterative phlebitis (Figure 1)^[14,20]. IgG4-positive plasma cells should be diffusely present in inflamed area (> 30 cells/high power field) (Figure 2). The rate of IgG4/IgG-positive plasma cells is at least over 40%, typically over 70%^[21]. As IgG4-positive plasma cell infiltration is not entirely specific for this condition, the ratio of them to IgG-positive plasma cells is important to avoid overdiagnosis. Histological findings contradicting a diagnosis of IgG4-related retroperitoneal fibrosis include neutrophilic infiltration, necrosis, discrete granuloma, and necrotizing arteritis.

IgG4-RELATED RETROPERITONEAL FIBROSIS: CLINICAL MANIFESTATION

Clinical presentations of IgG4-related retroperitoneal fibrosis are variable. About a half of the patients are believed to be symptom-free. Patients sometimes describe abdominal or back pain and edema of the lower extremities. Once ureters are blocked, symptoms related to hydronephrosis or renal failure may appear.

The spectrum of imaging features is also wide, including soft tissue masses sometimes involving the ureters or renal pelvis (Figure 3), aortic wall thickening involving adjacent soft tissue (Figure 4), or plaque-like diffuse fibrosis^[22-24]. On computed tomography (CT), the lesions exhibit a soft-tissue density. On magnetic resonance imaging (MRI), they show a low to intermediate signal intensity on T1-weighted images and various signal intensity patterns on T2-weighted images according to

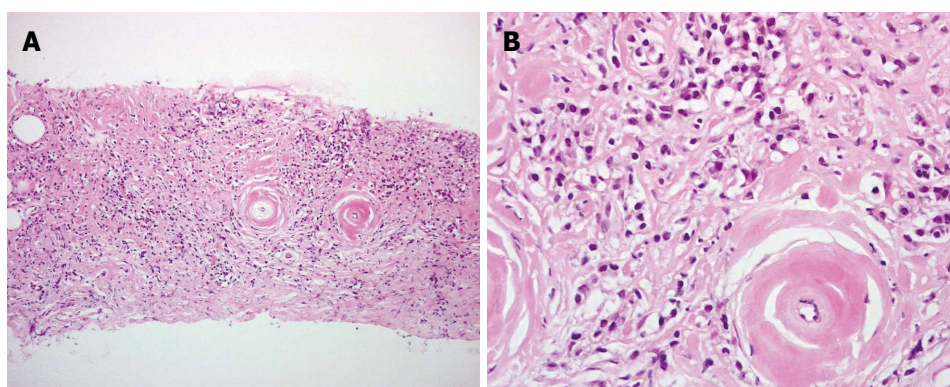


Figure 1 Histological findings with core needle biopsy: Fibrosis and inflammatory reaction with dense infiltration of abundant lymphocytes and plasma cells. A: Low magnification; B: High magnification.

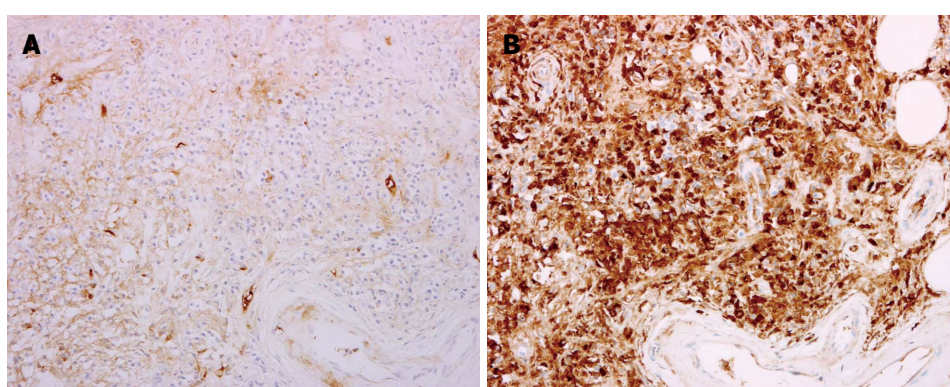


Figure 2 Immunopathological findings: infiltrating cells represent lymphocytes and IgG4-positive plasma cells. A: IgG4 staining; B: IgM staining.

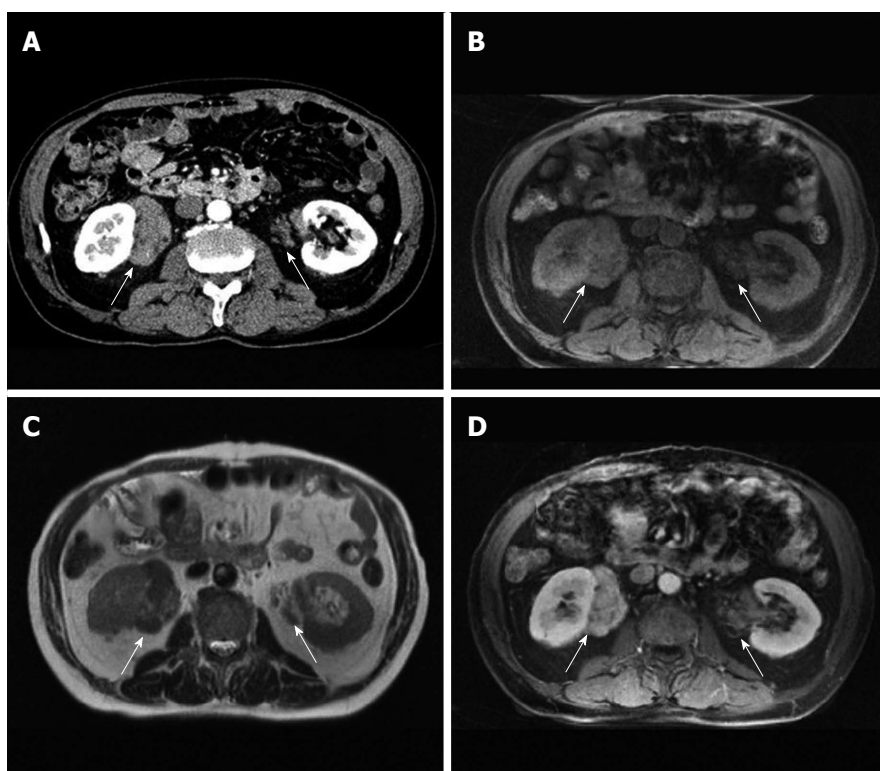


Figure 3 Localized pseudotumors (arrows) in a 67-year-old man with IgG4-related retroperitoneal fibrosis and autoimmune pancreatitis. A: Contrast-enhanced CT; B: T1-weighted; C: T2-weighted; D: Contrast-enhanced MRI.

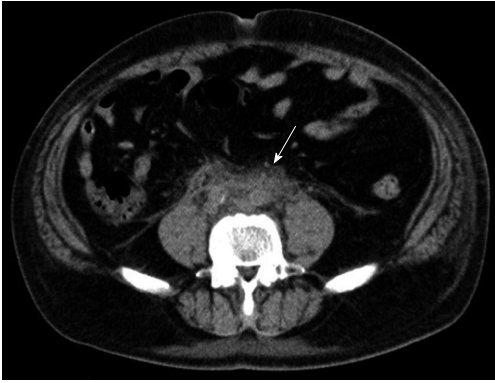


Figure 4 Pseudotumor spread in the retroperitoneum surrounding the abdominal aorta and vena cava (arrow).

the inflammatory activity (Figure 3). Contrast-enhanced images are useful to estimate the degree of fibrosis and inflammatory activity^[25,26].

IgG4-RELATED RETROPERITONEAL FIBROSIS: FUNCTIONAL PROBLEMS

Hydronephrosis related to retroperitoneal fibrosis impairs the renal function, eventually leading to renal insufficiency. Maeta *et al.*^[27] reported a rare case of acute renal failure due to IgG4-related retroperitoneal fibrosis. Ureteral obstruction was described in 45%-65% of reported patients with IgG4-related retroperitoneal fibrosis^[5,6,12,22,24,27,29-42]. Yet, assuming that many other patients are not reported, how often IgG4-related retroperitoneal fibrosis is associated with hydronephrosis remains unknown. Interestingly, we realized that the left kidney is more commonly affected by this complication in reported cases^[5,6,12,22,24,27,29-42]. Tsuboi *et al.*^[28] reported a 62-year-old man with IgG4-related systemic manifestations, who had left hydronephrosis due to a retroperitoneal pseudotumor. Hart *et al.*^[12] reported a 67-year-old man who had IgG4-related autoimmune pancreatitis and retroperitoneal fibrosis with hydronephrosis in the left kidney. Two additional cases also had left hydronephrosis^[29,30]. Given the fact that most of the patients had IgG4-related autoimmune pancreatitis, the left-sided predominance may be related to the anatomical location of the pancreas. Bilateral hydronephrosis can be also seen at the initial presentation, as described by Miura *et al.*^[31] and Takenaka *et al.*^[32]. Hydronephrosis is not common in retroperitoneal fibrosis affecting the renal hilum, as reported by Miyajima *et al.*^[23].

MANAGEMENT OF IgG4-RELATED RETROPERITONEAL FIBROSIS: PHARMACOLOGICAL THERAPY

The same as for other IgG4-related diseases, steroid therapy is highly effective for IgG4-related retroperitoneal fibrosis^[29-31]. However, for patients with ureteral obstruction, how to preserve the renal function is another aspect

of treatment. Because of the lack of consensus guidelines for this particular condition, we need to decide on the therapeutic plan (*i.e.*, corticosteroids, ureteral stent) based on the renal function of patients on a case-by-case basis. For patients without severe uremia or fluid retention, oral prednisolone is the most likely treatment of choice. Additional urological intervention such as ureteral stenting may be an option if the obstruction remains even after steroid therapy. Imaging studies involving CT are helpful to assess the effects of steroid therapy^[26,37,38].

According to the literature, initial doses vary from 20 mg to 100 mg once daily^[28-30,32,33]. A 52-year-old man with IgG4-related pancreatitis and periaortic retroperitoneal fibrosis without urinary tract obstruction was successfully treated with an initial dose of prednisolone of 30 mg^[33]. Tsuboi *et al.*^[28] reported a 62-year-old man with systemic disease, who had left hydronephrosis due to a pseudotumor, successfully managed with prednisolone of 30 mg. Miura *et al.*^[31] reported an 80-year-old man without pancreatic swelling. His bilateral hydronephrosis was treated successfully with prednisolone of 25 mg. A 51-year-old man with systemic manifestations and bilateral incomplete ureteral obstruction was successfully treated with 50 mg of prednisolone^[32]. Both the 67-year-old and 79-year-old men reported by Kikuno *et al.*^[29] and Nishimura *et al.*^[30] respectively, were also successfully treated with prednisolone of 30 mg. Low dose prednisolone therapy (initial dose: 0.5-0.6 mg/kg or 30 mg/body daily) with tapering has been the therapeutic standard with encouraging results (recovery rate greater than 90%), the same as for other manifestations of IgG4-related disease^[28,31,43].

MANAGEMENT OF IgG4-RELATED RETROPERITONEAL FIBROSIS: UROLOGICAL INTERVENTION

Marked effects of steroid therapy are usually expected in the first couple of weeks. When patients have advanced uremia, severe fluid retention, or ureteral obstruction with symptoms, however, an emergent ureteral stent or nephrostomy is required^[27]. Unilateral interventions may be sufficient for recovery from the life-threatening situation. Ureteral stenting is currently the interventional standard. Hart *et al.*^[12] reported a 67-year-old man who had IgG4-related autoimmune pancreatitis and retroperitoneal fibrosis with hydronephrosis of the left kidney. He underwent ureteral stenting for mild elevation of the serum creatinine level (1.6 mg/dL). Another 74-year-old Chinese man with hydronephrosis received ureteral stenting to relieve ureteral obstruction and associated pain^[38]. IgG4-related retroperitoneal fibrosis was thereafter diagnosed in this case. The stent was removed after confirming that pseudotumorous retroperitoneal fibrosis responded well to steroid therapy.

Unilateral nephrostomy needs to be considered for patients with anatomical problems in the lower urinary tract, including severe urethral stenosis and large pros-



Figure 5 Although a ureteral stent was correctly placed into the left renal pelvis (arrow), ipsilateral hydronephrosis did not improve in a 79-year-old woman with IgG4-related retroperitoneal fibrosis.

tatic hyperplasia; IgG4-related autoimmune pancreatitis is most frequently encountered in middle-aged to elderly men (mean age, 59–68 years, 4 to 7.5-fold higher rate compared to women)^[4,7,8,17,18]. In fact, we experienced a patient (79-year-old woman) with histologically proven IgG4-related retroperitoneal fibrosis, where ureteral stenting was not sufficient to alleviate ureteral obstruction and renal failure (Figure 5). She subsequently underwent nephrostomy, followed by steroid therapy. Ureteral stents might be constricted by the severe fibrotic process around the ureter.

IgG4-RELATED RETROPERITONEAL FIBROSIS MIMICKING MALIGNANCY

Abe *et al*^[41] reported a 39-year-old man, who showed an atypical clinical manifestation. He was diagnosed with a tumor in the left ureter. He underwent segmental ureterectomy, which led to the diagnosis of an IgG4-related periureteral pseudotumor in the distal ureter. Another 75-year old man who metachronously developed IgG4-related autoimmune pancreatitis and retroperitoneal fibrosis was also reported. He initially presented with left hydronephrosis and had surgery for possible ureteral cancer, and an IgG4-related pseudotumor was histologically diagnosed. Ten months later, he was diagnosed with autoimmune pancreatitis, which was successfully managed with prednisolone^[34]. These cases suggest that IgG4-related pseudotumorous retroperitoneal fibrosis is difficult to diagnose, particularly when it is the first or an isolated manifestation. Surgical resection is sometimes unavoidable for such patients.

Similar pseudotumorous lesions can develop in the more proximal urinary tract. Yoshino *et al*^[42] encountered a 71-year-old man, who had an IgG4-related pseudotumor mimicking renal pelvic cancer in his left kidney. In this case, urine cytology obtained by a retrograde catheter in the renal pelvis was negative for malignant cells. His serum IgG4 level was found to be elevated, and he was successfully treated with prednisolone. The diagnosis of IgG4-related pseudotumorous retroperitoneal fibrosis is

less difficult if patients have other organ manifestations. However, surgery has been conducted even for such patients^[33], suggesting that the clinical management of patients with IgG4-related disease requires close coordination between physicians and urologists.

OTHER ASPECTS OF IgG4-RELATED RETROPERITONEAL FIBROSIS

Pipitone *et al*^[22] suggested the utility of 18-fluorodeoxyglucose (FDG) positron emission tomography (PET) for the diagnosis and assessment of IgG4-related retroperitoneal fibrosis. This has been supported by additional studies^[24,26]. On the other hand, the definite diagnosis of IgG4-related retroperitoneal fibrosis usually requires tissue confirmation, but retroperitoneal biopsies are occasionally problematic due to expected adverse events or technical failure. Sampling error is always a possibility for patients with broad plaque-like lesions. Doe *et al*^[40] reported an interesting case: a 77-year-old man was diagnosed with IgG4-related retroperitoneal fibrosis and an elevated serum IgG4 level (398 mg/dL). Retroperitoneal biopsy was not performed because of his marked comorbidity, but lip biopsy revealed the periglandular infiltration of IgG4-positive plasma cells, leading to the diagnosis of IgG4-related disease.

ASSOCIATED CONDITIONS IN THE URINARY BLADDER

There has been no report on urinary bladder involvement in patients with proven IgG4-related disease. Crumley *et al*^[44] retrospectively examined biopsy samples of interstitial cystitis from the aspect of IgG4. Interstitial cystitis is a clinical entity previously called painful bladder syndrome, whose etiology and pathogenesis remain undetermined^[45,46]. Of 44 cases examined, 4 (9%) showed a significant increase in IgG4-positive plasma cells (greater than 30/hpf) with an IgG4/IgG ratio greater than 0.5. Those patients were characterized by an older age, severer inflammation, and smaller bladder capacity than the remaining 40 IgG4-negative patients. Serological data were not available because of the retrospective nature of the study. Further studies are necessary to conclude whether or not IgG4-related cystitis is a distinct entity.

ASSOCIATED CONDITIONS IN THE PROSTATE

Several case studies on IgG4-related prostatitis have been reported. Patients almost exclusively presented with lower urinary tract symptoms such as dysuria, pollakisuria, urinary urgency, and a feeling of incomplete emptying^[9,10]. The clinical presentation is similar to that in common benign prostatic hyperplasia or chronic prostatitis^[47,48]. The diagnosis of IgG4-related prostatitis may not be difficult once prostate biopsy is performed; biopsy of the pros-

tate is an established diagnostic routine^[49,50]. However, it depends on whether or not urologists, physicians, and pathologists are aware of this condition.

The first case of IgG4-related prostatitis was described in a case series of IgG4-related pancreatocholelitis reported in 2004^[4]. Two years later, Yoshimura *et al*^[9] described a 65-year-old man, in whom IgG4-related prostatitis was retrospectively diagnosed using IgG4 immunostaining 7 years after he received transurethral resection of the prostate to relieve bladder outlet obstruction. Nishimori and colleagues reported 2 additional cases^[10]. Both patients were initially diagnosed with common benign prostatic hyperplasia. One patient with IgG4-related pancreatitis showed the improvement of lower urinary tract symptoms with an alpha-adrenoceptor antagonist that is the first-line agent for men with benign prostatic hyperplasia^[48]. He was eventually diagnosed with IgG4-related prostatitis following prostate biopsy, which was performed because of an uptake in the prostate on FDG-PET. The other patient had isolated IgG4-related prostatitis, which was diagnosed by tissue examination of the transurethral resection specimen and elevated serum IgG4 level (473 mg/dL). Interestingly, he also showed FDG uptake in the prostate, while his pancreas was atrophic with no FDG uptake. Zaidan *et al*^[51] reported a man with long-standing IgG4-related retroperitoneal fibrosis, who was eventually found to have IgG4-related prostatitis following prostate biopsy. The biopsy was undertaken because of a significant FDG uptake.

Uehara *et al*^[52] histologically examined prostate tissue samples obtained from 6 cases, including one radical prostatectomy specimen. This study well addressed the histological characteristics of IgG4-related prostatitis. The histological features are basically similar to those of IgG4-related disease at other sites. Glands are replaced by the inflammatory process, consisting of lymphoplasmacytic infiltration, occasional eosinophils, and irregular fibrosis. Obliterative phlebitis was noted. IgG4-positive plasma cells were diffusely present in inflamed areas.

It is known that serum levels of prostate-specific antigen (PSA) are markedly elevated in men with bacterial prostatitis^[49,50], but whether or not IgG4-related prostatitis is associated with an elevated serum PSA remains unclear because of the limited number of cases. Patel and Szostek^[53] reported a man with systemic IgG4-related disease, in whom prostatic involvement was confirmed by biopsy. His serum PSA level was within the normal range. Hart *et al*^[54] reported a 55-year-old man with IgG4-related pancreatitis and prostatitis (the PSA level was normal: 0.67 ng/mL). Interestingly, his symptoms resolved when he was given a course of oral prednisone for monoarticular gout. In our experience, patients sometimes state that corticosteroids given for IgG4-related disease at other sites relieve their lower urinary tract symptoms, which suggests that IgG4-related prostatitis may be underdiagnosed.

In 2006, Taniguchi *et al*^[55] reported a 61-year-old man who presented with retroperitoneal and mediastinal fi-

brosis, and a mass in the left seminal vesicle. IgG4-related disease was diagnosed based on a high serum IgG4 concentration (583 mg/dL) and tissue examination. All lesions responded well to corticosteroids. This is probably the first reported case suggesting IgG4-related disease at this anatomical site.

In summary, men with IgG4-related pancreatitis sometimes present with IgG4-related prostatitis synchronously or metachronously. Lower urinary tract symptoms are common but not specific among elderly men. FDG-PET may be a useful diagnostic modality for IgG4-related prostatitis. Its urological features, such as efficacy of alpha-adrenoceptor antagonists for the alleviation of urinary symptoms and serum PSA elevation, remain unclear.

ASSOCIATED CONDITIONS IN THE TESTIS AND ACCESSORY ORGANS THEREOF

Bösmüller *et al*^[11] investigated 3 men (23, 25, and 52 years old) with paratesticular fibrous pseudotumors, and suggested that this condition may be a presentation of IgG4-related disease, although information about coexisting associated conditions in other organs was not provided. Hart *et al*^[12] subsequently reported a 67-year-old man who developed a similar condition during observation for IgG4-related autoimmune pancreatitis. Migita *et al*^[56] reported a 74-year-old man with bilateral IgG4-related sialadenitis. He had a history of left orchiectomy for a 4-cm paratesticular mass. A retrospective review of pathological slides confirmed an inflammatory mass with the massive infiltration of IgG4-positive plasma cells. de Buy Weniger *et al*^[57] reported a 57-year-old man with IgG4-related pancreatitis, who was found to have IgG4-related orchitis 7 years after pancreaticoduodenectomy. His main symptom was left testicular pain, and the left testis alone was affected. Dieckmann *et al*^[45] reported 2 young men with possible IgG4-related orchitis (28 and 18 years old). However, all four patients younger than 30 years (two cases each reported by Bösmüller *et al*^[11] and Dieckmann *et al*^[45]) showed no other organ involvement, which may challenge the diagnosis in young patients. Information on testicular functions such as testosterone levels and spermatogenesis was not provided in any case reports quoted in this section, and so further studies on endocrinological and functional outcomes of this rare condition are warranted.

CONCLUSION

IgG4-related autoimmune pancreatitis is frequently accompanied by relevant lesions in the retroperitoneum and genitourinary tract, leading to various clinical presentations and imaging abnormalities. Although surgical resection is occasionally unavoidable to rule out malignancy, such incidences will decrease as IgG4-related retroperitoneal fibrosis is more widely recognized and imaging findings are well characterized. We need to decide on the

therapeutic plan (*i.e.*, corticosteroids, ureteral stent) based on the renal function of patients on a case-by-case basis. Increasing evidence suggests that IgG4-related disease can affect the prostate and testis, and further studies are necessary to better understand reproductive-organ involvement of IgG4-related disease.

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WJG 20th Anniversary Special Issues (18): Pancreatitis

Diagnosis of autoimmune pancreatitis

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Abstract

Autoimmune pancreatitis (AIP) is a distinct form of chronic pancreatitis that is increasingly being reported. The presentation and clinical image findings of AIP sometimes resemble those of several pancreatic malignancies, but the therapeutic strategy differs appreciably. Therefore, accurate diagnosis is necessary for cases of AIP. To date, AIP is classified into two distinct subtypes from the viewpoints of etiology, serum markers, histology, other organ involvements, and frequency of relapse: type 1 is related to IgG4 (lymphoplasmacytic sclerosing pancreatitis) and type 2 is related to a granulocytic epithelial lesion (idiopathic duct-centric chronic pancreatitis). Both types of AIP are characterized by focal or diffuse pancreatic enlargement accompanied with a narrowing of the main pancreatic duct, and both show dramatic responses to corticosteroid. Unlike type 2, type 1 is characteristically associated with increasing levels of serum IgG4 and positive serum autoantibodies, abundant infiltration of IgG4-positive plasmacytes, frequent extrapancreatic lesions, and relapse. These findings have led several countries to propose diagnostic criteria for AIP, which consist of essentially similar diagnostic items; however, several differences exist for each country, mainly due to differences in the definition

of AIP and the modalities used to diagnose this disease. An attempt to unite the diagnostic criteria worldwide was made with the publication in 2011 of the international consensus diagnostic criteria for AIP, established at the 2010 Congress of the International Association of Pancreatologists (IAP).

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Key words: Autoimmune pancreatitis; Diagnosis; Criteria; Japanese; International consensus diagnostic criteria

Core tip: Autoimmune pancreatitis (AIP) was first reported in Japan in 1995. Since then, a large series of studies has been documented and the concept of AIP is now recognized worldwide. Two distinct subtypes of AIP occur with different incidences in Asian and western countries. Type 1 is often associated with IgG4-related systemic diseases and shares histological features of lymphoplasmacytic sclerosing pancreatitis. Type 2 is usually not associated with IgG4 abnormality and histologically shows idiopathic duct-centric pancreatitis with granulocytic epithelial lesions. Independent diagnostic criteria had previously been used in individual countries, but international consensus diagnostic criteria were published in 2011.

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INTRODUCTION

Autoimmune pancreatitis (AIP) was first documented in 1995 by Yoshida *et al*^[1], who reported a case of chronic

pancreatitis that fulfilled the definition of an autoimmune disease^[2] with respect to hyperglobulinemia, positive serum autoantibody, and steroid response. In 2001, Hamano *et al.*^[3] reported increased serum levels of IgG4 in Japanese patients with AIP. This disease is a form of chronic pancreatitis characterized by frequent presentation with obstructive jaundice, simultaneous and/or metachronal occurrences of extrapancreatic lesions, histology of lymphoplasmacytic infiltrates with fibrosis, and a dramatic response to corticosteroids^[4-9]. Symptoms, blood test data, and clinical images of the AIP often resemble those of pancreatic cancer (PC)^[10-12], malignant lymphoma^[1,13], and other types of pancreatitis. Therefore, differential diagnosis must be conducted carefully.

The first diagnostic criteria for AIP were established in Japan in 2002^[14], revised in 2006^[15], and revised again in 2011 (Table 1)^[16]. During this period, the concepts of AIP were well recognized worldwide and nationwide diagnostic criteria were proposed in South Korea^[17,18], the United States, Germany^[19], and Italy^[20]. The conditions and methodologies used in each criterion varied; hence, the cases diagnosed as AIP sometimes differed by country. AIP was later revealed to consist of two distinct subtypes: type 1 AIP, which is characterized by histology resembling that of “lymphoplasmacytic sclerosing pancreatitis (LPSP),” and type 2 AIP or “idiopathic duct-centric pancreatitis (IDCP)”^[21], with “granulocytic epithelial lesion (GEL)”^[8,22]. Type 1 AIP is now considered the pancreatic manifestation of systemic organ disorders termed “IgG4-related diseases (IgG4-RD)”^[23], while type 2 is usually not associated with IgG4 activity or extra-pancreatic lesions other than ulcerative colitis (UC). The proportions of type 1 and type 2 AIP vary substantially in western and eastern countries. Consensus meetings have been held and international criteria were established in Asia in 2008^[24], and on a worldwide scale (international consensus diagnostic criteria: ICDC) in 2011 (Tables 2-4 and Figures 1-3)^[25]. The ICDC are presently evaluated as the most sensitive and specific criteria for diagnosing AIP^[26].

CLASSIFICATION OF AIP

A worldwide survey of AIP^[27] indicated that most cases of AIP in Asia fit the histological profile of LPSP, or type 1 AIP, while European and American cases are a mixture of LPSP and idiopathic duct-centric pancreatitis (IDCP)^[21,27,28]. The necessity of adequate pancreatic specimens for histology makes accurate diagnosis of IDCP difficult before resection, and this is probably the reason for the limited number of reported cases of type 2 AIP. The two types of AIP also differ in characteristics depending on the geographical distribution, age and gender of the patients, serological findings, association with extra pancreatic lesions, and relapse ratios (Table 5).

Type 1 AIP

Type 1 AIP is histologically characterized as LPSP and is often associated with: (1) abundant lymphoplasmacytic

Table 1 Clinical diagnostic criteria for autoimmune pancreatitis in 2011 by Japan Pancreas Society (JPS-2011)^[16]

A: Diagnostic items
I: Enlargement of the pancreas:
(a) Diffuse enlargement
(b) Segmental/focal enlargement
II: ERP (endoscopic retrograde pancreatography) shows irregular narrowing of the main pancreatic duct
III: Serological findings
Elevated level of serum IgG4 (≥ 135 mg/dL)
IV: Pathological findings: Among (1)-(4) listed below
(a) Three or more are observed
(b) Two are observed
(1) Prominent infiltration of lymphocytes and plasmacytes and fibrosis
(2) More than 10 IgG4-positive plasmacytes per high-power microscope field
(3) Storiform fibrosis
(4) Obliterative phlebitis
V: Extra-pancreatic lesions: sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis/retroperitoneal fibrosis
(a) Clinical lesions
Extrapancreatic sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis (Mikulicz disease) or/retroperitoneal fibrosis
(b) Pathological lesions
Pathological examination shows characteristic features of sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis or/retroperitoneal fibrosis
<Option> Effectiveness of steroid therapy
A specialized facility may include in its diagnosis the effectiveness of steroid therapy, once pancreatic or bile duct cancers have been ruled out. When it is difficult to differentiate from malignant conditions, it is desirable to perform cytological examination using an endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). Facile therapeutic diagnosis by steroids should be avoided unless the possibility of malignant tumor has been ruled out by pathological diagnosis.
B: Diagnosis
I: Definite diagnosis
(1) Diffuse type
I a + III/IVb/V (a/b)
(2) Segmental/focal type
I b + II + two or more of < III/IVb/V (a/b) >
or
I b + II + < III/IVb/V (a/b) > + Option
(3) Definite diagnosis by histopathological study
IVa
II: Probable diagnosis
Segmental/focal type: I b + II + < III/IVb/V (a/b) >
III: Possible diagnosis ¹
Diffuse type: I a + II + Option
Segmental/focal type: I b + II + Option

When a patient with a focal/segmental image of AIP on CT/MRI without ERCP findings fulfill more than one of III, IVb and V (a/b) ERP criteria, he/she can be diagnosed as probable AIP only after the negative workup for malignancy by EUS-FNA, and confirmed as definitive one by an optional steroid response. ¹Possible diagnosis: A case may possibly be type 2, although it is extremely rare in Japan. AIP: Autoimmune pancreatitis; CT: Computed tomography; MRI: Magnetic resonance image.

infiltration with IgG4-positive cells [> 10 cells/high power field (HPF)]; (2) storiform fibrosis; and (3) obliterative phlebitis (Tables 1, 2 and 5). Type 1 AIP frequently occurs in elderly men and is geographically distributed in greater numbers in Asia^[29,30] than in western countries^[19,20,22,31]. Type 1 AIP is the pancreatic manifestation

Table 2 Diagnosis of definitive and probable type 1 autoimmune pancreatitis using international consensus diagnostic criteria^[25]

Diagnosis	Primary basis for diagnosis	Imaging evidence	Collateral evidence
Definitive type 1 AIP	Histology Imaging Response to steroid	Typical/indeterminate Typical Indeterminate Indeterminate	Histologically confirmed LPSP (level 1 H) Any non-D level 1/level 2 Two or more from level 1 (+ level 2 D ¹) Level 1 S/OOI + Rt or level 1 D + Level 2 S/OOI/H + Rt
Probable type 1 AIP		Indeterminate	Level 2 S/OOI/H + Rt
Criterion	Level 1		Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement)		Indeterminate (including atypical ³): Segmental/focal enlargement with delayed enhancement
D: Ductal imaging (ERP)	Long (> 1/3 length of the main pancreatic duct) or multiple strictures without marked upstream dilatation		Segmental/focal narrowing without marked upstream dilatation (duct size, < 5 mm)
S: Serology	IgG4, > 2 × upper limit of normal value		IgG4, 1-2 × upper limit of normal value
OOI: Other organ involvement	a or b a: Histology of extrapancreatic organs Any three of the following: (1) Marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration (2) Storiform fibrosis (3) Obliterative phlebitis (4) Abundant (> 10 cells/HPF) IgG4-positive cells b: Typical radiological evidence At least one of the following: (1) Segmental/multiple proximal (hilar/intrahepatic) or proximal and distal bile duct stricture (2) Retroperitoneal fibrosis		a or b a: Histology of extrapancreatic organs including endoscopic biopsies of bile duct ⁴ : Both of the following: (1) Marked lymphoplasmacytic infiltration without granulocytic infiltration (2) Abundant (> 10 cells/HPF) IgG4-positive cells b: Physical or radiological evidence At least one of the following (1) Symmetrically enlarged salivary/lachrymal glands (2) Radiological evidence of renal involvement described in association with AIP
H: Histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells		LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells
Response to steroid (Rt) ²	Diagnostic steroid trial Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestations		

¹Level 2 D is counted as level 1 in this setting; ²Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative workup for cancer including endoscopic ultrasound-guided fine needle aspiration; ³Atypical: Some AIP cases may show low-density mass, pancreatic ductal dilatation, or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP, and a thorough workup for cancer is negative (see algorithm); ⁴Endoscopic biopsy of duodenal papilla is a useful adjunctive method because ampulla often is involved pathologically in AIP. AIP: Autoimmune pancreatitis; ICDC: International consensus diagnostic criteria; HPF: High power field; LPSP: Lymphoplasmacytic sclerosing; OOI: Other organ involvement.

of IgG4-related disease (IgG4-RD)^[23,32]; consequently, a variety of systemic lesions with IgG4-positive cells infiltrates develop simultaneously or metachronously, in association with elevated level of serum IgG or IgG4 (> 135 mg/dL) and positive serum autoantibodies. These systemic lesions include sclerosing cholangitis (60%), sialadenitis (14%), retroperitoneal fibrosis (10%), interstitial pneumonitis (8%), and tubulointerstitial nephritis (8%)^[4], and many other organs are recognized as possible targets of IgG4-RD or type 1 AIP⁵ (Table 6). Response to corticosteroid therapy is usually excellent (97%-98%)^[33,34]; however, a high rate of relapse is also observed (56% in 1 year within steroid initiation and 92% within 3 years) (Table 5).

Type 2 AIP

Type 2 AIP is regarded as a specific pancreatic disease, characterized histologically by duct-centric pancreatitis with a GEL^[21,22,27,35]. Type 2 AIP patients are more frequently diagnosed in western countries, with a younger age of onset and without gender deviation, compared to type 1^[36]. Type 2 AIP occasionally coexists with inflammatory bowel disease (16%-30%)^[36,37]. Response to steroids is excellent, as in type 1, but type 2 AIP rarely relapse (Table 5)^[37].

Patients with type 2 AIP have no serological markers of autoimmunity. Therefore, the classification of type 2 AIP as a clinical entity of AIP is still debated. Nevertheless, the deposition of C3c and IgG in the basement

Table 3 Diagnosis of definitive and probable type 2 autoimmune pancreatitis using international consensus diagnostic criteria^[25]

Diagnosis	Imaging evidence	Collateral evidence
Definitive type 2 AIP	Typical/indeterminate	Histologically confirmed IDCP (level 1 H) or clinical inflammatory bowel disease + level 2 H + Rt
Probable type 2 AIP	Typical/indeterminate	Level 2 H/clinical inflammatory bowel disease + Rt
Criterion	Level 1	Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement)	Indeterminate (including atypical ²): Segmental/focal enlargement with delayed enhancement
D: Ductal imaging (ERP)	Long (> 1/3 length of the main pancreatic duct) or multiple strictures without marked upstream dilatation	Segmental/focal narrowing without marked upstream dilatation (duct size, < 5 mm)
OOI: Other organ involvement		Clinically diagnosed inflammatory bowel disease
H: Histology of the pancreas (core biopsy/resection)	IDCP	
	Both of the following: (1) Granulocytic infiltration of duct wall (GEL) with or without granulocytic acinar inflammation (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells	Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltrate (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells
Response to steroid (Rt) ¹	Diagnostic steroid trial Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in manifestations	

¹Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative workup for cancer including endoscopic ultrasound-guided fine needle aspiration; ²Atypical: Some AIP cases may show low-density mass, pancreatic ductal dilatation, or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP, and a thorough workup for cancer is negative (see algorithm). AIP: Autoimmune pancreatitis; ICDC: International consensus diagnostic criteria; IDCP: Idiopathic duct-centric pancreatitis.

Table 4 Diagnosis of autoimmune pancreatitis-not otherwise specified using international consensus diagnostic criteria^[25]

Diagnosis	Collateral evidence (case with only D1/2)
AIP-not otherwise specified	D1/2 + Rt

membrane of the pancreatic ducts and acini suggests an immune complex-mediated destruction of ducts and acini in type 2 as well as type 1 AIP^[38].

DIAGNOSTIC CRITERIA OF AIP

Diagnostic criteria, either nationwide^[9,16-20] or international^[24,25], consist mostly of common diagnostic items such as image findings of the pancreatic parenchyma, pancreatography, and extrapancreatic lesions; serological findings; histology of the pancreatic lesion; and response to steroid therapy (Tables 1-3). The diagnostic items are very similar, but the method or approach for analyzing each finding varies depending on the country. For instance, in Japan¹⁶, endoscopic retrograde pancreatography (ERP) is performed even by general clinicians but is usually precluded in western countries to avoid causing or worsening pancreatitis. In contrast, the Mayo Clinic in the United States^[9] routinely performs pancreatic core biopsy for diagnosing AIP. These differences in the methodology seem to reflect the diagnostic criteria or diagnostic algorithm used by individual country^[9,16-20].

Pancreatic parenchymal imaging

Focal or diffuse pancreatic enlargement is a common finding in both types of AIP. A dynamic study showed

that enhancement of the pancreatic parenchyma is repressed during the arterial to parenchymal phase and is recovered at the portal phase to delayed phase^[39]. This enhancement pattern is distinct from that of PC and is applied to contrast-enhanced EUS for the differentiation of AIP and cancer by analyzing time-intensity curves^[40,41]. Typically, a linear or band-like structure, depicted as low density by computed tomography (CT) and a hypo-intensity signal by T2-weight magnetic resonance image (MRI), appears at the margin of the enlarged pancreatic parenchyma and is referred to as a “capsule-like rim”, reflecting the fibrous tissue^[39,42]. Abdominal ultrasonography (US) and EUS show similar findings to those of early chronic pancreatitis, including hyperechoic foci (91%-100%), hyperechoic strands (30%-81%), lobularity (15%-53%), and a hyperechoic wall of the main pancreatic duct (30%) in cases with AIP, and these findings decrease after steroid therapy^[33,43]. Ultrasound of typical diffuse-type AIP shows a diffusely enlarged low-echoic pancreas without ductal dilation, or so-called “sausage-like appearance.” Elastographic studies have revealed inconsistent results regarding the hardness of pancreatic lesions associated with AIP^[44,45].

Pancreatographic imaging

An irregular narrowing of the main pancreatic duct (MPD), but not a complete stenosis or obstruction, is seen in cases of AIP. Nishino *et al*^[46] analyzed the differences in ERP findings between AIP and PC, and found a higher prevalence of narrowing of the MPD for ≥ 3 cm of its length and a higher prevalence for the presence of side branches in the narrowed portion of the MPD in the AIP group than in the PC group ($P < 0.001$ and $P < 0.001$,

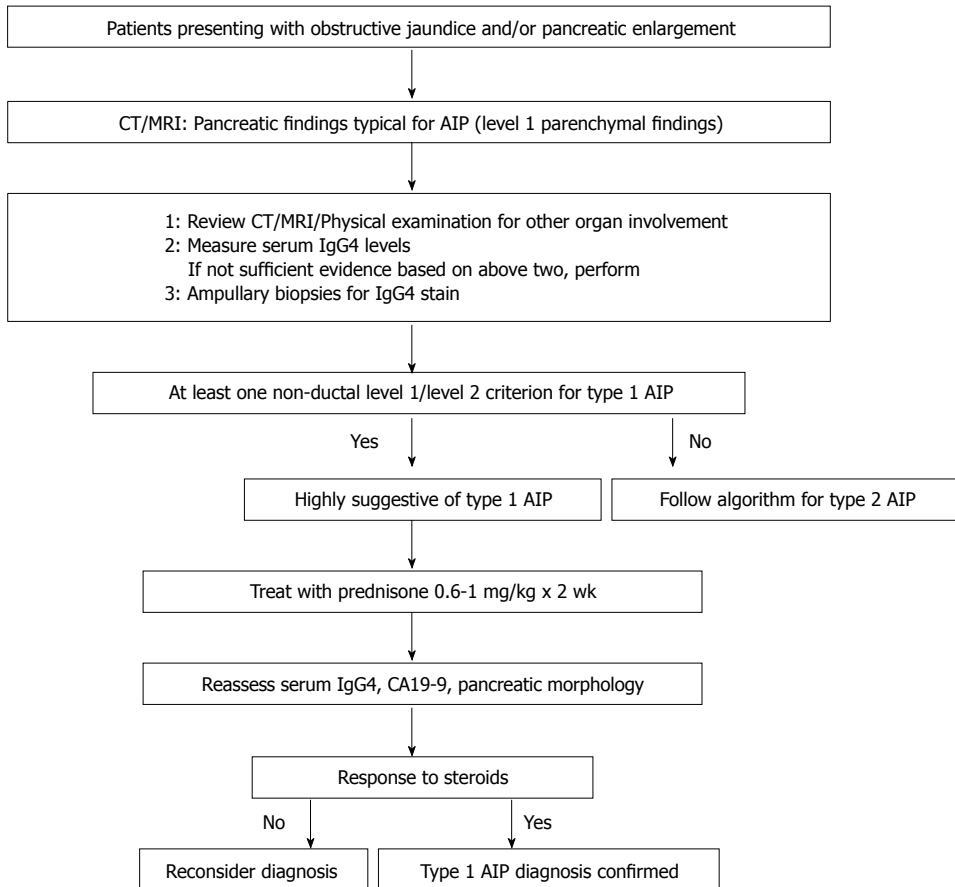


Figure 1 Algorithm of international consensus diagnostic criteria to diagnose type 1 autoimmune pancreatitis in subjects presenting with obstructive jaundice and/or pancreatic enlargement. This schematic drawing shows a flow to diagnose type 1 AIP with typical diffuse enlargement of the pancreas on CT/MRI (level 1 parenchymal findings)^[29]. AIP: Autoimmune pancreatitis; CT: Computed tomography; MRI: Magnetic resonance image.

respectively). In addition, an obvious dilation of the MPD (≥ 4 mm) upstream of the lesion was recognized in 87% of the PC cases, but this was seen in only 11% of the AIP cases ($P < 0.001$). The narrowed portion of the MPD is not visualized by magnetic resonance cholangiopancreatography (MRCP)^[47]; however, use of ERP is only mandatory in the Japanese criteria (Table 1). Either MRCP or ERP is acceptable in the Korean criteria^{17,18} and modality is not specified in the Mayo criteria (HISORT)^[9]. The ERCP finding seems to be extremely important in atypical cases^[10,33]; for instance, a case that does not show marked shrinkage following steroid therapy^[33,48] or a case of PC mimicking^[11] or accompanying^[12] AIP.

Serology

The most sensitive and specific serum marker for type 1 AIP is IgG4 (≥ 135 mg/dL, sensitivity: 86%, specificity to AIP against PC: 96%). However, IgG4 is not actually specific for AIP^[5], and elevated serum IgG4 or infiltrations of numerous IgG4-bearing plasma cells have also been reported in cases with PC (10%, 13/135)^[49]. Various antibodies appear in the sera of AIP patients, such as anti-lactoferrin antibody, anti-carbonic anhydrase II antibody, antinuclear antibody (ANA), and rheumatoid factor (RF) at respective frequencies of 75%, 55%, 60%, and 20%-30%^[50]. The sensitivity of a set of non-specific se-

rum markers (IgG + ANA + RF) (91%) is similar to that of IgG4, but the specificity (61%) is significantly lower than for IgG4^[5]. The SS-A (Ro) and SS-B (La) antibodies, which are markers of Sjögren's syndrome, are rarely seen in AIP patients, giving additional grounds for the idea that sclerosing sialadenitis seen in AIP patients is distinct from Sjögren's syndrome.

The level of serum markers is usually correlated with the autoimmune activity and a large number of systemic lesions are more often recognized in type 1 AIP with high levels of serum markers (IgG4, soluble IL2 receptor, *etc.*)^[51,52]. Relapse is also often recognized in cases with elevated levels of serum IgG^[33] or IgG4^[34]. Hence, these serum markers are also applicable to the clinical follow up of patients with type 1 AIP.

Extrapaneatic lesions (other organ involvement)

Extrapaneatic lesions are often associated with type 1 AIP and are correlated with disease activity. The most common extrapancreatic lesion seen in type 1 AIP is sclerosing cholangitis (bile duct), with other typical lesions including dacryoadenitis (lacrimal gland), sialadenitis (salivary gland), interstitial pneumonitis (lung), tubulointerstitial nephritis (kidney), retroperitoneal fibrosis (retroperitoneum), and lymph node lesions at the hepatic hilar portion. Many of reported extrapancreatic lesions

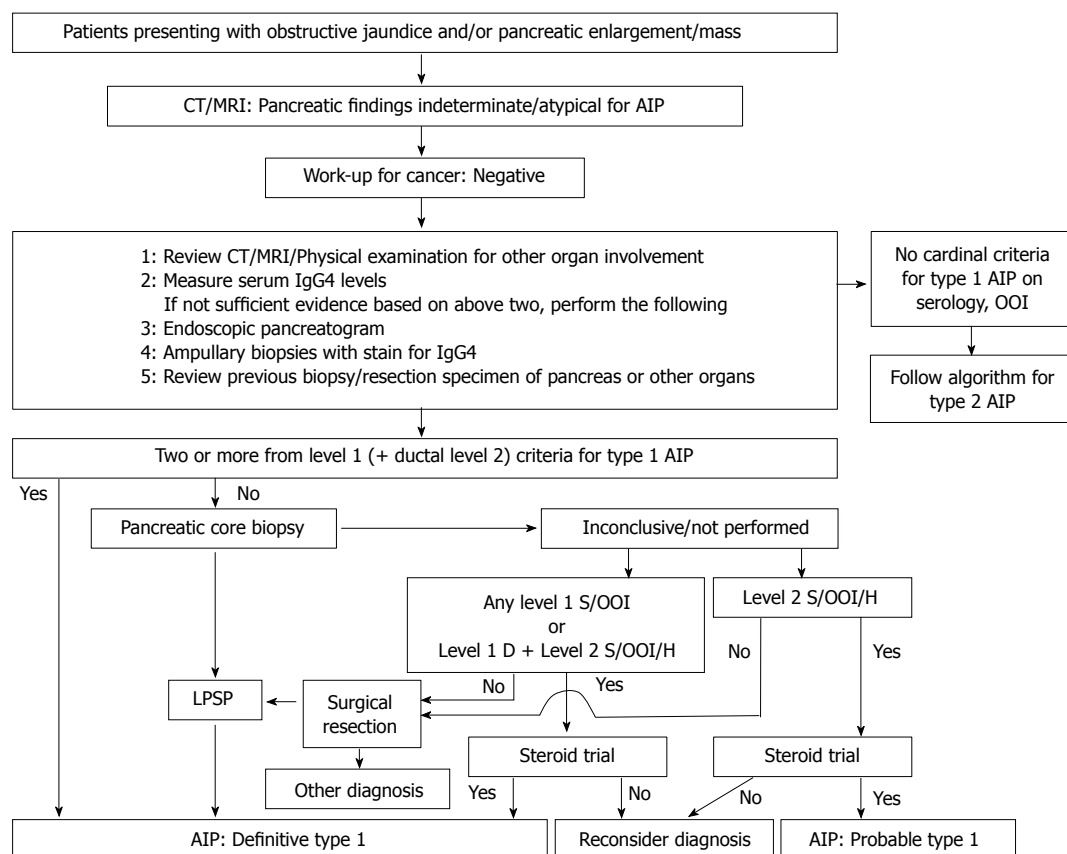


Figure 2 Algorithm of international consensus diagnostic criteria to diagnose type 1 autoimmune pancreatitis in subjects presenting with obstructive jaundice and/or pancreatic mass. This schematic drawing shows a flow to diagnose type 1 AIP with indeterminate or atypical findings of the pancreas on CT/MRI (level 2 parenchymal findings)^[29]. AIP: Autoimmune pancreatitis; CT: Computed tomography; MRI: Magnetic resonance image; OOI: Other organ involvement.

are summarized in Table 5 and classified as having close association or possible association with AIP. Representative extrapancreatic lesions have been reported as showing pathological findings similar to the pancreas, including massive lymphoplasmacytic infiltration and fibrosis, obliterating phlebitis, and presence of prominent IgG4 positive plasma cells^[7]. These lesions can be detected incidentally in cross-sectional images and whole body imaging such as ¹⁸F-Fluoro-deoxyglucose positron emission tomography (PET)^[53,54] and Gallium scintigraphy^[55]. These extrapancreatic lesions sometimes confuse the diagnosis; *i.e.*, type 1 AIP is sometimes accompanied by pseudotumor of the liver or lung, mimicking metastases from PC^[56]. The occurrence of OOI in AIP patients sometimes causes serious physical conditions, such as loss of consciousness due to swelling of the pituitary gland^[57] or hemorrhagic risk due to the decreased platelet numbers caused by autoimmune thrombocytopenic purpura in cases with anticoagulant intake^[58].

Histology of the pancreatic lesion

The pancreatic lesion of type 1 AIP histologically shows LPSP with 3 essential features: (1) a lymphoplasmacytic infiltrate surrounding small-sized interlobular pancreatic ducts that does not destroy the pancreatic ductal epithelium; (2) a swirling fibrosis centered around ducts and veins (storiform fibrosis); and (3) obliterative phlebitis

wherein the infiltrate surrounds and obliterates pancreatic veins. Destructive changes to the ducts and acini caused by infiltrating granulocytes are typically absent. Immunostaining reveals abundant IgG4-positive cells (> 10 cells/HPF)^[27,31].

Type 2 AIP histology typically shows IDCP (AIP with GELs)^[21,27,31], which is a distinct histological pattern from that of LPSP. The predominant interlobular stroma composed of lymphocytes plasma cells and reactive fibroblasts/myofibroblasts seen in type 1 AIP is replaced by the presence of GELs as the most distinctive feature of IDCP. These changes may lead to the destruction and obliteration of the duct lumen, seen in the medium to small-sized ducts and also in the acini. Infiltrates of IgG4-positive plasma cells are scant or absent in IDCP^[27,31]. Currently, a definitive diagnosis of type 2 AIP requires histology (Table 3 and Figure 3). This unique histological subtype could be distinguished from type 1 AIP by expert pathologists with high diagnostic ratio (concordances: 60%-100%, multirater kappa: 0.54) using the international consensus histopathological diagnostic criteria^[28].

The feasibility of arriving at a histological diagnosis for AIP using endoscopically obtained tissue samples has been argued^[59-62]. Several studies demonstrated that tissue samples obtained by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) enabled histological diagnosis of both type 1^[60-62] and type 2^[63,64] AIP.

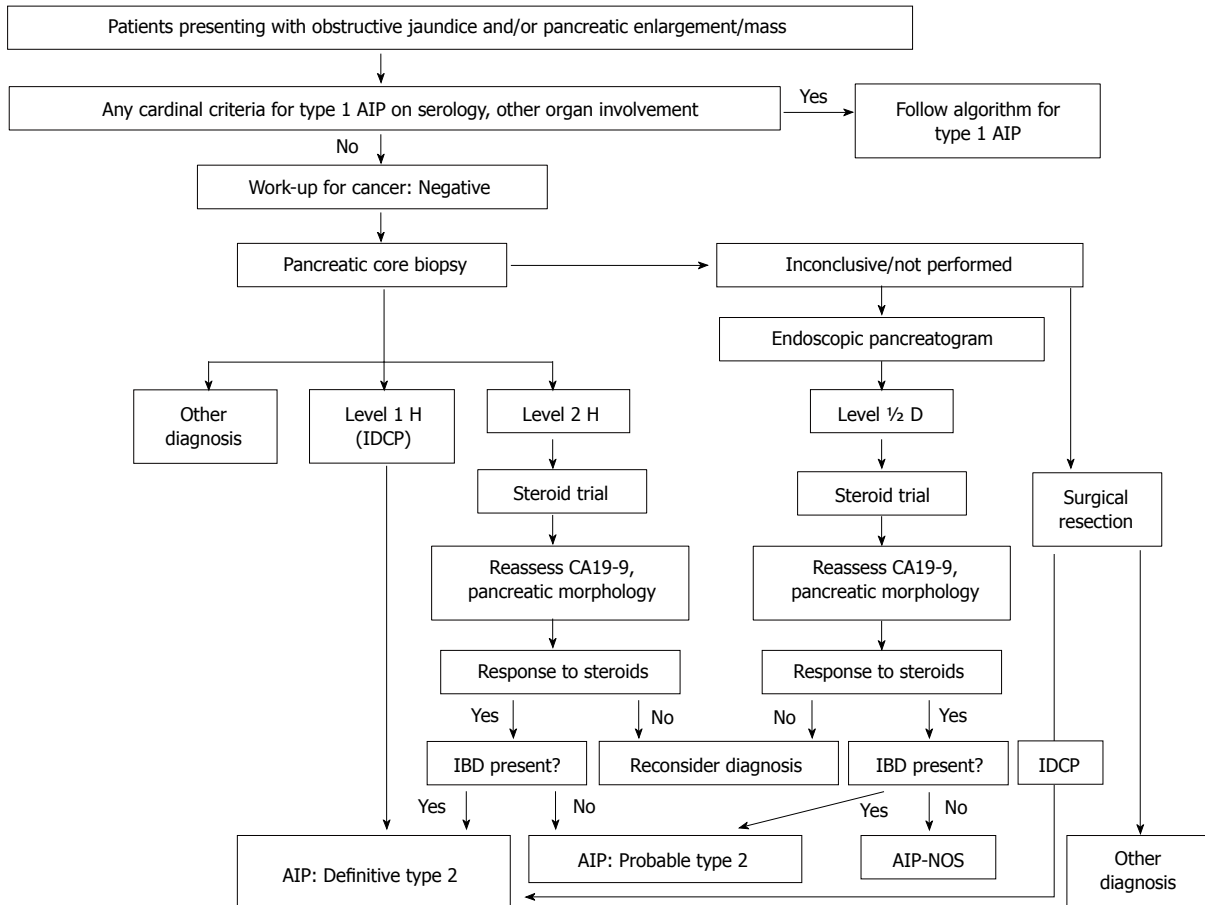


Figure 3 Algorithm of international consensus diagnostic criteria to diagnose type 2 autoimmune pancreatitis in subjects presenting with obstructive jaundice and/or pancreatic mass. This schematic drawing shows a flow to diagnose type 2 AIP with typical/indeterminate (atypical) findings of the pancreas on CT/MRI (level 1 and 2 parenchymal findings)^[25]. AIP: Autoimmune pancreatitis; IBD: Inflammatory bowel disease; IDCP: Idiopathic duct-centric chronic pancreatitis.

Exclusion of the pancreatobiliary malignancies

Exclusion of pancreatobiliary malignancies is necessary for the diagnosis of AIP, especially in atypical cases. Today, the diagnosis of pancreatic mass lesions by EUS-FNA provides a sensitivity for detecting PC tissue that exceeds 90% (91%-93%)^[59,65,66], making EUS-FNA the most effective tool for excluding pancreatic malignancies. However, core biopsy using a large-caliber needle^[60,61,67] may increase the chance of a definitive histological diagnosis of AIP. A Japanese nationwide survey published in 2012^[68] reported that histological confirmation was obtained in about 40% of AIP cases by EUS-guided tissue sampling, in 22% by resection, and in 18% by percutaneous biopsy. The choice of suitable modalities for histological evaluation can therefore eliminate non-necessary surgery in a large number of cases.

AIP is often associated with sclerosing cholangitis, which needs differential diagnosis from bile duct cancer. In this sense, periampullary forceps biopsy (and cytology) should be added in cases with biliary stricture, as this method has high sensitivity for confirming cancer tissue in the biliary cancer cases (77%^[69,70]-92%^[71]).

Response to steroid

Steroid response is seen in 97%-98% of both type 1 and

type 2 AIP cases^[33,34]; hence, it is considered a useful diagnostic tool. Moon *et al.*^[72] performed a 2 wk steroid trial on 22 consecutive patients with a pancreatic mass lesions atypical for AIP and used by CT and MRCP/ERCP to determine the steroid response. All 15 patients who responded to steroid were diagnosed with AIP, whereas all 7 patients who did not show a steroid response were confirmed as having PC^[72]. We also used abdominal US to analyze the steroid response of the pancreatic lesion of AIP, and we recognized a steroid response (shrinkage of the pancreatic lesion) in 86% of the cases in 2 wk and in 97% after one month^[33]. However, one case in this study showed no response by US and CT and required ERCP, which revealed an improvement in the narrowing of the MPD and the occurrence of hilar bile duct stenosis after the withdrawal of corticosteroid^[33,48]. Similarly, some cases of AIP fulfill the diagnostic criteria after cessation of steroid^[73], so that clinicians need to remain aware of this. Many diagnostic criteria including those for ICDC (Table 2) can include evaluation of a steroid response either in the pancreatic or extrapancreatic lesions^[9,17,18,25], but the diagnosis is worrisome when the steroid response is seen only in the extrapancreatic lesions and not in the pancreas.

Today, a “response to steroid” is a commonly evalu-

Table 5 Characteristics of clinicopathological findings in type 1 and type 2 autoimmune pancreatitis

	Type 1 AIP	Type 2 AIP
Geographical distribution	Asia > United States, Europe	Europe > United States > Asia
Age at presentation	60-70 s	40-50 s
Gender	Male >> Female	Male = Female
Symptoms	Jaundice, Abdominal pain	Jaundice, Abdominal pain
Serology	IgG4, IgG, Autoantibodies	Usually negative
Pancreatic images	Enlarged (focal, diffuse)	Enlarged (focal, diffuse)
Pancreatic histology	LPSP	IDCP with GEL
Extrapancreatic lesions	Sclerosing cholangitis, sialoadenitis, retroperitoneal fibrosis, interstitial nephritis, etc.	Inflammatory bowel disease
Steroid response	Excellent	Excellent
Relapse	High rate	Rare

AIP: Autoimmune pancreatitis; LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP with GEL: Idiopathic duct-centric pancreatitis with granulocyte epithelial lesion.

ated diagnostic item for AIP in almost all diagnostic criteria^[9,16-20,25]. However, it had not been included in the previous Japanese diagnostic criteria (2006)^[15] in order to avoid simplistic therapeutic diagnosis by a steroid response without exclusion of possible pancreatobiliary malignancies. Clinicians must be careful in making differential diagnoses, and when malignant conditions are difficult to differentiate, pathological examination by EUS-FNA is preferable.

CONCLUSION

AIP is a unique form of chronic pancreatitis consisting of two distinct subtypes and associated with various systemic disorders. An accurate diagnosis can only be obtained when clinicians have a good understanding well on this disease entity and need to make use of diagnostic items including clinical images for pancreatic parenchyma, pancreatography and extrapancreatic lesions, serum markers, histological examinations of the pancreatic lesion, and steroid responses.

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Table 6 Extrapancreatic lesions associated with type 1 autoimmune pancreatitis

Close association	Possible association
Lachrymal gland inflammation	Hypophysitis
Sialoadenitis	Autoimmune neurosensory hearing loss
Hilar lymphadenopathy	Uveitis
Interstitial pneumonitis	Chronic thyroiditis
Sclerosing cholangitis	Pseudotumor (breast, lung, liver)
Retroperitoneal fibrosis	Gastric ulcer
Tubulointestinal nephritis	Swelling of Papilla of Vater
	IgG4 hepatopathy
	Periaortitis
	Prostatitis
	Schonlein-Henoch purpura
	Autoimmune thrombocytopenia

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Antioxidative phytochemicals to ameliorate pancreatitis in animal models: An answer from nature

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Abstract

Despite enthusiastic efforts directed at elucidating critical underlying mechanisms towards the identification of novel therapeutic targets for severe acute pancreatitis (SAP), the disease remains without a specific therapy to be executed within the first hours to days after onset of symptoms. Although earlier management for SAP should aim to either treat organ failure or reduce infectious complications, the current standard of care for the general management of AP in the first hours to days after onset of symptoms include intravenous fluid replacement, nutritional changes, and the use of analgesics with a close monitoring of vital signs. Furthermore, repeated evaluation of severity is very important, as the condition is particularly unstable in the early stages. In cases where biliary pancreatitis is accompa-

nied by acute cholangitis or in cases where biliary stasis is suspected, an early endoscopic retrograde cholangiopancreatography is recommended. However, practice guidelines regarding the treatment of pancreatitis are suboptimal. In chronic pancreatitis, conservative management strategies include lifestyle modifications and dietary changes followed by analgesics and pancreatic enzyme supplementation. Recently, attention has been focused on phytochemicals or antioxidants as agents that could surpass the limitations associated with currently available therapies. Because oxidative stress has been shown to play an important role in the pathogenesis of pancreatitis, antioxidants alone or combined with conventional therapy may improve oxidative-stress-induced organ damage. Interest in phytochemicals stems from their potential use as simple, accurate tools for pancreatitis prognostication that could replace older and more tedious methods. Therefore, the use of antioxidative nutrition or phytochemicals may represent a new direction for clinical research in pancreatitis. In this review article, recent advances in the understanding of the pathogenesis of pancreatitis are discussed and the paradigm shift underway to develop phytochemicals and antioxidants to treat it is introduced. Despite the promise of studies evaluating the effects of antioxidants/phytochemicals in pancreatitis, translation to the clinic has thus far been disappointing. However, it is expected that continued research will provide solid evidence to justify the use of antioxidative phytochemicals in the treatment of pancreatitis.

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Key words: Acute pancreatitis; Chronic pancreatitis; Severe acute pancreatitis; Antioxidants; Phytochemicals

Core tip: In this review, the paradigm shift regarding the development of phytochemicals and antioxidants is introduced following a comprehensive description

of newer information pertaining to the pathogenesis of pancreatitis. Several animal models are discussed with regard to their role in efforts to develop efficient strategies against pancreatitis. Subsequently, newer therapeutic options with an emphasis on nutrients and phytochemicals are reviewed. Further discussion also focuses on the promise of studies evaluating the effects of antioxidants/phytochemicals in pancreatitis, the disappointing nature of translation of these agents to clinical settings, and the expected research advances that may support the use of antioxidative phytochemicals in the treatment of pancreatitis.

Park JM, Lee S, Chung MK, Kwon SH, Kim EH, Ko KH, Kwon CI, Hahm KB. Antioxidative phytochemicals to ameliorate pancreatitis in animal models: An answer from nature. *World J Gastroenterol* 2014; 20(44): 16570-16581 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16570.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16570>

INTRODUCTION

Acute pancreatitis (AP) is a relatively common clinical condition, presenting with variable severity from mild and self-limited attacks to severe attacks that contribute to mortality^[1]. Severity is associated mechanistically with the underlying pathogenesis of AP which includes pancreatic acinar cell injury in early stages after a local inflammatory reaction, subsequent acinar cell death in the form of apoptosis and necrosis, and the initiation of systemic inflammatory response syndrome (SIRS). An excessive SIRS leads to distant organ damage referred to as multiple organ dysfunction syndrome (MODS)^[2]. Recent insights changed a paradigm shift in understanding of AP that intra-acinar trypsinogen activation might lead to early pancreatic injury, but the inflammatory response of AP develops independently driven by early activation of enzyme activation^[3]. Whereas, though still effective, the concept that the pancreatic injury is initiated within pancreatic acinar cells subsequent to premature intracellular activation of digestive enzymes and these zymogen activations within acini early during AP was shown to be sufficient to induce AP, finally contributed to the development of chronic pancreatitis^[4,5]. Recently, Sah *et al*^[6] found that cerulean-induced chronic pancreatitis (CP) did not require intra-acinar activation of trypsinogen, whereas regulation of the inflammatory response by nuclear factor kappa B (NF-κB) might be involved in the pathogenesis of CP. Collectively, these data suggest a need for the development of novel compounds to either block the early activation of pancreatic enzymes or to ameliorate inflammation in order to limit or prevent complications of AP or inhibit the progression to CP or inflammation-associated fibrosis or carcinogenesis. The delay between the onset of pancreatitis and the development of the systemic response makes AP an ideal experimental and clinical model with which to study the role of inflammatory

mediators and to test novel therapies, as the elucidation of the key mediators involved in the pathogenesis of AP will facilitate the development of clinically effective anti-inflammatory therapies^[7].

Recent advances in understanding the pathogenesis of pancreatitis-induced SIRS and its complications

AP is an inflammatory disorder, as inflammation not only affects pathogenesis, but also determines the course of the disease from pancreatic acinar cell injury and death to the initiation of SIRS^[8]. As excessive SIRS culminates in the primary cause of morbidity and mortality associated with AP, distant organ damage (MODS), it is important to identify the molecules and factors involved in this process. Phospholipase A2 (PLA2), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, CINC/GRO-α, MCP-1, platelet activating factor (PAF), IL-10, CD40L, C5a, ICAM-1, MIP1-α, CCL5 (RANTES), substance P, and hydrogen sulfide (H₂S) have all been shown to play critical roles^[9]. The systemic effects of AP are similar to those of other conditions such as septicemia, severe burns, and trauma. For instances, AP in its severe form is complicated by MODS, most importantly by pulmonary complications which include hypoxia, acute respiratory distress syndrome, atelectasis, and pleural effusion^[10].

Novel pathogenic mechanisms relevant to newer therapeutics: Autophagy, apoptosis, and redox-associated transcriptional activators

Autophagy, the principal cellular degradative pathway for cellular protection, is impaired in pancreatitis and is associated with defective lysosomal function^[11]. Although research on autophagy in pancreatitis is now in its early stages, it is hoped that data regarding upstream mechanisms mediating autophagic dysfunction and downstream links to pancreatitis pathologies may provide insights into novel molecular targets and therapeutic strategies for the treatment of pancreatitis^[12]. In their detailed explanation of a profound dysfunction of key cellular organelles (lysosomes and mitochondria) in pancreatitis, Gukovsky *et al*^[13] described the cause of impaired autophagy in AP and attributed it to inefficient flux resulting from defective lysosomes. Additionally, they suggested that lysosomal dysfunction in pancreatitis could be attributed to either abnormal processing and activation of major lysosomal hydrolases such as cathepsins, or *via* a decrease in pancreatic levels of the key lysosomal membrane proteins LAMP-1 and LAMP-2. NF-κB inactivation is an additional key pathogenic concern in pancreatitis^[14]. NF-κB is a nuclear transcription factor responsible for regulating the transcription of a wide variety of genes involved in immunity and inflammation and plays a critical role in pancreatitis as well as extrapancreatic complications and pancreatic cancer^[15]. As seen in several animal models of pancreatitis, NF-κB has been critically implicated in either initiation or propagation of pancreatic inflammations, cerulean-induced pancreatitis^[16], taurocholate-induced pancreatitis^[17], and arginine-induced pancreati-

tis^[18]. Relevant to autophagy, NF- κ B pathway activation stimulated autophagy during induction of acute necrotizing pancreatitis, after which targeted inhibition of the NF- κ B pathway may provide novel therapeutic strategies for reducing the severity of pancreatitis^[19]. An additional novel mechanism relevant to newer therapeutics involves apoptosis. To test the hypothesis that preventive apoptosis execution would limit the propagation of necro-inflammations in pancreatitis, our group^[20] investigated the ability of natural products to induce apoptosis and ameliorate cerulean-induced pancreatitis. Bhatia^[21,22] concluded that apoptosis could be a favorable response to acinar cells and that interventions that favor induction of apoptotic, as opposed to necrotic, acinar cell death might reduce the severity of an attack of AP. Aside from pancreatic damage, accelerated acinar cell apoptosis can limit SIRS, as exemplified by honokiol, a low molecular weight natural product similar to *Artemisia*^[23]. The pathogenic roles of transforming growth factor- β (TGF- β) signaling^[24], H₂S bio-gas, and substance P have also come under scrutiny in order to identify potential therapeutic targets. H₂S, which plays important physiologic roles in the cardiovascular, central nervous, and gastrointestinal (GI) systems, has been associated with inflammation, especially gastritis and pancreatitis, through vasomodulation and neuromodulation^[25,26]. Substance P, a neuropeptide released from nerve endings after binding to neurokinin-1 (NK-1) receptors on the surface of effector cells, plays important roles in several inflammatory states including asthma, immune-complex-mediated lung injury, experimental arthritis, and inflammatory bowel disease, as well as A/CP through increasing microvascular permeability, promoting plasma extravasation, and mediating pain^[27]. Bhatia *et al.*^[28] investigated the interplay between the pro-inflammatory effects of H₂S and substance P in a murine model of cerulein-induced AP and suggested that the pro-inflammatory effects of H₂S may have been mediated by the substance P-NK-1 receptor pathway in AP. Lastly, oxygen free radicals in excessively high amounts are all very reactive chemically and can impose a detrimental influence on living organisms by provoking oxidative stress that can damage the pancreas^[28].

Recent updates on the pathogenesis of CP relevant to pancreatic inflammation

CP is an inflammatory disease of the pancreas characterized by progressive fibrotic destruction of the pancreatic secretory parenchyma. Genetic studies of hereditary, familial, and idiopathic forms of CP have provided much-needed insight into the pathogenesis of CP. The pivotal role of prematurely activated trypsin within the pancreas in the etiology of CP has been firmly established based on the identification of gain-of-function missense and copy number mutations in the cationic trypsinogen gene and loss-of-function variants in both the pancreatic secretory trypsin inhibitor and chymotrypsinogen C genes. In particular, variants in the gene encoding carboxypeptidase A1, CPA1, were found to be strongly associated with ear-

ly onset CP^[29,31]. Additionally, loss-of-function variants in the cystic fibrosis transmembrane conductance regulator and calcium-sensing receptor genes have also been shown to increase the risk of CP^[32]. In addition to these genetic preponderances, necrosis or apoptosis, and inflammation or pancreatic duct obstruction are known to be involved in the pathogenesis of CP. Furthermore, the fibrosing process ultimately leads to progressive loss of the lobular morphology and structure of the pancreas, deformation of the large ducts, and severe changes in the arrangement and composition of the islets. These changes in turn lead to pancreatic insufficiency and predispose patients to changes associated with carcinoma. Irrespective of etiological factors such as heredity, alcohol or nicotine consumption, and nutritional, efferent duct, immunological, and rare metabolic factors, the underlying inflammation and associated subsequent fibrotic destruction of the pancreatic secretory parenchyma are common pathogenic factors in CP that represent targets for prevention through modulation of pancreatic inflammation^[33]. Our understanding of CP pathogenesis has improved in recent years through important advances regarding the delineation of mechanisms responsible for the development of pancreatic fibrosis following repeated acute attacks of pancreatic necro-inflammation, also referred to as the necrosis-fibrosis concept^[34]. Although steroids can rapidly reduce symptoms in patients with autoimmune CP and micronutrient therapy to correct electrophilic stress is emerging as a promising treatment in the other patients^[35], steatorrhea, diabetes, local complications, and psychosocial issues associated with the disease represent additional therapeutic challenges. Such challenges may be resolved in part through intervention with potent anti-inflammatory/anti-oxidative phytochemicals. In this review, newer therapeutic nutrient-based options and phytochemicals will be introduced.

ANIMAL MODELS OF PANCREATITIS FOCUSED ON THE DEVELOPMENT OF NEW THERAPEUTICS

Failure to decrease the mortality rate attributable to pancreatitis or improve strategies to prevent CP over the past few decades indicate that current treatment options are limited and predominantly dependent on supportive therapy^[36]. Because a key feature of severe AP (SAP) is the presence of extensive tissue necrosis accompanied by inflammatory response syndromes, animal models of AP have become an essential investigative tool for developing potent anti-inflammatory agents. Therefore, a better understanding of the underlying pathophysiology of SAP may lead to more targeted therapeutic options, potentially leading to improved survival. Diverse animal models of AP, from the non-invasive gene knockout and *L*-arginine models as well as the hormone [cerulein as a cholecystikinin (CCK) analog]-, alcohol-, and immune-mediated-diet [choline deficient, ethionine supplemented,

Table 1 Rodent model to study acute and chronic pancreatitis

Acute pancreatitis
Cerulein ± lipopolysaccharide (LPS) or ethanol
Bile salt duct infusion
Duct obstruction ± secretagogues
Diet [choline-deficient ethionine-supplemented (CDE)]
Cytokines
Coxsackie virus group B (CVB)
Chronic pancreatitis
Cerulein (repeated dosing)
Alcohol
Duct infusion such as trinitrobenzene sulfonic acid or sodium taurocholate or dibutyltin dichloride
Duct obstruction
Genetic; Cox-2, CFTR, IKK2, LXRB, PERK, TGF-β1
Immunologic
Diet (CDE)
CVB

(CDE)]-induced models, to invasive models including pancreatic duct ligation (PDL), antegrade pancreatic duct perfusion, biliopancreatic duct injection of sodium taurocholate, combination of secretory hyperstimulation with minimal intraductal bile acid exposure, vascular-induced, ischaemia/reperfusion and duct ligation, are available^[37] (Table 1). Potential therapeutics can be developed with these animal models, as they share common aspects including the aforementioned pathogenesis of intracellular chemical activation, pancreatic secretion reflux, intracellular production of reactive oxygen species (ROS), and intracellular production of free radicals. As in CP, a special focus on pancreatic duct ligation, repetitive overstimulation with cerulein and chronic alcohol feeding, as well as specific genetic models has been applied^[38]. In this review, we will describe some of the animal models used in our efforts to develop efficient strategies against pancreatitis.

Cerulein-induced pancreatitis

Intravenous infusion of the synthetic CCK analog cerulein at a dose of 0.25 µg/kg per hour causes maximal stimulation of pancreatic exocrine secretion^[39]. The infusion of supramaximal doses of cerulein (5 µg/kg per hour and 10 µg/kg per hour) induces a significant increase in pancreatic enzymes in blood, as well as interstitial edema and inflammatory cell infiltration that leads to cerulein-induced edematous pancreatitis in rats, mice, dogs, and hamsters. Aside from intravenous infusion, repeated intraperitoneal injections can also be used to induce pancreatitis. In the early phase, large autophagic vacuoles result from fusion of zymogen granules, accompanied by an increase in lysosomal enzyme activity and activation of trypsinogen. However, since the degree of pancreatitis is generally mild, all animals survive the induction of pancreatitis and resolve completely within 6 d after induction. This model of experimental pancreatitis favors the analysis of intracellular events in the early phase of pancreatitis as seen in Figure 1A, which shows edematous pancreatitis, however, the addition of lipopolysaccharide injection or bile duct ligation can to wors-

en simple edematous mild pancreatitis as well as oxidative stress and result in acute hemorrhagic pancreatitis^[40-42].

Sodium taurocholate infusion; intraparenchymal or intrapancreatic ductal injection

Paran *et al.*^[43] are credited with the initial attempt to develop acute necrotizing pancreatitis through intraparenchymal injection of sodium taurocholate in rats. Sodium taurocholate was injected at a dose of 0.3 mL/100 g body weight in concentrations of 5% and 10% into the pancreatic parenchyma of 32 Wistar rats. Early pathological changes observed in the pancreas were focal hemorrhages, parenchymal necrosis, and neutrophil infiltration and at 72 h, the changes observed were acinar necrosis, edema, fibrin deposition and inflammatory cell infiltration. At later time points, changes such as fibrinoid necrosis and fibroblast proliferation were observed^[44]. High-pressure infusion of sodium taurocholate into the biliopancreatic duct of rats resulted in significant pancreatic and lung alterations^[45]. Taurocholate-induced pancreatitis is therefore a reliable model for severe necrotizing pancreatitis in mice with significantly greater pancreatic damage and systemic inflammatory responses as compared to cerulein-induced pancreatitis and correlate with the clinical observations of multisystem organ failure in AP and early changes in affected organs, suggesting that careful observation should be mandatory in patients with AP in order to institute supportive treatment^[46].

L-arginine-induced pancreatitis

In 1984, Hegyi *et al.*^[47] developed a new type of experimental necrotizing pancreatitis model in rats through the use of a high dose of L-arginine administered *via* intraperitoneal administration. This non-invasive model is highly reproducible and produces selective, dose-dependent acinar cell necrosis. Not only is this a good model to study the pathogenic mechanisms of acute necrotizing pancreatitis, but it is also excellent with regard to observing and influencing time course changes of the disease (Figure 1B). Subsequent intraperitoneal injection of 3 g/kg L-ornithine caused SAP and higher doses (4 to 6 g/kg) were lethal within hours^[48]. Serum and ascitic amylase activities were significantly increased and the increase in pancreatic trypsin activity correlated with the degradation of IκB proteins and elevated IL-1β levels. Oxidative stress in the pancreas was evident from 6 h, making this a simple, noninvasive model of acute necrotizing pancreatitis in rats *via* intraperitoneal injection of 3 g/kg L-ornithine. Compared with L-arginine, L-ornithine was even more effective in inducing pancreatitis. It should be noted that large doses of L-arginine produce a toxic effect on the pancreas attributable, at least in part, to the actions of L-ornithine.

PDL

AP may be induced by ligating the distal bile duct at the level of the duodenum, which causes the early development of AP, obstructive jaundice and cholangitis in

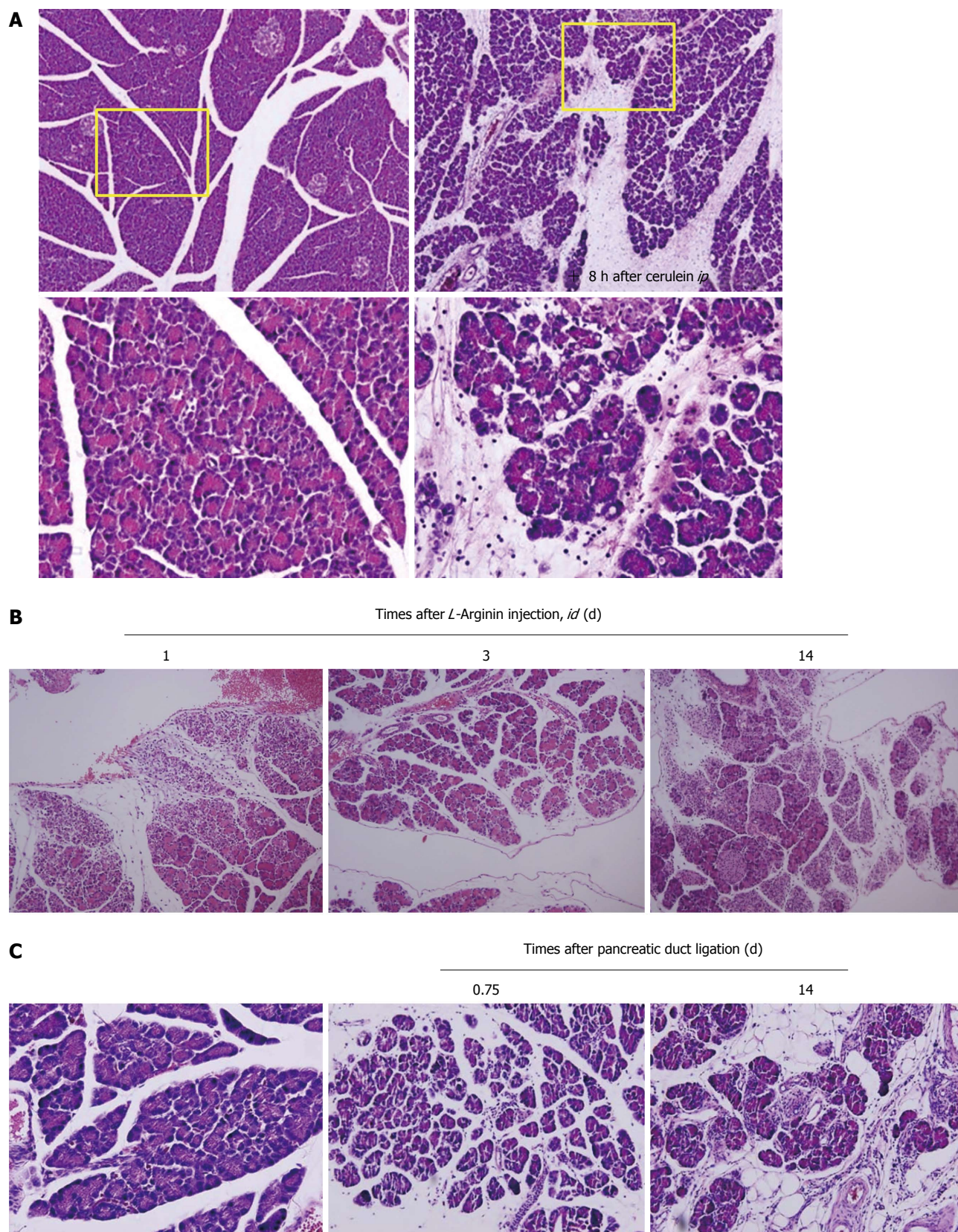


Figure 1 Animal models for pancreatitis. A: Cerulein-induced edematous pancreatitis. Caerulein-induced pancreatitis is a valuable experimental model for studying altered intracellular transport, compartmentation of lysosomal, and digestive enzymes, resulting in edematous pancreatitis. The formation of enlarged secretory vacuoles containing lysosomal and digestive enzymes is paralleled by the activation of lysosomes and degradation of cellular organelles in autophagosomes. On the level of secretory and autophagic vacuoles, activation of serine proteases occurs, which in addition to increasing lysosomal enzyme activities can represent the initial stage for acinar cell destruction and the development of pancreatitis; B: *L*-arginine-induced necrotizing pancreatitis. Parenchymal hemorrhage and widespread acinar cell necrotic changes were noted with *L*-arginine; C: Pancreatic duct ligation-induced pancreatitis. Morphologic examination of the pancreas showed massive interstitial edema, apoptosis, and necrosis of acinar cells with infiltration of neutrophil granulocytes and monocytes 0.75 d after pancreatic duct ligation. Two weeks later after periodontal ligation, the destructed parenchyma with fat replacement as well as some fibrotic changes were seen.

animals. The duct ligation model was developed in an attempt to resemble clinical conditions including gallstone formation, motility disorders of the sphincter, edema and strictures at the papilla, tumors of the papilla, and parasites impacting the terminal biliopancreatic duct. However, surgical ligation of the pancreatic duct alone usually causes only a mild to moderate degree of pancreatitis and has not been successful in inducing SAP. Instead, most laboratory animals developed chronic lesions in the pancreas characterized by atrophy and apoptosis of acinar and ductal tissue without significant necrosis or inflammation. Human CP is characterized by irreversible fibrosis, whereas pancreatic fibrosis in animal models is reversible (Figure 1C). Miyauchi *et al.*^[49] compared CP with fibrosis in three different animal models, the dibutyltin dichloride model, WBN/Kob rats, and PDL rats, and found that an imbalance between the synthesis and degradation of extracellular matrix molecules or the degree of stimulation over a certain period may lead to pancreatic fibrosis.

CDE diet-induced necrotizing pancreatitis

Female albino mice were fed a choline-deficient diet containing 0.5% DL-ethionine which was lethal within 5 d due to the development of an acute hemorrhagic pancreatitis accompanied by massive fat necrosis throughout the peritoneal cavity^[50]. Major findings included the accumulation of zymogen granules, vacuolation due to foci of cytoplasmic degradation, and alterations in the morphology of the zymogen granules (Figure 2A). Pancreatitis appeared to be due to the intraparenchymal activation of zymogens resulting from a synergistic action of choline deficiency with the basic toxicity of ethionine toward the acinar cells of the pancreas. Because this experimental model simulated the acute hemorrhagic pancreatitis with fat necrosis that occurs in humans, it may prove useful for exploring the pathogenesis of severe pancreatitis with SIRS (Figure 2B)^[51]. The diet model appears to be a good approximation of severe necrotizing human pancreatitis as well as CP with histological and biochemical similarities. Both the gross and histological appearance of the pancreatic and peripancreatic inflammation, as well as the clinical and biochemical course of diet-induced pancreatitis, resembled human disease and should be suitable for evaluation of potential clinically-applicable drugs^[52]. For example, our group developed ND-07, a novel drug candidate with potent antioxidative and anti-inflammatory properties, that effectively prevented necrotizing pancreatitis^[53].

Animal models for CP

Since CP is defined as a continuous or recurrent inflammatory disease of the pancreas characterized by progressive and irreversible morphological changes, pancreatitis followed by perilobular and intralobular fibrosis of the parenchyma, calcifications in the parenchyma as well as the formation of pseudocysts^[49]. Therefore, animal models of CP are not different from AP models, but need to overcome the acute fatal status according to models, adopting chronic PDL, repetitive overstimulation with

cerulean, chronic alcohol feeding, and chronic caring of *L*-arginine or CDE diet model. However, as seen in Figure 2C, irreversible fibrosis and pancreatic insufficiency following repeated acute attacks of pancreatic necroinflammation^[34], is accompanied.

LIMITATION OF CURRENT PHARMACOLOGIC TREATMENT OF ACUTE AND CHRONIC PANCREATITIS

AP and SAP

Though AP is a disease of variable severity that can lead to significant morbidity and mortality, current management has remained limited to only supportive measures and the treatment of complications. A myriad of pharmacologic therapies targeting various aspects of the underlying pathophysiology have been evaluated and tried over the last few decades, including anti-secretory agents, protease inhibitors, antioxidants, immunomodulators, non-steroidal anti-inflammatory drugs, and prophylactic antibiotics. Only a few of these therapies have demonstrated promise in significantly altering the progression of this disease, and therefore, further studies are necessary to clearly elucidate these benefits in patients at risk for poor outcomes^[54]. Regarding pharmacological prevention and treatment of AP, Bang *et al.*^[55] reported that somatostatin and octreotide inhibited the exocrine production of pancreatic enzymes and may therefore be useful as prophylaxis against post ERCP pancreatitis (PEP). Though the protease inhibitor gabexate mesilate has been used routinely as treatment for pancreatitis in some countries, randomized clinical trials and a meta-analysis have not supported this practice. Recently, the NSAIDs indomethacin and diclofenac have showed some potential as prophylaxis against PEP in randomized studies. Antibodies against TNF- α have been suggested as a potential rescue therapy, however, no clinical trials are being conducted at present^[56].

Chronic fibrosing pancreatitis

Because exocrine pancreatic insufficiency has been associated with changes in GI intraluminal pH, motility disorders, bacterial overgrowth, and altered pancreatic gland secretions, drug absorption in patients with CP may be affected by the degree of CP severity^[57]. Furthermore, the general health condition of CP patients is often quite poor, as most patients with CP limit their food intake due to the pain caused by eating and in some cases food intake may be more or less substituted with alcohol, tobacco and coffee. However, pancreatic fibrosis is a characteristic feature of chronic pancreatic injury, which is a result of the imbalance between the synthesis and degradation of extracellular proteins. As stellate cells are pivotal cells implicated in the TGF- β induction of collagens, our previous studies confirmed that antioxidant or antioxidative phytochemicals ameliorated the progression of fibrosing pancreatitis through suppressive actions on pancreatic stellate cells.

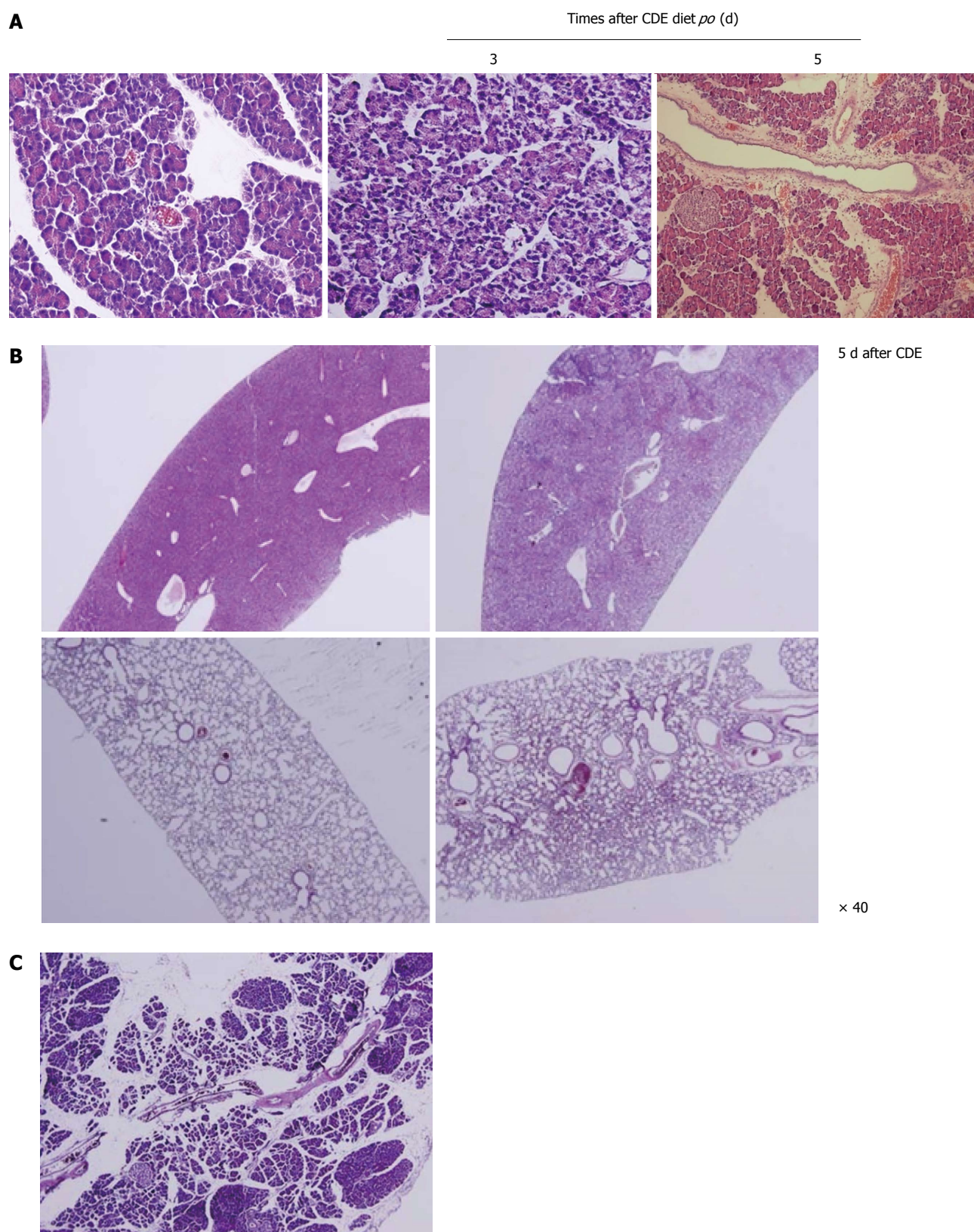


Figure 2 Animal model of choline-deficient, ethionine-supplemented diet-induced necrotizing pancreatitis. A: Choline-deficient, ethionine-supplemented (CDE) diet-induced necrotizing pancreatitis. Massive destruction of pancreatic parenchyma with focal necrotic foci was seen; B: Systemic inflammatory response syndrome hepatic necrosis and pneumonitis was seen; C: Chronic fibrosing pancreatitis was noted 2 mo after CDE diet administration.

APPLICATION OF ANTIOXIDATIVE PHYTOCEUTICALS TO AMELIORATE AP AND CP

Resveratrol

Resveratrol, a natural polyphenolic compound, was first discovered in the 1940s. Although initially used for cancer therapy, it has shown beneficial effects against most cardiovascular, cerebrovascular, and several inflammatory diseases^[58]. It is found in diverse forms of plant life, notably berry fruits, has positive effects on metabolism, and can increase the lifespans of various organisms. The effects of resveratrol have been attributed to its capacity to interact with multiple molecular targets involved in diverse intracellular pathways. One of the more well-known resveratrol interactions involves the activation of sirtuins, a class of NAD(+)-dependent deacetylases, and subsequent HDAC inhibition that affects multiple transcription factors and other protein targets^[59,60]. The intracellular pathways activated are crucial for anti-oxidant defense, regulation of the cell cycle, mitochondrial energy production, vascular tone, oncogene suppression, and many other phenomena. Meng *et al.*^[61] investigated whether resveratrol could effectively inhibit the expression of NF- κ B activation, alleviate the severity of SAP through its anti-inflammatory effects, and regulate inflammatory mediators. A study by Ma *et al.*^[62] found that the beneficial outcomes attributable to resveratrol were closely associated with anti-inflammatory, antioxidant, and chemopreventive effects, as well as the inhibition of platelet aggregation, in SAP. Through these effects, resveratrol was able to down-regulate pro-inflammatory cytokines, improve microcirculation, modulate cell apoptosis, and block calcium overload. Additionally, resveratrol inhibited NF- κ B activity and reduced concentrations of TNF- α , IL-6 and IL-1 β . It also regulated calcium and scavenged ROS capable of extensive tissue damage on extrapancreatic organs^[63]. Furthermore, resveratrol has been shown to ameliorate SIRS by improving underlying lung microcirculation dysfunction through decreasing leukocyte-endothelial interactions, reducing blood viscosity, improving the decrease in blood flow, and stabilizing erythrocytes in SAP rats^[61] and inactivated intraperitoneal macrophages^[64].

Artemisia extracts

Oxygen free radicals (ORFs) mediate an important step in the initiation of experimental AP. Additionally, several clinical findings have implicated OFRs as possible contributors to the pathogenesis of pancreatic fibrosis. To date, there are no studies reporting potential roles for OFRs in the development of CP with the prevention with antioxidants. Yoo *et al.*^[65] conducted a study designed to establish a mouse model of chronic fibrosing pancreatitis and to prove the involvement of OFRs in CP with fibrosis. Repeated intraperitoneal injection of cerulein provoked significant and severe chronic fibrosing pan-

creatitis after 5 wk. Following treatment with *Artemisia* extracts, the extent of pancreatic fibrosis was significantly decreased, as was the degree of pancreatic inflammation. Furthermore, the level of NF- κ B binding activity, which was increased in CP, was significantly attenuated after *Artemisia* extract treatment (Figure 3A). The levels of myeloperoxidase and iNOS activities were also significantly decreased in the *Artemisia*-treated group as compared to the pancreatitis only group. Conversely, cytoprotective proteins such as heat shock protein-70 and metallothionein were significantly increased in the *Artemisia*-treated group. In addition, *Artemisia* decreased the expression of alpha-SMA and type I collagen in cultured pancreatic stellate cells.

Other potential phytochemicals from nature

There have been published reports describing successful trials demonstrating the beneficial preventive or therapeutic effects of phytochemicals in diverse animal models of pancreatitis. As examples, rhubarb has been shown to significantly attenuate SAP by inhibiting activation of MAPKs and the expression of inflammatory mediators in taurocholate-induced pancreatitis^[66], *Nardostachys jatamansi* has been implicated as potentially protective in cerulein-induced pancreatitis *via* the induction of HO-1 expression^[67], and *Curcuma longa* has also been implicated as potentially protective against cerulein-induced AP and pancreatitis-associated lung injury *via* significant attenuation of inflammatory mediators such as IL-1 β and TNF- α ^[68]. Additional examples include the anti-inflammatory roles observed for cannabidiol and O-1602, the ligands of G protein-coupled receptor 55, in cerulein-induced AP in mice^[69] and the protective effects of *Scolopendra subspinipes mutilans* water extract in cerulein-induced pancreatitis *via* the deactivation of c-Jun NH₂-terminal kinase, p38, and NF- κ B and subsequent inhibition of high-mobility group box protein-1^[70]. Furthermore, attenuation of cerulein-induced AP by apamin, a component of bee venom, or α -pinene, has been observed and attributed to JNK inhibition^[71,72] and amelioration of AP by *Dachengqi* decoction has been observed and attributed to regulation of the necrosis-apoptosis switch in the pancreatic acinar cell and rat models^[73,74]. Protective effects of three Chinese herbal medicines containing ligustrazine, kakonein, and *Panax notoginsenosides* have been demonstrated on multiple organs in rats with SAP^[75] and protective effects of baicalin and octreotide have also been demonstrated on multiple organ injury in SAP^[76]. Beneficial pancreatic repair effects have been shown following the use of *Embllica officinalis*, a medicinal plant native to India, or melatonin in *L*-arginine-induced AP in rats^[77,78]. An improving effect of pentoxifylline and/or alpha lipoic acid on *L*-arginine-induced SAP has also been described and attributed to antioxidant and anti-inflammatory actions^[79]. Other research has shown effects of Korean red ginseng on superoxide dismutase inhibitor-induced pancreatitis in rats through inhibition of NF- κ B^[80] and the efficacy of *Salvia miltiorrhizae* injection in the treatment of

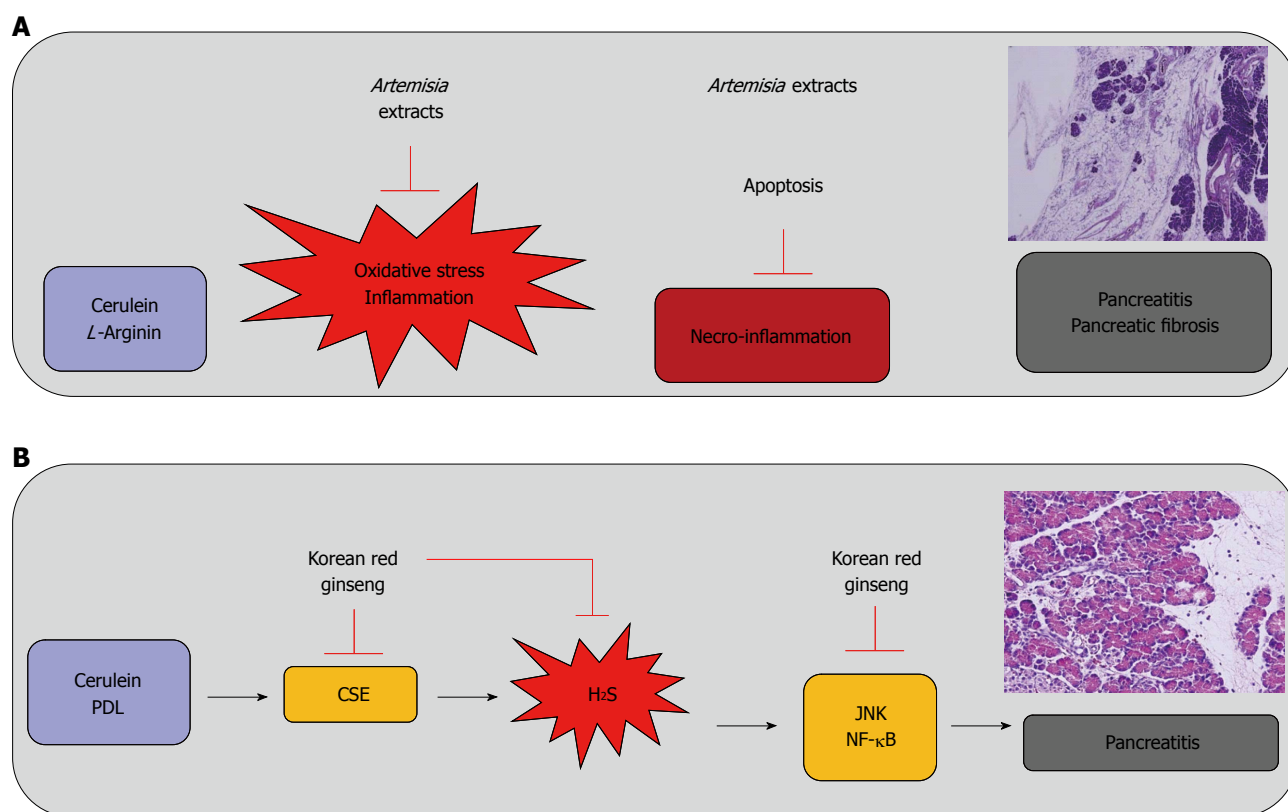


Figure 3 Therapeutic and preventive effect of antioxidative phytochemicals, Artemisia extract and Korean red ginseng against pancreatitis. A: Therapeutic effect of Artemisia extracts against cerulein or L-arginine-induced pancreatitis and chronic fibrosing pancreatitis; B: Korean red ginseng to ameliorate hydrogen sulfide (H₂S)-induced pancreatitis. NF-κB: Nuclear factor kappa B; PDL: Periodontal ligament; CSE: Cystathionine γ-lyase.

rats to promote *Bax*-mediated apoptosis in SAP^[81].

Antioxidants in the treatment of pancreatitis

Oxidative stress plays an important role in the pathogenesis of both AP and CP. Although its impact has been well documented and has been studied clinically in CP, it is less well defined in SAP. In their study of the pathophysiological aspects of oxidative stress in AP, Hackert and Werner^[82] showed that ROS not only participated in the inflammatory cascade, but also mediated inflammatory cell adhesion and consecutive tissue damage. Furthermore, ROS are known to be involved in the generation of pain, an additional important clinical feature of patients suffering from AP. Mechanistically, oxidative stress activates NF-κB, resulting in up-regulation of inflammatory cytokines in pancreatic acinar cells^[83]. This mechanism suggests that small-molecule antioxidants may be clinically useful anti-inflammatory agents *via* inhibition of oxidant-induced cytokine production^[84]. Similarly, the antioxidant pyrrolidine dithiocarbamate significantly attenuated SAP through inhibition of HMGB1^[85] and raxofelast, an inhibitor of lipid peroxidation, significantly reduced NF-κB activation and attenuated cerulein-induced pancreatitis^[86]. The potent antioxidant and anti-inflammatory functions of melatonin have also been demonstrated through their ability to ameliorate cerulein-induced pancreatitis by modulating the actions of Nrf2 and NF-κB^[87].

KOREAN RED GINSENG TO AMELIORATE PANCREATITIS VIA SUPPRESSION OF H₂S

Korean red ginseng (KRG) has been reported to reduce the risk of inflammation in diverse organs. In our previous studies^[88], we demonstrated significant inhibitory actions of KRG on *Helicobacter pylori*-induced H₂S synthesis and the pathogenic connections between H₂S synthesis and development of pancreatitis. Therefore, KRG may be a good example of a natural antioxidative phytochemical for use in ameliorating AP through the inhibition of H₂S synthesis. In one of our recent studies that tested the hypothesis that KRG prevents pancreatitis by mitigating H₂S generation and pancreatic inflammation, we performed *in vitro* experiments to document the inhibitory effects of KRG on H₂S-associated inflammation in pancreatic cells and *in vivo* experiments to document the therapeutic effect of KRG on cerulein-induced and PDL-induced AP. KRG was administered at a dose of 200 mg/kg 16 h and 1 h before the first cerulein injection and at a dose of 500 mg/kg 2 h and 4 h after the first cerulein injection by oral gavage. In the mice treated with KRG, pancreatic injuries as evidenced by pancreatic wet weight, histological examinations, serum levels of amylase and lipase, myeloperoxidase activities, serum and pancreatic levels of IL-6, immunohistochemical staining

of F4/80 for infiltrating macrophages, and H₂S synthesis, were all significantly ameliorated (Figure 3B). The novel finding that KRG decreased PDL-induced hyperamylasemia encouraged us to explore the possibility that KRG pretreatment may prevent ERCP-induced hyperamylasemia. These experiments are ongoing in our clinic.

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WJG 20th Anniversary Special Issues (18): Pancreatitis

Endoscopic prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis

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Abstract

Post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) is not an uncommon adverse event but may be an avoidable complication. Although pancreatitis of severe grade is reported in 0.1%-0.5% of ERCP patients, a serious clinical course may be lethal. For prevention of severe PEP, patient risk stratification, appropriate selection of patients using noninvasive diagnostic imaging methods such as magnetic resonance cholangiopancreatography or endoscopic ultrasonography (EUS), and avoidance of unnecessary invasive procedures, are important measures to be taken before any procedure. Pharmacological prevention is also commonly attempted but is usually ineffective. No ideal agent has not yet been found and the available data conflict. Currently, rectal non-steroidal anti-inflammatory drugs are used to prevent PEP in high-risk patients, but additional studies using larger numbers of subjects are necessary to confirm any prophylactic effect. In this review, we focus on endoscopic procedures seeking to prevent or decrease the severity of PEP. Among various cannulation methods, wire-guid-

ed cannulation, precut fistulotomy, and transpancreatic septostomy are reviewed. Prophylactic pancreatic stent placement, which is the best-known prophylactic method, is reviewed with reference to the ideal stent type, adequate duration of stent placement, and stent-related complications. Finally, we comment on other treatment alternatives, and make the point that further advances in EUS-guided techniques may afford useful PEP prophylaxis.

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Key words: Endoscopic retrograde cholangiopancreatography; Prevention; Pancreatitis; Pancreas stent; Cannulation; Fistulotomy

Core tip: Endoscopic prevention and/or reduction in the severity of pancreatitis (PEP) are considered to be an essential component of appropriate therapy for Post-endoscopic retrograde cholangiopancreatography patients, especially those at high risk. Numerous techniques and drugs have been developed. However, their proven benefits in terms of reducing the severity of pancreatitis are limited. Currently, one popular endoscopic method is prophylactic placement of a pancreatic stent. In this review, we focus primarily on the ideal type of stent, the timing of stent insertion, and the duration of stent placement adequate to prevent PEP. Also, we describe initial cannulation methods including wire-guided cannulation and precut fistulotomy (infundibulotomy), and the alternative techniques of percutaneous biliary drainage and recently emerging endoscopic ultrasonography-guided methodology.

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INTRODUCTION

Among complications of endoscopic retrograde cholangiopancreatography (ERCP), post-ERCP pancreatitis (PEP) is the most common, and the clinical course may be downhill. The prevalence of PEP depends on several factors, including the case mix, the thoroughness of follow-up evaluation, the PEP definition used, patient susceptibility factors, the type of instrumentation used, and the skill of the endoscopist^[1-4]. PEP occurrence is variable, developing after 1%-40% of all procedures, but typical PEP rates have been reported to range from 5%-15% in most prospective studies with unselected patients. Moreover, pancreatitis of severe grade is very rare, occurring after 0.1%-0.5% of ERCPs^[1-6].

Currently, the well-known risk factors for PEP include endoscopic papillectomy, sphincterotomy (including precut or pancreatic sphincterotomy), sphincter of Oddi dysfunction (SOD), younger age, female sex, balloon dilation of an intact biliary sphincter, a previous history of PEP, difficult cannulation or prolonged attempts to cannulate, repeated injection of pancreatic contrast medium, and acinarization^[1-11]. These risk factors can be divided into patient-related factors, procedural factors, and operator-related factors.

Although, ideally, PEP should be prevented, complete prevention may be impossible, and decreasing the severity of PEP may be a more realistic goal. Patient risk stratification prior to ERCP, adequate selection of patients using noninvasive diagnostic imaging methods such as magnetic resonance cholangiopancreatography (MRCP) or endoscopic ultrasonography (EUS), avoiding unnecessary procedures, pharmacological prevention and treatment, and the use of various endoscopic techniques to minimize complications, should all be considered. The presence of patient- or procedure-related risk factors allows possible complications to be predicted with reasonable accuracy. Thus, careful patient selection for high-risk and endoscopic procedures, conducted by experienced endoscopists, may reduce procedure-related complications. Pharmacotherapy has also been used widely in efforts to prevent PEP, but the results are inconclusive. Several pharmacological prophylactic treatments have been suggested; these include rectal diclofenac, octreotide, prednisone, and allopurinol^[12-17]. Effective prophylaxis of PEP has been demonstrated only using rectal diclofenac or indomethacin^[12,15]. However, larger-scale multicenter studies of non-steroidal anti-inflammatory drugs (NSAIDs), with consideration of racial and/or geographical differences, are necessary to confirm any prophylactic effect of PEP. Also, it remains unclear whether NSAIDs act synergistically with other prophylactic interventions including pancreatic stenting; this topic requires further work.

In this literature review, we focus on endoscopic aspects of PEP prevention or reduction in severity. We describe primary cannulation techniques including initial wire-guided cannulation, the use of precut fistulotomy (infundibulotomy) and transpancreatic septostomy when cannulation is difficult or as early rescue cannulation

techniques, and prophylactic pancreatic stent (PS) placement during the procedure. Also, we mention alternatives, including percutaneous transhepatic biliary drainage (PTBD), and the possibilities afforded by further advances in EUS-guided biliary drainage techniques.

DEFINITION AND MECHANISMS OF POST-ERCP PANCREATITIS

Cotton *et al.*^[8] reported a consensus classification of ERCP-related complications. In the cited report, PEP was defined as a clinical syndrome, consistent with acute pancreatitis, associated with a serum amylase level at least three times the normal value, measured more than 24 h after the procedure, and requiring hospital admission or prolongation of planned admission. The severity of PEP is based primarily on the length of hospitalization. Mild PEP is defined as the need for hospital admission or prolongation of planned admission for up to 3 d; moderate PEP is defined by the need for hospitalization for 3-to-10 d; and severe PEP is defined by hospitalization for more than 10 d, or development of significant complications.

The underlying pathogenesis of PEP is thought to be multifactorial, and remains unclear, but numerous mechanisms of PEP induction have been proposed. These include difficult biliary access caused by biliary sphincter hypertension, repeated inadvertent pancreatic duct cannulation and contrast injection, secondary prolonged papillary edema caused by mechanical injury attributable to difficult papillary manipulations, and thermal injury caused by sphincterotomy^[18,19]. Thermal injury may be caused by the electrocautery current applied during biliary or pancreatic sphincterotomy, endoscopic papillectomy, or ablation of neoplastic lesions in the region of the ampulla of Vater. Obstructions in the outflow of pancreatic juice may be caused by mechanical injury to the papilla and pancreatic sphincter attributable to use of instrumentation to manipulate the papilla. Chemical or allergic injury may be caused by instillation of contrast medium into the pancreas. Hydrostatic injury may occur after contrast injection into the pancreatic duct or infusion of water or saline through manometry catheters. Enzymatic injury may result from intraluminal activation of proteolytic enzymes as a result of introduction of foreign substances into the pancreatic duct. Infection may also play a role, after pancreatic instillation of flora from the intestine or from contaminated endoscopes or accessories. The results of all of these problems are varied, and include mechanical, chemical, and hydrostatic injury; and infection, triggering premature intracellular activation of proteolytic enzymes, which in turn causes further damage and stimulates local inflammation, as indicated by increased cytokine levels (those of interleukins 1, 6, and 8). If inflammation is severe, a systemic inflammatory response with multi-organ involvement may be activated^[1,20,21].

Most strategies for preventing PEP, or decreasing the severity of this condition, have sought to interrupt a step of the inflammatory cascade before, during, or after

ERCP. Endoscopic prevention of PEP seeks to remedy the obstruction of pancreatic outflow caused by the various factors described above.

WIRE-GUIDED CANNULATION

Basic catheterization accompanied by contrast injection was the first cannulation technique developed in the era of ERCP cannulae, and probably remains the most widely used initial cannulation method for ERCP. However, when the first attempts at contrast injection fail, a guidewire may be used as a crossover method to facilitate selective biliary access and to reduce complications caused by prolonged cannula manipulation or contrast injection into the pancreatic duct. Of procedure-related factors, selective cannulation of the common bile duct (CBD) *via* insertion of a guidewire may cause fewer complications than do conventional methods (which use contrast injection to access the bile duct). Ideally, accessing the bile duct with the aid of a guidewire may reduce traumatic injury to the pancreatic duct and papilla, and avoid the buildup of hydrostatic pressure associated with contrast injection, thereby reducing development of ERCP-related pancreatitis^[22-26].

Endoscopic technique

Technically, wire-guided cannulation is simple. Usually, a guidewire tipped with a hydrophilic substance, 0.035 or 0.025 inch in diameter, is preloaded into a pull-type papillotome. Next, the papillotome is oriented in the 11-to-12 o'clock position on the papilla, and bent to ensure correct alignment with the axis of the bile duct. In the direct contact method, after minimal insertion (2-3 mm) of the pull-type papillotome into the ampulla, the guidewire is carefully advanced through the CBD under fluoroscopic guidance until it is seen to enter the bile duct. It is also possible to attempt selective cannulation using the slightly (2-3 mm) protruding guidewire on the papillotome to make gentle contact with the papillary orifice. This non-contact method may seek to avoid direct injury caused by contact with the cannula or papillotome. If the pancreatic duct is entered, the guidewire is simply withdrawn and attempts are made to redirect it toward the CBD^[22]. However, neither an adequate extent of guideline insertion nor the time that should be permitted for pancreatic duct insertion of the guidewire including retrials, has yet been defined. If unintentional pancreatic duct insertion occurs three-to-five times, it is appropriate to consider switching to another method, such as double-guidewire-induced cannulation, prophylactic PS insertion followed by a precut, transpancreatic septostomy, or early precut fistulotomy, to minimize complications. A precut following prophylactic PS placement may optimally decrease the frequency or severity of PEP, in contrast to use of early precut fistulotomy or double-guidewire-induced cannulation only although these techniques may improve the success rate of selective biliary cannulation.

Clinical outcomes

The PEP-protective effects of wire-guided cannulation remain controversial. In the study by Lella *et al.*^[25], no patient in a cohort of 200 randomly selected for bile duct cannulation using a soft polytetrafluoroethylene-tipped guidewire (tipped with Teflon; DuPont, Wilmington, DE) developed pancreatitis (0% in the guidewire group *vs* 4.1% in the control group, $P < 0.01$). The cited authors concluded that wire-guided cannulation reduced the frequencies of pancreatic injuries by preventing unintentional injection of contrast media into the main pancreatic duct or the papilla *per se*. However, the authors did not assess PEP frequency with respect to the difficulty of CBD cannulation (the number of cannulation attempts made). Artifon *et al.*^[23] also showed that use of the guidewire technique for bile duct cannulation lowered the frequency of PEP (8.6% in the guidewire group *vs* 16.6% in the conventional group, $P = 0.02$). The cited authors assessed the difficulty of CBD cannulation, and the numbers of unintentional pancreatic duct cannulations, and concluded that the reduction in PEP was mainly attributable to prevention of injection of contrast media into the pancreatic ducts. The guidewire technique reduced the risk of pancreatitis by facilitating cannulation, by potentially limiting papillary trauma, and by reducing the need to conduct precut sphincterotomies. Although the ranges of cannulation attempts were given as 0 to 3, 4 to 6, and 7 to 10, the investigators did not report the frequencies of PEP development in these subgroups (thus by number of attempts). Even when soft wires tipped with hydrophilic material are used in cannulations, difficult wire passage or frequent pancreatic manipulations may cause injury to the papilla, increasing the risk of PEP. Another randomized study by Lee *et al.*^[22] showed that wire-guided cannulation reduced PEP development. Totals of 3 patients [2%; 1 mild, 1 moderate, 1 severe (in terms of disease)] in the wire-guided cannulation group and 17 (11.3%; 14 mild, 2 moderate, 1 severe) in the conventional group developed PEP ($P = 0.001$). However, the study population may have been a low-risk cohort. Only seven patients with suspected SOD were included. Among patients with SOD, PEP is a well-recognized complication, occurring at frequencies of 10%-20%^[8,19]. SOD independently increases the risk of PEP because of hypersensitivity of the papilla to trauma or an increase in hydrostatic pressure on the main pancreatic duct^[3,8,27]. On the contrary, in the study by Vandervoort *et al.*^[5] guidewire- or sphincterotome-mediated cannulation seem to have been used as rescue methods in high-risk patients who failed conventional cannulation. This explains why the PEP rate was higher when guidewire cannulation was used. Thus, in the cited work, PEP was more frequent in the wire-guided cannulation group (10.2% after wire-guided cannulation *vs* 6.1% after conventional cannulation, $P = 0.04$). However, a recent meta-analysis of the data of five randomized controlled trials showed that the wire-guided technique increased the primary cannulation

Table 1 Prospective randomized trials of wire-guided cannulation to reduce the incidence of post- endoscopic retrograde cholangiopancreatography pancreatitis

	<i>n</i>	Design	Pancreatitis (<i>n</i>)/accidental PD (<i>n</i>) (WGC <i>vs</i> CC) ¹	Post-ERCP pancreatitis <i>n/n</i> (%)		<i>P</i> value
				WGC	CC	
Lella <i>et al</i> ^[25]	200/200	Prospective/Randomized	0/82, 5/113	0/197 (0)	8/195 (4.1)	< 0.01
Artifon <i>et al</i> ^[23]	150/150	Prospective/Randomized	0/27, 4/21	13/150 (8.6)	25/150 (16.6)	0.02
Bailey <i>et al</i> ^[24]	202/211	Prospective/Randomized	NA	16/202 (7.9)	13/211 (6.2)	0.48
Katsinelos <i>et al</i> ^[26]	167/165	Prospective/Comparative	NA	9/167 (5.4)	13/165 (7.9)	0.37
Lee <i>et al</i> ^[22]	150/150	Prospective/Randomized	2/39, 8/44	3/150 (2)	17/150 (11.3)	0.001
Mariani <i>et al</i> ^[28]	678/571	Prospective/Comparative	15/99, 8/95	35/678 (5.2)	25/ 571 (4.4)	0.60
Kawakami <i>et al</i> ^[29]	199/201	Prospective/Randomized ²	NA	8/199 (4.0)	6/201 (2.9)	NS

¹The number of post-ERCP pancreatitis following accidental PD injection or cannulation in CC and WGC group; ²Multicenter RCT with a 2 × 2 factorial design. 0/82 *vs* 5/113, *P* = 0.08; 0/27 *vs* 4/21, *P* = 0.05; 2/39 *vs* 8/44, *P* = 0.09 by Fisher's exact test. PD: Pancreatic duct cannulation or contrast injection; WGC: Wire-guided cannulation; CC: Conventional cannulation; NS: Not significant; NA: Not available.

rate and reduced the risk of PEP compared to use of the standard contrast-injection method. Pooled analysis of PEP rates in wire-guided cannulation groups compared to those in groups treated using standard methods yielded an OR of 0.23 (95%CI: 0.13-0.41). Also, use of the wire-guided technique was associated with a significantly higher primary cannulation rate (OR = 2.05; 95%CI: 1.27-3.31). Although the meta-analysis included a relatively small number of studies, each work employed different cannulation difficulty criteria (involving cannulation times or numbers of attempts made), and, indeed, some studies did not define their criteria. Three well-designed studies using wire-guided cannulation techniques showed that use of such cannulation could reduce the development of PEP^[22,23,25]. However, other recent reported studies have yielded contrary results. Mariani *et al*^[28] found that the PEP rates in high- and low-risk patients did not differ between wire-guided cannulation and contrast injection groups (5.2% *vs* 4.4%). In a multicenter randomized study performed by Kawakami *et al*^[29], it was also shown that wire-guided cannulation did not reduce the incidence of PEP compared with use of a conventional method (Table 1). In both studies, trainees conducted (some) procedures. When used as an initial cannulation method, wire-guided cannulation seems to shorten cannulation times, as revealed in numerous studies, but any benefit in terms of reducing PEP development is now controversial.

The mechanisms by which guidewire cannulation reduces PEP risk remain uncertain. In the meta-analysis of Masci *et al*^[30], several technical issues, including multiple contrast injections into the pancreatic duct, difficult cannulation, precutting, pancreatic sphincterotomy, and balloon dilatation of the sphincter of Oddi, were identified as risk factors for PEP. Notably, the definition of “difficult” cannulation is imprecise, being both subjective and varying among studies. In the report on wire-guided cannulation by Lee *et al*^[22], the definition used was failure to achieve biliary access after attempting to do so for 10 min, or after more than five unintentional pancreatic cannulations. Artifon *et al*^[23] defined cannulation as difficult when 7-10 attempts were required to ultimately achieve cannulation. Recent studies suggest that the guidelines

for difficult cannulation should be stricter. Large, well-performed, randomized controlled studies aiming to establish cannulation difficulty criteria are needed to resolve these controversies. Also, wire-guided cannulation may not prevent PEP in patients with suspected SOD and who are subjected to unintentional pancreatic duct guidewire cannulation. In high-risk patients, such as those with SOD, repeated unintentional pancreatic duct guidewire cannulation may trigger PEP caused by mechanical trauma or increases in hydrostatic pressure attributable to repeated introduction of a guidewire into the main pancreatic duct. In instances of unintentional pancreatic duct guidewire cannulation, therefore, wire-guided cannulation followed by temporary placement of a PS may be preferred over wire-guided cannulation alone to prevent increases in pancreatic enzyme levels and to reduce the frequency or severity of PEP in high-risk patients^[5,22,31,32].

In summary, primary wire-guided cannulation in experienced hands can reduce cannulation time and facilitate successful biliary access, and may reduce the frequency and/or severity of PEP. However, more large-scale comparative studies that consider race, high-risk status, and operator experience, are required to confirm the existence of any prophylactic effect.

PRECUT SPHINCTEROTOMY

Pros and Cons

Precut sphincterotomy is an essential rescue technique in instances of difficult biliary cannulation. Irrespective of the technique used, the initial success rates of precut sphincterotomy have previously been reported to be as high as 90% during the first attempt, with success rates of 95%-99% following second attempts conducted 48-72 h later after edema and inflammation had subsided. In precut methods, various techniques including needle-knife sphincterotomy with or without PS guidance, fistulotomy (infundibulotomy), and transpancreatic sphincterotomy, are used, although few data are available to aid in the selection of a procedure^[33-40]. The overall complication rates after precut sphincterotomy have been reported to vary from 1.9% to 34%, compared to rates of 7%-14%

with conventional sphincterotomy. PEP is the most common and serious complication; the rates range from 2.1% to 14.9%, compared to the 1%-10% associated with conventional sphincterotomy^[3,33-46].

Although precut sphincterotomy may be an effective rescue technique, such sphincterotomy using a needle-knife has been directly implicated as a primary cause of PEP. Therefore, this technique has been considered potentially dangerous, especially when performed by less-experienced endoscopists. Most authorities recommend that only experts perform a precut. However, recent studies have shown that the complications of precut sphincterotomy are similar to those associated with conventional sphincterotomy, namely bleeding, PEP, perforation, and cholangitis^[33,43,46-49]. In terms of the endoscopist learning curve ensuring the safety and success of precut sphincterotomy, Akaraviputh *et al.*^[47] reported that the rate of procedure-related complications decreased significantly after the first 100 procedures were performed. Also, among all complications, the rate of immediate bleeding varied significantly, but the success of cannulation or the rate of PEP development did not differ with endoscopist experience. Lee *et al.*^[33] obtained similar results. The frequency of PEP in 159 patients who underwent precut fistulotomy did not differ by time interval. In the cited study, the risks associated with use of precut fistulotomy under circumstances where biliary cannulation was employed were not influenced by experience. Thus, the overall complication and PEP rates were similar; *i.e.*, not differing significantly, from those reported previously, at 10.7% and 5.7%, respectively, and the overall success rates were also similar, at 93.7%. No other serious complications were noted.

Consequently, most criticisms of the (supposedly) higher complication rates associated with precut sphincterotomy may be unwarranted. The high frequencies of post-procedural complications after such sphincterotomy may be associated with excessively edematous major papillae and extensive injuries caused by multiple or prolonged attempts to cannulate the CBD by standard methods before precut sphincterotomy. Huibregtse *et al.*^[49] showed that early implementation of precut increased successful biliary access on the first attempt, as well as the overall success rate, while reducing the rate of complications to 11.8% (pancreatitis: 0.5%). Previous repeat cannulation attempts, prolonged cannulation time, or numerous insertions of a guidewire into the pancreatic duct, may increase the risk of PEP. Freeman *et al.*^[3] reported that moderate numbers of cannulation trials (6-15), or more-than-moderate numbers (> 15), and use of more than one pancreatic contrast injection, were important in terms of the development of pancreatitis; multivariate analysis was used to arrive at these conclusions. Lee *et al.*^[33] showed that more than 15 attempts at cannulating the major papilla prior to precut fistulotomy was a risk factor for PEP development upon multivariate analysis (OR = 4.8, 95%CI: 1.178-19.580, *P* = 0.029). Bailey *et al.*^[41] also found that the number of attempts

at cannulating the papilla played a key role in guiding decision-making to minimize the risk of PEP. Thus, if precut fistulotomy is indeed a treatment candidate, early implementation of this approach may aid in successful selective biliary cannulation as well as reducing the severity of PEP. On the contrary, Cennamo *et al.*^[50] reported that the timing of precutting did not influence the operative success rate or the rate of complications associated with ERCPs. The cited authors showed that the rates of PEP did not differ between subgroups treated with early precutting (no more than 5 min of attempts at biliary cannulation using the standard approach, and three cannulations of the pancreatic duct) and delayed precutting (cannulation attempts lasting 25 min). However, the cited study had a small sample size and, thus, a low statistical power. A recent meta-analysis of early precut studies (although including precuts performed at different times and the use of various techniques including needle-knife precutting starting at the orifice, and fistulotomy) showed that early precut implementation reduced the PEP risk (to 2.5% *vs* 5.3%, OR = 0.47, 95%CI: 0.24-0.91) but not the overall complication rate^[51].

Theoretically, the greater number of complications could have resulted from direct thermal injury caused by the needle-knife *per se*, especially during precut sphincterotomy, in which incisions commenced at the papillary orifice. Avoidance of thermal injury to the pancreatic duct, by making incisions above the papillary orifice during precut fistulotomy, minimizes the risk of pancreatitis^[35,36,46]. However, too small a papilla, a short papillary roof, distortion caused by invasion of a tumor or a mass, or location of the ampulla of Vater on the inner center or ridge of a huge periampullary diverticulum, may preclude use of precut fistulotomy^[33].

In summary, although some aspects of the timing and optimal type of precut sphincterotomy remain controversial, as does the need for endoscopist experience, use of early precut fistulotomy in patients for whom cannulation is difficult may not exacerbate PEP to an extent greater than conventional methods. In instances of persisting papillary contact or prolonged cannulation time, early precut fistulotomy may minimize the severity of PEP by decreasing mechanical trauma. However, a definition of a "difficult" cannulation, and adequate training in precut sphincterotomy are required, as are data from more large-scale multicenter studies.

Transpancreatic sphincterotomy/septostomy

Transpancreatic sphincterotomy or septostomy is a technique involving cutting of the septum that separates the pancreatic duct from the bile duct, through the pancreatic orifice^[52]. Unlike a freehand technique such as use of a needle-knife, transpancreatic papillary septostomy in patients for whom cannulation is difficult, or who experience unintentional pancreatic duct cannulation, can be performed using a papillotome, without exchange of devices, after guidewire introduction into the pancreatic duct; or indeed without a guidewire. When unintentional

Table 2 Studies for the use of pancreatic stents to prevent post-endoscopic retrograde cholangiopancreatography pancreatitis

Study	Design	Indications	PEP rate		
			<i>n</i>	Non-stent/stent (%)	<i>P</i> value
Smithline <i>et al</i> ^[63]	RCT	Biliary ES for SOD, small ducts, or precut	93	18/14	0.229
Aizawa and Ueno ^[31]	Retrospective case-control	Biliary balloon dilatation for stone	40	6/0	0.110
Fogel <i>et al</i> ^[18]	Retrospective case-control	Biliary ± pancreatic ES for SOD	436	28.2/13.5	< 0.05
Fazel <i>et al</i> ^[32]	RCT	Difficult cannulation, biliary ES, SOD	76	28/5	< 0.05
Freeman <i>et al</i> ^[19]	Prospective case-control	Consecutive high-risk ERCP in which a major papilla PD stent was attempted	225	66.7/14.4	0.060
Harewood <i>et al</i> ^[58]	RCT	Endoscopic ampullectomy	19	33/0	0.020
Sofuni <i>et al</i> ^[64]	RCT	All consecutive ERCP (excluding pancreatic cancer, pancreas divisum, PD therapy cases)	201	13.6/3.2	0.020
Tsuchiya <i>et al</i> ^[66]	RCT	All consecutive ERCP irrespective of risk factors	64	12.5/3.1	> 0.05
Saad <i>et al</i> ^[70]	Retrospective nonrandomized	Suspected SOD and normal manometry	403	9/2.4	0.006
Lee <i>et al</i> ^[59]	RCT	Difficult biliary cannulation	101	29.4/12	0.031

ES: Endoscopic sphincterotomy; SOD: Sphincter of Oddi dysfunction; RCT: Randomize controlled study; PD: Pancreatic duct; PEP: Post-ERCP pancreatitis.

pancreatic duct cannulation has occurred, the procedure is relatively easy. Wire-guided septostomy is performed after introducing a soft guidewire into the pancreatic duct, and sphincterotomy follows, maintaining the bile duct orientation at 11 o'clock. If the septum between the pancreatic and bile ducts is incised, the biliary and pancreatic orifices become separately visible^[52-54]. Another useful option for septostomy is a precut following placement of a prophylactic PS along the stent. This may primarily prevent PEP and also facilitates selective biliary access. This means that the second procedure, the precut from the orifice, is relatively easy; the operator is more comfortable in such circumstances than is the case when a freehand technique such as fistulotomy is to be performed. Either a precut from the orifice or fistulotomy is possible, but precutting from the orifice in the biliary direction along a supporting stent may be more feasible than use of the freehand technique. The prophylactic effects of PSs are described below.

PROPHYLACTIC PLACEMENT OF PANCREATIC STENTS

Pancreatic stents were originally introduced to treat pancreatic ductal pathology such as benign or malignant strictures and ductal leaks after trauma or surgery. The exact mechanism by which PSs may reduce the risk of PEP is but poorly understood. The stents probably preserve pancreatic drainage that otherwise might be impaired by mechanical injury to the pancreatic sphincter caused by prolonged or repeated manipulations of catheters and guidewires and thermal injury caused by biliary and pancreatic sphincterotomy or snare papillectomy. Many clinical trials and a meta-analysis have shown that placement of PSs in high-risk patients effectively reduces the incidence and/or severity of PEP. Recent studies have found that prophylactic placement of a PS reduces the frequency and severity of PEP in particular high-risk groups, including those with known or suspected SOD; those who have undergone papillectomy, precut

sphincterotomy, or pancreatic sphincterotomy; those with a history of PEP; or those for whom cannulation is difficult (Table 2)^[18,31,32,55-70]. Prophylactic placement of PSs is now increasingly adopted to reduce the risk of PEP. PS placement also reduces the frequency of severe PEP^[18-21,31,32,55-68].

Presently, the routine use of PSs in high-risk patients and in procedures conducted at advanced centers has changed attitudes toward ERCP; the incidence and severity of PEP have been reduced to more acceptable levels. However, few data are available on the effects of prophylactic PSs, especially in terms of technical difficulties in the context of cannulation time or the frequency of papillary contact^[56,61,69]. Also, the sizes and lengths of the stents employed have been variable, and no guideline or consensus yet exists on the optimal type, diameter, or length of a PS.

Ideal types of pancreatic stents

PSs vary in terms of diameter, length, and shape. An ideal PS should completely prevent development of PEP, be easily deployed, spontaneously dislodge after exerting an adequate preventative effect, and not cause ductal or parenchymal pancreatic changes^[71]. In terms of such changes, a retrospective analysis of 34 patients with 38 PSs placed to deal with disrupted ducts, isolated strictures, pancreas divisum, and hypertensive pancreatic sphincters, found that 36% of all patients exhibited subsequent ductal changes^[72]. Also, a study on the dog pancreas showed that polyethylene PSs caused histopathological changes in normal tissues attributable to stent occlusion or local stent-induced trauma^[73]. These results suggest that PSs may cause permanent changes to the pancreatic duct or parenchyme. If the placement time is too short, a smaller and shorter stent may not sufficiently protect against PEP development. Short stents (less than 3 cm long) are generally preferred to longer stents to avoid stenting across the neck of the pancreatic duct. However, longer stents should be considered when the pancreatic duct is angulated in the head of the pancreas. Stents may be straight, or may have a single pigtail or partial curl in the

Table 3 Efficacy of 3- vs 5-F pancreatic stents in preventing post-endoscopic retrograde cholangiopancreatography pancreatitis

	Technical success	Spontaneous migration	PEP	Stents
Rashdan <i>et al</i> ^[61] (3 F vs 4, 5, 6 F)	NA	86%/73%/67%/65% ¹ ($P < 0.01$)	7.5%/10.6%/9.8%/14.6% ($P = 0.047$)	COOK, 4-12 cm
Chahal <i>et al</i> ^[56] (3 F vs 5 F)	91%/100% ($P = 0.0003$)	88%/98% ($P = 0.0001$) ²	14%/9% ($P = 0.3$)	3 F, 8 and 10 cm/5 F, 3 cm
Zolotarevsky <i>et al</i> ^[69] (3 F vs 5 F)	97.5%/100%	75%/68.4% ($P = 0.617$) ²	17.5%/10.5% ($P = 0.519$)	COOK, Zimmon 3 F, 3 cm/ 5 F, 5 cm

¹10-14 d; ²2 wk. PEP: Post-ERCP pancreatitis; NA: Not available.

duodenum, to prevent proximal migration. Short stents without proximal flaps facilitate early spontaneous migration (within 1 wk). Thus, establishment of drainage may be not assured when stents without proximal flaps are used because of the potential for very early stent migration. However, stents with flaps require endoscopic removal at a later date. Another option is to place longer (> 7 cm) stents of small diameter (3 or 4 F) that have no proximal flaps. This practice has the potential advantages of less ductal trauma and spontaneous distal migration; repeat endoscopy is not necessary^[74]. A large retrospective study suggested that unflanged longer-length (8-10 cm) 3 F polyethylene stents with single duodenal pigtailed were associated with significantly higher spontaneous dislodgement rates compared to larger-caliber, shorter unflanged 4 or 5 F stents. The cited study also reported a somewhat lower incidence of PEP in patients who received 3 F compared with 5 F stents, although the difference was not statistically significant^[61]. Another study by Chahal *et al*^[56] compared use of long 3 F and short 5 F stents and showed that the spontaneous dislodgement rate of unflanged, short 5 F PSs (98%) was significantly higher than that of unflanged, long 3 F stents (88%) after 14 days in patients at high risk for PEP development ($P < 0.01$). Placement of short stents reduced the need for later endoscopic stent removal. Higher rates of PS placement failure (0% in the 5 F group but 8.3% in the 3 F group, $P = 0.0003$) and PEP (14% in the 3 F group and 9% in the 5 F group, $P = 0.3$) were observed in patients with 3 F stents. Recently, Zolotarevsky *et al*^[69] reported that placement of 5 F compared to 3 F PSs for PEP prophylaxis was easier, more rapid, and required fewer wires. However, no statistically significant differences in spontaneous passage rates (68.4% in the 5 F group; 75.0% in the 3 F group; $P = 0.617$) or PEP rates ($P = 0.519$) were evident (Table 3).

The prophylactic utility of placing smaller 3 F stents during difficult biliary cannulations has undergone little evaluation. Technically, the failure rates in previous studies involving placement of 3 F PSs after therapeutic ERCP have been rather high (9%-10%)^[56,63]. The main problem is that a guidewire of smaller diameter than the standard 0.035-inch wire must be used. Deployment of long 3 F PSs is technically more difficult because of the need to use smaller caliber (0.018- or 0.021-inch) guidewires, which can be difficult to maneuver around tortuous pancreatic ducts compared to a hydrophilically tipped 0.035-inch guidewire. Placement of long stents also re-

quires deeper guidewire access into the main pancreatic duct, which may not be possible in patients with highly angulated or tortuous ducts. Thus, usually, placement of a 5 F PS using a 0.035-inch guidewire may be valuable to allow easy negotiation of the pancreatic duct and stent deployment. However, one recent randomized controlled trial evaluating the feasibility and utility of smaller and shorter (4-8 cm) 3 F stents showed that placement of a 3 F PS was technically feasible, significantly reduced the rate of PEP developing after difficult biliary cannulations, and that a higher rate of distal spontaneous dislodgement (94%) was evident within 7 d. The technical failure rate when experts operated was low (4%), and no complications resulted from PS placement^[59]. The use of smaller-sized guidewires may require extensive endoscopic experience and skilled assistance.

Timing of pancreatic stent placement

It is unclear whether stents should be placed before or after therapeutic procedures such as sphincterotomy, stone extraction, and biliary stent placement, but early placement of a PS may be beneficial because various procedure-related factors may contribute to development of PEP. A retrospective study by Fogel *et al*^[18] found that pancreaticobiliary sphincterotomy with PS placement was associated with a lower rate of pancreatitis than was biliary sphincterotomy alone. The cited authors noted a tendency for pancreatitis rates to be lower when a PS was placed before major papillar pancreatic or biliary sphincterotomy (10.7%), than after sphincterotomy (19.2%). Another retrospective study reported similar complication rates upon traction minor papillotomy followed by PS placement, compared with needle-knife surgery after PS placement (8.3% vs 7.8%)^[75]. A recent randomized trial comparing use of the needle knife and pull-sphincterotome techniques for pancreatic sphincterotomy in high-risk patients showed that PEP was significantly more frequent among patients undergoing pancreatic sphincterotomy with a pull sphincterotome followed by placement of a PS than in those treated with needle-knife pancreatic sphincterotomy performed after placement of a PS [7 of 24 (29%) vs 0 of 24 (0%), $P < 0.01$]. Forty patients undergoing major papillar pancreatic sphincterotomy for manometrically documented SOD were randomized to traction sphincterotomy using a blended current followed by placement of a PS vs needle-knife sphincterotomy after placement of a stent; all patients received long, unflanged 3 F stents^[76].

Access to the pancreatic duct after biliary sphincterotomy or other biliary therapy such as balloon dilatation or stone extraction is sometimes very difficult. Failure usually occurs either because the pancreatic orifice cannot be identified or a guidewire cannot be deeply advanced into the pancreatic duct. Also, deep pancreatic cannulation can be difficult or impossible when, anatomically, looping or tight angulations are evident in the distal pancreatic duct. For such reasons, it is recommended to access the pancreatic duct with a guidewire early in the procedure and to maintain wire access until a stent has been placed in high-risk cases in which PS placement is believed to be warranted^[19,74]. However, sometimes, repeat procedures such as stone extraction using a retrieval balloon, or mechanical lithotripsy, may dislodge the guidewire or preloaded stent even though the stent was placed using a guidewire.

Usually, prophylactic PS placement, rather than only maintaining a guidewire, may be reasonable before any therapeutic procedure. This suggestion is based on the data of the studies reported above, but further large-scale, prospective studies are warranted.

Duration of pancreatic stent placement

Few data are available to indicate the duration for which a PS should remain in place to effectively prevent PEP. Cha *et al.*^[55] reported that the rates of pancreatitis were significantly higher in patients from whom PSs were removed immediately after needle-knife precut sphincterotomy compared to those in whom the stents were left in place for 7-10 d (21.3% *vs* 4.3%, $P = 0.027$). These data suggest that placement and maintenance of a PS when needle-knife precut sphincterotomy is performed reduces the frequency and severity of PEP. The cited study also showed that excessively early removal of a PS might not effectively prevent development of pancreatitis. However, no data regarding the adequacy of the duration of stenting that is needed to consistently prevent PEP are available. This may be anywhere from a few hours to a week or more. The precise duration of PS placement required to effectively reduce the risk of PEP is not well known.

In general, stent removal at the end of ERCP is not recommended. Excessively early removal of a PS may increase the risk of pancreatitis. However, removing a stent too late may increase the risk of ductal or parenchymal change. Ideally, the PS should be in place for a minimum of 24 h or more, and then dislodge spontaneously^[2].

Pancreatic stent-related complications

Relapsing acute and chronic (painful) pancreatitis can develop in patients with pancreatic stent-induced injuries. However, the long-term outcomes of PS placement have not yet been thoroughly investigated although it is assumed that most ductal injuries are transient, eventually resolving spontaneously, without clinical symptoms. A large-scale retrospective study suggested that unflanged longer (8- to 10-cm), 3 F polyethylene stents with single duodenal pigtailed were associated with a substantially

reduced frequency of ductal change (24% for 3 and 4 F stents compared to 80% for 5 and 6 F polyethylene stents)^[61]. This may indicate that use of smaller-caliber stents is associated with a reduced risk of ductal injury. Ductal and parenchymal changes may be most prominent in patients with traditional 5 or 7 F stents, because which may be of similar caliber to the native main pancreatic duct. The stent diameter should be less than that of the pancreatic duct.

Summary of use of prophylactic pancreatic stents

In prophylactic PS placement, a long PS of smaller diameter (3 F) may dislodge spontaneously within a few weeks without any ductal change, but small guidewires (0.018 or 0.021 inch) are required, and such small guidewires may be difficult to handle and to insert deeply into the tail portion. On the other hand, placement of short (2-3 cm) 5 F unflanged stents can commonly be achieved using 0.035-inch guidewires that can be handled relatively easily by endoscopists. Also, over 90% of such stents dislodge spontaneously. However, a stent that is too short may migrate soon after insertion, thus failing to prevent PEP and (perhaps) causing injury to the duct genu because of the short length. To effectively prevent PEP, the duration of stent placement that is adequate, without causing ductal or parenchymal change, should be determined. Finally, careful study of an ideal stent design, and the material used, is warranted. All of easy stent insertion, risk reduction, and spontaneous dislodgement in a timely fashion without ductal injury, are required. We suggest that short 5 F, or long 3 F, stents without inner flanges should be used to stent a normal pancreatic duct. The stent diameter should be less than that of the targeted pancreatic duct. However, endoscopists should remember that technical failure of PS insertion might aggravate the severity of pancreatitis, so that the procedure *per se* unfortunately becomes a risk factor.

OTHER ALTERNATIVES

Repeat or delayed ERCP

Repeat or continuing attempts at cannulation increase the risk of PEP, as explained above. When primary cannulation fails, the alternatives include PTBD, repeat ERCP conducted by same or another endoscopist (perhaps in a more advanced institution) after 2-3 d of delay, or surgical exploration^[77,78]. Of these approaches, delayed ERCP performed 2-3 d later by the same endoscopist may increase the success rate of selective cannulation and also reduce the complication risk to within an acceptable range by avoiding excessive papillary manipulation or unintentional ductal injury. Delayed ERCP may afford a good visual field, without papillary edema or bleeding, and reduce the rapid bowel movement that develops with longer procedure times, in turn reducing the need for additional procedures and enhancing successful biliary cannulation. However, excessively prolonged manipulations during primary cannulation attempts are inevitably

associated with complications. A decision to interrupt a procedure should be considered as early as possible.

Percutaneous transhepatic biliary drainage

Percutaneous transhepatic biliary drainage (PTBD) is the most common salvage procedure used to access the biliary tract after failure of ERCP. Especially in patients with advanced malignant hilar biliary strictures, percutaneous drainage may be more feasible than endoscopic drainage. To palliate jaundice in patients with non-resectable malignant hilar biliary strictures, the biliary obstruction pattern (particularly the Bismuth type) should be considered before selection of an optimal drainage method. Endoscopic biliary drainage and stenting is recommended as the first-line drainage procedure in Bismuth type II patients, considering that this approach is efficacious and relatively noninvasive. However, internal stent insertion and drainage through the PTBD tract may be the best option for Bismuth type IV^[79] patients. One retrospective study found that the success of biliary drainage was significantly higher when drainage was percutaneous rather than endoscopic (93% *vs* 77%, $P = 0.049$)^[80]; no between-group differences in overall complication rates or the median survival time of successfully drained patients were evident. The goal of palliative drainage of hilar cholangiocarcinoma patients is drainage of an adequate liver volume (50% or more), irrespective of unilateral, bilateral, or multisegmental stenting. In patients of Bismuth types III or IV, the percutaneous approach was preferred over the endoscopic approach in a document detailing Asia-Pacific consensus recommendations^[81]. However, PTBD-related adverse event rates of 9%-33%, and mortality rates of 2%-15%, have been reported^[82-85]. Furthermore, in terms of quality of life, long-term placement of external catheters is very uncomfortable for patients. Also, recent studies on bilateral metallic stenting have enjoyed high levels of technical success, and a reduced revision rate, even in patients with advanced hilar cholangiocarcinoma^[86-88].

The choice of an endoscopic approach or PTBD may depend on endoscopist experience and institutional guidelines. In the near future, advanced endoscopic techniques and newly developed devices may improve endoscopic methods. However, in terms of complications, in particular PEP, primary PTBD does not irritate the ampulla of Vater. Accordingly, in difficult cases, and in advanced hilar cholangiocarcinoma patients requiring adequate drainage, PTBD can be both an alternative option and a rescue method.

FUTURE ADVANCES IN ENDOSCOPIC PROCEDURES

Repeated cannulation attempts and pancreatic duct manipulations on the ampulla of Vater are associated with PEP development, caused by inevitable contact with the papilla. Thus, theoretically, PTBD, or EUS-guided biliary drainage (EUS-BD), may serve as alternatives to ERCP

when it is performed to inhibit development of pancreatitis by avoiding direct contact with the ampulla of Vater. Recent studies have shown that EUS-BD is an effective alternative to PTBD after failure of ERCP. Also, a potential benefit of EUS-BD is internal drainage, thereby avoiding long-term external drainage in patients who are expected to enjoy longer survival and in those for whom external PTBD drainage catheters cannot be internalized. However, EUS-BD with transluminal stenting is inherently complex in procedural terms, requiring several multi-step processes, thus prolonging procedure times, in turn associated with the possible development of several adverse events, including stent migration and bile peritonitis^[89-93]. Also, EUS-guided drainage techniques have been but recently developed and no dedicated devices or guidelines are yet available. Procedure-related complications including bile peritonitis or pneumoperitoneum are not uncommon. To date, the procedure has been performed by only experienced endoscopists in advanced endoscopy centers, usually as a salvage method rather than as a form of primary biliary drainage. Further development of technical devices and establishment of standard techniques minimizing complications are needed. Also, further long-term follow-up in the context of large-scale studies (including primary intervention to ensure biliary drainage) are required before the technique can be recommended for primary use.

SUGGESTED ALGORITHM FOR ENDOSCOPIC PREVENTION OF POST-ERCP PANCREATITIS

Prior to ERCP, patient selection considering risk stratification, operator-related factors, and hospital circumstances, should be considered, and efforts should be made to avoid unnecessary ERCP by diagnostic replacement with EUS or MRCP, if possible. Trainee involvement must be taken into account. If possible, pharmacological prophylaxis - such as rectal NSAIDs - should also be considered. We recommend wire-guided rather than conventional cannulation as the initial cannulation method. If unintentional pancreatic duct cannulation occurs more than three times, it may be wise to consider changing to double-guidewire cannulation or transpancreatic septostomy to enhance biliary access. However, in such instances, precutting from the orifice following early prophylactic PS placement may be more effective to reduce the severity of PEP. If attempts at double-guidewire cannulation persist for some time or a technical difficulty is encountered, an early switch to a precut following prophylactic PS placement should be considered. Also, the use of a double-guidewire cannulation technique may increase the risk of complications caused by additional frequent papillary contact, or pancreatic duct cannulation, even though use of the method may facilitate selective biliary cannulation. Transpancreatic sphincterotomy may be also a risk factor for PEP if pancreatic juice passage is disturbed.

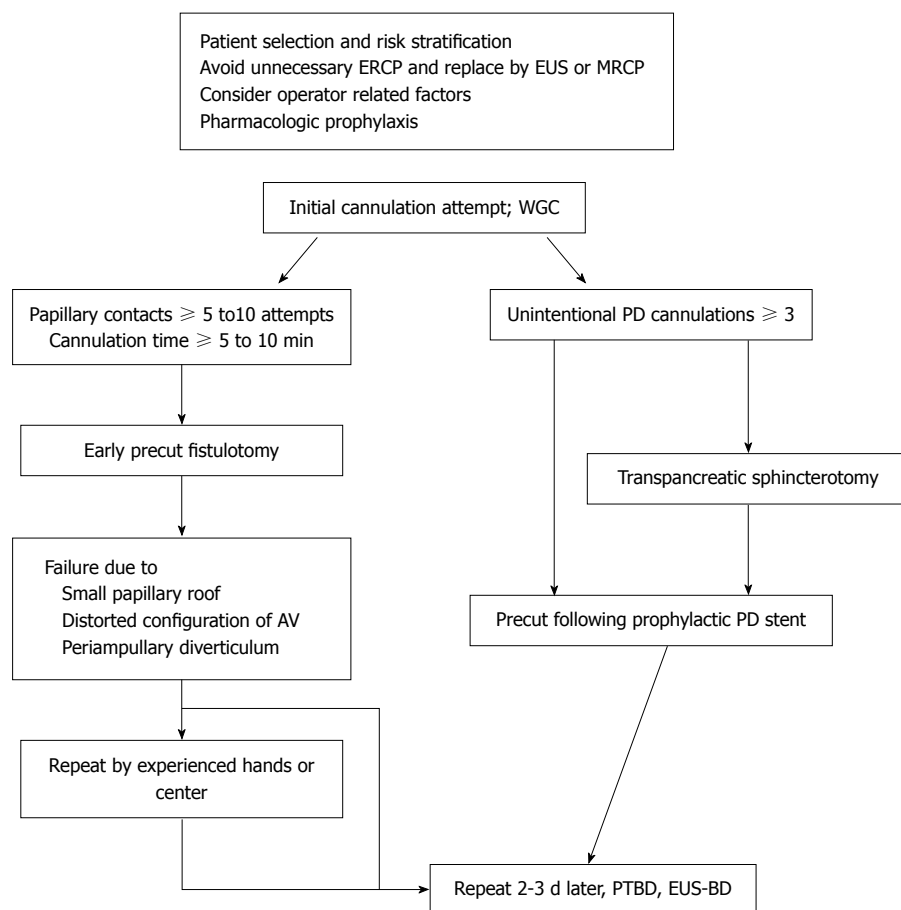


Figure 1 Suggested endoscopic algorithm for decreasing the severity of post-endoscopic retrograde cholangiopancreatography pancreatitis and facilitating biliary access. WGC: Wire-guided cannulation; AV: Ampulla of Vater; PD: Pancreatic duct; PTBD: Percutaneous transhepatic biliary drainage; EUS-BD: EUS-guided biliary drainage.

Thus, risky operative conditions, such as a prolonged procedure time (more than 5-10 min), or technical failure of selective cannulation, should trigger consideration of prophylactic pancreatic stenting. Otherwise, if frequent papillary contacts persist (if more than 10 cannulation attempts, or at the very most up to 15 attempts, are made), or the cannulation time is more than 5-to-10 min without unintentional pancreatic cannulation, early precut fistulotomy can be considered. However, if the papilla is too small, the segment of the papillary roof short, a periampullary diverticulum present, or the ampulla is located in the center of the ridge of the diverticulum, a precut may be disturbed. In those cases, PTBD, EUS-BD, the rendezvous technique, repeat ERCP performed by a senior experienced endoscopist, or delay in ERCP for 2 or 3 d, should be considered. Use of such a step-wise algorithm may enhance successful biliary access and avoid unnecessary prolongation of procedure time (Figure 1). However, such options should be considered against a background of hospital circumstances and the availability of endoscopists.

CONCLUSION

Various endoscopic or interventional techniques includ-

ing primary wire-guided cannulation, precut fistulotomy, transpancreatic septostomy, prophylactic PS placement, or alternatives such as PTBD or EUS-BD, have been described above as prophylactic methods for the decreasing severity or frequency of PEP. Till now, prophylactic PS placement in high-risk patients or those treated with certain procedures may be the single most effective method to reduce the severity and/or frequency of PEP. Improvements in stent design and the materials used in stent construction are to be expected. Also, the optimal timing of stent placement and its duration require study. Wire-guided cannulation and precut fistulotomy should be compared using strict definitions of “difficult” cannulation, endoscopist experience, and racial or regional characteristics. Furthermore, as either alternative or primary methods, PTBD or more advanced EUS-guided techniques may be available in difficult or failed cannulation. Finally, recently emerging pharmacological prophylaxis, such as rectal NSAIDs, should be considered either in combination, or alone, in large-scale comparative studies.

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WJG 20th Anniversary Special Issues (19): Capsule endoscopy

Colon capsule endoscopy: Current status and future directions

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Core tip: Colon capsule endoscopy is a promising, minimally invasive wireless technique for the visualization of the colon. With the second generation, the diagnostic accuracy of Colon capsule endoscopy has significantly improved for polyp detection. Preliminary data suggest that colon capsule endoscopy may be useful to monitor mucosal inflammation in patients with inflammatory bowel disease. Limitations include the inability to take biopsies and the procedural costs. However, given the potentially higher acceptance within an average risk colorectal cancer (CRC) screening population, its usefulness as a screening tool with regard to CRC prevention should be further evaluated.

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Abstract

Colon capsule endoscopy (CCE; PillCam Colon; Given Imaging; Yoqneam, Israel) is a minimally invasive wireless technique for the visualization of the colon. With the recent introduction of the second generation colon capsule the diagnostic accuracy of CCE for polyp detection has significantly improved and preliminary data suggest it may be useful to monitor mucosal inflammation in patients with inflammatory bowel disease. Limitations include the inability to take biopsies and the procedural costs. However, given the potentially higher acceptance within an average risk colorectal cancer (CRC) screening population, its usefulness as a screening tool with regard to CRC prevention should be further evaluated.

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Key words: Capsule endoscopy; Endoscopy; Colon capsule endoscopy; Colonoscopy; Colorectal cancer; Colon; Colonic capsule endoscopy; Inflammatory bowel disease

INTRODUCTION

Endoscopic screening for colorectal cancer (CRC) has been shown to be effective in reducing mortality from the disease^[1,2]. However, while the decrease in CRC mortality is primarily attributable to the use of colonoscopy, its acceptability is still low among patients. Therefore, less-invasive screening methods with comparable sensitivity for the detection of polyps and cancer are highly desired.

Colon capsule endoscopy (CCE) was first introduced in 2006 as a wireless, minimally invasive technique for the imaging of the large bowel that does not require sedation or gas insufflation^[3]. However, while capsule endoscopy of the small bowel has quickly found its place as a first-line imaging device for patients with obscure gastrointestinal bleeding, CCE was immediately met with skepti-

Table 1 Comparison of technical features of first and second generation colon capsules

CCE	Year of introduction	Size (mm)	Field of view	Frame rate (images/s)	Frame rate in the upper intestines	Special features
PillCam Colon 1 (CCE-1)	2006	31 × 11	156°	4	Sleeping mode 1 h 45 min	-
PillCam Colon 2 (CCE-2)	2009	31.5 × 11.6	172°	4-35	14/min until first frame of small bowel	Adaptive Image rate, Graphic interface, Live imaging

CCE-1: First generation colon capsule endoscopy; CCE-2: Second generation colon capsule endoscopy.



Figure 1 First and second generation colon capsules. CCE-1: First generation colon capsule endoscopy; CCE-2: Second generation colon capsule endoscopy.

cism due to high procedural costs, the need for extensive bowel cleansing in order to gain reasonable polyp detection rates and the inability to take biopsies, thus requiring additional conventional colonoscopy to confirm finding and remove polyps. To increase the accuracy of CCE, the second-generation colon capsule (CCE-2) was recently developed that has an increased angle of view for each of the two cameras involved allowing for a panoramic view and an adjustable frame rate ranging from 4 to 35 images per second^[4]. The increased demand for this CE-marked minimally invasive technique has recently prompted the European Society of Gastrointestinal Endoscopy (ESGE) to publish a consensus guideline on the standardized use of CCE^[5]. In February 2014, FDA approval has been granted for CCE basing on data from a 16-site clinical trial involving 884 patients that assessed the safety and effectiveness of CCE in detecting adenomas at least six millimeters in size^[6].

In this review, the current and future role of CCE, its indications and limitations will be discussed.

COLON CAPSULE ENDOSCOPY - TECHNICAL FEATURES AND SAFETY

PillCam colon capsule endoscopy (Given Imaging Ltd, Yoqneam, Israel) is now available in its second generation (CCE-2; Figure 1). CCE-2 is 11.6 mm × 31.5 mm in size and features two head cameras that each have a 172° angle of view, allowing for almost 360° visual coverage of the colon. While the first-generation colon capsule had a flat frame rate of 4 images per second only, CCE-2

comes along with an improved image acquisition and an adaptive frame rate from 4 to 35 images per second (Table 1). This means that the camera is able to capture up to 35 pictures while in motion whereas 4 images per second are captured when it is virtually stationary to save battery power. The transit time of the capsule from the small bowel into and through the colon is relatively long, and the battery power of the capsule must therefore not be overused. To transfer the capsule optimally through the small bowel and colon, a laxative (booster) is ingested to accelerate the transit of the capsule through the small bowel into the colon (hence the name booster). Automatic detection of the small bowel mucosa triggers the timing of booster ingestion and is signaled to the patient by the data recorder. This is optimized by the CCE-2 and new data recorder technique and this technical innovation has been shown to be highly reliable in clinical studies^[7].

The resolution of CCE-2 imaging is below 0.1 mm, with a magnification of about 1 to 8. Polyp size can be estimated with the graphic interface tool of the included Rapid 8™ software. This tool, however, has not yet been verified in patients. Additional software features such as the, Flexible spectral imaging color enhancement* (FICE) technology permit enhanced visualization of detected lesions.

CCE has so far been shown to be a safe procedure and complications were almost all attributed to bowel cleansing and/or performance of colonoscopy including therapeutic interventions. In the two prospective studies that have compared CCE-2 with conventional colonoscopy, adverse events were reported from 6.8% and 8% of patients, respectively^[4,8]. However, fatigue reported from two patients in the study by Spada *et al.*^[8] was the only adverse event directly related to the CCE procedure itself. An overview of reported adverse events is shown in Table 2.

BOWEL PREPARATION FOR COLON CAPSULE ENDOSCOPY

A thorough bowel cleansing procedure is indispensable for the success of CCE. Accurate polyp detection can only be achieved when the colon is completely free of solid stool because unlike in conventional colonoscopy, a washing or sucking device is not available. In addition, a clean bowel promotes capsule propulsion for a complete bowel investigation which otherwise has to rely on longitudinal large bowel contractions which only occur a few times each day. For a better description of bowel cleanli-

Table 2 Complication rates reported from studies involving both first and second generation colon capsules *n* (%)

Ref.	Year	<i>n</i>	Complications		Major complications in detail
			Minor	Major	
Schoofs <i>et al</i> ^[3]	2006	41	0	0	-
Eliakim <i>et al</i> ^[37]	2006	98	0	1	Perforation at colonoscopy
Van Gossum <i>et al</i> ^[10]	2009	320	26 (2.9%)	0	Associated to bowel preparation: 22/26
Eliakim <i>et al</i> ^[4]	2009	104	8 (7.7%)	1 (0.96%)	7/8 associated to bowel preparation 1/1 urinary retention
Pilz <i>et al</i> ^[38]	2010	59	1 (1.69%)	1 (1.69%)	1/1 perforation nach Koloskopie 1/1 skin reaction from capsule electrodes
Gay <i>et al</i> ^[39]	2010	128	0	0	-
Sacher-Huvelin <i>et al</i> ^[11]	2010	545	19 (3.5%)	3 (0.5%)	Heart failure, potentially associated to bowel preparation: patient died Bleeding at mucosectomy Perforation at colonoscopy
Spada <i>et al</i> ^[8]	2011	109	8 (6.8%)	1 (0.85%)	5/8 associated to bowel preparation 2/8 fatigue 1/8 pain 1/1 perforation at colonoscopy
Herrerías-Gutiérrez <i>et al</i> ^[40]	2011	144	0	0	-
Hartmann <i>et al</i> ^[13]	2012	50	4 (8%)	1 (2%)	3/4 associated to bowel preparation 1/1 perforation at colonoscopy
Kakugawa <i>et al</i> ^[14]	2012	64	1 (1.56%)	0	1/1 associated to bowel preparation
Total	-	1621	67 (4.1%)	8 (0.49%)	-

Most complications are suspected to derive from colonoscopy and/or bowel preparation regimen and not related to CCE. CEE: Colon capsule endoscopy.

Table 3 Four-point grading scale for objective description of the level of cleanliness of the colon during colon capsule endoscopy^[41]

Cleansing level scale	Description	Categories
Poor	Inadequate; Large amount of fecal residue precludes a complete examination	Inadequate
Fair	Inadequate but examination completed Enough feces or turbid fluid to prevent a reliable examination	Quality of the investigation is significantly compromised
Good	Adequate Small amount of feces or turbid fluid not interfering with examination	Adequate
Excellent	Adequate No more than small bits of adherent feces	Quality of the investigation is not significantly compromised

ness in clinical trials, a 4-point grading scale ranging from poor to excellent has been proposed (Table 3).

For optimal bowel preparation, the ESGE guidelines recommend a split-dose regimen of at least 4 L of polyethylene glycol (PEG) solution to be administered on the evening before and during the morning of the exam itself^[5]. This bowel cleansing preparation should be preceded by a clear liquid diet on the day before the procedure. More recently, a prospective, randomized study has shown equal efficacy of a one-day cleansing regimen *vs* a two-day protocol^[9].

There is ample evidence that boosters of low-dose sodium phosphate (NaP) should be added to the PEG-based bowel preparation to accelerate transit time and enhance capsule visibility (Table 4)^[10,11]. Currently, the recommended dose of NaP booster is 30 mL diluted with one liter of water to be taken when the capsule has entered the small bowel and a second booster of 15–25 mL NaP with 500 mL of water 3 h later if the capsule has not been egested by that time^[5]. Higher doses of NaP were associated with an increased risk of side effects and NaP should be avoided in elderly patients as

well as patients with hypovolemia, renal insufficiency, active colitis, and those taking specific medications including ACE inhibitors^[12].

Hartmann *et al*^[13] observed good cleanliness following PEG plus ascorbic acid as the booster but incomplete investigations in 24% of cases. Finally, Mg-Citrate has also been recommended as a booster in a recent investigation^[14]. Thus, a cleansing formulation with little or no toxicity and a broad patient tolerability still needs to be defined.

INDICATIONS AND CONTRAINDICATIONS FOR COLON CAPSULE ENDOSCOPY

The acceptance of conventional colonoscopy as a screening tool for colorectal cancer is generally low despite the fact that colorectal carcinoma associated mortality may be significantly reduced^[15,16]. Therefore, the main interest for CCE development was its use as a minimally invasive, widely accepted screening tool for polyp detection.

Table 4 Diagnostic accuracy of colon capsule endoscopy for the detection of significant colon polyps (≥ 6 mm or ≥ 3 polyps)

Ref.	Year published	Colon capsule	Number of patients included	Sensitivity	Specificity	PPV	NPV
Schoofs <i>et al</i> ^[3]	2006	CCE-1	36	77%	70%	59%	84%
Eliakim <i>et al</i> ^[37]	2006	CCE-1	84	50%	83%	40%	88%
Van Gossum <i>et al</i> ^[10]	2009	CCE-1	320	64%	84%	-	-
Gay <i>et al</i> ^[39]	2010	CCE-1	126	87.5%	76%	79%	85%
Pilz <i>et al</i> ^[38]	2010	CCE-1	56	79%	54%	63%	71%
Sacher-Huvelin <i>et al</i> ^[11]	2010	CCE-1	545	39%	88%	47%	85%
Eliakim <i>et al</i> ^[4] second gen	2009	CCE-2	98	89%	76%	46%	97%
Spada <i>et al</i> ^[8]	2011	CCE-2	109	84%	64%	-	-
Rex <i>et al</i> ^[6]	2013	CCE-2	689	81%	93%	-	-

CCE-1: First generation colon capsule endoscopy; CCE-2: Second generation colon capsule endoscopy; PPV: Positive predictive value; NPV: Negative predictive value.

Indeed, it was recently reported that screening participation increased by fourfold when CCE was offered as an alternative to conventional colonoscopy even with the knowledge that a later colonoscopy could be necessary^[17]. A number of prospective studies have compared CCE to conventional colonoscopy as the gold standard for the detection of significant polyps (polyp size ≥ 6 mm or ≥ 3 polyps), a widely accepted surrogate marker for advanced neoplasia (Table 4). Published studies that used the first generation colon capsule (CCE-1) for comparison with conventional colonoscopy reported sensitivities and specificities for the detection of significant polyps in the range of 39.0%-87.5% and 54.0%-88.0%, respectively. Two meta-analyses of CCE-1 studies involving 7 and 8 studies, respectively, have since been published^[18,19]. They showed overall sensitivities and specificities of 69% and 68% and 86% and 82%, respectively, for the detection of significant polyps.

With the introduction of the second-generation CCE-2 in 2009 and implementation of more standardized bowel cleansing protocols the detection of colonic lesions has significantly increased diagnostic accuracy. To date, two studies have been published on polyp detection by CCE-2 compared to conventional colonoscopy^[4,8] while a third study involving 884 patients has only been published in abstract form^[6]. For the detection of significant findings, sensitivities and specificities ranged from 81%-89% and 64%-93%, respectively. In the latter study which is the largest investigation of CCE-2 so far, a sensitivity of 88% (95%CI: 82%-93%) was found for the detection of adenomas ≥ 6 mm and 92% (95%CI: 82%-97%) for adenomas ≥ 10 mm with respective specificities of 82% (95%CI: 80%-83%) and 95% (95%CI: 94%-95%).

Finally, a recent study suggests that CCE-2 may be better at detecting flat lesions compared to conventional colonoscopy. In this retrospective analysis of 16 patients it was shown that 25 out of 27 flat lesions ≥ 6 mm detected with conventional colonoscopy were correctly detected by CCE-2. Where conventional colonoscopy categorized only 15 of these lesions as polypoid, CCE-2 classified 24 of these as polypoid. This discrepancy may have been caused by air insufflation during conventional

colonoscopy and it suggests that the currently widely used Paris classification for polyps may not be adoptable for CCE. The sensitivity and specificity for detection of flat lesions by CCE-2 in this study were 90% and 96%, respectively^[20].

Conventional colonoscopy represents the gold standard for the examination of the colon, and a complete investigation that includes visualization of the cecum and/or terminal ileum may be attained in over 95% of cases^[21], but may be as low as about 60% in some cohorts^[22]. In most of these cases, difficult anatomical conditions, bowel adhesions and previous surgical interventions result in incomplete colonoscopic examinations. Thus, CCE may play a particular role in patients who have undergone incomplete colonoscopy. Other indications may involve unwillingness to undergo conventional colonoscopy for personal or religious reasons and contraindications for sedation. A number of recent studies suggest that there is increased interest to study the usefulness of CCE in these heterogeneous patient groups.

In a recent French multicenter study, 72% of 102 patients were investigated by CCE-1 following incomplete colonoscopy and 28% for contraindications for colonoscopy^[23]. Overall, significant findings (carcinoma, inflammatory bowel disease, angiectasia, and others) were observed in 34% of cases and treatment decision was subsequently influenced in 59% of these patients. Several other studies have reported similar percentages of significant findings and influence on treatment decisions (Table 5). However, several reports of capsule retentions suggest that CCE should be used with caution on patients with suspected malignancies unable or unwilling to undergo conventional colonoscopy.

Mucosal healing as assessed by optical colonoscopy is increasingly employed as an endpoint in inflammatory bowel disease (IBD) treatment studies as well as in clinical practice^[24]. Monitoring of mucosal inflammation by CCE may play a role as a more widely accepted diagnostic tool to guide treatment decisions in IBD patients. Therefore, a number of recent studies have investigated the role of CCE in the assessment of mucosal inflammation^[25-29]. In the study conducted by Sung *et al*^[25], the sensitivity and specificity of CCE for the detection of active

Table 5 Colon capsule endoscopy for incomplete colonoscopy or patients with contraindications for colonoscopy

Ref.	Year	n	CCE	Complete visualization of the colon by CCE + colonoscopy	Treatment decision influenced in ...	Significant findings	Capsule retention
Pioche <i>et al</i> ^[23]	2012	102	CCE-1	93%	59%	34%	12 cases
Alarcón-Fernández <i>et al</i> ^[42]	2012	34	CCE-1	85%	59%	23.5%	-
Negreanu <i>et al</i> ^[43]	2013	67	CCE-2	77% (CCE) 90% (CCE + colonoscopy)	-	34%	2 cases
Triantafyllou <i>et al</i> ^[44]	2013	75	CCE-1	91%	-	44%	-

CCE: Colon capsule endoscopy; CCE-1: First generation colon capsule endoscopy; CCE-2: Second generation colon capsule endoscopy.

ulcerative colitis was 89% and 75%, respectively when compared to conventional colonoscopy. However, more recent studies showed that CCE was clearly inferior compared to conventional colonoscopy for the assessment of disease activity and extent^[26]. At present, conventional colonoscopy should therefore be the first choice to guide treatment decisions while the role of CCE in IBD needs further clarification.

Contraindications for CCE are similar to those defined for small bowel capsule endoscopy^[30]. These include swallowing disorders, prior abdominal surgery of the gastrointestinal tract, known or suspected bowel obstruction, presence of a cardiac pacemaker and pregnancy. So far, colon capsule retention has only been reported in studies involving patients with incomplete endoscopy or those who were unwilling or unable to undergo conventional colonoscopy and those with suspected gastrointestinal malignancies, inflammatory bowel disease or prior radiation history. In all but two patients, capsules could eventually be evacuated by flexible endoscopy without the need for surgery. In two cases reported by Negreanu and colleagues, surgery for bowel cancer was decided upon capsule findings and was subsequently performed without complications and the capsules were evacuated during the procedure. This, however, emphasizes the need to carefully select patients who can undergo CCE without the risk of complications. Finally, patients who are at risk of NaP toxicity should undergo alternative booster preparations such as Mg-Citrate^[5,31,32].

CONCLUSION

Colon capsule endoscopy has shown to be a feasible and exceptionally safe procedure for the visualization of the entire colon. Its acceptance among patients and accuracy for the detection of pathologic findings has been studied for a variety of indications including the detection of polyps and adenomatous lesions as well as for monitoring inflammatory bowel disease. With the introduction of the second-generation colon capsule the sensitivity of the procedure for polyp detection has been markedly increased when compared to standard colonoscopy, which is mainly explained by the improved optical setup. In addition, CCE may be useful in patients with ulcerative colitis to monitor disease activity. Finally, patients unable or unwilling to undergo conventional colonoscopy are currently the main focus of attention and the indication

for CCE should be discussed in these patients on an individual basis as outlined in the ESGE guidelines.

However, CCE is limited as a first-line diagnostic device due to the inability to take tissue samples and to predict histology upon polyp detection. Thus, patients in whom significant findings are made during CCE still need referral to colonoscopy for clarification. In addition, even the improved second-generation colon capsule holds a sensitivity that is short of 90% in comparison to conventional colonoscopy for the detection of significant findings. Some authors argument that conventional colonoscopy itself might be an imperfect golden standard and that CCE might surpass detection rate of colonoscopy in some instances^[33]: *e.g.*, limitations of CCE in study results may be explained by the mismatch of polyp-size estimation between CCE and conventional colonoscopy, which served as the gold standard in these studies. That is, polyps, which were “overestimated” in size by CCE, may in fact have been “underestimated” by colonoscopy. Thus, currently it remains unclear how CCE might find a place in CRC prevention in the long-term.

Finally, the overall accuracy of CCE largely depends on bowel cleanliness. Indeed, split-regimens based on polyethylene glycol with additional booster preparations to be administered during the procedure are required to obtain adequate bowel cleanliness. It was shown in several studies that a complete visualization of the bowel mucosa as well as high capsule egestion rate is preferably obtained with sodium phosphate boosters. The downside of this cleansing regimen is its responsibility for most of the adverse events during CCE. Another issue that needs further clarification is the cost-effectiveness of CCE in different indications. However, it has been suggested that CCE may be cost-effective in a CRC screening program if the uptake of CCE as a screening tool is higher than that of colonoscopy^[34]. Future approaches to CCE are aiming at the improvement of polyp characterization, mainly *via* improvement of the software setup for polyp size estimation and by integration of chromoendoscopy techniques and/or confocal imaging with near infrared light for virtual histologic characterization^[35,36]. In addition, externally rechargeable batteries or even battery-free capsules are being developed.

Taken together, CCE is a safe and feasible method for the minimally visualization of the colon. Current indications aim at patients in whom conventional colonoscopy cannot be or has been incompletely performed.

Given the poor acceptance of screening colonoscopy, CCE should be tested in large-scale screening programs. For patients unable to undergo conventional colonoscopy, randomized comparisons with other non-invasive imaging modalities (*e.g.*, virtual colonoscopy) are certainly required.

CONFLICT OF INTEREST

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Capsule endoscopy in pediatrics: A 10-years journey

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although experience has been limited, the patency capsule may help lessen the potential of capsule retention; and newly researched protocols for bowel cleaning may further enhance CE's diagnostic yield. However, further research is needed to optimize the use of the various CE procedures in pediatric populations.

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Key words: Capsule endoscopy; Children; Small bowel; Pediatric endoscopy

Core tip: Recent investigations using capsule endoscopy as a tool to monitor mucosal change with therapy and to improve bowel cleaning (which potentially will increase the diagnostic yield) and new capsules to evaluate the esophagus and colon present an enhanced value to be gained from capsule endoscopy, 10 years after investigations began in pediatrics.

Abstract

Video capsule endoscopy (CE) for evaluation the esophagus (ECE), small bowel (SBCE) and the colon (CCE) is particularly useful in pediatrics, because this imaging modality does not require ionizing radiation, deep sedation or general anesthesia. The risk of capsule retention appears to be dependent on indication rather than age and parallels the adult experience by indication, making SBCE a relatively safe procedure with a significant diagnostic yield. The newest indication, assessment of mucosal change, greatly enhances and expands its potential benefit. The diagnostic role of CE extends beyond the SB. The use of ECE also may enhance our knowledge of esophageal disease and assist patient care. Colon CCE is a novel minimally invasive and painless endoscopic technique allowing exploration of the colon without need for sedation, rectal intubation and gas insufflation. The limited data on ECE and CCE in pediatrics does not yet allow the same conclusions regarding efficacy; however, both appear to provide safe methods to assess and monitor mucosal change in their respective areas with little discomfort. Moreover,

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INTRODUCTION

Since 2001, capsule endoscopy (CE) has been used to evaluate small bowel pathology in adults. In patients of 10 to 18 years of age, these evaluations began in January 2004^[1]. In 2009, CE was also approved by United States Food and Drug Administration (FDA) for use in children 2 years of age and older^[2]. As a result, the use of CE has expanded in the pediatric population over the past decade, largely because of the possibility of avoiding ionizing radiation, deep sedation and general anesthesia. That success has prompted CE evaluations of the esophagus and colon as well while broader indications and applica-

Table 1 Clinical indications, outcomes and adverse events by different age groups

	Adult	Pediatric	< 8 yr
Indications (%)			
OGIB + IDA	66	15	36
CD/UC/IC	10	63	24
Abdominal pain	11	10	14
Polyps/Neoplasms	3	8	-
Other	10	4	25
Outcomes (% positive findings for different indications)			
OGIB + IDA	61	42	-
CD/UC/IC	55	65	-
Abdominal pain	23	43	-
Polyps/Neoplasms	56	75	-
Overall	59	61	67
Adverse events (%)			
Capsule retention	1.4	2.6	0.5
Incomplete examinations	16	13	7
Other	1.1	0.9	-

CD: Crohn's disease; IDA: Iron deficiency anemia; OGIB: Obscure gastrointestinal bleeding; IC: Indeterminant colitis; UC: Ulcerative colitis.

tions of CE are being defined in the diagnosis and monitoring of gastrointestinal disease.

SMALL BOWEL CAPSULE ENDOSCOPY

Because the small intestine was often considered as the mysterious “black box” of the GI tract, small bowel capsule endoscopy (SBCE) has become particularly valuable for pediatric patients to achieve a definite diagnosis in cases with small bowel pathologies [*i.e.*, Crohn's disease (CD)] or obscure gastrointestinal bleeding) such that the small bowel is no longer the frontier that it had been in the past^[3].

Indications

The American Society for Gastrointestinal Endoscopy developed indications for SBCE^[4]. However, the relative frequency of indications in compiled pediatric reports differs from that in data regarding adults. In adults, 66% of CEs have been for obscure gastrointestinal bleeding (OGIB) including iron deficiency anemia (IDA); 11% for clinical symptoms only (*e.g.*, pain, diarrhea, and weight loss without OGIB); 10% for CD; with the balance (13%) for other indications^[5-25]. In pediatric patients, 60% of CE have been for CD, 15% for OGIB, 10% for abdominal pain/diarrhea, and 8% for polyposis^[15,16]. More than half of the procedures for IBD indications are related to evaluation of CD and colitis, with 44% due to the suspicion of CD, 16% related to evaluation of known CD, 2% to differentiate indeterminate colitis (IC), and 1% to further evaluate ulcerative colitis (UC). Abdominal pain and diarrhea account for another 10% of the procedures and might be considered as evaluations for the same indications.

Even within the pediatric population, these clinical indications are age-stratified (Table 1). In a review of 83 procedures in children aged 1.5-7.9 years (when CD is less prevalent), the most common indication for CE was

OGIB, accounting for 30 (36%) procedures, with positive yields in 16 (53%)^[21]. Suspicion of CD accounted for 20 (24%) procedures, with positive findings in 11 (55%). Abdominal pain accounted for another 12 procedures (14%), and CD was the indication in 3 patients. CD was found in 14 (31%) of the patients where a positive diagnosis was made. Investigation of malabsorption and protein loss required 12 and 9 procedures (14% and 11%), respectively, with positive findings in 6 each. In contrast, OGIB in older children (age 10-18 years) accounted for only 13%-24% of all indications^[5,9,11,17,19,23].

Additionally, SBCE is being used to identify eosinophilic enteropathy (with areas of erythematous, denuded mucosa)^[9]; an ulcerative inflammatory enteropathy in cystic fibrosis^[26] graft-vs-host disease^[8]; monitoring medical therapy in CD^[5-27]; and to evaluate the graft's integrity after small bowel transplantation^[7,8].

Preparation

The inability to establish the exact location of the capsule in the small intestine, and the inability to flushing or suction fluids make adequate bowel cleaning of particular importance for SBCE. Debris, biliary secretion, bubbles and blood, especially in the distal small bowel, and failure of the capsule to reach the cecum have the potential to limit the diagnostic yield^[28].

Since cleaning the small intestine prior to examination may improve the diagnostic yield, CE-preparation regimens-mainly using the same products adopted for colonoscopy preparation-have been proposed^[29]. But the optimal preparation regimen is yet to be established^[30]. A clear liquid diet the evening before CE and an overnight fast appears to be associated with poor visibility of the terminal ileum in the majority of patients^[30]. Since simethicone seems to improve mucosal visualization and tolerability by reducing air bubbles, flammable gas (namely, hydrogen) and abdominal discomfort^[31], a combination of simethicone and polyethylene glycol (PEG) has frequently been promulgated as an effective means to increase the visibility of the small intestine (SB)^[4,32-35].

The only pediatric study to date prospectively evaluated 198 patients with five different preparation regimens^[35]. The mucosal visibility of the SB was assessed at five equal time points. After preparation with PEG and simethicone, discomfort was lessened and mucosal visualization improved significantly in the distal ileum, which is the portion most often affected by debris. However, the overall diagnostic yield was not affected except in the last section of SB.

The least amount of PEG solution tested, 1.75 g/25 mL per kg (up to 1 Lt) of PEG solution (70 g/1000 mL) the night before the procedure plus 20 mL (376 mg) oral simethicone 30 min before capsule ingestion appears to be the preparation of choice for SBCE in children. No significant differences were found regarding gastric and small-intestinal transit times or in the proportion of patients in whom the cecum was not visualized. However, intestinal transit is much faster in children than adults

and therefore bowel preparation might not impact intestinal transit time in the pediatric age group compared to adults.

Patient outcomes

A meta-analysis^[5] and additional reports from the pediatric literature^[6,7], comprised 995 patients who underwent 1013 CE procedures with positive findings in 511 (61.4%; 95%CI: 52.7%-69.7%). Studies were complete (*i.e.*, the capsule reached or passed the ileocecal valve by the end of the recording period) in 846 procedures. (86.0%; 95%CI: 81.6%-89.9%)^[5-7]. In the studies for which ingestion was reported, a total 824 (88.4%) children swallowed the capsule uneventfully (95%CI: 86.4%-90.3%)^[15]. The youngest child to swallow the capsule was 4 years old^[23]. Only 1 patient in the reports could not swallow the capsule and refused endoscopic placement, although the inability to swallow the capsule or the fear of gagging and choking doing so are not infrequent occurrences in clinical practice^[11].

A new diagnosis was established in 162 patients (66.0%; 95%CI: 45.4%-83.9%) including patients where the capsule did not enter the colon^[12,17,18,23]. A change in therapy followed for 115 of the patients (71.3%; 95%CI: 45.2%-91.5%) where those parameters were quantified.

CD was the most prevalent diagnostic outcome of SBCE studies performed in the pediatric population, based on the criteria of at least 3 mucosal ulcers as previously reported by Fireman and colleagues^[36] and Mow and colleagues^[37]. In one study, SBCE examination reclassified 4 of 5 patients with UC and 1 of 2 patients with IC (total 5 of 7, or 71%) to CD due to newly recognized SB mucosal lesions^[12]. In various studies, a change in medical therapy resulted for 75%-92% of patients with known CD^[12,13,17].

A recent pilot study evaluating dietary intervention in pediatric CD^[27] assessed small bowel mucosal change using CE since 38% of pediatric CD is isolated to the small intestine and 80% of pediatric CD have small bowel involvement^[38]. Using the Lewis score, a validated, weighted index of 3 parameters (stenosis, ulceration and villous edema)^[39], mucosal improvement was seen at 12 and 52 wk from baseline, providing objective evidence of mucosal change, which can be used to complement standard clinical IBD research scoring methods that can be affected by the subjective reports from the patients and their families. In pediatric patients investigated for OGIB or IDA by SBCE, 38.4% had confirmed diagnoses^[14]. This compares with 59.4% positive results in adults^[40]. Forty-six lesions were diagnosed by SBCE^[8-11,13,18]: 15 vascular malformations, 7 CD; 14 nonspecific enteropathies; 3 polyps; 2 marked lymphoid hyperplasias; and 1 case each of Meckel's diverticulum, nonsteroidal anti-inflammatory drug-induced lesions, lymphangiectasia, leukemia-related and graft-versus-host disease. In patients younger than age 8 years, there were 4 cases of polyps, 2 of angiodysplasias, 2 blue rubber bleb hemangiomas, 2 Meckel's diverticulae, 1 anastomotic ulcer, and 1 intestinal duplica-

tion^[23]. In the adult meta-analysis, vascular abnormalities also were the most common cause of OGIB (50%), followed by inflammation and ulcers (27%), and neoplasia (9%)^[40]. Evaluation of polyposis syndromes, accounted for 8.0% of the indications in 81 pediatric patients, with positive results in 80.2% of procedures compared to adult diagnostic yield of 55.9% for neoplastic lesions^[41].

Although SBCE is rarely performed for evaluation of malabsorption, it is useful since intestinal lymphangiectasia can appear beyond the reach of the endoscope^[5]. The infrequency of celiac disease seen in pediatric patients may reflect the infrequency of CE use for evaluation of malabsorption in this population^[4] or the decreased time of gluten exposure with potentially patchy or very subtle mucosal changes in childhood at histological levels of Marsh I or II, for which the sensitivity of CE is low^[42]. Although lymphonodular hyperplasia and intussusceptions are often seen, they are normally non-pathogenic conditions indigenous to the pediatric population^[5].

Adverse events

Capsule retention in the SB occurred in 18 and gastric retention occurred in 4 of 1013 procedures in the meta analysis, producing a pooled retention rate of 2.3% ($n = 22/1013$; 95%CI: 1.5%-3.4%)^[5-7,15]. Endoscopy was used to remove 5 capsules including 4 from the stomach^[9-15] and 1 from an ileal pouch^[5]; 13 were retrieved surgically while taking appropriate measures to mitigate the cause of the retention^[8,10,13,17]. A retained capsule was successfully evacuated by bowel prep at 22 d post-ingestion^[10].

The greatest risk factors for capsule retention include known IBD (5.2% risk), previous small bowel follow-through (SBFT) demonstrating small bowel CD (35.7% risk) and a body mass index below the fifth percentile combined with known IBD (43% risk), although retention has occurred despite the absence of stricture on SBFT^[14]. Among 4 patients with CD having capsule passage lasting longer than 5 d (with 3 continuing on to retention), age was significant (18.8 ± 0.9 vs 14.6 ± 3.5), but not height or weight, compared to patients who did not have retention^[17]. Retention rates for indications of OGIB, CD, and neoplastic lesions were 1.2% (95%CI: 0.9%-1.6%), 2.6% (95%CI: 1.6%-3.9%), and 2.1% (95%CI: 0.7%-4.3%), respectively, with a pooled rate of 1.4% (95%CI: 1.2%-1.6%) for those procedures^[43]. On a per-procedure basis, this pattern is similar in adults, where retention in OGIB, CD, and polyps occurs at rate of 1.4%, 2.2%, and 1.2%, respectively^[40]. Thus, it appears that the risk of retention is dependent on the clinical indication, with an higher incidence in patients with suspected chronic small bowel obstruction^[43]. Rare cases of perforation, aspiration, or small bowel obstruction have been reported in adults but none have been reported in children. Minor mucosal trauma has occurred in children in which capsules were placed with the Roth net^[21]. A specific capsule placement device is now available (AdvanCE, United States Endoscopy, Mentor, OH^[44]).

Patency capsule: The majority of capsule retentions have occurred in patients with normal small bowel radiological studies, yet functional patency may be present in patients with radiologically documented strictures. To avoid this concern, an identically sized patency capsule (PC) containing a mixture of barium, lactose and a radio-frequency identity tag was developed. The first version had a single timer plug that degraded at 40 h. The currently available version has dual timer plugs that gradually implodes if passage does not occur within 30 h.

Both a retrospective^[5] and a prospective study^[45] have been performed in pediatric IBD using the first iteration of the PC prior to SBCE. Of the 19 who were evaluable in the retrospective analysis, patency was established and subsequent CE was performed successfully in all but 1 patient who had a retained capsule from CE the following week. The prospective trial of 18 patients (age 10-16 years) who ingested the PC, 15 of whom excreted an intact PC (mean 34.5 h) without any PC or CE retentions or adverse events^[45]. CD was eventually diagnosed in all patients having PC transit of more than 40 h and in 9 of 12 who passed the patency capsule in 40 h or less. There were no capsule retentions or adverse events. Thus, the PC can serve as a useful guide and may lessen the likelihood of CE retention, particularly in known CD where the risk of retention is greatest.

Esophageal and colon capsules: Esophageal capsule endoscopy (ECE) was approved by the US FDA and introduced for clinical use in 2004 with a second iteration (ESO 2; Given Imaging) released in 2007. However, clinical trials and apparent pediatric use (or at least, the reporting of that use) have been limited. Only 2 small pediatric trials of the first ECE capsule have appeared. Both focused on portal hypertension, finding that variously sized varices and other esophageal and duodenal findings could be seen despite a rapid transit time in pediatric patients^[46-48].

Similarly, colon capsule endoscopy (CCE; Given Imaging Ltd, Yoqneam, Israel)^[49-54] has been aided by a recently released, second-generation CCE device (CCE-2)^[51,52]. Consensus guidelines of ESGE on CCE have proposed that CCE-2 may be useful to monitor inflammation in UC, which may help guide therapy^[53]. To date, there have been few studies conducted in adults, with only one using the second generation of CCE^[54-57]. There is only one pilot study using CCE-2 in 29 pediatric patients with ulcerative colitis^[58]. Sensitivity of CCE-2 in detecting disease activity was 96% (95%CI: 79%-99%) and specificity was 100% (95%CI: 61%-100%), corresponding to an overall accuracy of 97% (95%CI: 90%-100%). The positive and negative predictive values were 100% (95%CI: 85%-100%) and 85% (95%CI: 49%-97%), respectively. Optimal preparation is yet to be adequately studied or established.

CONCLUSION

SBCE is a useful diagnostic tool that has particular ben-

efit in pediatrics because it does not usually require ionizing radiation, deep sedation or general anesthesia. The risk of retention appears to be dependent on indication rather than age and parallels the adult experience by indication, making SBCE a relatively safe procedure with a significant diagnostic yield. Recent investigations to improve bowel cleaning and establish CE as a useful tool to monitor mucosal change may further expand its utility.

The limited data on ECE and CCE in pediatrics do not warrant the same conclusions as yet; however, both appear to provide safe methods to assess and monitor mucosal change in their respective anatomic areas with little discomfort. However, further investigations are needed to maximize the impact of this burgeoning area of mucosal assessment and to determine whether CE can pre-empt traditional studies in order to lessen cost and improve tolerability of needed procedures.

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Rethinking elective colectomy for diverticulitis: A strategic approach to population health

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Abstract

Diverticulitis is one of the leading indications for elective colon resection. Surgeons are trained to offer elective operations after a few episodes of diverticulitis in order to prevent future recurrences and potential emergency. However, most emergency surgery happens during the initial presentation. After recovery from an episode, much of the subsequent management of diverticulitis occurs in the outpatient setting, rendering inpatient "episode counting" a poor measure of the severity or burden of disease. Evidence also suggests that the risk of recurrence of diverticulitis is small and similar with or without an operation. Accordingly, contemporary evaluations of the epidemiologic patterns of treatments for diverticulitis have failed to demonstrate that the substantial rise in elective surgery over the last few decades has been successful at preventing emergency surgery at a population level. Multiple professional societies are calling to "individualize" decisions for elective colectomy and there is an international

focus on "appropriate" indications for surgery. The rethinking of elective colectomy should come from a patient-centered approach that considers the risks of recurrence, quality of life, patient wishes and experiences about surgical and medical treatment options as well as operative morbidity and risks.

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Key words: Diverticulitis; Colectomy; Colostomy; Indications; Elective; Appropriate; Quality of life; Laparoscopy

Core tip: Over the last decade, the relationship between elective and emergency surgery has come into question. With most emergency resections being performed in patients without a prior hospitalization, it has become apparent that diverticulitis recurrences are a poor predictor for future emergency operation at the population level. In addition, the rate of diverticulitis recurrence appears to be small and similar for those who do and do not undergo resection. This evidence suggests a need to rethink the factors that should be considered when deciding on elective colectomy for diverticulitis.

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INTRODUCTION

With the aging of the population, the management of diverticulitis is becoming an increasingly important problem^[1-3]. In the United States alone, care related to diverticulitis results in an estimated 1.5 million inpatient days and 300000 admissions each year^[1]. While 10%-20% of people admitted to the hospital for diverticulitis un-

dergo emergency resection^[4,5], regardless of whether or not patients undergo surgery during initial presentation, they remain at lifetime risk for recurrent episodes and hospitalizations. Surgeons play an integral role in counseling patients on the risks of recurrence *vs* the risks of an operation, adhering to the premise that elective, “prophylactic” colectomy can prevent future episodes of diverticulitis and emergency colostomy^[6,7]. Accordingly, diverticulitis is one of the leading indications for elective colon resection^[8,9].

Over the last decade, the relationship between elective and emergency surgery has come into question. With most emergency resections being performed in patients without a prior hospitalization^[4,5,10], it has become apparent that diverticulitis recurrences are a poor predictor of need for future emergency operation at the population level. In addition, the rate of diverticulitis recurrence appears to be small and nearly identical, whether or not patients undergo resection^[4,10]. Furthermore, the observed increase in the rates of elective colectomy has not correlated with decreases in emergency colectomy^[11,12].

In light of this evidence, a number of international professional societies have indicated that surgery should no longer be performed based on the number of prior episodes of diverticulitis^[1,6,13,14]. What is emerging is a rethinking of the factors that should be considered when deciding about the role of elective surgery.

RETHINKING THE RISK OF RECURRENCE

Most emergency surgery occurs during the initial presentation for diverticulitis^[4,5,10] and treatment options at that time are directed towards controlling the source of infection. The more challenging clinical decision making begins after recovery from an acute episode, because all patients remain at lifetime risk for recurrence and emergency colectomy and/or colostomy. Elective colectomy has conventionally been recommended after the second episode of diverticulitis^[15-17] and after the first episode in patients younger than 50^[7,10,17-20].

A number of population-level studies in the last decade, however, have shown the rates of recurrent hospitalizations for diverticulitis after non-operative management (4%-13%)^[10] are similar to the rates in those who have had a colectomy (5%-11%)^[4]. Additionally, even elective resection carries a 1%-3% risk of anastomotic failure requiring “rescue colostomy”^[13,21]. Incorporating these risks into a modeled analysis demonstrated that delaying elective surgery until after at least episodes resulted in a lower rate of colostomy and cost savings^[5].

The relationship between elective and emergency surgery also needs to be better understood. Across Washington State, our group has tracked age- and sex-adjusted rates of hospitalization and colectomy for diverticulitis since the 1980s^[12]. Our review of the over 84000 patients hospitalized for diverticulitis between 1987 and 2012 demonstrated that the age- and sex- adjusted rates (adjusted to the 2000 census population of the state) for

elective colectomy nearly tripled, rising from 7.9 to 17.2 per 100000 people^[22]. This rise, which was most pronounced in the early 2000s, has not been accompanied by decreases in emergency surgery (which rose from 7.1 to 10.2 per 100000 people), percutaneous interventions (from 0.1 to 3.7 per 100000) or emergency admissions for diverticulitis (from 34.0 to 85.0 per 100000). Given that 80%-90% of emergency surgery happens at the first episode of diverticulitis, these findings suggests that the practice of routine elective colectomy does not prevent future emergency surgery at a population level.

In studies of patients with diverticulitis, the most common outcomes assessed are hospitalizations for recurrent disease and whether or not patients had an emergency operation or a colostomy. Focusing only on hospitalized diverticulitis has limited assessments about current practice patterns because in the last 2 decades there has been an important shift towards outpatient management of recurrent disease^[1,4,20]. Diverticulitis is now one of the leading reasons for outpatient visits related to the gastrointestinal (GI) tract^[2] and outpatient management is 3 times more common than inpatient care^[23]. Researchers may have not previously evaluated outpatient care for diverticulitis because outpatient information is not as readily available as inpatient data and because of concerns about coding accuracy when using outpatient diagnostic codes^[23], such as co-existence of diverticulitis or its symptoms with other outpatient GI conditions like irritable bowel syndrome. While including unconfirmed cases of diverticulitis may lack specificity, a counting approach that captures presumed episodes of outpatient diverticulitis is consistent with the way clinicians and patients both experience and “count” recurrences.

RETHINKING SEVERITY OF DIVERTICULITIS

The historic recommendation for early resection in young patients^[7,18,24] was based not only on the time at risk for recurrence, but also the belief that presentation at a young age indicated a more virulent disease and an increased likelihood for more severe recurrences^[10,15,19,20]. This “more virulent” nature of diverticulitis in young patients is has been contested by newer evidence^[20,25-27], but it appears that younger patients in the last decade are undergoing resection for diverticulitis more often than older patients^[28,29]. Whether this stems from a greater relative impact of diverticulitis on the quality of life (QoL) of younger patients or whether decisions are based on younger patients’ comparatively good health remains to be determined. However, this issue has become more relevant in the last 2 decades with reported rates of diverticulitis rising significantly in the young^[19,28-30].

Severity of diverticulitis and complicated diverticulitis are problematic to measure with administrative databases, as diagnostic and billing codes for abscess, peritonitis and perforation are often secondary and inconsistently recorded. Increasing outpatient manage-

ment suggests those requiring inpatient hospitalizations today are “sicker” than they were in the past^[1,4,20]. However, studies looking at complicated diverticular disease in hospitalized populations have found relative stability of patients with “complicated” diverticulitis^[5,31,32]. Additionally, the proportion of emergency admissions having surgery, perhaps the ultimate measure of disease severity, appears to be decreasing at a population-level^[11,30,32,33]. Unfortunately, even this measure can be misleading—while the proportion of admitted patients having surgery may be decreasing, the overall rate (or incidence) of surgery can still be increasing. This is attributable to the overall increased number of patients admitted for diverticulitis, even after adjustment for age and sex^[5,11,12]. A number of plausible explanations exist for the decreasing proportion requiring emergency surgery, including increased percutaneous interventions^[12,32], more refined classification of abscesses and contained perforations with improved imaging^[33], and a shift to delayed elective surgery^[4]. However, the inconsistent definition of diverticulitis severity makes it a difficult metric to track and justify as an indication for elective colectomy at a population level.

RETHINKING THE THRESHOLD TO OPERATE

One hypothesis to explain the disconnect between elective and emergency surgery for diverticulitis is that the adoption of laparoscopy is responsible for the dramatic rise in surgery, rather than changes in the incidence or severity of the disease^[30]. Laparoscopic techniques for colorectal surgery were introduced in the early 1990s^[34]. However, it was not until the early 2000s that training programs began incorporating it and several randomized trials of laparoscopy for colon cancer were published^[35,36]. Population-level studies have ascribed the growth of elective surgery during this period to the availability and adoption of laparoscopy by the colorectal community^[11,12,28,30], similar to what occurred with the introduction of laparoscopic gall bladder surgery in the late 1980s^[37,38].

Laparoscopic colectomy improves outcomes through lower morbidity, fewer complications and quicker discharge from the hospital^[36,39], and has been recommended as the approach of choice for elective resection for diverticulitis^[1]. Use of laparoscopic colon surgery (LCS) is increasing with some studies estimating that approximately half of elective colectomies for diverticulitis are currently performed laparoscopically^[30,31,40], especially among younger patients^[11,28]. However, it appears that that countries with greater use of laparoscopy have higher rates of elective surgery for diverticulitis^[40,41], and some “early adopters” of laparoscopy also had a dramatic rise in right-sided resections for diverticulitis, a previously an uncommon procedure^[42]. While none of these studies can absolutely causally attribute the rise in surgery to laparoscopy, the evidence has reinforced speculation that the threshold for surgeons to recommend, and for

patients to undergo, elective surgery has been lowered by the availability of LCS.

RETHINKING INDICATIONS FOR ELECTIVE SURGERY

The evolution of evidence around diverticulitis over the last decade has put a new emphasis on defining “appropriateness” metrics for elective surgery for this disease. Indeed, with an international focus on cost of healthcare and estimates that 1 in 3 healthcare dollars is spent on care that doesn’t appear to add value^[43,44], there has been increasing interest in establishing appropriateness criteria for many surgical procedures^[45–49]. The production of such guidelines and criteria by both professional societies and insurance companies^[50–54] have had mixed effects in reducing rates of procedures that do not meet the designated criteria. For many surgical diseases, including diverticulitis, assessing compliance with these recommendations has been problematic because detailed information about the indications for surgery is lacking from existing registries.

Increasingly guidelines have recommended individualizing the decision for elective colectomy^[1,13,14]. However, it remains to be determined which discrete measures of patient experience should be used to assess whether a resection is appropriate. Surgeons often report that their patients may not meet the professional recommendations but have reasonable indications for elective colectomy such as anxiety related to the possibility of an emergency, fear of travel, uncertainty about insurance and childcare coverage, intolerance of oral antibiotics or lingering symptoms and impaired QoL.

QoL impact appears to be driven by symptoms as well as the fear of recurrence, uncertainty about travel, concerns about chronic antibiotic use and lost productivity related to time away from work. These are factors that may also be particularly relevant among younger patients. Evidence for these “non-clinical” impacts of diverticulitis is found on social media websites for patients with diverticulitis (<http://diverticulitis.supportgroups.com>, <http://www.dailystrength.org>). Patients on these sites commonly report lingering symptoms after recovery from an episode of diverticulitis as one of the drivers for elective surgery. Recurrent symptoms include fevers, chills, decreased appetite, abdominal bloating and changes in bowel habits. These have been variously referred to in the literature as “smoldering”, “residual”, or “ongoing, symptomatic uncomplicated diverticular disease”^[55]. Unfortunately, there is no generally accepted taxonomy for this and considerable overlap in symptoms with other conditions^[56,57]. Attempts to quantify the drivers of impaired QoL have been limited to small cohorts and suffer from response rates as low as 50%^[4,58]. Accounting for these symptoms is problematic without the use of standard evaluations that have not been a part of most prior studies. To address this issue, patient-centered outcomes research is needed to assess competing patient experi-

ences with and without surgery.

CONCLUSION

Contemporary evaluations of the epidemiologic patterns of diverticulitis and treatments for diverticulitis suggest a disconnect between the use of elective colectomy for prevention of emergency surgery at a population level. Recommending elective surgery based on the number of prior episodes is no longer supported. Rather, a patient-centered approach to counseling for elective colectomy should consider the risks of recurrence, QoL burden, patient wishes and experiences about surgical and medical treatment options and operative morbidity and risks. To guide decision making, studies incorporating this spectrum of relevant metrics should be performed and incorporated into new guidelines aimed at accomplishing more appropriate care.

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Evidence or eminence in abdominal surgery: Recent improvements in perioperative care

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Abstract

Repeated surveys from Europe, the United States, Australia, and New Zealand have shown that adherence to an evidence-based perioperative care protocol, such as Enhanced Recovery After Surgery (ERAS), has been generally low. It is of great importance to support the implementation of the ERAS protocol as it has been shown to improve outcomes after a number of surgical procedures, including major abdominal surgery. However, despite an increasing awareness of the importance of structured perioperative management, the implementation of this complex protocol has been slow. Barriers to implementation involve both patient- and staff-related factors as well as practice-related issues and resources. To support efficient and successful implementation, further educational and structural measures have to be made on a national or regional level to improve the standard of general health care. Besides postoperative morbidity, biological and physiological variables have been quite commonly reported in previous ERAS studies. Little information, however, has been obtained on

cost-effectiveness, long-term outcomes, quality of life and patient-related outcomes, and these issues remain important areas of research for future studies.

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Key words: Enhanced recovery; Surgery; Fast track; Perioperative management; Postoperative outcome

Core tip: There is a strong and evolving evidence base to support Enhanced Recovery After Surgery (ERAS) programs in abdominal surgery. Such pathways are safe and efficient in enhancing recovery and reducing morbidity. However, patient-related outcomes, cost effectiveness and long-term benefits from ERAS protocols need to be studied more carefully in the future. To support efficient and successful implementation, further educational efforts have to be performed on a national or regional level to improve the standard of care in the general population.

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INTRODUCTION

Traditional perioperative care is heterogeneous and often based on regional and local traditions or even individual preferences of the surgeon, anesthesiologist or other staff. Enhanced Recovery After Surgery (ERAS) is an evidence-based, structured, multi-modal program for optimal perioperative care, initially described and developed by Professor Henrik Kehlet, Copenhagen, Denmark, for patients undergoing colonic surgery^[1]. The ERAS recom-

Table 1 Enhanced recovery after surgery elements

Preoperative	Pre-admission counseling Stopping smoking and alcohol abuse Optimize nutrition and glucose control No oral bowel preparation
Intra-operative	Preoperative carbohydrate loading Avoiding sedative premedication Thromboembolism and antimicrobial prophylaxis Epidural or other regional anesthesia Balanced fluid therapy avoiding overhydration Active warming Minimally invasive surgery PONV prophylaxis
Postoperative	No abdominal drains or nasogastric drains Multimodal analgesia to avoid opioids Early removal of urinary catheter Early oral feeding and intense mobilization No intravenous infusions Support of GI function (laxatives/prokinetics) Nutritional supplements Audit

PONV: Postoperative nausea and vomiting; GI: Gastrointestinal.

mendations cover multiple aspects of the pre-, peri- and postoperative periods, with the aim of reducing surgical stress, maintaining physiological functional capacity, and facilitating postoperative recovery^[2-4]. ERAS is a dynamic concept according to the best available evidence. The main items are: preadmission counseling, no bowel preparation, preoperative carbohydrate loading to avoid preoperative fasting and dehydration, balanced perioperative fluid management, multimodal analgesia avoiding opioids using epidural or other regional anesthesia, minimally invasive surgery, no abdominal or nasogastric drains and early removal of urinary catheter, early oral feeding, intense mobilization and support of gastrointestinal function (Table 1).

ADVANTAGES OF THE ERAS CONCEPT

That the ERAS program, compared with traditional perioperative management, results in enhanced recovery, shorter hospital stays, and reduced postoperative morbidity has been convincingly shown in repeated randomized controlled trials and meta-analyses^[5,6].

Patient-related symptoms^[7,8], quality of life^[9] and cost-effectiveness^[10] have been less commonly reported but are likely to be improved by ERAS when compared with traditional care. Laparoscopic and minimally invasive procedures will further improve outcomes compared with open surgery in ERAS pathways^[11], although remarkably early recovery can also be obtained after open abdominal surgery^[12]. Limited data are available on post-discharge and late postoperative outcomes. Studying the process of implementation will provide valuable information on the importance of individual items on outcomes from surgery, and issues related to ERAS implementation and evidence-based perioperative medicine on a broader basis in general health care. Structured implementation of ERAS

in the Breakthrough project, which included a third of all hospitals in the Netherlands, was reported to be successful and resulted in an improved standard of care and a 3-d reduction in length of stay^[13]. National incentives, such as in the Netherlands^[13] and the NHS Enhanced Recovery partnership program in England^[14] to support the implementation of ERAS on a national basis, are imperative to obtain a major improvement in general health care.

ADOPTION OF ERAS BY OTHER SURGICAL DISCIPLINES

The convincing data from colorectal surgery has encouraged an accelerating spread of the ERAS concept to other surgical disciplines. Published guidelines from the ERAS Society cover recommendations for perioperative care, not only for colorectal surgery^[3,4], but also pancreaticoduodenectomy^[15] and radical cystectomy for bladder cancer^[16]. In addition, enhanced recovery protocols have safely and successfully been implemented for other major elective abdominal procedures such as liver resections^[17], esophagectomy^[18,19], gastrectomy^[20], bariatric surgery^[21], hysterectomy^[22], and emergency surgery^[23,24].

PATIENT PERSPECTIVE

A recent systematic review studied how recovery outcomes were reported when comparing fast track pathways with traditional care^[25]. The studies focused on in-hospital biological and physiological variables such as the return of gastrointestinal function and postoperative complications. In contrast, patient-reported symptoms, functional status, and quality of life were less commonly studied, in particular post-discharge. Nevertheless, when patient satisfaction and quality of life were reported in randomized trials, fast track programs were either superior or equal to traditional care, but never inferior^[26-33]. The use of heterogeneous measures, however, hinders comparisons across studies. Recently, the SF-36 was also validated as a measure of postoperative recovery after colorectal surgery^[34]. The need for better outcome measures, including the patient's experiences (*i.e.*, core outcome sets or composite outcomes), has been emphasized^[8,35].

ADHERENCE TO THE ERAS PROTOCOL

Despite increasing awareness of the importance of structured perioperative management, implementation of this complex protocol has been slow^[36]. Several large surveys have been performed to study the adoption of the concept in different countries. The surveys report a wide variation in adherence to fast track protocols, and methods that are harmful for the patient and prolong postoperative recovery are still commonly used. Nevertheless, a somewhat higher acceptance of evidence-based methods seems to be reported in questionnaires concerning the surgeon's preferences than in surveys based on the actual registration of clinical parameters and ERAS items. One

Table 2 Key points in this paper

Key points
Traditional unstructured perioperative care is still common
The ERAS protocol is an evidence-based structured perioperative regime
The ERAS program improves postoperative recovery and reduces morbidity
More research is needed on cost-effectiveness, long-term outcomes, quality of life, and patient-related outcomes
Regional and national strategies to support the implementation of evidence-based perioperative care in general health care are warranted

could speculate that questionnaire surveys may reflect what physicians believe should be done rather than what they actually would do in clinical practice. In two previous surveys among surgeons and anesthesiologists in five countries in Europe^[37,38], prevailing routines for colonic surgery deviated considerably from the best available evidence, with a wide variation between countries. In another survey on colonic surgery, conducted in 295 hospitals in the United States and Europe (United Kingdom, France, Germany, Italy and Spain), most centers still adhered to traditional perioperative care^[39]. Bowel cleansing methods, for example, were used in > 85% of cases and nasogastric tubes were retained for several days postoperatively in 40% *vs* 66% of the patients in the United States and Europe, respectively. Traditional perioperative care was reflected in the postoperative length of stay; over 10 d in the European countries and 7 d in the United States. This could be compared with discharge from hospital 2-5 d after colonic surgery, reported from trials performed in dedicated centers with a successful implementation of fast track programs^[40,41].

Similar to colonic surgery, traditional approaches in perioperative care were common for rectal surgery in a large survey covering 461 institutions in Germany and Austria from 2006^[42]. In a more recent survey among colorectal surgeons in Great Britain and Ireland published in 2008, it was concluded that routine adherence to ERAS was relatively high, indicating a general trend among colorectal surgeons to comply with ERAS interventions. There remained, however, a potential for improvement^[43]. In a survey from 2011 in New Zealand and Australia, some, but not all, ERAS interventions were routinely used according to a questionnaire recently distributed to colorectal surgeons^[44].

All members of the health care/multidisciplinary team must be included in the repeated educational efforts necessary for successful implementation of the ERAS concept^[45]. A recent survey among senior anesthesiologists from mainly European countries showed a low level of knowledge about ERAS pathways^[46]. Current routines differed from the ERAS guidelines in > 50% of the centers concerning fluid infusion policy, fasting, postoperative opioids, premedication, and the use of prokinetics.

BARRIERS TO IMPLEMENTATION

Today, it is still the case that the change in practice from

a more traditional approach to evidence-based perioperative care appears to be slow^[40]. On its own, a protocol is not sufficient to introduce necessary fast track recovery routines^[36]. Some studies have, therefore, explored possible barriers to ERAS protocol compliance. A qualitative interview-based study identified four key areas important for the implementation process: patient-related factors, staff-related factors, practice-related issues, and resources^[47]. This highlights the need for multidisciplinary efforts to reach a high level of compliance and the involvement of hospital management. In a questionnaire survey from Toronto, surgical residents reported some barriers to the early discharge of patients, which included patient and family expectations, surgeon preferences, and the beliefs of the health care team^[48]. Other reported issues were that some ERAS items may seem to be too time consuming, and there was a lack of co-specialty and institutional support^[49].

ADHERENCE AND OUTCOME

At our own institution, the ERAS program has been chosen for all patients undergoing colorectal surgery since 2002. In 2004-2005, a second round of educational efforts was made, as were other measures to enforce the process of implementation^[50]. Thus, as published by Gustafsson *et al.*^[50] compliance with the ERAS protocol at our institution improved from 43% to 71%. Interestingly, in this cohort of 953 patients undergoing major colorectal cancer surgery, improved adherence to the ERAS protocol was significantly associated with improved clinical outcomes^[50].

In addition to patient perspective and physiological outcomes, evaluation of the possible economic advantages of enhanced recovery pathways is warranted. A cost reduction from the decrease in morbidity and hospital length of stay may promote the implementation of fast-track programs and increase adherence to the protocol. Available data are sparse, but do support the cost-effectiveness of fast-track programs^[51,52].

CONCLUSION

Key points in this paper are summarized in Table 2. There is a strong and evolving evidence base to support ERAS programs in abdominal surgery. Such pathways are safe and efficient in enhancing recovery and reducing morbidity. The implementation of ERAS pathways in new surgical procedures needs to be audited and carefully evaluated in clinical studies since the evidence base for different ERAS items may vary depending on the selected surgical procedure. To support efficient and successful implementation, further educational efforts have to be performed on a national or regional level to improve the standard of care in the general population. Patient-related outcomes, cost effectiveness and long-term benefits from ERAS protocols need to be studied more carefully in the future.

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Surgical treatment of familial adenomatous polyposis: Dilemmas and current recommendations

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Abstract

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited syndrome characterized by multiple adenomatous polyps (predisposing to colorectal cancer development) and numerous extracolonic manifestations. The underlying genetic burden generates variable clinical features that may influence operative management. As a precancerous hereditary condition, the rationale of performing a prophylactic surgery is a mainstay of FAP management. The purpose of the present paper is to bring up many controversial aspects regarding surgical treatment for FAP, and to discuss the results and perspectives of the operative choices and approaches. Preferably, the decision-making process should not be limited to the conventional confrontation of pros and cons of ileorectal anastomosis or restorative proctocolectomy. A wide discussion with the patient may evaluate issues such as age, genotype, family history, sphincter function, the presence or risk of desmoid disease, potential complications of each procedure and chances of postoperative surveillance. Therefore, the definition of the best moment and the choice of appropriate procedure constitute an individual decision that must take into consideration patient's preferences and

full information about the complex nature of the disease. All these facts reinforce the idea that FAP patients should be managed by experienced surgeons working in specialized centers to achieve the best immediate and long-term results.

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Key words: Familial adenomatous polyposis; Surgical treatment; Restorative proctocolectomy; Ileal pouch-anal anastomosis; Ileorectal anastomosis; Adenocarcinomas

Core tip: This paper is an extensive review of the literature focusing the options and criteria for surgical treatment of patients with Familial Adenomatous Polyposis. The author put together a great number of dilemmas and the current recommendations in order to help readers to understand how complex the disease is and to summarize the current knowledge.

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INTRODUCTION

Familial adenomatous polyposis (FAP) is a genetic complex syndrome that may affect 1-8 per 10000 persons. Clinically, it is characterized by early development of a wide range of colorectal adenomatous polyps after the second decade of life and many extracolonic manifestations. If not treated by prophylactic colectomy, patients will have an almost 100% risk of developing a colorectal cancer (CRC).

Classic FAP and the attenuated form (AFAP) result from germline APC (*adenomatous polyposis coli*) mutations. FAP is an inherited autosomal dominant disease in which the majority of patients will be affected in a context of familial history, and almost 30% may have a “*de novo*” mutation. The less aggressive variant AFAP exhibits fewer colorectal adenomatous polyps (usually 10-100), later age of adenoma appearance and a lower cancer risk^[1]. A subset of patients will exhibit a mutation in the base-excision-repair MUTYH gene, whose carriers may present a less severe and later polyposis when compared to FAP. Discovered in 2002, this recessive syndrome is called MUTYH associated polyposis (MAP), and CRC are frequent and discovered at the same moment as the polyposis^[2].

FAP patients must be operated due to the almost inevitable adenoma-carcinoma sequence, and surgery has a positive impact on life expectancy^[3]. In the past, most patients died from CRC, but this risk has been gradually reduced since the founding of the first Polyposis Registry by Lockhart-Mummery in St Mark's Hospital in 1925 (Figure 1). Subsequently, the establishment of National Polyposis Registries in many countries changed morbidity patterns through early diagnosis and prophylactic colectomy, with a resulting improved prognosis^[4]. Besides this, extracolonic manifestations such as desmoid disease, duodenal and pouch neoplasia may still pose important challenges to patients and surgeons.

Genetic testing and familial counseling should follow clinical and endoscopic diagnosis. Certain genotypes have been linked to specific extracolonic manifestations and may influence polyposis severity; moreover, it has been suggested that these associations should be considered in surveillance and therapeutic decisions (Table 1).

After diagnosis, surgery represents the mainstay of treatment for FAP patients (Figure 2). Surgical options include total colectomy with ileorectal anastomosis (IRA), total proctocolectomy with ileostomy (TPI), and restorative proctocolectomy (RPC) with or without mucosectomy and ileal-pouch anal anastomosis.

SURGICAL DECISION MAKING

As a rare and complex genetic disease, it is well recognized that FAP is better managed by a collaborative group of specialists. Several clinical and genetic features may influence surgical decisions regarding timing and extension of resection.

The present manuscript aims to raise and discuss several controversial issues concerning the surgical treatment of patients diagnosed with FAP.

Timing of surgery

There are no guidelines regarding the timing of surgery and most classical FAP patients undergo surgery between 15 and 25 years of age. Preferably prophylactic, an elective resection may be planned considering individual and family features, as well as patient's preferences^[12]. The



Figure 1 British surgeon John Percy Lockhart-Mummery (1875-1957) who established the famous Polyposis Registry in St. Marks Hospital (London).

main factors involved in timing of surgery are listed in Table 2.

Obviously, adenoma-associated symptoms such as diarrhea, bleeding, malnutrition or growth retard may encourage surgical indication on the next available opportunity. Also, specific endoscopic and histological features (presence of numerous adenomas, sized lesions or high-grade dysplasia) represent good reasons to indicate a prophylactic colectomy as soon as possible. Even asymptomatic patients with severe polyposis should not have their surgical procedure postponed.

It is important to emphasize that those patients with severe polyposis or CRC are more likely to be symptomatic. Among our own patients, CRC incidence was much lower in asymptomatic patients (1.1% *vs* 65.8%). Moreover, patients without CRC presented a shorter length of symptoms (15.2 mo *vs* 26.4 mo) and less frequent weight loss (11.4% *vs* 33.9%)^[13]. Similarly, Bülow *et al*^[14] reported a 60% incidence of CRC in patients with symptomatic FAP.

Besides symptoms, age is also an important factor related with CRC risk. In untreated patients, the mean age of CRC diagnosis and subsequent death have been reported to be 39 and 42 years, respectively^[15]. In a review of 1073 patients from European countries, the risk of having a carcinoma at an age less than 20 years was estimated to be approximately 1%^[16]. Within our own series, average age of patients without CRC was lower at treatment (29.5 years *vs* 40.0 years, $P = 0.001$)^[13]. Age distribution revealed a cumulative incidence of 1.9% and 32.1% in patients with less than 20 and 30 years of age, respectively. This data is similar to the 15% incidence in patients before the age of 25 years at the St Mark's Hospital^[17].

These data explain why surgery may be deferred till the late teens or beginning of the third decade in most patients^[18], after physical, emotional and social maturation are established^[19]. Once FAP is diagnosed, there is a recommendation for immediate surgery for severe cases (> 1000 colonic and/or > 20 rectal polyps) as soon as practicable^[20]. A similar approach should be employed in patients with family history of severe disease^[12].

In another situations, FAP diagnosis does not require

Table 1 Phenotype-Genotype correlations in familial adenomatous polyposis patients

Phenotype	Mutations	Authors
AFAP	APC extreme ends (exons 3,4,5) and exon 9	Spirio <i>et al</i> ^[5] 1993
Profuse polyposis (approximately 5000 polyps)	Between codons 1250-1464	Nagase <i>et al</i> ^[6] 1992
Severe polyposis and early CRC onset	Deletion in codon 1309	Caspari <i>et al</i> ^[7] 1995
Desmoid tumor	Between codons 1444-1580	Caspari <i>et al</i> ^[7] 1995
CHRPE	Between codons 463-1387	Olschwang <i>et al</i> ^[8] 1993
Thyroid cancer	5' to codon 1220	Cetta <i>et al</i> ^[9] 2000
Duodenal adenomas	Between codons 976-1067	Bertario <i>et al</i> ^[10] 2003
Rectal cancer	Codons 1250 to 1464	Bertario <i>et al</i> ^[11] 2000

AFAP: Attenuated familial adenomatous polyposis; APC: Adenomatous polyposis coli; CRC: Colorectal cancer; CHRPE: Congenital hypertrophy of the retinal pigment epithelium.

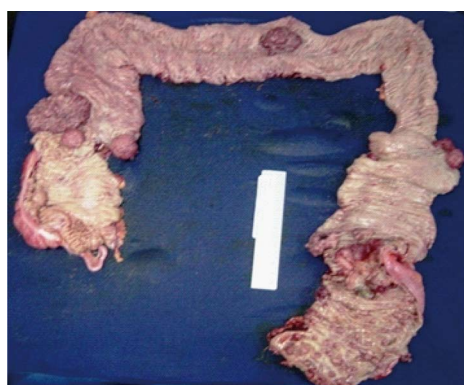


Figure 2 PAF specimen showing numerous polyps along the colon and at least seven simultaneous malignant lesions.

the need to perform surgery immediately. For example, an elective procedure may be delayed if an asymptomatic (at-risk or mutation carrier) young patient is compliant with surveillance and polyposis is not severe, since he understands there is a risk for CRC. In this setting, a personal decision based on psychological well-being and patient/family preferences related to school education or professional issues may also affect the planning of surgery. Considering that most patients will be treated around their twenties, doctors should discuss with young patients the possibility of sexual dysfunction following rectal resection, a technique-associated complication due to pelvic plexus lesion occurring in less than 4% of the patients^[1].

Also, patients presenting an AFAP (by phenotype or genotype) or with a suggestive family history may be operated later, as CRC will generally occur in the early to mid-50s^[21]. Another reason to delay surgery would be a preoperative diagnosis or the treatment of high-risk patients (positive family history or genetically susceptible) for desmoid tumor, if there were no imminent risk for CRC^[22].

Selecting the best procedure: Ileal-anal or IRA?

The decision-making process to surgical alternatives must be tailored to the disease severity as well as to patient's age, clinical conditions and personal preferences. Certainly, surgeon's experience and technical skills may influence

the final choice^[21]. The factors that may affect surgical decision are listed in Table 3.

Proctocolectomy with ileostomy is the less common operation performed and must be reserved for patients with low rectal cancer, sphincter dysfunction, when a mesenteric desmoid prevents pouch construction or when it is impossible to pull the pouch down to the pelvis. This operative choice may lead to profound body image and emotional alterations related to the stoma and sexual dysfunction after pelvic dissection. Differently, surgical decision for most patients will confront IRA and RPC, and until now the published literature reached no consensus about this issue, making the choice between RPC and IRA a continuous matter of debate^[23]. Discussion of the pros and cons of these techniques must not be oversimplified, cause several operative, oncological and functional variables must come into debate (see Table 4).

IRA is generally recommended for patients with few rectal polyps, with AFAP, a family history of mild phenotype and for those young women with desire to be pregnant. IRA should not be performed in patients with a diseased rectum (adenomas > 3 cm, with severe dysplasia, cancer or sphincter dysfunction) or colon cancer. Patients with other features should undergo RPC^[24-26].

Besides being a rectal sparing prophylactic colectomy, IRA provides good surgical and functional outcomes, but requires long-term follow-up of the rectum. During the pre-pouch era, metachronous rectal cancer rates varied from 15% to 40%, decreasing to less than 10% after pouch surgery came into practice^[21,25]. After IRA, development of rectal cancer risk depends on several risk factors such as length of follow-up, chronologic age and local of APC mutation^[11,26].

Certainly, bad selection criteria in the era before RPC accounts for the high rates of rectal recurrence after IRA. Today it is possible to select better candidates for IRA, once the risk of proctectomy is much lower in those presenting less than 20 rectal or 1000 colonic polyps^[27]. In a series of 776 IRA from a multicenter Scandinavian study, proctectomy was performed in 229 of 576 patients (40%) during the prepouch period *vs* 26 of 200 (13%) during the pouch period^[25].

Thus, IRA may provide good results in AFAP, MAP

Table 2 Factors to be considered in the timing decision for surgery

Reasons to indicate or postpone surgery	Timing for surgery
Presence of symptoms (> risk of CRC)	As soon as possible
Asymptomatic patient with mild disease	Discuss opportunity (before 20 years?)
Sized lesions or with high-grade dysplasia, not amenable to endoscopic resection	Immediately
Severe disease at colonoscopy or by family history/genotype	As soon as practicable
Attenuated polyposis at colonoscopy or by family history/genotype	Personal decision (16-20 years if mild or 21-25 years if attenuated polyposis)
Preoperative diagnosis, positive family history or genetically susceptible for desmoids	Delay surgery (after evaluating CRC risk)

CRC: Colorectal cancer.

Table 3 Patient's and disease factors affecting operative choices

Patient	Disease
Age, sex, obesity, prior surgery	Number and location of polyps
Genetics - family history	Colorectal cancer or metastatic disease
Female fecundity	Presence or risk of desmoid disease
Compliance with follow-up	AFAP
Acceptance of a temporary stoma	MAP

MAP: MYH associated polyposis; AFAP: Attenuated familial adenomatous polyposis.

and mild FAP patients that agree to undergo follow-up, and RPC should be reserved for those with profuse polyposis^[21,22]. Consequently, a policy of blanket RPC is not a good idea for patients with mild or attenuated disease, mainly for those asymptomatic and at a young age.

During the last decades, RPC progressively turned out to be the most common procedure despite its surgical morbidity. For this reason, candidates to RPC must be aware of its technical complexity and the reported high complication rates^[28]. Comparative studies with IRA have reported higher complications after RPC^[29,30], although this has not been a unanimous finding^[31]. In our own series^[26], we also observed more complications after RPC (48.1%) compared to proctocolectomy with ileostomy (26.6%) and IRA (19.0%) ($P = 0.03$).

Furthermore, pelvic dissection may lead to urinary and sexual dysfunction such as decreased fecundity in women (but it doesn't risk pregnancy) and male impotence^[32,33]. Indeed, it has been reported a 50% reduction in female fecundity after RPC^[32]. As IRA doesn't involve pelvic dissection, young females of childbearing age must be informed of this fact in order to refine the decision-making process.

Although not yet proven, RPC has been associated with a greater risk of desmoid disease when compared to IRA^[34]. If this information turns out to be true, this chance must be critically evaluated as desmoid disease is an important cause of mortality among FAP patients.

Long-term functional results have been generally better after IRA^[35,36]. In a meta-analysis of 12 selected studies (1002 FAP) comparing functional outcome and quality of life between RPC and IRA^[37], bowel frequency, night defecation and use of incontinence pads were signifi-

cantly less in the IRA group, although fecal urgency was more frequent with IRA compared with ileal pouch-anal anastomosis (IPAA). Reoperation within 30 d was more common after IPAA. There was no significant difference between the procedures in terms of sexual dysfunction, dietary restriction or postoperative complications (bowel obstruction, hemorrhage, intra-abdominal sepsis, and anastomotic leak). Rectal cancer was only observed in the IRA group (5%). In addition, abdominal reoperation on the rectum was more frequent after IRA (28%) *vs* IPAA (3%). The study demonstrated the individual merits and weaknesses of IRA and IPAA. Generally, better functional results are attributed to IRA, although there are no significant differences regarding quality of life^[35,38].

Another capital issue regarding RPC is oncological. Although it was initially thought that RPC would abolish the risk of neoplasia, adenomas may develop within the ileal pouch many years after the surgical treatment^[39]. Furthermore, there is a risk of malignant transformation attested by reports of cancer at the ileal pouch or at the ATZ^[40,41]. These data clearly reflect that RPC is not a cancer-free procedure.

Then, surgical experience with RPC assumes a great importance. FAP patients should be advised to have the operation performed in medical centers that are familiar with FAP and by surgeons with proper training to perform this procedure.

CONTROVERSIAL ISSUES

Should genetic guide surgery?

As already stated, the final decision depends on many factors that include a fully informed patient. However, no consensus has been reached regarding the use of genotype to guide surgical option, mainly in patients with a polyp-free rectum. This idea is based on the relations between APC genotype and colonic polyposis. There are mutations associated with severe (between codons 1250 and 1464, especially at codon 1309), mild (extreme ends of the gene and in the alternatively spliced part of exon 9) and intermediate (in the remaining parts of the gene) forms of the disease^[42].

In an important study containing data from four National Polyposis Registries, there were analyzed cumulative risks of proctectomy and cancer twenty years after

Table 4 Recommendations for surgical treatment based on clinical and genetic features

Operation	IRA	RPC
Indications	Mild FAP or MAP (< 20 rectal or < 1000 colonic polyps) AFAP by family history, endoscopy or genetic testing No colorectal carcinoma Young women without definitive offspring Metastatic CRC	Many (> 20) rectal adenomas or > 3 cm or high-grade dysplasia Severe colonic phenotype (> 1000) or family history Colorectal carcinoma Mutations in codon 1309 Mesenteric desmoid or family history or APC mutation (codons 1403-1578)
Pros	Technically simple, good function, low morbidity, no pelvic dissection	
Cons	Metachronous rectal cancer	Technically demanding High morbidity

AFAP: Attenuated familial adenomatous polyposis; MAP: MYH associated polyposis; IRA: Ileorectal anastomosis; CRC: Colorectal cancer; RPC: Restorative proctocolectomy; APC: Adenomatous polyposis coli.

primary colectomy in four hundred and seventy-five polyposis patients^[43]. The authors registered cumulative risks of secondary proctectomy of 10%, 39%, and 61% in the attenuated, intermediate, and severe genotype groups, respectively ($P < 0.05$). Risks of cancer were not different. This data clearly shows that mutation analysis may be used to predict the risk of secondary proctectomy, and thus may help select the best option in patients with a few rectal adenomas.

Surgical traumas, hormonal influences in women, family history and genotype (APC mutation between codons 1445 and 1578) have been listed into a desmoid risk scoring system for FAP patients^[44]. Recognition of this genotype-phenotype association has influenced surgical decision-making and has also served as an alert to delay or avoid surgery in order to prevent desmoid disease. This recommendation is especially important in those with 3' APC mutations, which are associated with a 65% risk of developing mesenteric desmoids^[45].

Handsewn or mechanical anastomosis?

IPAA may be accomplished either by handsewn or stapled anastomosis, and the relative benefits of each technique are a source of intense debate, as these two options may impact the outcome after RPC.

At the beginning, IPAA was performed manually after anorectal mucosectomy above the dentate line. Subsequently, this supposed advantage of removing all at-risk mucosa in ulcerative colitis and FAP patients proved to be insufficient after reports of islands of rectal mucosa left behind during dissection. A natural technical advance in FAP surgery was achieved with the introduction of stapled anastomosis. Besides being technically easier and faster, it promotes better function when compared to hand-sewn anastomosis due to the less sphincter manipulation and the preservation of a small rectal cuff above the anastomosis.

Against the premise that RPC would eliminate cancer risk, there is accumulating evidence that adenomas may develop within the pouch in 8%-74% and also at the anal transition zone (ATZ). Still controversial, potential factors for the development of pouch polyposis have been

investigated. Ileal pouch adenomas are more common in patients older than 50 years of age or those presenting more than 1000 colonic adenomas. Age of the pouch is also important, with risk varying from 7%-16% after 5 years to 35%-42% after 10 years and 75% after 15 years^[40,46].

As shown in Table 5, the incidence of polyps at the ATZ is usually lower after handsewn when compared to double-stapled anastomosis^[36,47,48,49]. The presumable thinking that the remnant mucosal left behind after stapled anastomosis carries the same risk of malignization as the original disease, one could think that mucosectomy is probably the ideal choice. The risk of malignization within the pouch is probably low, as there are only thirty cases of cancer reported both at the anastomotic site and in the ileal pouch so far^[39]. But since invasive cancer has been reported to occur either from the preserved ATZ or from retained mucosal remnants, a reduction of the cancer risk should not guide solely the operative choice. Consequently, as the natural course and significance of pouch polyps are better understood, the facts discussed here raise the need for long-term endoscopic pouch surveillance regardless of the anastomosis performed.

Another important question regarding handsewn and stapled anastomosis is the confrontation of functional results and morbidity. As already reported, even though mucosectomy may reduce the risk of adenoma and cancer formation, it has been related to worse functional outcomes^[50].

Is it safe to perform an ileal-pouch anastomosis without a diverting ileostomy?

Despite its intrinsic technical complexity, RPC is safe (mortality: 0.5%-1%) and carries an acceptable risk of non-life-threatening complications (10%-25%), achieving good long-term functional outcome with excellent patient satisfaction (over 95%).

A temporary protective ileostomy proximal to pouch has been classically performed in order to mitigate the effects of anastomosis leakage and to prevent pelvic sepsis (reported in 6% and 37%), fistulization and thus compromise pouch function. Consequently, it should also

Table 5 Incidence of adenomatous polyps at the anal transition zone or anastomotic site after handsewn or double-stapled anastomosis

Study	Handsewn anastomosis	Double-stapled anastomosis	Follow-up (yr)
Remzi <i>et al</i> ^[47]	21% in the pouch 14.3% in the ATZ	11% in the pouch 28% in the ATZ	5.8
Von Roon <i>et al</i> ^[48]	27%	54%	10
Friederich <i>et al</i> ^[49]	29%	64%	7
Van Duijvendijk <i>et al</i> ^[56]	10%	31%	7

ATZ: Anal transition zone.

prevent the need for re-laparotomy and, most importantly, pouch failure^[51]. Although most patients exhibit a very good acceptance of this temporary stoma, it may be a source of several complications.

Ileostomy omission has been advocated in selected cases, with the rationale that it is associated with similar rates of septic complications and may also provide economic advantages^[52]. Selection criteria should exclude clinical (high doses of steroids, malnutrition, toxicity or anemia) and technical factors (difficult procedures with intraoperative complications). Furthermore, surgeons must be sure that the ileoanal anastomosis is tension-free, that it is supplied with adequate blood flow, that the tissue rings are intact and that air leaks are absent^[53,54].

Several studies identified the underlying disease (ulcerative colitis) as a risk factor for pouch-related sepsis^[50,51]. Within this context, its omission is attributed to the general fewer RPC complications rates in FAP than in ulcerative colitis. At diagnosis, FAP patients usually exhibit few symptoms and good general conditions, a different picture from those with ulcerative colitis. And when comparing septic complications with and without ileostomy, most cases have been attributed to steroid use.

In a paper from Saint Antoine Hospital^[55], the authors reported their experience with 71 patients (38 females) who underwent laparoscopic RPC between November 2004 and February 2010. Indications were FAP (34), ulcerative colitis (35), indeterminate colitis (1) and Lynch syndrome (1). Laparoscopic RPC was performed as a one-stage procedure in 49 patients, and after a sub-total colectomy in 22. Seven patients in each group underwent the formation of a diverting stoma. Sixteen patients experienced at least one postoperative complication. The postoperative morbidity was 29% ($n = 4/14$) and 21% ($n = 12/21$) in patients with and without a stoma ($P = 0.8$), and the rate of fistula was 21% and 5%, respectively ($P = 0.08$). Seven percent of patients with a stoma and 16% without stoma had an intra-abdominal collection ($P = 0.7$). Nine patients required reoperation, which was not influenced by the presence or absence of a diverting stoma. The results of this study are similar to other laparoscopic RPC series.

Omission of ileostomy may have a great impact on young patients at school age. Once large-bowel techniques are evolving rapidly, the selection criteria for not

performing an ileostomy after laparoscopic RPC, especially in FAP, still needs to be clarified. López-Rosales *et al*^[56] reported good results in eight out of ten patients who underwent IPAA without protection. Ky *et al*^[57] registered eleven postoperative complications and three reoperations among 32 patients. In our own series, one patient who had undergone one-stage procedure developed a postoperative fistula successfully treated with intestinal deviation. So far, we have preferred to perform laparoscopic RPC with ileostomy, and this choice is also based on the potential risk of desmoid tumors in FAP, which has been associated with surgical trauma among other predictive factors^[58].

The review of the pertinent literature leads to the recognition that selective omission of a protective ileostomy may be safe and associated with similar septic complications and failure rates when compared with stoma patients. However, this finding forces us to critically evaluate FAP patient selection criteria, in which an experienced surgical team, a patient with a good clinical status and a procedure without adverse intraoperative outcomes should necessarily be included^[59].

Laparoscopic or open approach?

During the last decade, surgical technique has evolved significantly, mainly with the crescent incorporation of laparoscopic techniques to accomplish complex procedures such as total abdominal (procto) colectomy. Acceptance of laparoscopic extensive resections is still controversial due to obstacles such as technical intraoperative difficulties, greater length of surgical procedure and the need for specialized training and instruments. Furthermore, most of the current literature includes case-matched studies and case series that deal with patients suffering from different diseases.

Regardless of these limitations, many publications have demonstrated the feasibility, safety, and good functional outcome in patients with ulcerative colitis and FAP. These patients are ideal candidates for a minimally invasive approach as these diseases usually affect young, motivated and body image conscious patients. Thus, the laparoscopic approach may be a useful mean to minimize morbidity in this population^[60].

A review of the literature concerning laparoscopy usually reveals greater operative time, no difference in mortality, complication, reoperation and readmission rates; higher cosmesis scores and less blood loss have also been widely reported^[61-63]. In our own experience with 49 patients, we registered a very low conversion rate (2%) and no patient required blood transfusion. Moreover, we observed 24.5% complications, 2% mortality and only 14.3% reoperations. Median length of hospital stay was only 6.2 d^[58].

Besides lessening the body image impact, another potential advantages may be associated with laparoscopy. There exists an amount of evidence suggesting a reduction in abdominal and pelvic adhesions, which could result in less small bowel obstruction (SBO)^[64]

and improved fertility^[65]. But the idea regarding a lower incidence of SBO has been critically debated^[66,67]. RPC is known to be associated with postoperative infertility in open surgery, which may be caused by pelvic adhesions affecting the fallopian tubes. However, fertility after laparoscopic IPAA has been rarely assessed. The group from Paris analyzed 63 patients aged 45 or less by questionnaire^[65]. The results were compared with those of controls undergoing laparoscopic appendectomy. Most patients (73%) suffered from ulcerative colitis. The authors found that 73% of the patients who attempted pregnancy after IPAA were able to conceive, and suggest that the infertility rate appears to be lower after laparoscopic IPAA than after open surgery.

Another important conclusion came from another institution^[68] where 50 patients were evaluated by questionnaire after attempting to conceive. This study grouped patients with FAP (12), ulcerative colitis (37) and colonic ischemia (1). Comparison of open (23; 46%) and laparoscopic (27; 54%) RPC revealed a higher pregnancy rate after laparoscopic ileal anal-pouch anastomosis (log-rank, $P = 0.023$), suggesting laparoscopy to be the best approach in young women.

Taking into account all the controversial issues, one could argue whether those complex procedures should be performed only in specialized centers and by skilled and experienced surgical teams. The analysis of a greater number of patients allocated in randomized controlled trials may adequately elucidate the real dimension of the supposed advantages of laparoscopy for extended colorectal resections.

Is there a role for chemoprevention?

When facing an inherited cancer syndrome, management may be accomplished by genetic counseling, screening for at-risk lesions, chemoprevention, prophylactic surgery and lifetime surveillance. Current guidelines recommend that patients at risk for FAP should initiate endoscopic examination at 10-12 years of age, with continuing regular endoscopic surveillance until colectomy is advisable due to polyp burden, size or degree of dysplasia^[16]. During this period, all significant sized adenomas should be removed if surgery has not been advised yet.

Ideally, FAP treatment would be pharmacological, as the NSAIDS, sulindac, celecoxib (selective cyclo-oxygenase-2 inhibitor) and aspirin may cause regression of established adenomatous polyps in individuals with FAP and may also reduce the number and size of colorectal adenomas. Thus, these drugs may act as an adjunct to postpone surgery in patients with mild polyposis or after ileal-rectal anastomosis. Besides these potential effects, chemoprevention alone is not suitable since CRC may develop even in patients with polyp suppression with NSAIDS^[16].

Otherwise, chemoprevention with celecoxib may be an acceptable therapeutic option in cautiously selected FAP patients who present a high risk of rectal or duodenal cancer but a low risk of cardiovascular and thrombo-

embolic events^[69]. Within a context of primary therapy, it may be indicated for those who refuse surgery or have a high surgical risk, for patients with extensive desmoid disease or for ileal pouch polyposis whose treatment means an end-ileostomy^[70].

Due to these properties, celecoxib was approved by the Food and Drug Administration (FDA) in 1999 and by the European Medicines Agency (EMA) in 2003 to reduce the number of adenomatous colorectal polyps in individuals with FAP in conjunction with usual care (e.g., endoscopic surveillance and surgery)^[69]. As an alternative to chemoprevention with celecoxib, it has been recognized that sulindac may present a stronger effect on the number of colorectal adenomas (although it doesn't prevent them), and that its gastrointestinal-related toxicity may be managed with proton pump inhibitors^[16].

CONCLUSION

All the data presented here clearly show how complex the decisions regarding FAP surgical treatment are. In this context, many disease and patient factors must be considered when taking the final choice. As a genetic disease associated with a great risk of CRC, the rationale of performing a prophylactic colectomy is a mainstay of FAP management. Patients should undergo an appropriate clinical evaluation and receive psychological support, since a great part of this population is young and recognize they suffer from a hereditary condition that usually affects other family members and deserves surveillance for life. In this way, the challenge of the working team (surgeon, gastroenterologist, genetic counselors and others) is to take individual decisions throughout the disease evolution based on the best available evidences and recommendations.

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Advances in non-surgical management of primary liver cancer

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide. There have been great improvements in the diagnosis and treatment of HCC in recent years, but the problems, including difficult diagnosis at early stage, quick progression, and poor prognosis remain unsolved. Surgical resection is the mainstay of the treatment for HCC. However, 70%-80% of HCC patients are diagnosed at an advanced stage when most are ineligible for potentially curative therapies such as surgical resection and liver transplantation. In recent years, non-surgical management for unresectable HCC, such as percutaneous ethanol injection, percutaneous microwave coagulation therapy, percutaneous radiofrequency ablation, transcatheter arterial chemoembolization, radiotherapy, chemotherapy, bio-

therapy, and hormonal therapy have been developed. These therapeutic options, either alone or in combination, have been shown to control tumor growth, prolong survival time, and improve quality of life to some extent. This review covers the current status and progress of non-surgical management for HCC.

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Key words: Ablation therapy; Biotherapy; Hepatocellular carcinoma; Hormonal therapy; Percutaneous ethanol injection; Percutaneous microwave coagulation therapy; Radiofrequency ablation; Radiotherapy; Transcatheter arterial chemoembolization; Chemotherapy

Core tip: In recent years, there has been considerable progress in the development of non-surgical management for unresectable hepatocellular carcinoma. These therapeutic options, either alone or in combination, have been shown to control tumor growth, prolong patient survival, and improve quality of life to some extent. Some of these strategies have been extensively used in clinical practice as the preferred approaches for advanced primary liver cancer.

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INTRODUCTION

Primary liver cancer, with hepatocellular carcinoma (HCC) being the most common form, is the fifth most common cancer and the third most common cause of cancer-related death worldwide^[1]. It was predicted that the incidence of liver cancer in China would increase over the next few

Table 1 American Joint Committee on Cancer Tumor Node Metastasis staging system

Stage	Tumor	Node	Metastasis
Stage I	T1: Solitary tumor without vascular invasion	N0: No regional lymph	M0: No distant metastasis
Stage II	T2: Solitary tumor with vascular invasion or multiple tumors, size < 5 cm	Node metastasis	
Stage III A	T3: Multiple tumors with size > 5 cm or tumor involving a major branch of the portal or hepatic vein(s)		
Stage III B	T4: Tumor that invades adjacent organs other than the gallbladder or perforates visceral peritoneum		
Stage III C	Any T	N1: Regional lymph node metastasis	
Stage IV	Any T	Any N	M1: Distant metastasis

Table 2 Okuda staging system¹

Criteria	Positive	Negative
Tumor size ²	> 50%	< 50%
Ascites	Clinically detectable	Clinically absent
Albumin	< 3 mg/dL	> 3 mg/dL
Bilirubin	> 3 mg/dL	< 3 mg/dL

¹Stage 1: No positive criteria; Stage 2: 1-2 positive criteria; Stage 3: 3-4 positive criteria; ²Measured from the largest cross-sectional area of tumor to the largest cross-sectional area of the liver.

years^[2]. Thus, liver cancer poses a heavy burden for our community. In the United States, it was reported that the number of new HCC cases has increased over the past several years, with the incidence rate increasing significantly from 2.7/100000 in 2001 to 3.2/100000 in 2006^[3].

At present, surgery-based comprehensive therapy plays a dominant role in the treatment of HCC. However, the majority of patients lost their opportunities for surgical treatment when diagnosis was confirmed. Moreover, only 15% of patients may benefit from surgical excision.

In clinical practice, the type of treatment for HCC is largely dependent on how advanced the tumors have developed. Thus, tumor staging is a crucial basis for the selection of surgical and non-surgical therapeutic interventions and has a significant impact on therapeutic outcomes. Many different staging systems have been developed, including the American Joint Committee on Cancer Tumor Node Metastasis staging system (Table 1), Okuda staging system (Table 2)^[4], Cancer of the Liver Italian Program Scoring System (Table 3)^[5,6], Barcelona Clinic Liver Cancer (BCLC) System (Table 4)^[7,8], Chinese University Prognostic Index (Table 5)^[9], Japan Integrated Staging Score, and Groupe d' Etude et de Traitement du Carcinoma Hepatocellulaire. However, each of these systems have their advantages and disadvantages, and no worldwide consensus as to which is the more preferred prognostic staging system for HCC has been established.

Regardless of which staging system is used in clinical practice, non-surgical approaches have shown great promise in the management of primary hepatic carcinoma. Among all non-surgical approaches, percutaneous ethanol injection (PEI), percutaneous microwave coagulation therapy (PMCT), and percutaneous radiofrequency ablation (RFA) have become the three most widely used

Table 3 Cancer of the Liver Italian Program staging system

Criteria	Points
Child-Pugh stage	
A	0
B	1
C	2
Tumor morphology	
Uni-nodular and extension ≤ 50%	0
Multinodular and extension ≤ 50%	1
Massive or extension > 50%	2
Alpha-fetoprotein level	
< 400 ng/mL	0
≥ 400 ng/mL	1
Portal vein thrombosis	
No	0
Yes	1

techniques for the treatment of HCC less than 5 cm in diameter and/or having a tumor number less than 3. In this review article, we aim to summarize the recent advances in non-surgical therapeutic approaches for HCC.

ABLATION THERAPY

Ablation therapy is considered the best treatment choice for patients with early but unresectable liver cancer^[10]. Most commonly used ablation therapies include PEI, RFA, microwave coagulation therapy (MCT), high intensity focused ultrasound (HIFU), interstitial laser photocoagulation, and freezing treatment.

PEI

In this procedure, 95% alcohol is slowly injected into the tumor mass *via* a puncture needle previously inserted under the guidance of ultrasound. The high concentration of ethanol infiltrates the tumor tissue where it dehydrates the tumor cells and causes protein degradation and coagulative necrosis of the tumor and surrounding tissues. This procedure is simple, convenient, and less costly. PEI is an effective treatment for small HCC. The efficacy of PEI for HCC tumors smaller than 3 cm in diameter is significantly better than for those larger than 5 cm in diameter. It has been reported that in HCC patients whose tumor mass was less than 3 cm, a complete response rate of 70%-80% and a 5-year survival of 40%-60% have been achieved^[11].

Table 4 Barcelona Clinic Liver Cancer staging system

Stage	PST ¹	Tumor stage ² /cancer symptoms	Hepatic function	Recommended treatment
0 (very early)	0	Single nodule < 2 cm	Child-Pugh A; normal portal pressure; normal bilirubin	Resection
A (early)	0	Single nodule < 5 cm Up to 3 nodules, < 3 cm each	Child-Pugh A; elevated portal pressure and/or elevated bilirubin	Liver transplantations or PEI/RFA ^{3,4}
B (intermediate)	0	Large, multinodular; no cancer symptoms	Child-Pugh A-B	TACE
C (advanced)	1-2	Portal invasion, extrahepatic disease, or cancer symptoms	Child-Pugh A-B	New anti-tumoral agents
D (terminal)	> 2	Any of the above	Child-Pugh C	Symptomatic treatment

¹PST evaluated using the World Health Organization's performance status scoring system (also known as the Eastern Cooperative Oncology Group System or the Zubrod system); ²N1 or M1 under American Joint Committee on Cancer's Tumor Node Metastasis staging system; ³Recommended in the absence of associated diseases; ⁴PEI/RFA is recommended in the presence of associated diseases. PEI: Percutaneous ethanol injection; PST: Performance status; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization.

Table 5 Chinese University Prognostic Index risk groups in hepatocellular carcinoma

Parameter	Weight (CUPI score)			
Bilirubin (mg/mL)	< 1.9	0	1.9-2.8	3
Ascites	Present	3		
Alkaline phosphatase	≥ 1 ka	3		
TNM stage	I and II	-3	IIIa and IIIb	-1
AFP (ng/mL)	≥ FP	2	IVa and IVb	0
Disease symptoms on presentation	None	-4		

Adapted from Leung *et al*^[9]. CUPI: Chinese University Prognostic Index; TNM: Tumor Node Metastasis; AFP: Alpha fetoprotein.

The therapeutic efficacy of PEI for HCC has been adversely linked to tumor size, Child-Pugh score, BCLC staging, and serum alpha fetoprotein levels^[12]. PEI is strongly recommended to HCC patients in whom the tumors are located near major bile ducts, gallbladder, and diaphragm, or in whom the tumor size is < 1.5 cm in diameter^[13].

The biggest drawback of PEI is the high recurrence rate, usually around the tumor margin^[14]. Multiple injections and large amounts of alcohol are sometimes required to achieve a better therapeutic effect, but this may cause cumulative damage and even cirrhosis in hepatic parenchyma.

RFA

RFA is a minimally invasive treatment for solid tumors such as HCC. In RFA, the heat generated by high frequency alternating current (in the range of 350-500 kHz) is transduced into the tumor tissues through an electrode probe. The transduced heat will then cause necrosis and scarring in the tumor tissues. RFA is usually conducted in the outpatient setting, using either local anesthetics or conscious sedation anesthesia. Insertion of radiofrequency probes is usually done through percutaneous, laparoscopic, or open intraoperative ultrasound guidance.

RFA is commonly indicated for: (1) small HCC patients unsuitable for resection; (2) a single tumor with a

maximum diameter ≤ 5 cm or multiple but fewer than 3 tumors with a maximum diameter ≤ 3 cm; (3) HCC patients with no lymphovascular invasion or neighboring organ invasion; and (4) patients with Child-Pugh Class A or B liver function^[15]. In a report involving 88 cases of small HCC treated with RFA^[16], the 3-year local recurrence rate was 4.8%, 3- and 5-year survival rates were 83.0% and 70.0%, respectively, and the 3- and 5-year disease-free survival rates were 34.0% and 24.0%, respectively.

The major disadvantages of RFA include: (1) dissipation of RF heat through nearby major blood vessels, thereby potentially reducing the curative effect and damaging adjacent organs; and (2) in large tumors, the rate of necrosis is low. The following are independent risk factors of recurrence after RFA treatment: (1) tumor diameter is > 3 cm; (2) tumor is located near the intrahepatic vasculature; (3) subcapsular tumors; and (4) PT extends over 3 s. The effect of RFA can be improved if these risk factors are taken into account in clinical practice^[17].

Ultrasound-guided RFA is a relatively safe, well-tolerated, and versatile treatment option that offers excellent local control of primary and metastatic liver tumors. The appropriate use of percutaneous, laparoscopic, and open surgical RFA is beneficial in the management of patients with liver tumors in a variety of situations^[18]. Randomized controlled trials have shown that RFA offers a higher complete response at fewer treatment sessions and a better survival compared to ethanol injection^[11].

PMCT

MCT is a relatively new type of ablative approach for the treatment of liver cancer. MCT can efficiently induce coagulative necrosis in tumor tissues, and tumors with unfavorable location or those larger than 3 cm in diameter are also suitable for MCT without further risk of local tumor recurrence^[19,20].

In a recent study, a novel 915 MHz system was used to treat 47 patients with 80 tumor nodules (average tumor size 2.6 ± 0.9 cm) in 51 treatment sessions^[20]. The treatment was delivered laparoscopically in 20 cases and percutaneously in 31 cases. High-risk conditions (defined as unfavorable tumor location such as those invisible by na-

tive transabdominal ultrasound, superficial tumors, or risk of heat sink phenomena) were found in 28 cases (53%). Local recurrence rate was 17% on a per-patient basis and 12% on a per-tumor basis ($n = 9$). One patient died of uncontrollable upper gastrointestinal bleeding during the postoperative hospital stay. No MCT-associated complications occurred. Median follow-up period was 20 mo.

By univariate logistic Cox regression, it was revealed that tumor size, procedure access, and high-risk location were significant prognostic factors for local tumor recurrence. However, by multivariate reiteration, only chosen access to MCT and tumor size was significantly correlated with local recurrence.

The commonly encountered complications of MCT include skin burns, liver capsule bleeding, and severe pain. In cases where the tumor size is > 5 cm in diameter, cancer cells may become thermoresistant and active proliferation may occur, thereby favoring tumor metastasis and recurrence. Nevertheless, MCT may be superior to other therapeutic approaches for HCC.

HIFU

HIFU is a highly precise procedure that applies high-intensity focused ultrasound energy to locally heat and destroy diseased or damaged tissue through ablation. Thus, HIFU is a hyperthermia therapy, a class of clinical therapies that use temperature to treat diseases. This minimally invasive therapeutic procedure directs acoustic energy into the disease tissues^[21]. Although the application of HIFU technology in the management of patients with hepatocellular carcinoma is still in its early stages, several studies concerning HIFU treatment of liver tumors have been reported. In one published study^[22], 39 patients with cirrhosis Child A or B and unresectable HCC adjacent to major hepatic veins were treated with HIFU. These patient/tumor characteristics would be ineligible for other ablation treatments such as RFA or PEI. Following one session of HIFU treatment, more than 50% of the patients developed complete tumor necrosis, indicating that HIFU can achieve complete tumor necrosis even when the lesion is located adjacent to major hepatic blood vessels. No major complications were observed and the overall survival rates at 1, 3, and 5 years were 75.8%, 49.8% and 31.8 %, respectively. In a similar study^[23], Orsi *et al*^[23] showed that in six HCC patients whose tumors were located in difficult locations (*i.e.*, adjacent to a main hepatic blood vessel, heart, bowel, stomach, gall bladder, or bile ducts), treatment with HIFU achieved complete response in all patients without any complications.

Targeted cryoablation therapy

Helium cryoablation is a minimally-invasive freezing technique used to treat solid tumors through extremely low temperature. Within a few seconds, the tip temperature of the therapeutic device can drop to -140°C , and then quickly rise to $20-45^{\circ}\text{C}$. The unique freeze-thaw cycles could more completely destroy the tumor tissues, regulate the presentation of tumor antigens, and activate the

body's anti-tumor immune response. Cryoablation kills tumor cells and induces necrosis in tumor tissues primarily through two mechanisms, namely cellular damage and vascular injury^[24]. Cell damage occurs immediately in the freeze-thaw process, whereas vascular injury is the result of blood stagnation and further microcirculation failure.

The therapeutic effect of cryoablation on target tissue could be influenced by many factors, including freezing temperature, freezing rate, thawing rate, and the frequency that the freeze-thaw cycle is applied. Cryogenic treatment not only effectively kills all tumor cells in the frozen region, but also maximally preserves the normal liver tissues. Based on a long-term follow-up study, cryosurgery could achieve a survival rate comparable to that of liver resection, in addition to reducing overall mortality and improving quality of life^[25].

TRANSCATHETER ARTERIAL CHEMOEMBOLIZATION

Normal liver has a dual blood supply system: 25%-30% of said blood supply comes from the hepatic artery and 70%-75% from portal vein system. In the case of HCC, 90%-99% of the tumor blood supply comes from the hepatic artery, whereas only a small portion of the tumor tissue is nourished by the portal vein. Transcatheter arterial chemoembolization (TACE) causes tumor necrosis by blocking the tumor blood supply with the emulsion of chemotherapy drugs and lipiodol while exerting minimal impact on the normal liver. TACE is the first choice for unresectable advanced liver cancer, and is one of the preferred therapies for small HCC.

Major indications of TACE include: (1) HCC patients with good liver function reserve but incapable of having their tumors radically resected; (2) no thrombosis in the portal vein trunk; (3) tumor occupies less than 70% of the whole liver; (4) de-bulking the size of huge liver cancer for later resection; (5) palliative control of pain, bleeding, and arteriovenous fistula caused by the tumor; and (6) as a preventive therapy after tumor resection^[26]. In a study involving 8510 cases of unresectable HCC, the median survival time following TACE treatment was approximately 34 mo, and 1-, 3-, 5-, and 7-year survival rates were 82%, 47%, 26%, and 16%, respectively^[27]. In patients with portal vein thrombosis, the average survival time can still be extended by appropriate TACE therapy^[28,29]. Overall, TACE shows satisfactory results on small HCC (< 5 cm in diameter)^[30].

Incomplete necrosis of tumor tissues is the major drawback of TACE. Therefore, multiple treatments are needed. Pathological examination of surgical specimens after TACE showed live cancer cells around most tumors. This is mainly due to drug resistance of tumor cells, incomplete tumor embolization, and re-established collateral blood supply.

According to 2013 NCCN guidelines on HCC, all tumors, irrespective of location, may be amenable to arterially-directed therapies, provided that the arterial blood

supply to the tumor may be isolated without excessive non-target treatment. Arterially-directed therapies include transarterial blood embolization (TAE), chemoembolization (TACE plus drug-eluting beads), and radioembolization with Yttrium-90 microspheres. All arterially-directed therapies are relatively contraindicated in patients with bilirubin > 3 mg/dL unless segmental injections can be performed. Radioembolization with Yttrium-90 microspheres has an increased risk of radiation-induced liver disease in patients with bilirubin over 2 mg/dL.

Arterially-directed therapies are relatively contraindicated in patients with main portal vein thrombosis and patients with liver function classified as Child-Pugh Class C. In HCC patients, if there is evidence of a residual/recurrent tumor not amenable to other local therapies, and provided that the patients have adequate liver function or their bilirubin return to baseline level, sorafenib may be an appropriate choice following arterially-directed therapies. The safety and efficacy of using sorafenib concomitantly with arterially-directed therapies and/or ablation is being investigated in ongoing clinical trials. Arterially-directed or systemic therapy should be considered in patients with unresectable/inoperable lesions > 5 cm^[31-33].

RADIOTHERAPY

It was previously believed that liver cancer is generally insensitive to radiotherapy, while liver tissue is sensitive to radiation; therefore, when used to treat liver tumors derived from chronic viral hepatitis, radiotherapy may cause radiation-induced liver injury.

Studies over the past few years have shown that radiation therapy may have potential therapeutic benefits in patients with advanced HCC. It has been verified that HCC is almost equally sensitive to radiation therapy as poorly-differentiated nasopharyngeal squamous cell carcinoma^[34]. Some recently developed stereotactic radiotherapy techniques (including gamma knife, X knife, three-dimensional conformal radiotherapy (3DCRT), and intensity modulated radiation therapy) may improve irradiation capacity and minimize X-ray damage to normal liver tissue. Image guided radiotherapy techniques shows even more enhanced therapeutic effects, as this technique takes into account the displacement error caused by the breathing movement of the target organ and uses the concept of 4D radiation therapy.

In a study of 70 cases of primary liver cancer treated with 3DCRT, 54.3% of cases had a reduction in their primary tumor lesions, 39% had portal vein tumor thrombus cleared or shrunk, and the median survival period was extended to 11.2 mo^[35]. Radiation therapy can also be applied to the palliative treatment of large HCC and very late HCC, either alone or in combination with other treatment modalities^[36,37]. For the palliative therapy for larger or metastatic tumors, radiotherapy can help relieve major symptoms such as pain. For HCC complicated with local (*e.g.*, hepatic hila) or distant lymph node metastasis, radiotherapy can be applied to palliatively treat the tumor

thrombus of the portal vein and inferior vena cava, as well as the lymph node and distant metastasis, provided that the primary tumors are well under control^[38-40]. However, the cirrhotic liver may have a reduced tolerance to radiation therapy. Thus, the correct safe dosage and partition of radiation have not yet been standardized. At present, in order to improve efficacy and reduce adverse reactions, radiation is usually given in a small and extended course, with the presumption that the accumulated total dose is therapeutically sufficient.

Monoclonal antibodies carrying radioactive material have been shown to achieve some therapeutic effect. For example, intraoperative injection of Yttrium-spherical particles *via* the hepatic artery has been shown to shrink the tumor, relieve symptoms, and prolong patient survival, and in a minority of patients tumor resection was possible after the therapy^[41,42].

CHEMOTHERAPY

Liver cancer is a chemoresistant tumor, but the underlying molecular mechanisms are unclear. Altered biological characteristics of the cancer cells and the perturbed pharmacokinetic properties of the liver, as well as the inherent resistant nature of the cancer cells, may all play a role.

p53 is an important tumor suppressor gene and is a critical regulator for chemotherapeutic drug-induced apoptosis. Inactivation of the *p53* pathway has been causally linked to primary drug resistance of the cancer cells^[43]. *p53* mutation occurs frequently in liver cancer. Hepatitis B virus (HBV) infection and chemical drugs have been shown to induce *p53* mutation. Over-expression of DNA topoisomerase II alpha in HCC is likely responsible for the observed resistance of liver cancer cells to Adriamycin^[44].

Reduced number of functional liver cells, impaired liver microcirculation, and compromised detoxifying capacity of the liver (*e.g.*, due to reduced activity of CYP450 system) all contribute to the poor absorption, distribution, and bioavailability of conventional chemotherapeutic drugs. As a result, it can be difficult for chemotherapeutic drugs to achieve the therapeutically relevant level, the diseased liver may have an increased susceptibility to developing liver dysfunction, and patients are vulnerable to developing complications such as infection, jaundice, ascites, and gastrointestinal bleeding. The innate resistance of cancer cells, particularly cancer stem cells, may be related to the increased expression of drug efflux genes such as the multidrug resistance gene^[45].

So far, there is no convincing evidence that chemotherapy can improve overall survival of patients with advanced HCC^[46]. For example, single agent doxorubicin may be effective in 10%-15% of cases, but it does not improve overall survival, and serious adverse reactions such as neutropenia are a hurdle for more aggressive treatment^[47]. Other chemotherapeutic drugs such as cisplatin, etoposide, epirubicin, 5-FU, gemcitabine, irinotecan, and liposomal doxorubicin also showed no significant

effect in addition to having adverse effects can be severe^[48-50]. Combinatorial chemotherapies have also failed to improve overall survival in HCC patients^[51,52].

BIO THERAPY

Immunotherapy

In recent years, tremendous progress has been made in immunotherapy for HCC. Interferon is the cornerstone of treatment for viral hepatitis, but its application in the management of advanced HCC is still controversial. A high dose of interferon (2.5×10^7 - 50×10^7 IU/m², 3 times per week) has been found to improve overall survival of HCC patients in 30% of cases^[53]. The main drawback of interferon treatment is its adverse reactions, but these can be minimized when interferon is used at a lower dose (3×10^6 IU/m², 3 times per week). A combination of monoclonal antibody and single chain antibody variable region gene (scFv) derived from tumor tissues has shown some anti-tumor effect^[54]. Likewise, lymphoid immune therapy could improve the survival of patients with primary liver cancer^[55,56].

Targeted molecular therapy

With the enhanced understanding of the molecular mechanisms governing the development of HCC and treatment resistance, many molecular drugs have been developed. These agents may target one or more key signaling pathways that are important for cancer development and progression, such as cell proliferation, apoptosis, and angiogenesis^[57]. Of most relevance to clinical practice is the multi-kinase angiogenesis inhibitor sorafenib, a FDA approved agent for the treatment of advanced HCC that has shown promising results^[58,59]. However, large clinical trials have revealed that less than 50% of patients respond to sorafenib treatment, and in said responders this agent only increases mean patient survival by 4.2-6.5 mo and the long-term response is lacking^[59]. More importantly, rapid resistance will develop after the termination of drug administration^[60,61]. Expansion of liver cancer stem cells in the hypoxic environment may be partially responsible for sorafenib resistance in clinical practice, while tumor aggressiveness and patient survival were correlated with the proportion of cancer stem cells^[62].

Other molecular agents such as bevacizumab (monoclonal antibody against vascular endothelial growth factor), erlotinib, and cetuximab (epidermal growth factor receptor blocking agents) have all been tested in various stages of clinical trials, but their therapeutic effects remain to be further determined^[63-65].

HORMONAL THERAPIES

Sex steroid hormones can interact with growth receptors and promote the growth of cancer cells. As such, hormonal therapy has been explored as a potential treatment option for many types of solid tumors such as breast, endometrial, and prostate cancers. In HCC, sex hormone

receptors such as estrogen receptor, progesterone receptor, and androgen receptor are all expressed^[66]. The liver is sensitive to sex hormone stimulation, which may play an important role in the development of liver cancer^[67]. Consequently, hormone receptor blockers have been attempted in the treatment of advanced HCC. Unfortunately, prospective randomized controlled trials have failed to demonstrate an improved overall survival in patients with advanced HCC who were treated with hormone receptor blockers^[68]. Meta-analysis of the published data on the use of hormonal therapy also failed to demonstrate a survival advantage for patients with advanced HCC^[69]. Thus, there is a lack of sufficient evidence to prove the therapeutic advantage of hormonal therapy for liver cancer.

COMBINED MODALITY THERAPIES

Since single agent treatments only have limited therapeutic benefits, it is reasonable to assume that a combination of more than one treatment option may produce better therapeutic outcomes. However, no standard combinatorial protocols are available. It is generally believed that combinatorial treatment for liver cancer should be individualized^[10].

CONCLUSION

Although a definite non-surgical therapy for HCC is not available, many treatment modalities have been developed. Which therapeutic approach is most appropriate to a given patient is dependent on several factors, in particular tumor staging, patient age, co-morbidities, and availability of treatment modalities. The reasonable selection of available treatment options is key to improving therapeutic outcome and patient survival.

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Gut microbiota in alcoholic liver disease: Pathogenetic role and therapeutic perspectives

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Abstract

Alcoholic liver disease (ALD) is the commonest cause of cirrhosis in many Western countries and it has a high rate of morbidity and mortality. The pathogenesis is characterized by complex interactions between metabolic intermediates of alcohol. Bacterial intestinal flora is itself responsible for production of endogenous ethanol through the fermentation of carbohydrates. The intestinal metabolism of alcohol produces a high concentration of toxic acetaldehyde that modifies gut permeability and microbiota equilibrium. Furthermore it causes direct hepatocyte damage. In patients who consume alcohol over a long period, there is a modification of gut microbiota and, in particular, an increment of Gram negative bacteria. This causes endotoxemia and hyperactivation of the immune system. Endotoxin is a constituent of Gram negative bacteria cell walls.

Two types of receptors, cluster of differentiation 14 and Toll-like receptors-4, present on Kupffer cells, recognize endotoxins. Several studies have demonstrated the importance of gut-liver axis and new treatments have been studied in recent years to reduce progression of ALD modifying gut microbiota. It has focused attention on antibiotics, prebiotics, probiotics and synbiotics.

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Key words: Alcoholic liver disease; Bacterial translocation; Dysbiosis; Prebiotics; Probiotics; Synbiotic; Gut microbiota; Endotoxin

Core tip: A close anatomical and functional relationship between gut and liver exists. Blood circulated in the portal vein transfers various toxic compounds for filtration by liver. Endotoxin is a lipopolysaccharide derived from the cell wall of Gram negative bacteria presents in the intestine, which is absorbed from intestinal epithelium and transported to the liver and Kupffer cells through the portal vein. A qualitative (dysbiosis) and quantitative (bacterial overgrowth) alteration of intestinal microbiome are the causes of an increase of endotoxins and subsequently, liver damage. The new treatments try to contrast dysbiosis and bacterial overgrowth decreasing evolution of alcohol liver disease.

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INTRODUCTION

Alcoholic liver disease (ALD) is the cause of a high rate of morbidity and mortality worldwide. It is the common-

est cause of cirrhosis in many Western countries^[1]. It accounted for 3.8% of all deaths in 2004^[2]. ALD consists of several types of disease such as fatty liver (steatosis), steatohepatitis, fibrosis, cirrhosis and ultimately hepatocarcinoma (HCC). Steatosis is reversible with alcohol abstinence, but it is considered a risk factor for progression to fibrosis and cirrhosis^[3,4].

The metabolism of alcohol is also regulated by intestinal bacteria ("bacteriocolonial" metabolism of ethanol). In 1984 Bode *et al*^[5] demonstrated a qualitative and quantitative significant difference between flora in people with alcoholism and gut microflora of a control group. Intestinal homeostasis is influenced by several factors such as gut motility, gastric acidity, immunological defence factors, bile salts, and colonic pH^[6].

The liver strategic position confers it with the important role of translating physiological and pathological processes within the gastrointestinal tract into metabolic and immunologic outcomes^[7].

PATHOGENESIS OF ALCOHOLIC LIVER DISEASE

Alcoholic steatohepatitis (ASH) and severe ALD occur in approximately 30% of heavy drinkers^[8]. The pathogenesis of ALD is a dynamic and unknown process characterized by several interactions that involve the immune system and metabolic intermediates of alcohol. The poor understanding of these interactions contrasted with the progress in developing specific treatments for ALD^[9-11]. Ethanol metabolism-associated oxidative stress, abnormal methionine metabolism, ethanol-mediated induction of leakage of gut endotoxins, and activation of Kupffer cells are all involved in the pathogenesis of ALD^[12-14].

The fermentation of carbohydrates made by bacterial intestinal flora is itself responsible for production of endogenous ethanol. This is strongly enhanced in the presence of gut dysmotility (*e.g.*, from obesity, diabetes, or chronic alcohol use) or an excess of carbohydrates in the diet^[15]. The intestinal oxidation of alcohol results in increasing concentrations of acetaldehyde^[16,17], the first and most toxic product of ethanol metabolism responsible for alteration of intestinal permeability (gut leakiness) and microbiota homeostasis.

Apart from the liver, several organs contribute to ethanol metabolism resulting in acetaldehyde production, such as the pancreas, gastrointestinal tract, heart and brain^[18-20]. Acetaldehyde is produced by bacterial alcohol dehydrogenase^[21] and metabolised by aldehyde dehydrogenase in the colon^[22]. In a recent study Kwon *et al*^[23] evaluated the role of aldehyde dehydrogenase 2 deficiency in mouse in the progression of alcohol liver disease. They showed the role of acetaldehyde in hepatic inflammation and fibrosis.

Acetaldehyde is itself responsible for mitochondrial dysfunction and altered acetaldehyde metabolism that leads to its accumulation. It determines direct hepatocyte damage forming adducts with proteins and DNA

by the interactions with amino, hydroxyl, and sulfhydryl groups^[24]. Acetaldehyde is also responsible for increased paracellular intestinal permeability because of a redistribution of tight junction proteins (occluding and ZO-1) and adherent junction (E-cadherin and β -catenin) proteins inhibiting their phosphorylation by protein tyrosine phosphatase^[25-27] (Figure 1).

The leakiness of gut activates the transcription of nuclear factor kappaB (NF- κ B) gene and over-expression of nitric oxide (NO) synthesis.

NO is synthesized from *L*-arginine by nitric oxide synthases (NOS). Three isoforms of nitric oxide synthases exist: neuronal NOS (nNOS), endothelial NOS (eNOS), defined as constitutive NOS (cNOS), and inducible NOS (iNOS)^[28]. NO production by cNOS is responsible for epithelial cell barrier integrity^[29,30]. Otherwise NO produced by iNOS occurs in inflammation and it may contribute to aggravate integrity of the intestinal barrier^[31].

iNOS is expressed in endothelial cells, hepatocytes, macrophages, neutrophils, and many other cell types^[32]. An increased expression of iNOS and consequent production of NO is responsible for an augmented nitration and oxidation of tubulin. This leads to a decreased stability of tubulin and damage of the microtubule cytoskeleton with disruption of barrier function. Besides, the increased synthesis of NO results in oxidative stress in hepatocytes^[33,34].

Epidermal growth factor (EGF) contrasts this process, promoting growth and differentiation of gastrointestinal mucosa. EGF stabilizes the cytoskeleton through down regulation of activity of iNOS^[35,36].

INTESTINAL MICROFLORA

The intestinal microflora changes after fetal development and the major changes occur after weaning^[37]. The microbiota is composed by more than 500 species of bacteria; some of them are fixed in the intestine, while the others only pass through the intestine^[38]. According to the study by Neish, 109 CFU/mL and 1012 CFU/mL of bacteria may be found, respectively, in the terminal ileum and colon. Gram negative bacteria and anaerobes are dominant species in the intestinal lumen which are estimated to be 100 to 1000 times more than aerobic ones. *Bacteroides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus*, *Clostridium* and *Escherichia coli* (*E. coli*) are the most frequent ones^[39]. The intestine also provides residence to more than 15 species-level bacteria phylotypes and in a healthy state they have a symbiotic relationship with its host. However, in each person, the pattern of the microorganism population is unique and different^[40] (Table 1).

There is a close anatomical and functional relationship between the gut and the liver known as the gut-liver axis and in patients with liver cirrhosis, the intestinal balance is compromised.

Blood circulating in the portal vein transfers various toxic compounds such as bacteria and their derivatives

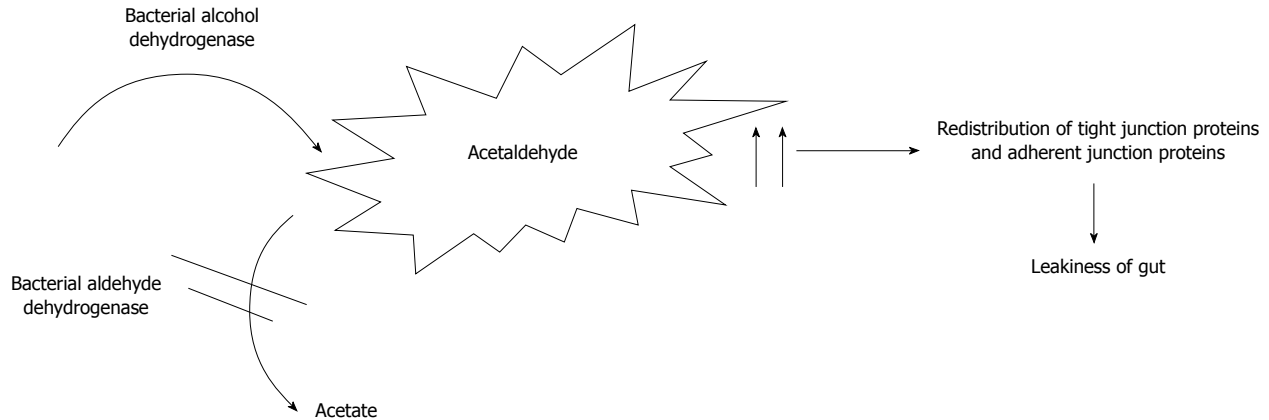


Figure 1 Metabolism of alcohol.

Table 1 Intestinal microbiota in the gastrointestinal compartments

Gastrointestinal tract	Microbiota
Oesophagus	<i>Streptococcus</i> , <i>Prevotella</i> , <i>Veilonella</i>
Stomach	<i>Streptococcus</i> , <i>Staphylococcus</i> , <i>Lactobacillus</i> , <i>Helicobacter pylori</i>
Duodenum	<i>Streptococcus</i> , <i>Staphylococcus</i> , <i>Lactobacillus</i> , <i>Helicobacter pylori</i> , <i>Veilonella</i> , <i>Yeasts</i>
Jejunum	<i>Streptococcus</i> , <i>Staphylococcus</i> , <i>Lactobacillus</i> , <i>Helicobacter pylori</i> , <i>Veilonella</i> , <i>Yeasts</i>
Ileum	<i>Bifidobacterium</i> , <i>Bacteroides</i> , <i>Veilonella</i> , <i>Clostridium</i> , <i>Enterobacteriaceae</i>
Colon	<i>Bacteroides</i> , <i>Bifidobacterium</i> , <i>Clostridium</i> , <i>Streptococcus</i> , <i>Ruminococcus</i> , <i>Peptostreptococcus</i> , <i>Eubacterium</i> , <i>Faecalibacterium</i>

(ethanol, ammonia, and acetaldehyde) for filtration by liver and modulates Kupffer cells activity and cytokine production. The increase of pathogen-associated molecular patterns and accumulation of metabolites in the liver can cause the liver harm. In return, the liver secretes bile acids to the intestine and modulates its activities^[41].

Alterations in the type and amount of microorganisms are important elements in the dysfunctions of the liver; in fact liver disease causes quantitative (bacterial overgrowth) and qualitative (dysbiosis) changes in the intestinal microflora^[42].

Dysbiosis is the alteration of intestinal homeostasis. Several studies have shown the role of continuous ethanol assumption in the breakdown of this balance. Bull-Ottersen *et al*^[43] studied the temporal effects of chronic ethanol consumption on commensally intestinal bacteria in a mouse model. They demonstrated that alcohol consumption over a long period elevates the growth of Gram negative bacteria and causes a decrease of both *Bacteroidetes* and *Firmicutes*, and an increase of *Actinobacteria* and *Proteobacteria*. *Proteobacteria* are Gram negative bacteria and include several pathogenic species such as *Salmonella*, *Helicobacter*, *Vibrio* and *Escherichia*, one of the main bacteria in the gut. Similar results were obtained by Mutlu *et al*^[44]. They noted higher levels of *Proteobacteria* and lower abundance of *Bacteroidetes* in subjects with chronic alcohol

consumption.

The breakdown of microbiota balance is responsible for different negative consequences (endotoxemia, translocation of lipopolysaccharides) that leads to hyperactivation of the immune system.

LPS is a constituent of the wall of Gram-negative bacteria^[45] which induces macrophages to release proinflammatory cytokines, such as IL-1 β and tumour necrosis factor (TNF)^[46].

Endotoxin is a LPS, a component of the outer membrane Gram negative bacteria present in the gut. Generally only a little part of endotoxin is absorbed from the intestinal epithelial lining reaching the liver and the Kupffer cells inside the portal vein. In chronic alcohol consumption, bowel flora releases a bigger amount of endotoxins, responsible for the altered intestinal barrier and activation of the inflammatory process that leads to the progression of ALD^[47], cirrhosis and HCC^[48].

Hyper-permeability of the intestine following alcohol consumption leads to endotoxemia, which is filtrated by the liver and triggers the proinflammatory pathways for causing ASH.

Endotoxemia is responsible for elevated plasma levels of LPS-binding protein (LBP). The augmentation of endotoxins can prime and activate both hepatic and extra hepatic macrophages to overproduce inflammatory cytokines such as TNF- α , IL-6, IL-1 and IL-8^[12].

Systemic endotoxemia and the cytokines produced in the inflammatory process increase intestinal permeability altering tight junctions. This leads to endotoxins passing into the circulation, creating a vicious cycle^[49,50].

ALD also results in quantitative alterations of the intestinal microbioma. The small intestinal bacterial overgrowth (SIBO) is another cause of bacterial translocation.

To make a diagnosis of SIBO, it is necessary to find $\geq 1 \times 10^3$ bacteria (*i.e.*, CFU) per mL of proximal jejunal aspiration^[51]. Bacterial overgrowth is advantaged by intestinal stasis that permits the proliferation of coliform bacteria^[52]. Therefore, the bacteria generally recognized as SIBO are gram negative aerobes and anaerobes such as *E. coli*, *Enterococcus* spp. and *Proteus mirabilis*^[53,54]. The main

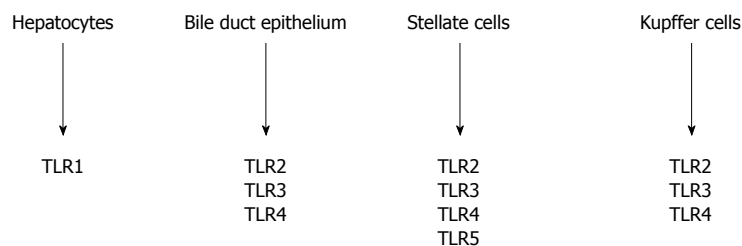


Figure 2 Liver cell types and their receptors. TLR: Toll-like receptor.

causes of SIBO are gastric achlorhydria, gastrocolic or coloenteric fistula and small intestine motility disorder^[52]. Ethanol decreases intestinal motility which favours proliferation of luminal bacteria^[16].

ROLE OF THE IMMUNE SYSTEM

Chronic ethanol consumption has been associated with immune suppression and increased morbidity and mortality^[55]. Alcohol ingestion alters both the innate and adaptive immune system.

Ethanol increases the susceptibility of the gastrointestinal tract to bacteria through the suppression of natural killer cell activity and antibody-dependent cell-mediated cytotoxicity by lymphocytes^[56,57].

The host immune system has an important role in the defence of the intestine. Several molecules are responsible for the limited expansion of pathogenic microorganisms, such as reactive oxygen species, IgA, β -defensins and cryptidins^[39,58]. The innate immune system is also composed by Toll-like receptors (TLRs) that recognize specific pathogen-associated molecular patterns (PAMPs) such as LPS, lipoteichoic acid, peptidoglycan, unmethylated DNA and double-stranded RNA^[59].

In humans 10 TLRs have been recognized. Multiple cells in the liver express significant levels of multiple TLRs and have long been recognized to be critical determinants in the pathogenesis of cirrhosis^[60,61]. Every type of liver cell expresses specific TLR: TLR1 was found in hepatocytes, TLR2, 3, and 4 in stellate cells, bile duct epithelium and particularly in Kupffer cells. Bile duct epithelium expresses TLR5 too (Figure 2).

Endotoxins produced in the body cause an inflammatory reaction, activating Kupffer cells through their link with two types of receptors, cluster of differentiation 14 (CD-14) and TLR-4. These receptors are both essential to determine liver injury, but they present different structures. CD-14 is a surface receptor without a cytoplasmic domain, while TLR4 is a transmembrane protein with a cytoplasmic domain that can be associated with a soluble protein, MD-2, through a not covalently link.

CD14 binds LPS and this complex is recognized by TLR4. CD14 also has a soluble form that facilitates the transfer of LPS to the TLR4/MD-2 receptor complex^[62]. The association between LPS and CD14 is facilitated by a soluble shuttle protein, LPS-binding protein (LBP)^[63].

LPS recognition by TLR4 on macrophages and other cell types in the liver determines activation of down-

stream signaling pathways responsible for activation of transcription factors such as NF- κ B and activator protein-1 (AP-1). This process causes an increased inflammatory cytokine production such as interferon gamma (IFN γ), TNF- α , interleukin-6 (IL-6), IL-1, chemokines and reactive oxygen species^[64,65].

Furthermore LPS/TLR4 promotes fibrogenesis by sensitizing hepatic stellate cells (HSCs). The sensitised HSCs induce NF- κ B activation, up-regulate gene expression of some chemokines (IL-8 and monocyte chemoattractant protein-1) and promote transforming growth factor beta (TGF β) release by Kupffer cells^[66]. The activated TLRs can enroll adapter molecules like myeloid differentiation factor-88 (MyD88)^[67].

The CD14/TLR4 receptor complexes activate MyD88 dependent and MyD88 independent pathways that modulate survival and replication of apoptosis cells^[68]. Furthermore, the MyD88-signaling pathway leads to production of oxidative stress and pro-inflammatory cytokines that causes hepatocellular damage^[69-71].

The effects of alcohol are exerted on organs different from liver too. Blanco *et al.*^[69,70] demonstrated the role of ethanol in neuroinflammation. They showed that ethanol can directly induce downstream iNOS expression and activation of NF- κ B through the translocation of TLR4 into lipid rafts. The activation of NF-signalling is also determined by acetaldehyde^[71].

TREATMENT

Abstinence from alcohol is the foundation for treatment of alcoholic liver diseases. In every stage of liver damage, the cessation or marked reduction in alcohol consumption has been demonstrated to improve the histology and/or survival of patients^[72].

In patients with elevated alcohol ingestion, high levels of plasma endotoxin may be determined by: (1) excessive production of endotoxin in the intestine through overgrowth of intestinal bacteria; (2) gut permeability; and (3) delayed clearance of endotoxin by Kupffer cells. Actually, the main treatments, such as antibiotics, prebiotics, probiotics and synbiotics, try to prevent endotoxemia by inhibiting the intestinal Gram negative overgrowth and preserving intestinal permeability.

Antibiotic

Acute and chronic ingestion of alcohol causes an increased endotoxin plasma level in humans and mice

Table 2 Properties of ideal probiotic strains

Properties of ideal probiotic strains
Resistance to bile
Resistance to hydrochloric acid
Resistance to pancreatic juice
Ability to tolerate stomach and duodenum conditions and gastric transport
Stimulation of the immune system
Improvement of intestinal function <i>via</i> adhering and colonizing the intestinal epithelium
Competition with pathogens
Modulation of permeability
Anticarcinogenic and antipathogenic activity

models^[73,74]. Alcohol consumption causes changes in gut microbiota and it is associated with upper gastrointestinal bacterial overgrowth^[75,76].

The antibiotic treatment controls large bowel bacterial overgrowth improving the prognosis of ALD^[77]. However, despite improvement of liver function, prolonged use of antibiotics alters gut flora and this may favour pathogenic bacterial colonization.

Antibiotic treatment should be based on bacterial sensitivity testing to particular antibiotics, but this will require an excessive use of culture therefore it should be targeted at those intestinal bacteria generally responsible for SIBO^[78,79].

Several antibiotics are considered suitable against overgrowth of Gram negative aerobes and anaerobes such as rifaximin, amoxicillin/clavulanate, metronidazole, ciprofloxacin, norfloxacin, and cephalexin. The fundamental role of rifaximin in the treatment of hepatic encephalopathy has been recently demonstrated, but it seems to have a role in treatment of ALD too^[80].

The main advantage of rifaximin is that it is not absorbed and therefore presents few side effects. Furthermore there is little evidence for resistance^[57,81-83].

Prebiotics

Antibiotics produce quantitative alterations of intestinal microflora whereas prebiotics act against dysbiosis. The prebiotics promote selectively the growth of protective gut bacteria (*Bifidobacteria* and *Lactobacilli*) increasing the body's natural resistance to invading pathogens^[84].

Prebiotics are identified as “non digestible food ingredients that, when consumed in sufficient amounts, selective stimulate the growth and/or activity of one or a limited number of microbes in the colon, resulting in documented health benefits”^[85].

They are complex carbohydrates that reach the small bowel because they cannot be metabolized by pancreatic and intestinal enzymes in gastrointestinal tract^[86]. All prebiotics are resistant to gastric acidity but are susceptible to the metabolism by gut microbiota. While probiotics show strain specific beneficial effects, prebiotics of the same family present similar properties, though their degree of polymerisation distribution linkage type may differ^[87].

The most commonly commercialized prebiotics are

lactulose, fructo-oligosaccharides (FOS) and galacto-oligo-saccharides (GOS). GOS are nondigestible oligosaccharides derived from lactose, chains of galactose monomers that are naturally found in human milk. GOS, like other prebiotics, simulate pathogen binding sites present on the surface of gastrointestinal epithelial cells inhibiting enteric pathogen adhesion and successive infection^[86,88-91].

FOS is naturally present in vegetables such as onions, asparagus, wheat, artichokes *etc.* They modulate gut microbiota, prevent pathogens adhesion and colonization, induce anti-inflammatory effects and regulate lipid and glucose metabolism. These prebiotics can exercise these effects thanks to their structural resistance to mammalian digestive enzymes.

Probiotics

Actually probiotics are defined as “monocultures or mixed culture of live microorganisms that, if administered to a person, positively influence the host by improving the properties of his/her own microflora”. Probiotics modulate intestinal microbiota, favouring an anti-inflammatory milieu that contrast bacterial translocation, endotoxin production and improve intestinal barrier integrity.

The mechanisms by which probiotics exert their effects are largely unknown. Different actions have been reported in literature. They control inflammation reducing gut pH and compete with pathogens for binding and receptor sites^[92-94]. To do this, they have to show specific characteristics, in particular they should be resistant to bile, hydrochloric and pancreatic juice in order to reach the small bowel (Table 2). Tolerating stomach and duodenum conditions, probiotics can stimulate the immune system and improve intestinal function *via* adherence and colonization of the intestinal epithelium.

The most common probiotics are lactose-fermenting *Lactobacilli* and *Bifidobacteria*. *Lactobacillus* strains, LAP5 and LF33 exert their effects by inhibiting the growth of *E. coli* and *Salmonella typhimurium in vitro*^[95]. Furthermore, other studies have demonstrated that *Lactobacillus acidophilus* strain NP51 reduces the number of *E. coli* O157:H7 in the fecal samples of beef cattle^[96,97]. *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal cells from the inflammation caused by *E. coli*^[98]. Finally it has been demonstrated that *Lactobacillus* GG, administered to rats, reduced plasma levels of endotoxin and severity of liver injury^[99].

They have been reported to stabilize mucosal barrier function and modulate the gut microflora, limiting the growth of pathogenic bacteria, by acidifying the gut lumen, competing for nutrients, and producing antimicrobial substances^[100-103].

Developing nutritional practices, mucosal barrier repairing, apoptosis prevention due to providing of short chain acids, and improving intestinal epithelial viability are other probiotic effects which stabilize physiological luminal permeability together with lowering ammonia adsorption^[104]. These functions alleviate tight junction

disturbance by pathogens^[105], and are essential agents for lowering bacterial translocation. BT is also affected by probiotics because of their induction of anaerobes and gram positive bacteria growth, limiting gram negative bacteria, and preventing pathogen adherence^[101].

Controlling flora bacteria quantity can lead to decreased endotoxins and other toxic compounds derived from bacteria such as ethanol, phenol, indoles which cause injury to the liver. Decreased levels of these substances in the liver result in lowering of proinflammatory production such as TNF- α , IL-6, and IFN γ via down-regulation of NF- κ B^[106]. On the other hand, they can depress urease activity of microflora bacteria followed by ammonia production and release into the portal system. Furthermore, probiotics decrease fecal pH value and reduce ammonia adsorption^[107]. Therefore, probiotics determine an improvement in hepatic encephalopathy through a reduction of bacterial ammonia reaching the portal vein.

In 2010 Foster *et al.*^[108] demonstrated that probiotics effects on mental status were maintained during the wash out period.

Synbiotic

The synbiotic is a compound of probiotics and prebiotics that exercises its beneficial effects stimulating the growth of protective intestinal bacteria^[102,103,109].

Prebiotics stimulate the growth of beneficial bacteria (*i.e.*, *Bifidobacteria* and *Lactobacilli*) in the gut and their effectiveness increases when they are used in association with probiotics^[110,111]. On the other hand, the mixture of prebiotics and probiotics might enhance the survival and activity of probiotics.

CONCLUSION

Several studies have demonstrated the central role of microbiota in the pathogenesis and development of liver disease^[65,112]. For this reason therapeutic strategies to control ALD are focussed on the gut microbiome. Obviously the beneficial effects of probiotics depend upon a number of factors such as the duration, frequency and quantity of probiotics consumption and the health of the patients at the beginning of the treatment.

Probiotics have been shown to have several beneficial effects on intestinal function. They prolong remission in ulcerative colitis, maintaining and improving intestinal barrier integrity and stimulate mucosal immunity^[113-116].

The treatment could prevent alcohol-induced gut leakiness and development of AHS and the possible mechanisms are the reduction of alcohol-induced intestinal and systemic oxidative stress. Actually the beneficial effects of probiotics in ALD are supported by numerous laboratory results and several studies have shown their potential, however, despite their demonstrated effects on intestinal barrier integrity, there is no high-quality clinical evidence. This is an important limit that does not always permit recommendation of the use of probiotics in clinical practice^[117,118].

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Could the improvement of obesity-related co-morbidities depend on modified gut hormones secretion?

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Abstract

Obesity and its associated diseases are a worldwide epidemic disease. Usual weight loss cures - as diets, physical activity, behavior therapy and pharmacotherapy - have been continuously implemented but still have relatively poor long-term success and mainly scarce adherence. Bariatric surgery is to date the most effective long term treatment for morbid obesity and it has been proven to reduce obesity-related co-morbidities, among them nonalcoholic fatty liver disease, and mortality. This article summarizes such variations in gut hormones following the current metabolic surgery procedures. The profile of gut hormonal changes after bariatric surgery represents a strategy for the individuation of the

most performing surgical procedures to achieve clinical results. About this topic, experts suggest that the individuation of the crosslink among the gut hormones, microbiome, the obesity and the bariatric surgery could lead to new and more specific therapeutic interventions for severe obesity and its co-morbidities, also non surgical.

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Key words: Obesity; Bariatric surgery; Gut hormones; Nonalcoholic fatty liver disease; Microbiome

Core tip: It is important to emphasize the role of the major peptides released by the enteroendocrine system, which promote satiety and modulate energy homeostasis and utilization, as well as those that control fat absorption and intestinal permeability. Bariatric surgery could be the most effective treatment for obesity and co-morbidities, often within days after surgery, independently of weight loss and it is currently the only therapy available for obesity which results in long-term, sustained weight loss. We hypothesize that gut hormones might play a role in induction and long-term maintenance of weight loss, could determine the improvement of obesity-related co-morbidities and could help to identify new drug targets and improved surgical techniques.

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INTRODUCTION

Obesity epidemic in the United States (US), as well as

all over the world, is steadily rising^[1,2]. According to National Health and Nutrition Examination Survey in the 2007-2008, a percentage as high as of US population was found to be obese [body mass index (BMI) ≥ 30 kg/m²], of which about 5.7% was found to be of severe obesity (BMI ≥ 40 kg/m²)^[1,2]. Obesity rates have also been advancing worldwide, and more than 700 million people will be obese by 2015, as predicted by the World Health Organization.

OBESITY RISK

Obesity is associated to comorbidities as hypertension, hyperlipidemia, heart failure, type 2 diabetes mellitus (T2DM), obstructive sleep apnea, thromboembolic disease, osteoarthritis, gastroesophageal reflux disease, asthma, polycystic ovary disease, nonalcoholic fatty liver disease (NAFLD), as well as several cancers^[1,3]. Metabolic syndrome, which involves the combination of risk factors for cardiovascular disease such as insulin resistance, visceral obesity, dyslipidemia, glucose intolerance, and hypertension, has often been associated with more severe liver^[4]. Insulin resistance and chronic low-grade inflammation appear to be key points of this anomalous condition.

In addition, a number of other features contribute to the obesity development, including social factors, such as the accessibility of calorie-dense foods (elevated energy intake) and the sedentary lifestyle (reduced energy expenditure), as well as genetic and epigenetic predispositions^[1,5].

MECHANISM OF ENERGY

HOMEOSTASIS: DEFENDING A WEIGHT SET POINT

Body weight remains usually constant during the life. This condition manifests an overall balance between energy intake and expenditure. Kennedy, 50 years ago, proposed a negative feedback system of peripheral fat on food intake^[6]. The adipose tissue role in the complex systems of energy homeostasis has been increasingly understood over the last decade.

Central control of feeding

Humans take food in discrete episodes during the day. The energy intake is determined on the basis of frequency and caloric amount of each meal. Hunger and food-seeking behaviour before a meal are stimulated by various short-term polypeptide hormonal signals from the periphery (orexigen hormones, *e.g.*, ghrelin *etc.*), whereas other peripheral stimuli promote satiety and/or meal cessation [anorectics; *e.g.*, glucagon-like peptide-1 (GLP-1), peptide tyrosine tyrosine 3-36 (PYY3-36), cholecystokinin (CCK), oxyntomodulin (OXM), and several others] (Figure 1)^[7]. The efficacy of short-term signals to defend against deviations from a set weight point can be modu-

lated by long-term signals related to total body energy stores (*e.g.*, insulin, leptin, ghrelin, *etc.*)^[1,8].

Numerous important areas of the brain have been associated with the regulation of energy balance. Insulin, leptin, and PYY inhibit, while ghrelin stimulate the arcuate nucleus (ARC) of the hypothalamus that contains populations of neuropeptide Y/Agouti-related protein (NPY/AgRP)-expressing neurons^[9]. Orexigenic neurons, identified in the lateral hypothalamic area and perifornical area, are stimulated by NPY/AgRP. Increased feeding and weight gain are derived by stimulation of NPY/AgRP neurons^[10]. The ARC includes neurons that can express the proopiomelanocortin and cocaine- and amphetamine-regulated transcript. Leptin stimulate these cells, while NPY/AgRP neurons inhibit^[11]. In rodent and human researches, the reduction of food intake and weight loss derived by treatment with leptin. The ARC of the hypothalamus and the lateral hypothalamic area acquire and integrate information about overall energy stores from the periphery and relay this information to higher centers, such as the ventral tegmental area of the mesolimbic system, which is involved in the hedonic experience of feeding, as reported by Harvey *et al.*^[1].

Peripheral control of feeding

Hormonal, nutritional, and neuronal afferent feedback from the periphery to the central nervous system (CNS) depend on ability to increase or decrease calorie intake in response to changes in energy needs. Adipokines, including leptin and adiponectin, which are hormonal signals from adipose tissue provide feedback signals to the brain (adipose-brain axis), whereas feedback signals from the intestinal tract (the gut-brain axis) include polypeptide hormones (enterokinase) and neuronal signals *via* the vagus and other autonomic afferents, as reported by Harvey *et al.*^[1] (Figure 2). Basal levels of insulin and leptin proportionally develop following the increase of the fat mass, providing a long-term signal of body energy stores to the brain. On the contrary, basal ghrelin levels are inversely related to the body energy storage. Short-term fluctuations in numerous gut hormones including ghrelin, insulin GLP-1, PYY3-36, and CCK, as well as vagal signals regulate mealtime, as reported by Harvey *et al.*^[1].

HORMONAL CHANGES AFTER BARIATRIC SURGERY

Bariatric surgery consists of various interventions that have been subdivided as purely restrictive, purely malabsorptive, or combined restrictive and malabsorptive.

The number of bariatric interventions (*i.e.*, metabolic surgery) for the treatment of obesity is in exponential increase. This is partly due to the most effective and durable weight loss; in addition, a good deal of improvement of co-morbidities after surgery compared with medical treatments such as diet and physical activity^[12], or actually applicable pharmacologic^[13] or endoscopic^[14] treatments was observed. Actually, bariatric surgery is greatly ap-

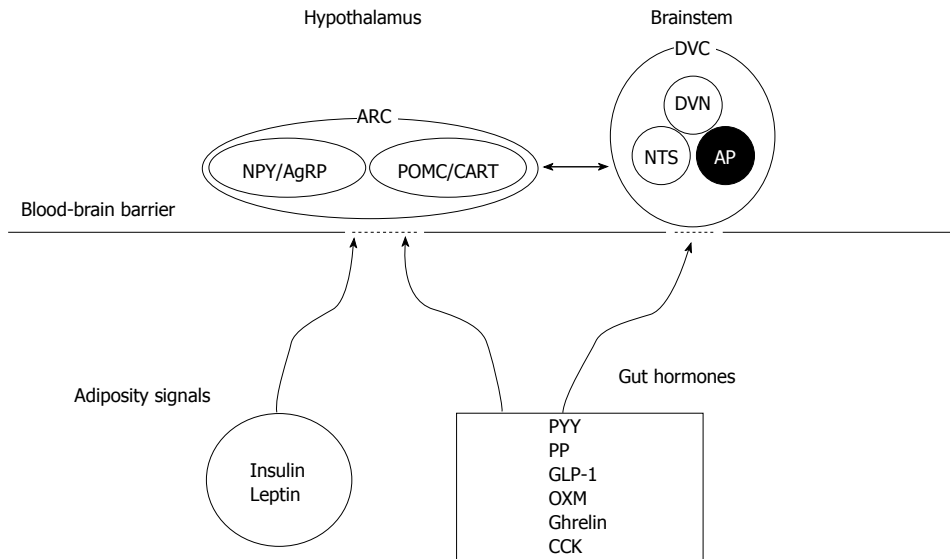


Figure 1 Humoral signals implicated in the physiological regulation of food intake. Diagram summarising the major signalling pathways which converge on the hypothalamus and brainstem in order to regulate food intake. ARC: Arcuate nucleus; NPY/AgRP: Neuropeptide Y and agouti-related peptide; POMC/CART: Proopiomelanocortin and cocaine- and amphetamine-regulated transcript; DVC: Dorsal vagal complex; DVN: Dorsal motor nucleus of vagus; NTS: Nucleus of the tractus solitarius; AP: Area postrema; GLP-1: Glucagon-like peptide-1; CCK: Cholecystokinin; PP: Pancreatic polypeptide; PYY: Peptide YY; OXM: Oxyntomodulin.

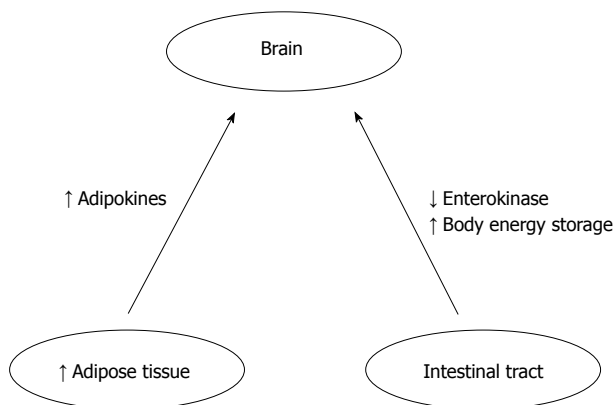


Figure 2 Peripheral feedback signals.

proved by patients and physicians because the techniques are minimally invasive and well finished and standardized^[15].

Each bariatric technique results in multiple effects, according to the intervention on the gastrointestinal anatomy (Figure 3). For example, sleeve gastrectomy (SG) has been formerly considered exclusively restrictive with the capacity of the stomach greatly reduced and the absorptive surface of the small unchanged. Nevertheless, the largest production of ghrelin, an appetite-stimulating hormone, occurs in the fundus and body of the stomach, and the ghrelin reduced production following SG probably plays an important role in decreasing appetite.

Bypass-type interventions, like Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion, with or without duodenal switch (BPD/DS), are usually regarded as a combination of restrictive and malabsorptive outcomes: bypassing or removing a large part of the stomach, these interventions divert chyme away from the duodenum

and proximal small bowel rapidly presenting nutrients to the distal gut, as reported by Harvey *et al*^[1]. The adjustable gastric band (AGB), which places a constrictive ring around the gastric inlet and is usually considered to be an exclusively restrictive intervention, results in prolonged satiety; the mechanism is yet partially understood^[1,16].

Reduction caloric intake and gut hormone expression modifications are determinants for weight loss and improvement of the metabolic derangements associated with obesity. Therefore, there are two considerations that supported this hypothesis. Firstly, following bariatric surgery, while patients are in a state of negative energy balance and are quickly losing weight, they do not complain of feeling hungry, but rather have decreased appetite and precociously satiety^[1,17]. Secondly, following different types of metabolic surgery, patients with T2DM show a rapid improvement in glycemic control, occurring after surgery; before significant weight loss can to appear.

Here we will consider actual knowledge of the role of specific hormones believed to be essential in energy homeostasis and the expression of which is modified after bariatric surgery (Table 1).

Ghrelin

Ghrelin is a 28-amino acid peptide produced in different sites of the endothelial system. It was identified in 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a) in the pituitary gland^[1,18]. Subsequently, in rodents it was observed that ghrelin determine an increase in feeding and weight gain and develop an orexigenic role in energy balance^[10].

The ghrelin is principally produced from cells located in the oxyntic glands of the fundus and body of the stomach (also known as P/D1 cells). Ghrelin levels support an ultradian rhythm-rising encode a corresponding

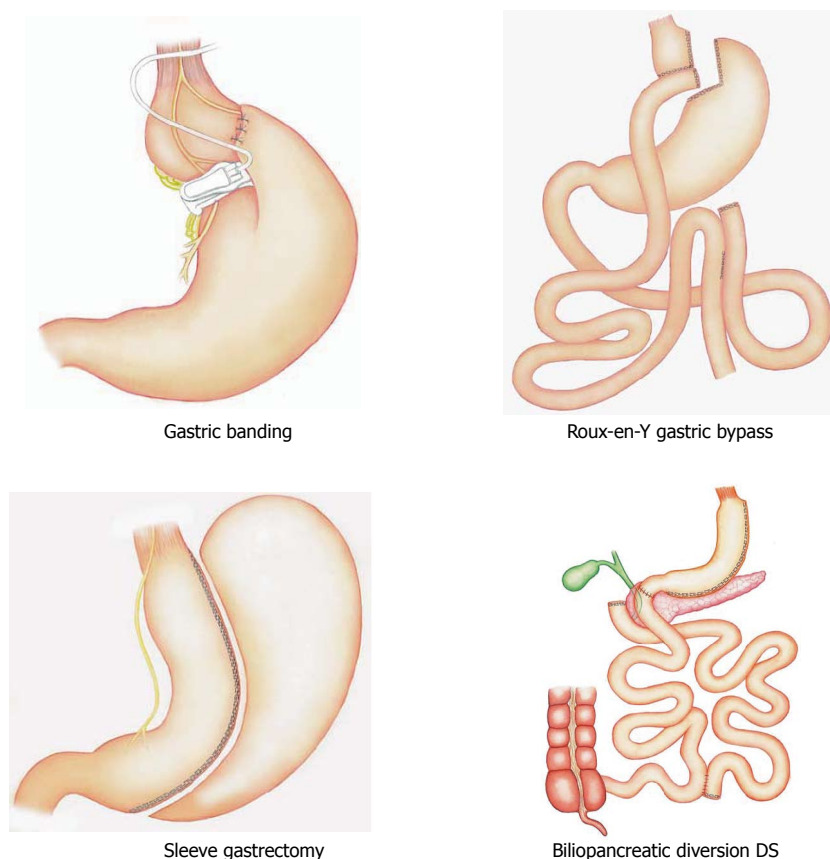


Figure 3 Gastric banding: an adjustable gastric band is used to divide the stomach into a small proximal compartment taken down a few centimeters distal to the compartment (pouch) and a larger distal compartment (residual stomach). Roux-en-Y gastric bypass: The stomach is gastric inlet. The jejunum is divided 50 cm beyond the ligament of Treitz, and its aboral end is connected to the small gastric pouch. Some 150 cm distal to this point, the other end of the small bowel is sewn to a loop that has been pulled up to meet it (so-called Roux-en-Y reconstruction). Surgical effects: restriction, with an additional malabsorptive component. Sleeve gastrectomy: More than 80% of the stomach is resected, and the gastric remnant is tubularized, with an initial filling volume of less than 100 mL. Surgical effects: Restrictive and hormonal mechanisms. Biliopancreatic diversion duodenal switch (DS): The stomach is reduced in size as in sleeve gastrectomy. Duodenum is divided distal to the pylorus, and the jejunum is divided 250 cm proximal to the ileocecal valve and anastomosed to the duodenum. The other end is connected to the ileum 100 cm proximal to the ileocecal valve. Mechanism of effect: A combination of restrictive gastric surgery with a considerable degree of malabsorption.

pattern of NPY discharge for daily meal patterning^[1,19]. It has been shown that ghrelin cells express the clock genes *PER1* and *PER2*, and the ghrelin release is entrained to peak just before regular feeding times^[1,20]. A rising serum ghrelin level induces an increase in food seeking behavior and the initiation of feeding. After food intake, ghrelin levels are quickly suppressed^[19]. Ghrelin effects on the brain are mediated in part by the vagus nerves and in part by a humoral action.

Numerous sites of the CNS, that include the ARC, the nucleus accumbens, the parabrachial nucleus, dorso-medial and lateral hypothalamic area and the subfornical part of the circumventricular organs, contain ghrelin receptors or ghrelin-stimulated activity that play a significant role in energy homeostasis. In the ARC, after stimulation of NPY/AgRP neurons it observe ghrelin orexigenic action^[10].

Obese subjects show low ghrelin levels while lean subjects show high ghrelin levels. Ghrelin fasting levels appear rise in diet-induced or exercise-induced weight loss, and this may result in augmented sensation of hunger with failure to lose excess weight^[21]. Thus, maintaining a weight set point might depend from ghrelin in,

particularly in the condition of weight loss resistance.

Ghrelin levels, before and after RYGB, are reported in twenty-three studies about (Tables 2 and 3). A significant early decrease in ghrelin was found in five out of the seven studies that reported ghrelin levels within the first 14 d after surgery. Some of these studies compared patients undergoing RYGB with a control group of patients undergoing other non resective gastric operations such as AGB or Nissen fundoplication^[1,22,23]. Only RYGB resulted in a significant reduction in postoperative ghrelin levels.

There are not consistent researches of longer-term changes in ghrelin levels after RYGB (Tables 2 and 3). Several studies reported preoperative and postoperative circulating ghrelin levels between 1 mo and 2 years after surgery.

In some of these researches the evaluation of a single serum ghrelin concentration in the morning after an overnight fast was performed; in others the meal-associated suppression of ghrelin was assessed. Some researchers evaluated "active" ghrelin, the metabolically active isoform including a unique post-translational octanoylation of the serine-3 hydroxyl group^[1,18], while other group also

Table 1 Anatomic and physiologic changes due to bariatric surgery

	Stomach	Duodenum	Proximal intestine	Distal intestinal
RYGB	Decreased capacity to 15-30 mL Chyme bypasses 90% of stomach	Bypassed by chyme	Proximal jejunum bypassed by chyme Rapid exposure of distal jejunum to chyme	Earlier exposure to chyme
AGB	Decreased gastric transit time Restrictive band around gastric cardia Direct pressure on vagus nerves	No alterations	Bile mixes with chyme in proximal ileum No alterations	No alterations
BPD/DS	Decreased capacity to 60-150 mL 85%-90% of stomach removed	Bypassed by chyme	Chyme bypasses jejunum Rapid exposure of ileum to chyme	Rapid exposure to chyme
SG	Accelerated gastric emptying by 2-fold to 3-fold Decreased capacity to 60-150 mL 85%-90% of stomach removed Accelerated gastric emptying by 2-fold to 3-fold	More rapid exposure to chyme	Bile mixes with chyme in distal ileum Possibly earlier exposure to chyme	Possibly earlier exposure to chyme

AGB: Adjustable gastric band; BPD/DS: Biliopancreatic diversion with or without duodenal switch; RYGB: Roux-en-Y gastric bypass; SG: Sleeve gastrectomy.

Table 2 Short term (< 3 mo) fasting serum hormonal changes after bariatric surgery

	RYGB	AGB	SG	BPD/DS
Ghrelin	↓ [22,26,72,74,75, 87-91] = [23-25,36,45,88,89] ↑ (abs. .inf.)	↓ (abs. .inf.) = [24,25,27] ↑ [27]	↓ [26,27,45] = (abs. .inf.) ↑ (abs. .inf.)	↓ [108] = [108,111,112] ↑ [109-111]
GLP-1	↓ (abs. .inf.) = [23,24,26,36,37,89,115-117] ↑ (abs. .inf.)	↓ (abs. .inf.) = [24] ↑ (abs. .inf.)	↓ (abs. .inf.) = [26] ↑ (abs. .inf.)	↓ (abs. .inf.) = [112,122-125] ↑ (125,126)
PYY	↓ [23] = [24,25,36,46,76,89,91] ↑ (abs. .inf.)	↓ (abs. .inf.) = [24,25] ↑ (abs. .inf.)	↓ (abs. .inf.) = [46] ↑ [26,45]	↓ (abs. .inf.) = (abs. .inf.) ↑ (abs. .inf.)
Insulin	↓ [22,23,25,26,87,89,90,115,117,118,127-129] = [25, 46,89,91,116] ↑ [88]	↓ [21,22] = [25] ↑ (abs. .inf.)	↓ [26,48] = (abs. .inf.) ↑ (abs. .inf.)	↓ [122,123] = (abs. .inf.) ↑ (abs. .inf.)
Leptin	↓ [23-25,89,90,117,127-129] = (abs. .inf.) ↑ (abs. .inf.)	↓ [25] = [24] ↑ [21]	↓ (abs. .inf.) = (abs. .inf.) ↑ (abs. .inf.)	↓ [111,112,122] = (abs. .inf.) ↑ (abs. .inf.)
Adiponectin	↓ [72, 87] = [87] ↑ (abs. .inf.)	↓ (abs. .inf.) = (abs. .inf.) ↑ (abs. .inf.)	↓ (abs. .inf.) = (abs. .inf.) ↑ (abs. .inf.)	↓ (abs. .inf.) = [122] ↑ (abs. .inf.)

↓: Decreased; =: Not significant; ↑: Increased; abs. .inf.: Absence of information. The number inside the Table indicate the related references. RYGB: Roux-en-Y gastric bypass; AGB: Adjustable gastric band; SG: Sleeve gastrectomy; BPD/DS: Biliopancreatic diversion with or without duodenal switch; GLP-1: Glucagon-like peptide-1; PYY: Peptide tyrosine-tyrosine.

detected the inactive des-acyl isoform^[1].

This variability in the methods as well as the differences among assays restricts the studies comparisons.

Although considerable physiologic variations in serum ghrelin levels may contribute to conflicting findings, two observations emphasize the expected decreased ghrelin levels in patients after RYGB. Cummings *et al*^[24] determined the 24-h plasma ghrelin profiles, body composition, insulin levels, leptin levels, and insulin sensitivity in 13 obese subjects before and after a six-month dietary program for weight loss. The 24-h ghrelin profiles were also determined in 5 subjects who lost weight after RYGB and 10 normal-weight controls; 5 of the 13 obese subjects who participated in the dietary program were matched with the subjects in the gastric-bypass group as obese controls^[24]. The increase in the plasma ghrelin level with the subsequent diet-induced weight loss is consistent with the hypothesis that ghrelin plays a role in the long-

term regulation of body weight^[24]. RYGB is correlated with considerably suppressed ghrelin levels, probably contributing to the weight-reducing effect of the procedure^[24]. Nevertheless, the findings reported by Cummings *et al*^[24] is not in line with other investigators: some studies have found an unchanged ghrelin spike before a meal and normal suppression following a meal after RYGB^[25,26]. These inconsistencies may be also due to different techniques in performing RYGB.

Korner *et al*^[26] showed that the differences in levels of gut hormones may play a role in promoting greater weight loss and insulin sensitivity after RYGB compared with AGB.

The changes in ghrelin after SG were measured in different studies: fasting ghrelin levels are decreased at all time points up to 5 years of follow-up. Moreover, some studies tried to evaluate and compare the effects of RYGB^[27] or AGB^[28,29] with the SG on fasting ghrelin lev-

Table 3 Long term (> 3 mo) fasting serum hormonal changes after bariatric surgery

	RYGB	AGB	SG	BPD/DS
Ghrelin	↓ [77,85,92,97,100] = [24,25,45,72, 87-89,91,95,99,101] ↑ [45, 73,78-83,86,88,93,94,96,99]	↓ (abs. inf.) = [24,25,103,104,106] ↑ [28,27,92,101,102,104,105,107]	↓ [28,27,45,107,114] = (abs. inf.) ↑ (abs. inf.)	↓ [54,97] = [96,113] ↑ [111,112]
GLP-1	↓ [120] = [24,25,26,37,80,89,95,116,119,121] ↑ (abs. inf.)	↓ [120] = [24,25,106,120] ↑ [102]	↓ (abs. inf.) = (abs. inf.) ↑ (abs. inf.)	↓ (abs. inf.) = (abs. inf.) ↑ (abs. inf.)
PYY	↓ (abs. inf.) = [24,25,89,91] ↑ [25,45,81,96,119,120]	↓ (abs. inf.) = [24,25] ↑ [120]	↓ (abs. inf.) = (abs. inf.) ↑ [45]	↓ (abs. inf.) = (abs. inf.) ↑ (96)
Insulin	↓ [25,78,78,87,89,74,94,95,97,98,99,100,102,120,121,127,129,135] = [24,25,80,89,91] ↑ [82,84,95,99,130]	↓ [25,92,102,104,120,131] = [106] ↑ (abs. inf.)	↓ [48] = (abs. inf.) ↑ (abs. inf.)	↓ [97,113,126] = (abs. inf.) ↑ (abs. inf.)
Leptin	↓ [24,25,78,79,89,92-94,96-99,101,127,129,132,135] = (abs. inf.) ↑ (abs. inf.)	↓ [25,92,101-104,106,132,136] = [24] ↑ (abs. inf.)	↓ (abs. inf.) = (abs. inf.) ↑ (abs. inf.)	↓ [97,111-113] = [54,96] ↑ (abs. inf.)
Adiponectin	↓ (abs. inf.) ↑ [78,79,84,87,90,93,94,97,99,131,133,134]	↓ (abs. inf.)	↓ (abs. inf.) = (abs. inf.) ↑ (abs. inf.)	↓ (abs. inf.) = [54] ↑ [97]

↓: Decreased; =: Not significant; ↑: Increased. abs. inf.: Absence of information. The number inside the Table indicate the related references. RYGB: Roux-en-Y gastric bypass; AGB: Adjustable gastric band; SG: Sleeve gastrectomy; BPD/DS: Biliopancreatic diversion with or without duodenal switch; GLP-1: Glucagon-like peptide-1; PYY: Peptide tyrosine-tyrosine.

els, which showed to be decreased.

These studies lend credence that the ghrelin suppression after both SG and RYGB may be part of the mechanism that contributes to T2DM remission.

Glucagon-like peptide-1

Glucagon-like peptide-1 (7-36) amide (GLP-1) is a 30-residue peptide hormone released from intestinal L cells following nutrient consumption, as reported by Donnelly^[30]. It potentiates the glucose-induced secretion of insulin from pancreatic beta cells, increases insulin expression, but also inhibits beta-cell apoptosis. Donnelly^[30] has reported that GLP-1 promotes beta-cell neogenesis and satiety, reduces glucagon secretion, delays gastric emptying, and increases peripheral glucose disposal. Intact peptide is inactivated during passage across the hepatic bed by the enzyme dipeptidyl peptidase-4 (DPP-IV) associated with the hepatocytes, and further degraded by the peripheral tissues, while the kidney is important for the final elimination of the metabolites. GLP-1 is also a neurotransmitter located in numerous areas of the brain (paraventricular nucleus and dorsomedial hypothalamus, dorsoventral complex, thalamus, and pituitary). The GLP-1 receptor is G-protein-coupled and is expressed in the pancreatic islets, lung, hypothalamus, stomach, heart and kidney^[31].

Peripheral infusion of GLP-1 or PYY3-36 reduces food intake in healthy, obese, and diabetic subjects^[32]. These two hormones, in combination with other anorectic gut hormones, act as peripheral sensory inputs, integrated in the CNS, modulating appetite and energy expenditure.

GLP-1 and PYY3-36 directly act on the stomach to slow gastric emptying and acid secretion functioning as an “ileal brake” mechanism to intestinal motility, prevent-

ing an exceedingly rapid transit and allowing time for digestion and absorption of nutrients in the proximal intestine^[1,33].

GLP-1, one of the gut hormones called incretins, has an important role in enhancing insulin secretion by the pancreas^[1]. The increased insulin response to enteral as opposed to parenteral glucose is defined the “incretin effect”^[34]. Both *in vivo* and *in vitro* researches have showed that GLP-1 increase insulin secretion in the beta cell. Moreover, glucagon secretion is inhibited by GLP-1 while insulin sensitivity is increased.

Apoptosis is contrasted by GLP-1 while beta cell regeneration is promoted^[35]. Diabetic patients show a blunted first-phase insulin response following a meal, partly due to a decreased incretin response^[1,36].

Exenatide, a GLP-1 receptor agonist, is currently used for the treatment of T2DM. A strong GLP-1 response was reported 10 years after RYGB, suggesting a long-lasting effect. This phenomenon may play a key role in maintaining T2DM remission and weight loss after RYGB. Following RYGB, fasting GLP-1 levels are usually unchanged (Tables 2 and 3). Nevertheless, the GLP-1 response to a glucose or mixed meal is consistently enhanced (Tables 2 and 3). This effect occurs within two days following surgery^[37] and remains stable for one year^[25,26,38].

Jiménez *et al.*^[39] showed that an enhanced GLP-1 response to meal intake in T2DM patients is not sufficient to maintain normal glucose tolerance in the long term after RYGBP.

Similar to RYGB, a large number of studies have shown unchanged fasting GLP-1 and a significant increase in response to a glycemic challenge.

Peterli *et al.*^[27] prospectively evaluated GLP-1 changes following SG in randomized patients to receive either SG or RYGB. Curiously, following a test meal, GLP-1 results

augmented in the SG patients and PYY response similar to RYGB patients. Another, there was a rapid resolution of T2DM in both groups. Their results do not confirm the foregut theory that hypothesizes that bypassing the duodenum and proximal small intestine is important to achieve the diabetes remission following bariatric surgery. Another hypothesis suggests that the rapid gastric transit arises as consequence of both early exposure of the distal intestine to nutrients and stimulation of the “ileal brake” mechanism^[1,33]. This combination of effects influences digestive process and feeding behavior.

Peptide tyrosine tyrosine

PYY1-36 is co-secreted with GLP-1 and OXM by L-cells of the distal small bowel and colon in proportion to calorie content of the meal^[1]. The main circulating and active form, PYY3-36, is formed by cleavage of PYY1-36 by the enzyme DPP-IV^[40]. Serum levels rise within 15 min after initiating a meal and peak 1-2 h later, remaining elevated for various hours^[41]. An anorectic effect of PYY3-36 has also been reported^[42]. Similar to GLP-1, an anorectic CNS action was reported and it acts by reducing gastric emptying and acid production, by inhibiting pancreatic exocrine function, and by slowing intestinal transit time. Obese subjects show a reduced PYY3-36 response to feeding that might represent a cause of excessive appetite^[43].

PYY3-36 has the anorectic action on the ARC through the Y2 receptors expressed at NPY/AgRP neuronal level, beyond in the nucleus of the solitary tract and the nodose ganglion of the vagus. Most of PYY3-36 anorectic effect is likely mediated by the vagus nerve^[44].

Following RYGB, similar to GLP-1, there is a forward and particular increase in PYY3-36 response to a glycemic challenge (Tables 2 and 3). Along with GLP-1, this contributes to decreased appetite and rapid weight loss^[40,45]. Three studies also reported a significantly increased fasting level of PYY3-36 following RYGB. Following the AGB, two studies^[25,26] failing to demonstrate a variation in either fasting PYY or meal-stimulated response. This aspect showed no agreement with the enhanced response following RYGB.

Three researches^[27,46,47] have showed the changes in meal-stimulated PYY3-36 response before and after SG reinforcing Peterli's conclusions regarding an increased GLP-1 response to SG; the researches showed an elevated meal-stimulated PYY3-36 response, that was similar to the change in PYY3-36 after RYGB.

Insulin

Insulin develops numerous roles in energy metabolism and is hormone more widely. Elevated serum glucose levels following a meal stimulate, particularly, insulin release by pancreatic beta cells. Insulin release is also induced by several amino acids (alanine, glycine, and arginine), acetylcholine from vagal nerve endings, and some incretins, including GLP-1 and glucose-dependent insulinotropic polypeptide (GIP)^[1].

Insulin, among its various functions, is central to energy balance. Postprandial secretion of insulin lowers spikes of glucose levels following a meal by storing glucose as glycogen in muscle and liver and as triglycerides in adipose tissue. *Vice versa*, low serum insulin levels in fasting situations end up in mobilization of glucose from the liver and fatty acids from muscle. Consequently a major ATP amount is produced by β -oxidation, from adipose tissue.

Insulin has both long- and short-term effects on energy homeostasis. Insulin shows a negative feedback role on feeding by direct effects on the CNS^[1]. In animal studies, decreased food intake and weight loss is caused by insulin after injected at central level. NPY/AgRP neurons in the ARC are inhibited by insulin. Its levels have also been suggested as long-term indicators of overall energy stores. Overweight compared with lean subjects showed higher basal and meal-stimulated insulin levels^[48].

Fasting insulin levels are decreased due to RYGB and BPD/DS (Tables 2 and 3). Obese patients with T2DM shows a rapid improvement in glycemic control or impaired glucose tolerance. This is the result of: (1) a restored first-phase insulin response to a glycemic challenge due to an enhanced incretin effect; and (2) an improved insulin sensitivity related to weight loss.

Fasting insulin levels are also reduced after AGB. Nevertheless, the enhanced incretin effect in response to a meal is not observed. In diabetic patients, insulin sensitivity improves with weight loss.

SG also results in rapid resolution of diabetes in T2DM patients with decreased fasting insulin levels and a rapid improvement of the first phase insulin response and insulin sensitivity^[27,49].

Leptin

Leptin is a 167-amino acid protein produced in several sites in the body, in particular by adipocytes - and it acts as a signal of overall body energy stores rising in proportion to total body fat mass^[50].

Leptin is produced mainly in white adipose tissue, although it is also produced in brown adipose tissue, placenta, ovaries, skeletal muscle, stomach, breast, bone marrow, pituitary, and liver^[1].

Several researches have showed that leptin acts on the hypothalamus, where it inhibits NPY/AgRP receptor neurons in the ARC while stimulates α -MSH neurons^[1,9,11]. The OB-Rb receptor would seem to be binded to leptin. The JAK-STAT and MAPK signal transduction pathways would seem to be activated by leptin^[1].

Leptin acts on appetite and energy balance oppositely to the action of ghrelin. Although in mice leptin appears to act as a significant physiological brake on appetite, its role in humans requires further studies, because of minimal weight loss obtained in obese patients taking leptin analogues, as reported by Harvey *et al.*^[1].

Harvey *et al.*^[1] has reported that leptin resistance in obesity has been proposed as one possible interpretation of this finding. Another hypothesis is that, in humans,

relative leptin deficiency is essentially the more potent stimulus at times of negative energy balance, such as starvation and diet-induced weight loss, as reported by Harvey *et al*^[1]. In this way the weight loss recovery is facilitated.

In different studies the serum leptin measurement after metabolic surgery was regularly performed; the results demonstrated its decrease with weight^[45]. At the moment, there are no data demonstrating strong differences among leptin levels after equivalent weight loss due to RYGB or diet/exercise. Therefore, it should be excluded a direct bariatric surgery effect on serum leptin concentration.

Adiponectin

High-molecular-weight adiponectin is a 244-amino acid protein produced in white adipose tissue^[1]. Serum adiponectin levels are inversely proportional to body fat mass, BMI, waist-to-hip ratio, serum insulin, and glucose levels^[1]. In some studies, obese subjects compared with lean controls show a reduction of fasting adiponectin levels^[51]. Nevertheless, the response to a meal appear to be exaggerated in obese subjects^[51].

In the pathogenesis of insulin resistance in T2DM would seem to have a role adiponectin. In rodents, hepatic gluconeogenesis is improved by adiponectin administration while fatty acid utilization in muscle cells appears to be increased^[52]. Moreover, in a study comparing Pima Indians - a population with a high propensity for obesity and T2DM - and Caucasians, hypo adiponectinemia is more closely related to the degree of insulin resistance^[53], high levels being protective against developing T2DM^[54].

About the adiponectin levels before and after RYGB, just one study reported an increase in fasting adiponectin. No significant difference in adiponectin levels after BPD/DS were found in three out of four studies^[51-54], despite 44% decrease of total body weight reported by Kotidis *et al*^[53]. The differences could be related, among other things, to the presence of adiponectin gene mutations that alter its physiological functional role and affect its levels in the body. For this reason, we suggest to perform molecular investigations in order to clarify the mutational state of adiponectin gene and the effect of possible mutations on level outcomes.

Following AGB, adiponectin levels also rise as the weight reduction occurs. To our knowledge, no study has investigated changes in adiponectin following SG.

Tables 2 and 3 summarize short and long term effect of bariatric procedures on serum fasting gut hormones considered in this review.

ADAPTED BARIATRIC SURGERY

Actually our knowledge on the mechanisms of energy homeostasis are increased, newer bariatric procedures have been developed to achieve specific metabolic goals. Surgical procedures designed to treat T2DM in non-mor-

bidity obese patients, such as the ileal interposition^[56] and duodenal-jejunal bypass^[57], are being evaluated in Institutional Review Board-approved researches. Bariatric surgery improve strongly diabetic as showed in most of the studies. Discontinuation of antidiabetic medications and remission of T2DM after bariatric surgery were put in evidence in 86.8% and 64.7% of the patients, respectively, with fasting plasma glucose and glycated hemoglobin (HbA1c) slightly above normal range^[57]. Furthermore, bariatric surgery provided adequate glycemic control for 30.1% of the patients using insulin prior to surgery^[57]. It has been showed, in some studies, that malabsorptive bariatric techniques have higher diabetes remission rates than restrictive ones^[57-59]. T2DM usually finds the solution within few days to some weeks following malabsorptive procedures such as RYGB and BPD; in both cases a significant weight loss is previously achieved^[57]. Although the exact mechanism is not yet completely understood, some studies support the idea that malabsorptive procedures involving rerouting of food might control high glucose levels in T2DM by bursting insulin sensitivity and/or by improving β -cell function^[57]. Obviously, both mechanisms favor losing weight and reducing caloric intake^[59-62]. Some studies have described that acute insulin response to intravenous glucose and early phase insulin response to oral glucose load significantly improve within a month following gastrointestinal bypass surgery^[57,60,63]. Mechanisms for these mutations could be due to the important decrease in insulin resistance and the increase in GLP-1 postprandial plasma levels immediately after surgery^[57]. Actually, two hypotheses, named hindgut and foregut theory, have been proposed to explain T2DM improvement after metabolic surgery and surgical-induced weight loss. The former theory claims that surgical nutrient rerouting to the small intestine distal part ends up in elevated GLP-1 secretion and concomitant GLP-1 glucose-lowering effects; the latter hypothesis emphasizes that surgical bypass of the foregut prevents the release of a not yet well-defined nutrient, that functions as a pro-diabetes stimulus in predisposed subjects^[64]. The weight loss effect of metabolic surgery on T2DM in not severely obese patients ($\text{BMI} \geq 35 \text{ kg/m}^2$) might be lower than that on T2DM with a higher BMI ($\geq 40 \text{ kg/m}^2$)^[57]. The understanding of the above mechanism is the key to success in metabolic surgery. There is no strong evidence describing the effectiveness of metabolic surgery on long-term follow-up in obese diabetic individuals^[57].

LINKING PHARMACOTHERAPY AND BARIATRIC SURGERY

The therapeutic effect of metabolic surgery are not observed in all patients. For example, up to 15% of patients fail to achieve a weight loss over 30%^[1,65] and a minority of diabetic patients will still have inadequate glycemic control following after bariatric surgery^[1,66]. In others, with weight regain the presurgical hormonal conditions comes back. A hormonal panel may point to a particu-

lar feedback system that is limiting the overall success. Targeted pharmacotherapy might be necessary and sufficient to suppress the system and achieve the metabolic goal. As clearly depicted in their work, Le Roux *et al*^[45] enforced jejunoileal bypass on rats and compared these to sham-operated rats. Increased PYY3-36 caused weight loss in jejunoileal bypass rats. Antagonism of the PYY with a specific neutralizing antibody led the rats to regain weight, whereas administration of PYY3-36 resulted in additional weight.

Outcome of bariatric surgery on insulin sensitivity and secretion is different in relation to the type of surgery methods that was performed^[67]. In fact, while RYGB enhances insulin secretion after a meal, thus improving glucose metabolism, BPD/DS acts through the improvement in insulin sensitivity allowing the subsequent reduction of insulin hypersecretion, a typical feature of the insulin resistance state^[67]. Gastric banding action is expected to be mediated only through weight loss, and the effect of sleeve gastrectomy remains still to be clarified. Incretin secretion is mostly elevated under nutrient stimulation after gastric bypass, likely leading to an overstimulation of pancreatic β -cells, and resulting in the elevated insulin secretion.

RYGB intervention affects fasting GLP-1, PYY or ghrelin levels and produces greater improvement in insulin sensitivity compared with diet at equivalent weight loss in T2DM patients^[68]. No beneficial effect was observed in nondiabetic subjects at this early time-point.

No change in fasting GLP-1 concentrations after massive weight loss achieved with bariatric surgery was reported. In particular, after biliopancreatic diversion in morbidly obese patients without diabetes mellitus^[69].

All hormones showed changes from baseline: some variations have been identified soon after surgery (ghrelin, leptin, adiponectin), whereas others were preserved in the long term (GLP-1, PYY, ghrelin, leptin, adiponectin), as summarized in Tables 2 and 3^[70-136]. At present no clinical studies are available combining bariatric procedures with specific pharmacotherapy for metabolic complications. However this topic appears to be of increasing interest, especially if it is implemented by pharmacogenomic data that could illustrate the specific pattern of drug susceptibility belonging to each patient.

NAFLD AND BARIATRIC SURGERY

Bariatric surgery was historically related with reduced prevalence and severity of liver disease. However, in some studies, the progression of liver disease in patients postoperatively was reported. In the early age of bariatric surgery, jejunoileal bypass was the most usually enforced procedure. A considerable portion of patients developed advanced liver disease, probably due to bacterial overgrowth and endotoxemia in the bypassed intestine, resulting in bacterial translocation and liver disease. Nevertheless, the jejunoileal bypass surgery has rarely been enforced in recent years due to the multiple complications arising from this procedure.

In a recent review the association between bariatric surgery (RYGB) and ghrelin reduction was underlined; ghrelin is known to stimulate insulin counter regulatory hormones, reduce adiponectin, and block hepatic insulin signalling^[137]. Moreover, RYGB is also associated with GLP-1 high levels; consequently it enhances glucose tolerance by enhancing insulin secretion, suppressing glucagon production, inhibiting gastric emptying, and increasing B cell mass^[137]. For this reason, bariatric surgery is probably to have potential benefit in ameliorating this gut hormones that strongly contribute to NAFLD pathogenesis^[137]. Nevertheless, Hafeez *et al*^[137] concluded that further investigations are needed to determine (1) the benefit of bariatric surgery in NAFLD patients at high risk of developing liver cirrhosis; and (2) the role of bariatric surgery in modulation of NAFLD complications such as diabetes and cardiovascular disease. The outcomes of the future researches will evaluate whether bariatric surgery will be one of the prescribed choice for treatment of the most progressive NAFLD type^[137].

GUT MICROBIOTA AND BARIATRIC SURGERY

Bariatric surgery is generally the only available treatment for severe obesity that regularly develops and sustains considerable weight loss^[138]. This surgery leads to some mutations in acid exposure to the gastric remnant and proximal small bowel. It also acts by restricting the amount and types of food that can be easily ingested, by promoting a modest degree of nutrient malabsorption by shortening the length of the small bowel. In addition, the RYGB may result in intestinal dysmotility. Actually, very little is known about the mutations in the gut microbiome that occur after RYGB, and, Kong *et al*^[139] showed an increase in gut microbiome richness and in the number of associations between gut microbiome and white adipose tissue genes. Variations of gut microbiome were correlated with mutations in white adipose tissue gene expression^[139]. These findings stimulate deeper explorations of the mechanisms linking gut microbiome and white adipose tissue pathological alterations in human obesity and its variations after weight loss^[139]. In this field, genomic, transcriptomic and bioinformatics analysis could offer a potent integrated tool to clarify the majority of unclear aspects.

Osto *et al*^[140] showed that RYGB surgery might differently modify the gut microbiome composition in the three distinct anatomical sections of the small intestine compared to sham surgery. RYGB induced variations in the microbiota of the alimentary limb and the common channel resembling those seen after prebiotic treatment or weight loss by dieting, as reported by Osto *et al*^[140]. These variations may be associated with altered production of intestinal hormones known to control energy balance. Postsurgical modulation of gut microbiome may significantly contribute to the beneficial metabolic effects of RYGB surgery^[140], not excluding those on NAFLD.

Ashrafian *et al.*^[141] reported that, in rats, surgically induced metabolic shifts identify some of the potential mechanisms that contribute toward bariatric cardio-protection through gut microbiota ecological fluxes and an entero-cardiac axis to shield against metabolic syndrome of cardiac dysfunction.

Recently, Sweeney and Morton^[142], in their review, have been provided the practicing surgeon with (1) an update on the state of a quickly innovating branch of clinical bioinformatics, specifically, the micro-biome; (2) a new understanding of the micro-biome changes after RYGB and weight loss; and (3) a basis for understanding further clinical applications of studies of the distal gut micro-biome, such as in Crohn's disease, ulcerative colitis, and infectious colitis.

We suggest that the application of bioinformatics tools for better understanding the most significant micro-biome features could be cross-linked with the results of the expression hormone gene analysis conducted in the patients after weight loss. In this way it is possible to interpret the actual contribute of micro-biome in treatment outcomes.

FUTURE DIRECTIONS

Bariatric surgery leads to certain variations in the hormonal milieu that develop weight loss in obese subjects acting on energy balance regulation, particularly on food intake. Bariatric surgery is also related to some potential beneficial effects on co-morbid diseases correlated with obesity such as T2DM, metabolic syndrome^[71,143,144] and NAFLD. Translational researches about the relative contribution of abounding signaling pathways in energy homeostasis will probably appear in combined treatment strategies containing both pharmacotherapy and surgery. Future research in these areas is warranted.

Surgery aimed principally at diseases such as diabetes and not weight loss are referred to as "metabolic surgery", as reported by Cohen *et al.*^[145]. Cohen *et al.*^[145] showed that metabolic surgery has been proven to be safe and effective, and although more data are needed, it is unquestionable that a new discipline has been founded. Metabolic surgery can effectively treat T2DM in individuals with any BMI, including $< 35 \text{ kg/m}^2$, who do not respond to standard medical therapy^[145].

Bariatric surgery can be enforced safely with acceptable low complication rate and mortality by specialized teams.

Changes in gut hormones, containing increases in GLP-1, PYY, and oxyntomodulin, decrease in GIP and ghrelin, or the combined action of all these hormones, might have a role in induction and long-term maintenance of weight loss^[146]. In particular, it has been recently demonstrated by Holst^[147] that GLP-1 and PYY appear to contribute tightly to the reduction in food intake after bypass and, thus, to the weight loss.

Actually, there are no data indicating that a reduced secretion of the hormones is associated with the pathogenesis of obesity and/or T2DM, but impaired secretion

usually observed in obesity (and hence also in diabetes) may contribute to the development, as reported by Holst^[147]. These hormones have subsequently become attractive novel targets for the development of obesity and T2DM therapies^[148].

The previous study of the hormones mutational state with molecular tools could relate the pathogenesis aspects to the therapy aspects: if the presence of pathogenic mutations is highlighted by genomic analysis, its effects could affect or impair the hormone physiological role. In this case also the therapy outcomes could be influenced by the genes variations; consequently therapy strategy must be design to overcome the problem.

In fact, by recapitulating the gut hormone secretion changes after bariatric surgery, drugs based on gut hormones represent an exciting possibility for the treatment of obesity and T2DM^[149]. Nevertheless, the timing, the exact variations of gut hormones and the relative importance of these ones in the metabolic improvement post-bariatric surgery remain to be further clarified^[150].

Dixon *et al.*^[151] has reported that bariatric surgery, in addition to its profound weight-reduction effects, leads to a durable resolution of T2DM. Therefore, Dixon *et al.*^[151] has hypothesized that a cure for this disease may be obtainable, both by surgery as well as with drugs or devices that mimic surgery effects.

All these studies are safely interesting and will probably improve our knowledge on the pathogenesis of several metabolic diseases. This aspect is positively related to the possibility to identify new and exciting therapeutic opportunities. However, high-level controlled trials evaluating in particular long term benefits of bariatric surgery in obesity and its co-morbid diseases such as T2DM and the metabolic syndrome are required^[152].

Recently, Lee *et al.*^[153] suggested that the novel anorexic hormone nesfatin-1 and another new hormone, the obestatin, might contribute to the marked improvement in glycemic homeostasis and weight loss in diabetics after RYGB and SG.

Nesfatin-1 is a recently identified 82-amino-acid peptide derived from the precursor protein, nucleobindin2 (NUCB2)^[154]. The brain distribution of NUCB2/nesfatin-1 at the mRNA and protein level along with functional studies in rodents support a role for NUCB2/nesfatin-1 as a novel satiety molecule acting through leptin-independent mechanisms^[154].

Consequently may be very significant the quantification of nesfatin levels in obese patients by using the appropriate methods transcriptomic-based.

Obestatin is a recently discovered 23-amino acid peptide encoded by the ghrelin gene^[155,156]. Although in the original Zhang's works obestatin appeared to suppress food intake and decrease gastric emptying^[155-157], consequently antagonizing the orexigenic effect of ghrelin, subsequent researches in rodents showed controversial results^[155-161]. Obestatin is present not only in the gastrointestinal tract, but also in the spleen, mammary gland, breast milk, and plasma^[155,156,160]. Obestatin seems to function as part of a complex gut-brain network whereby

hormones and substances from the stomach, intestine and the brain about satiety or hunger^[155,156,160].

Lee *et al.*^[153] concluded that RYGB and SG produce differential influences with regards to circulating nesfatin-1 and obestatin levels in non-morbidly obese, T2DM patients. Circulating nesfatin-1 may modulate glucose homeostasis in two surgical procedures, and participate in regulating body weight in SG. However, many questions are still to be answered, in particular the receptor involved in the peptide's actions, and the processing of NUCB2 to nesfatin-1 in hypothalamic or gut tissues still remain elusive. Furthermore, signaling mechanisms directly associated with the action of nesfatin-1 have been little explored^[153].

CONCLUSION

It is important to emphasize the role of the major peptides released by the enteroendocrine system, which promote satiety and modulate energy homeostasis and utilization, as well as those that control fat absorption and intestinal permeability, as reported by Mells *et al.*^[162]. Clarifying new functions for enteroendocrine system-related peptides and developing pharmacologic peptide analogues offer future pharmacologic chances for obesity-related human disease, of which NAFLD is such important a part^[4].

Bariatric surgery could be the most effective treatment for obesity and co-morbidities, often within days after surgery, independently of weight loss^[163] and it is actually the only therapy available for obesity which results in long-term, sustained weight loss^[148].

We hypothesize that gut hormones might induce and maintain the weight loss to long-term, could determine the improvement of obesity-related co-morbidities and could help to detect new drug targets and improved surgical procedures.

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Estradiol agonists inhibit human LoVo colorectal-cancer cell proliferation and migration through p53

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estrogen receptors (ER) or direct administration of ER agonists on human colorectal cancer.

METHODS: LoVo cells were established from the Bio-resource Collection and Research Center and cultured in phenol red-free DMEM (Sigma, United States). To investigate the effects of E2 and/or ER selective agonists on cellular proliferation, LoVo colorectal cells were treated with E2 or ER-selective agonists for 24 h and 48 h and subjected to the MTT (Sigma) assay to find the concentration. And investigate the effects of E2 and/or ER selective agonists on cell used western immunoblotting to find out the diversification of signaling pathways. In order to observe motility and migration the wound healing assay and a transwell chamber (Neuro Probe) plate were used. For a quantitative measure, we counted the number of migrating cells to the wound area post-wounding for 24 h. We further examined the cellular migration-regulating factors urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA) and matrix metalloproteinase (MMP)-9 in human LoVo cells so gelatin zymography that we used and gelatinolytic activity was visualized by Coomassie blue staining. And these results are presented as means \pm SE, and statistical comparisons were made using Student's *t*-test.

RESULTS: The structure was first compared with E2 and ER agonists. We then treated the LoVo cells with E2 and ER agonists (10^{-8} mol/L) for 24 h and 48 h and subsequently measured the cell viability using MTT assay. Our results showed that treatment with 17 β -estradiol and/or ER agonists in human LoVo colorectal cancer cells activated p53 and then up-regulated p21 and p27 protein levels, subsequently inhibiting the downstream target gene, cyclin D1, which regulates cell proliferation. Taken together, our findings demonstrate the anti-tumorigenesis effects of 17 β -estradiol and/or ER agonists and suggest that these compounds may prove to be a potential alternative therapy in the treatment

Abstract

AIM: To investigate the effects of 17 β -estradiol *via*

of human colorectal cancer. These results demonstrate that 17 β -estradiol and/or ER agonists downregulate migration-related proteins through the p53 signaling pathway in human LoVo colorectal cancer cells. These findings suggest that p53 plays a critical role in the 17 β -estradiol and/or ER agonist-mediated protective activity against colorectal cancer progression. In addition, 17 β -estradiol and/or ER agonists dramatically inhibited cell migration and reduced the expression of u-PA, t-PA and MMP-9 as well as MMP-2/9 activity in LoVo cells, which regulate cell metastasis. Moreover, we observed that pretreatment with a p53 inhibitor significantly blocked the anti-migration effects of E2 and/or ER agonists on LoVo cells. That E2 and/or ER agonists may impair LoVo cell migration by modulating migration-related factors *via* the p53 tumor suppressor gene.

CONCLUSION: Direct ER treatment may prove to be an attractive alternative therapy in the treatment of human colorectal tumors in the future.

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Key words: Estrogen; Estrogen agonist; Estrogen receptors; Human colon cancer cell; p53

Core tip: The present study is to investigate the effects of 17 β -estradiol *via* estrogen receptors or directly administration of ERs agonist on the development of human colorectal cancer, and to elucidate whether the effect was regulated by tumor suppressor gene p53. Here, our results showed that 17 β -estradiol and/or ERs agonist treatment in human LoVo colorectal cancer cells could activate p53, then up-regulated p21 and p27 protein levels, subsequently inhibited downstream target gene, cyclin D1, which regulated the cell proliferation.

Hsu HH, Kuo WW, Ju DT, Yeh YL, Tu CC, Tsai YL, Shen CY, Chang SH, Chung LC, Huang CY. Estradiol agonists inhibit human LoVo colorectal-cancer cell proliferation and migration through p53. *World J Gastroenterol* 2014; 20(44): 16665-16673 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16665.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16665>

INTRODUCTION

Epidemiological studies suggest that cancers of the lung/bronchus, prostate and colon/rectum in men and cancers of the lung/bronchus, breast and colon/rectum in women continue to be the most common cancers in the United States. Colorectal cancer is the third most common cause of cancer death^[1]. Colorectal cancers include nonhereditary and hereditary types. Hereditary colon cancers include familial adenomatous polyposis and hereditary non-polyposis colon cancer (HNPCC). HNPCC is the most common form of colorectal cancer, accounting

for 5%-10% of total hereditary colorectal cancers, and it occurs as early as age 25 with an average age of 45 years at diagnosis^[2].

The role of the female sex hormone, 17 β -estradiol, in tumorigenesis has been studied for many years. It has been proposed that the lower incidence of colorectal cancer (CRC) in women might be due to the influence of female sex steroid hormones^[3]. Many studies have confirmed that hormone replacement therapy (HRT) in postmenopausal women reduces the incidence of colorectal cancer^[4], whereas only one study has reported an adverse effect of HRT^[5].

One might ask what is the mechanism behind this protective effect of female sex hormones against cancer cell proliferation and carcinogenesis? The biological activity of 17 β -estradiol is mediated mainly by its binding to two specific receptors: estrogen receptor alpha (ER α), the prevalent form in the breast, cardiovascular system and liver, and estrogen receptor beta (ER β), the prevalent form in the gastrointestinal tract^[6]. Both ER α and ER β exist in colorectal cancer cells^[7].

Various proteases are expressed in cancer progression and metastasis^[8]. The systems primarily responsible for extracellular matrix (ECM) degradation *in vivo* are matrix metalloproteinase (MMP) and plasminogen activator (PA) systems^[9]. MMPs are a family of functionally related zinc-containing enzymes that include interstitial collagenases, gelatinases, metalloelastase and membrane-type MMPs^[10,11]. The gelatinases MMP-2 and MMP-9 have been implicated in colorectal cancer progression and metastasis in animal models and patients^[12]. In the proteolytic plasminogen system, the up-regulation of urokinase-type plasminogen activators (u-PAs) and tissue-type plasminogen activators (t-PAs) has been shown to activate MMPs and is involved in colon cancer progression^[13,14]. In addition, a mutation in the adenomatous polyposis coli (APC) tumor suppressor gene occurs in most colorectal tumors, resulting in the accumulation of β -catenin due to reduced ubiquitin-mediated proteolysis, which may play a causal role in promoting carcinogenesis^[15,16]. The current results indicate that the accumulation of nuclear β -catenin can be used as a prognostic marker in patients with stage IIA colon cancer^[17].

The p53 tumor suppressor gene mediates many cellular processes, including cell cycle regulation, DNA repair, differentiation and apoptosis, in response to various extracellular and intracellular signals^[18,19]. In contrast, it is well known that p53 mutations contribute to the malignant progression of colorectal cancer and resistance to anticancer therapy^[20-22]. Interestingly, the precise anti-metastasis mechanisms underlying the protective effects of 17 β -estradiol/ERs on colorectal cancer *via* the p53 tumor suppressor protein remain unclear. This study examines the effects of 17 β -estradiol and/or ER agonists on the regulation of cell proliferation and migration in human LoVo colorectal cancer cells. The roles of p53 and the precise molecular mechanisms behind this protective property are identified.

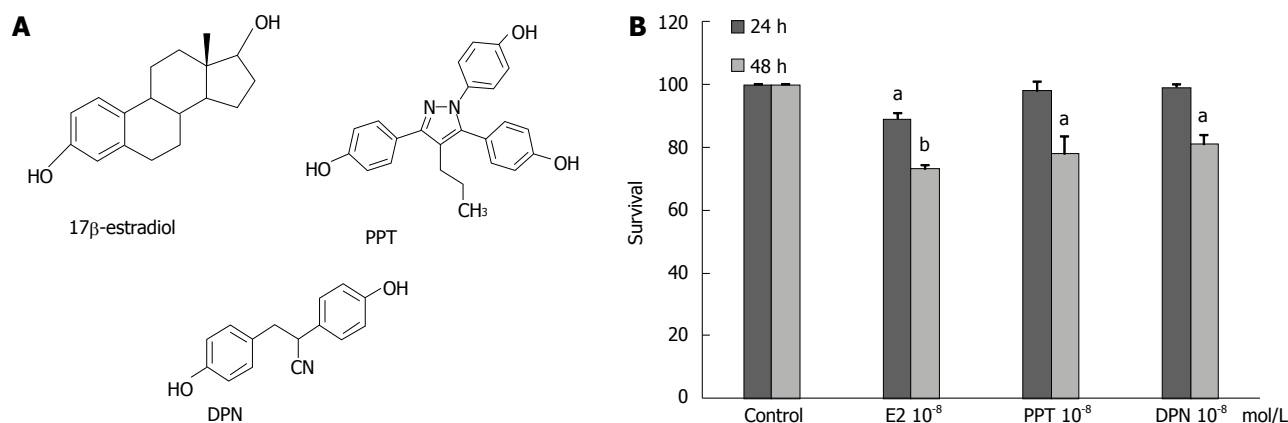


Figure 1 Estradiol and ER selective agonists impair cell proliferation in human LoVo cells. A: Structures of 17 β -estradiol, propylpyrazole-triol (PPT), a selective agonist of ER α , and diarylpropionitrile (DPN), a selective agonist of ER β ; B: LoVo cells cultured in DMEM were treated with 17 β -estradiol, PPT and DPN for 24 h and 48 h and were then analyzed for cell viability by the MTT assay. ^a $P < 0.05$ vs control; ^b $P < 0.001$ vs control (means \pm SE, $n = 3$).

MATERIALS AND METHODS

Cell, chemicals and materials

The human colon cancer cell line LoVo was obtained from the Bioresource Collection and Research Center (BCRC). LoVo cells were established from a metastatic nodule resected from a 56-year-old Caucasian male colon adenocarcinoma patient.

The following reagents were used for experiment: 17 β -estradiol (E2) (Sigma, Louis), an ER α -selective agonist [propylpyrazole-triol (PPT)], an ER β -selective agonist [diarylpropionitrile (DPN)] (Figure 1A), an ER α -selective antagonist [methyl-piperidinopyrazoledihydrochloride (MPP)], an ER β -selective antagonist 4-[2-Phenyl-5,7-*bis* (trifluoromethyl) pyrazolo [1,5-*a*] pyrimidin-3-yl] phenol (PHTPP), the ER antagonist ICI 182780 (ICI) (all from TOCRIS), and the p53 inhibitor Pifithrin- α , *p*-Nitro, Cyclic (Merck).

Cell culture

LoVo colon cancer cells were cultured in phenol red-free DMEM (Sigma, United States) supplemented with 1.5 g/L sodium bicarbonate, 3.5 g/L glucose, 1% penicillin-streptomycin and 10% cosmic calf serum (Hyclone, United States) in a humidified atmosphere at 37 °C with 5% CO₂. The medium was changed to phenol red-free DMEM with 0% serum 4 h before the experiment was started.

Cell proliferation assay

To investigate the effects of E2 and/or ER selective agonists on cellular proliferation, LoVo colorectal cells were treated with E2 or ER-selective agonists for 24 h and 48 h and subjected to the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay. The blue formazan crystal absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay plate reader.

Western immunoblotting and antibodies

Total proteins were extracted using lysis buffer [50 mmol/L Tris-base, pH = 7.5, 0.5 mol/L NaCl, 1.0

mmol/L EDTA, pH = 7.5, 10% glycerol, 1 mmol/L β -Mercaptoethanol and a proteinase inhibitor cocktail (Roche Molecular Biochemicals)]. The cell lysate proteins were analyzed using SDS-PAGE. The following primary antibodies were used for incubations: MMP-9 (Chemicon), Cyclin D1 sc-246, β -Catenin sc-7963, GSK-3 β sc-9166, p53 sc-1311, u-PA sc-14019, t-PA sc-5239, α -Tubulin sc-5286, and β -actin sc-47778 (all from Santa Cruz Biotechnology). Following primary antibody incubations, membranes were incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG) (all from Santa Cruz Biotechnology).

Wound healing assay

LoVo cells were seeded into six-well plates at 1×10^5 cells/well in culture medium. Confluent monolayers were scratched with a sterile micro-pipette tip and then washed with PBS to remove floating cells in serum-free medium. Then, the cells were serum starved for 4 h. Wound healing was performed by treatment with E2, ER-selective agonists, a p53 inhibitor (10 μ mol/L), ER-selective antagonists or ICI182780 (1 μ mol/L), respectively. For a quantitative measure, we counted the number of migrating cells to the wound area post-wounding for 24 h.

Cell migration and invasion assays

The cell migration assay was carried out using a modified Boyden chamber consisting of a trans well chamber (Neuro Probe) plate with 8- μ m pore size polycarbonate membrane filters^[23]. Serum-deprived LoVo cells were added to the upper part of the Boyden chamber, and the bottom chamber was filled with DMEM containing 10% serum. After incubation for 48 h, the cells were allowed to migrate to the underside of the membrane. The cells on the membrane filter were then fixed with methanol and stained with 0.05% Giemsa (Sigma). The number of migrated cells was quantified by cell counting in at least three random fields (magnification, $\times 200$) per filter.

Gelatin zymography

LoVo cells cultured in DMEM were treated with E2 (10⁻⁸

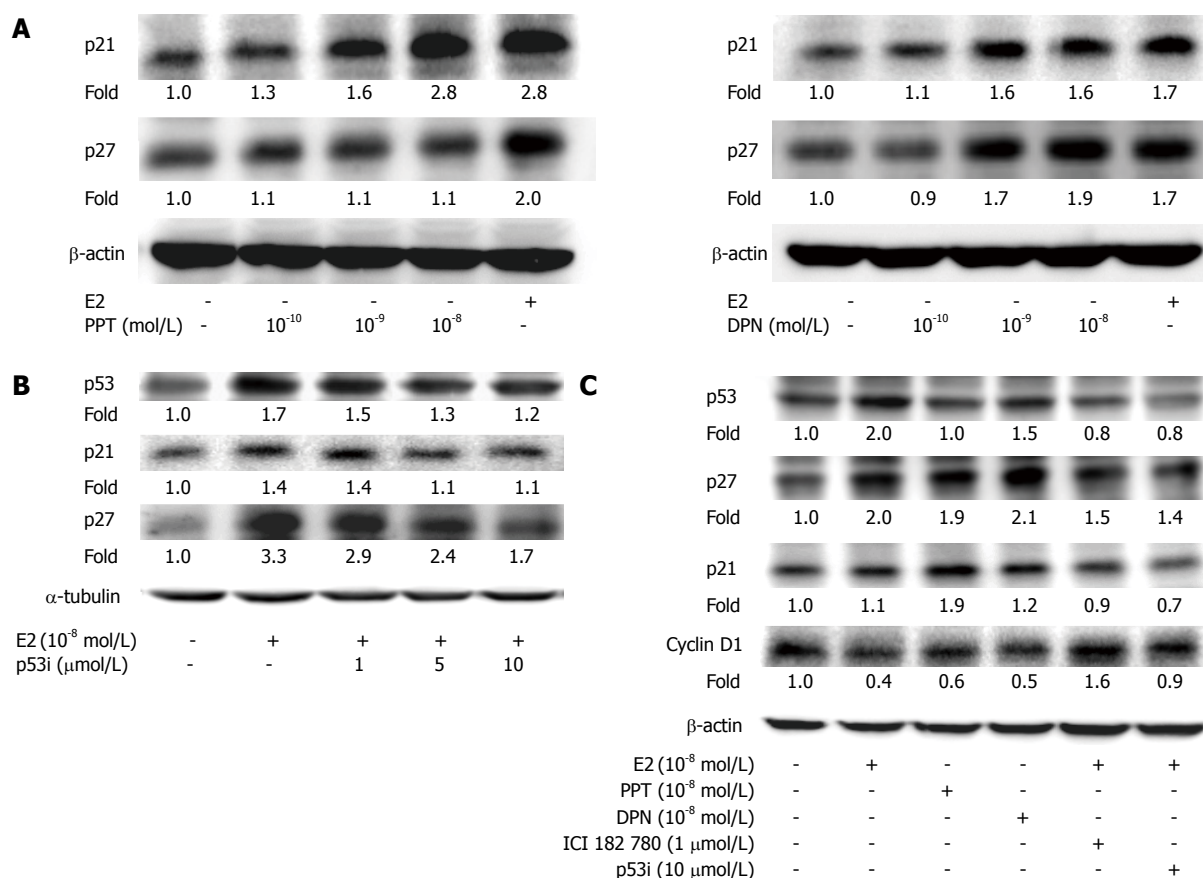


Figure 2 17 β -estradiol and/or ER selective agonists upregulate p53 signaling proteins to change the cell cycle progression in human LoVo colorectal cancer cells. A: LoVo cells were treated with E2 or various concentrations (10⁻¹⁰ mol/L, 10⁻⁹ mol/L and 10⁻⁸ mol/L) of PPT or DPN for 48 h; B: LoVo cells were pretreated with various concentrations (1 μmol/L, 5 μmol/L and 10 μmol/L) of a p53 inhibitor for 1 h followed by E2 (10⁻⁸ mol/L) treatment for 48 h; C: LoVo cells were pretreated with vehicle, the ER antagonist ICI 182780 (ICI), or a p53 inhibitor for 1 h, followed by E2 administration for 48 h. Subsequently, cells were harvested and measured by Western blotting. A β -actin standard was used as a loading control for all proteins. All experiments were repeated twice with identical results.

mol/L) for 24h and subsequently collected in conditional medium. Samples were electrophoresed without reduction (no DTT) on 8% SDS polyacrylamide gels copolymerized with 0.1% gelatin. When the tracking dye at the front reached the bottom of the gel, the gel was removed and shaken gently for 30 min in 2.5% Triton X-100 to remove SDS. The gels were then transferred to a bath (without Triton X-100) and washed for 30 min to remove Triton X-100. The gels were then incubated overnight (37 °C) in reaction buffer containing 40 mmol/L Tris-HCl (pH = 8.0), 0.01% NaCl and 10 mol/L CaCl₂. Finally, gelatinolytic activity was visualized by Coomassie blue staining.

Statistical analysis

Each experiment was repeated at least twice with identical results. The results are presented as means \pm SEs, and statistical comparisons were made using Student's *t*-test. Significance was presented as a *P* < 0.05 or *P* < 0.01.

RESULTS

Effects of 17 β -estradiol and ER selective agonist on cell proliferation and viability in human LoVo colorectal cancer cells

To determine the effects of E2 and ER-selective agonists

on the proliferation of human LoVo colorectal cancer cells, the structure was first compared with E2 and ER agonists. We then treated the LoVo cells with E2 and ER agonists (10⁻⁸ mol/L) for 24 h and 48 h and subsequently measured the cell viability using MTT assay. The results showed a significant reduction in LoVo colorectal cancer cell viability, with a reduction of approximately 28.0% following E2 treatment for 48 h, 21.0% following PPT treatment for 48 h and 15.8% following DPN treatment for 48 h (Figure 1B). We further examined the level of p53 signaling and downstream proteins through Western blotting. After LoVo cells were treated with E2 (10⁻⁸ mol/L) or various concentrations (10⁻¹⁰ mol/L, 10⁻⁹ mol/L and 10⁻⁸ mol/L) of PPT or DPN for 24 h, we observed a significant dose-dependent reduction in the expression of p21 and p27 (Figure 2A). In LoVo cells, administration of a p53 inhibitor (1 μmol/L, 5 μmol/L, 10 μmol/L) significantly inhibited the E2-induced activation of p53, p27 and p21 in a dose-dependent manner (Figure 2B). The serum-starved human LoVo colorectal cancer cells were pretreated with ICI or p53 inhibitor (1 μmol/L), which significantly inhibited the E2-induced increases in p53, p27 and p21 protein levels and blocked the E2-dependent reduction in the protein levels of cell cycle-regulating proteins such as cyclin D1. At the same time,

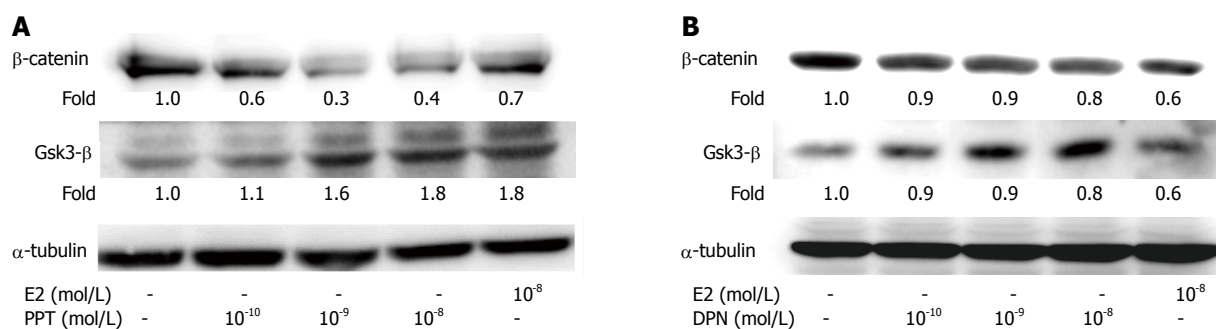


Figure 3 17 β -estradiol and ER selective agonists impair the Wnt- β -catenin signaling pathway in human LoVo colorectal cancer cells. LoVo cells were treated with E2 (10⁻⁸ mol/L) or various concentrations (10⁻¹⁰ mol/L, 10⁻⁹ mol/L and 10⁻⁸ mol/L) of PPT (A) or DPN for 24 h (B). Cells were harvested and measured by Western blotting. An α -tubulin standard was used as a loading control for all proteins. All experiments were repeated twice with identical results.

we observed that treatment with the ER agonists PPT or DPN alone also had the same effect as E2 treatment on human LoVo cells (Figure 2C). These results suggest that the administration of 17 β -estradiol or ER agonist can inhibit cell proliferation in human LoVo cells by modulating p53 signaling and its downstream cell cycle target gene, cyclin D1.

Suppression of the Wnt- β -catenin signaling pathway by E2 and ER-selective agonists in human LoVo colorectal cancer cells

After LoVo cells were treated with various concentrations (10⁻¹⁰ mol/L, 10⁻⁹ mol/L and 10⁻⁸ mol/L) of PPT or DPN for 24 h, the level of the Wnt- β -catenin complex protein was investigated. The GSK-3 β protein content was dramatically increased, and the β -catenin level was decreased in a dose-dependent manner (Figure 3). These results might indicate that E2 or ER agonist treatment can inhibit colorectal cancer cells growth *via* Wnt/wingless signaling suppression.

17 β -estradiol and/or ER selective agonists down-regulate the u-PA, t-PA and MMP-9 protein levels in LoVo cells

It is known that proteolytic plasminogen system activation by u-PA and t-PA is involved in the up-regulation of downstream MMPs in cancer cells^[24]. We therefore further examined the cellular migration-regulating factors u-PA, t-PA and MMP-9 in human LoVo cells. In our studies, we observed that a significant reduction in u-PA, t-PA, MMP-2 and MMP-9 protein expression was induced by E2 (10⁻⁸ mol/L) treatment within 24 h. Subsequently, the quantitative results showed that PPT (10⁻¹⁰ mol/L, 10⁻⁹ mol/L and 10⁻⁸ mol/L) treatment alone also significantly reduced the u-PA, t-PA and MMP-9 levels. DPN (10⁻¹⁰ mol/L, 10⁻⁹ mol/L and 10⁻⁸ mol/L) treatment alone reduced the u-PA and MMP-9 levels in a dose-dependent manner (Figure 4A). Administration of a p53 inhibitor (1 μ mol/L, 5 μ mol/L, 10 μ mol/L) to LoVo cells significantly blocked the E2-induced inhibition of u-PA and t-PA in a dose-dependent manner (Figure 4B). The serum-starved human LoVo colorectal cancer cells that were pretreated with ICI or p53 inhibitor (1 μ mol/L)

significantly blocked the E2-dependent reduction in t-PA protein levels (Figure 4C). Therefore, these results suggest that 17 β -estradiol or ER agonist administration can inhibit cellular migration-regulating factors, including u-PA, t-PA and MMP-9, in a p53-dependent manner.

17 β -estradiol inhibits MMP-2/9 activities in LoVo colorectal colon cells

It is known that MMPs function as proteases in extracellular matrix protein degradation^[25]. Thus, MMP activities play important roles in cell cycle regulation. We therefore examined whether E2 can suppress MMP-2/9 expression and activity in LoVo cells. In Figure 5, gel images from gelatin zymography show that MMP-2 (72 kDa) and MMP-9 (92 kDa) activities were significantly suppressed in LoVo cells treated with E2.

17 β -estradiol and/or ER agonists inhibit human LoVo colorectal cancer cell motility through the tumor suppressor gene p53

We observed that the migration ability of LoVo cells could be inhibited after E2 (10⁻⁸ mol/L) treatment. We further determined that E2 and/or ER agonists played a role in LoVo cell migration ability. We then cultured LoVo cells with E2 (10⁻⁸ mol/L) in the presence or absence of ICI 182780, a p53 inhibitor, ER α antagonists (MPP) or ER β antagonists (PHTPP). We also treated LoVo cells with PPT or DPN (10⁻⁸ mol/L), which are ER agonists, alone for 48 h. We observed the migration ability in LoVo cells using scratch motility and migration assays. In the scratch motility (Figure 6A) and migration assays (Figure 6B), we observed that ICI 182780 and the p53 inhibitor dramatically blocked the inhibitory effects of E2 on cellular migration in LoVo cells. In addition, treatment with either of the ER agonists, *i.e.*, PPT or DPN, alone also significantly inhibited LoVo colorectal cancer cell migration by approximately 24% and 39%, respectively. Both ER α and ER β are involved in down regulating cellular mobility in human LoVo colorectal cancer cells. These findings showed that p53 mediates the E2-ER-modulated migration ability of human LoVo cells and that ER agonist treatment alone has the same ability to inhibit LoVo cell migration.

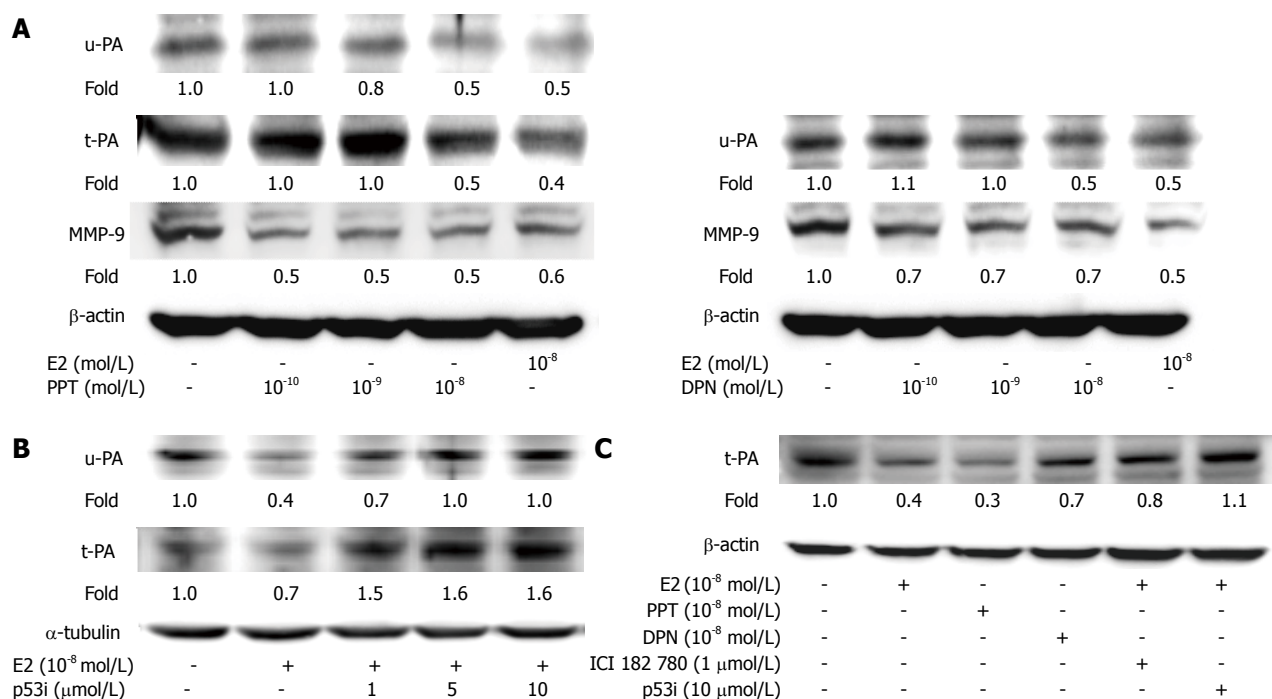


Figure 4 17 β -estradiol and/or ER selective agonists down-regulate the protein levels of u-PA, t-PA and MMP-9 in LoVo cells *via* p53. A: LoVo cells were treated with E2 or various concentrations (10^{-10} mol/L, 10^{-9} mol/L and 10^{-8} mol/L) of PPT or DPN for 24 h; B: LoVo cells were pretreated with various concentrations (10^{-10} mol/L, 10^{-9} mol/L and 10^{-8} mol/L) of a p53 inhibitor for 1 h, followed by E2 administration for 24 h; C: LoVo cells were pretreated with vehicle, the ER antagonist ICI 182780, or a p53 inhibitor for 1 h, followed by E2 administration for 24 h. Cells were harvested and examined by Western blotting. A β -actin or α -tubulin standard was used as a loading control for all proteins. All experiments were repeated twice with identical results.

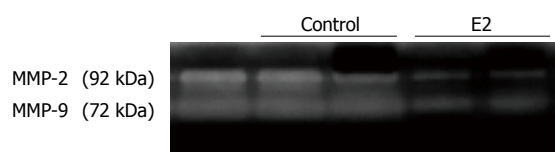


Figure 5 17 β -estradiol inhibits MMP-2/9 activity in LoVo colorectal colon cells. LoVo cells cultured in DMEM were treated with E2 (10^{-8} mol/L) for 24 h, and then, the culture medium was collected. Samples were electrophoresed without reduction on 8% SDS polyacrylamide gels copolymerized with 0.1% gelatin.

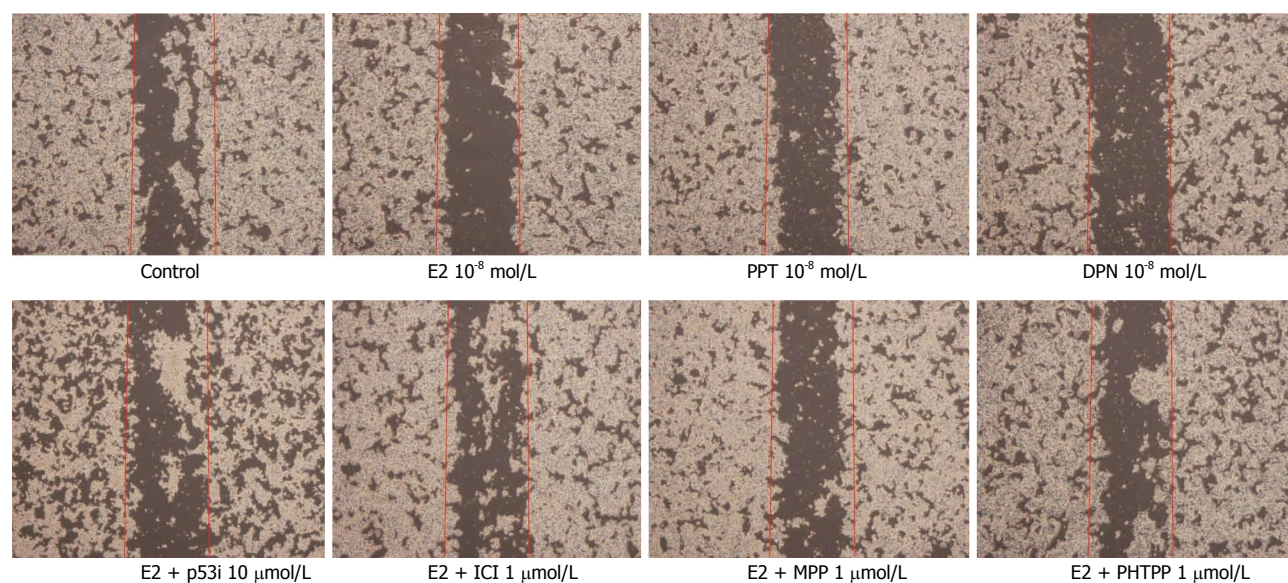
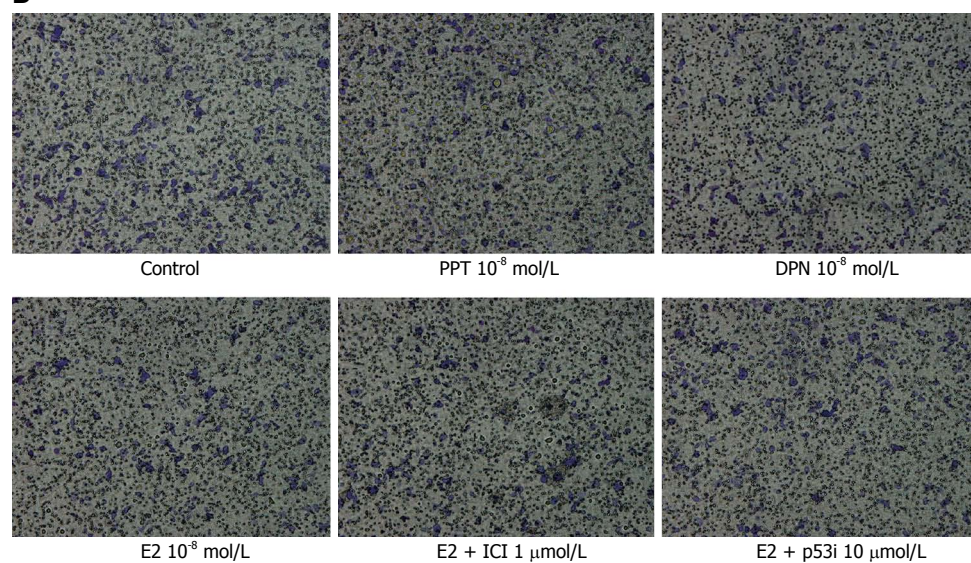
DISCUSSION

Previous studies have shown that E2 binding to ERs can regulate tissue and cellular responses *via* multiple signaling pathways^[26]. The ERs act at estrogen-responsive target genes to either trans-activate or trans-repress gene expression^[27]. In our previous studies, we found that over-expressed ER α induces apoptosis and inhibits the proliferation of human LoVo colorectal cancer cells^[28]. The apoptotic effects of over-expressed ER β acted in a ligand-dependent manner^[29]. These published results provide evidence that E2 and/or the over-expression of ERs plays a critical role in inducing the apoptosis of human LoVo colorectal cancer cells. Here, we were interested in further determining the anti-motility effects of the ER α - and ER β -selective agonists PPT and DPN on LoVo colorectal cancer cells.

The major findings of this study can be summarized as follows: (1) treatment with E2 and/or ER agonists (10^{-10} mol/L, 10^{-9} mol/L and 10^{-8} mol/L) significantly

inhibits human LoVo colon cancer cell proliferation by increasing p53, p21 and p27 protein levels and decreasing the expression of the downstream target gene cyclin D1. These results suggest that E2 and/or ER agonists (10^{-8} mol/L) greatly suppress cell proliferation by activating the p53 signaling pathway and modulating cell cycle-regulating factors in human LoVo cells; (2) the motility of LoVo colorectal cancer cells is significantly suppressed by E2 and/or ER agonist treatment. We simultaneously observed that the decrease in the migratory ability of LoVo cells due to treatment with E2 (10^{-8} mol/L) and/or ER agonists (10^{-10} mol/L, 10^{-9} mol/L and 10^{-8} mol/L) was accompanied by the down regulation of migration-related proteins, including u-PA, t-PA and MMP-9, as well as the suppression of MMP-2/9 activities (Figure 5); and (3) the inhibitory effects of u-PA, t-PA and MMP-9 in human LoVo cells that had been treated with E2 and/or ER agonists were completely inhibited by a p53 inhibitor. These results demonstrate that E2 and/or ER agonists down regulate migration-related proteins through the p53 signaling pathway in human LoVo colorectal cancer cells. These findings suggest that p53 plays a critical role in the E2- and/or ER agonist-mediated protective activity against colorectal cancer progression.

Changes in the expression of cell cycle regulators, such as cyclin D1, are a critical step in tumor development and progression, which are the most critical events in colorectal cancer^[30]. The cell cycle regulator Cyclin D1 is a key intracellular regulator that is involved in cell cycle progression through the G1 phase and is over-expressed in colorectal carcinoma, leading to a worse prognosis^[31].

A

B


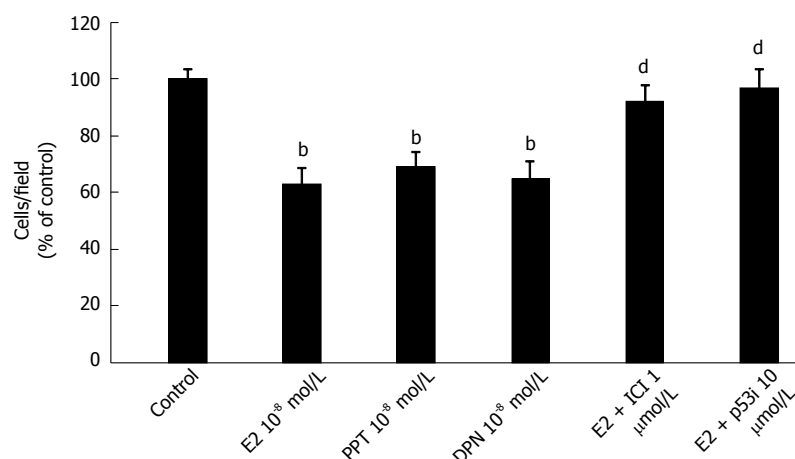


Figure 6 17 β -estradiol and/or ER selective agonists inhibit human LoVo colorectal cancer cell motility through p53 signaling. LoVo cells cultured in DMEM were pretreated with vehicle, ICI 182780 (ER antagonist 1 μ mol/L), p53 inhibitor (10 μ mol/L), MPP (ER α antagonist 1 μ mol/L), or PHTPP (ER β antagonist 1 μ mol/L) for 1 h, followed by treatment with E2 (10⁻⁸ mol/L), PPT (10⁻⁸ mol/L) or DPN (10⁻⁸ mol/L) administration for 48h. The migration ability of LoVo cells was analyzed by a scratch motility assay (A) and a migration assay (B). The results were observed and analyzed with a fluorescence microscope. All experiments were repeated twice with identical results. ^a*P* < 0.05 vs control; ^b*P* < 0.01 vs control; ^d*P* < 0.01 vs E2.

In our study, we observed that cell proliferation and the expression of cyclin D1 in LoVo cells was significantly inhibited by E2 and/or ER agonist treatment. These findings suggest that E2 and/or ER agonists may protect against colorectal cancer proliferation by modulating cell cycle regulators.

Many studies have reported that increased MMP levels contribute to ECM remodeling and tumor cell motility, thus leading to the progression of malignant tumors^[8,12]. Activation of the plasminogen activator system, which includes u-PA and t-PA, is reported to be involved in MMP activation and colorectal cancer development. Expression of u-PA and t-PA is considered a marker of malignant colon cancer^[13,14,32,33]. Here, we found that the administration of E2 and/or ER agonists dramatically inhibited cell migration and reduced the expression of u-PA, t-PA and MMP-9 as well as MMP-2/9 activity in LoVo cells. Moreover, we observed that pretreatment with a p53 inhibitor significantly blocked the anti-migration effects of E2 and/or ER agonists on LoVo cells. These findings suggest that E2 and/or ER agonists may impair LoVo cell migration by modulating migration-related factors *via* the p53 tumor suppressor gene.

Taken together, our results suggest that the tumor suppression protein p53 may mediate downstream signaling when E2 binds to ERs and, further, that it modulates E2-mediated anti-tumorigenic properties by inhibiting the expression of u-PA, t-PA and MMP-9. In addition, ER agonists directly activate ER α or ER β Anti-migration effects were observed after E2 treatment of human LoVo colorectal cancer cells. These results show that direct ER treatment may prove to be an attractive alternative therapy in the treatment of human colorectal tumors in the future.

and colon/rectum in men and cancers of the lung/bronchus, breast and colon/rectum in women continue to be the most common cancers in the United States. Colorectal cancer is the third most common cause of cancer death.

Innovations and breakthroughs

This study examines the effects of 17 β -estradiol and/or ER agonists on the regulation of cell proliferation and migration in human LoVo colorectal cancer cells. The roles of p53 and the precise molecular mechanisms behind this protective property are identified.

Applications

ER agonists directly activate ER α or ER β Anti-migration effects were observed after E2 treatment of human LoVo colorectal cancer cells. These results show that direct ER treatment may prove to be an attractive alternative therapy in the treatment of human colorectal tumors in the future.

Peer review

The authors mainly focus on to explore whether estrogen or estradiol agonists inhibit human LoVo colorectal-cancer cell proliferation and migration through p53. Their findings showed that treatment with 17 β -estradiol and/or ER agonists in human LoVo colorectal cancer cells activated p53 and then up-regulated p21 and p27 protein levels, subsequently inhibiting the downstream target gene, cyclin D1, which regulates cell proliferation.

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COMMENTS

Background

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Glytan decreases portal pressure *via* mesentery vasoconstriction in portal hypertensive rats

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Author contributions: Du QH and Han L contributed equally to this work and are co-first authors in this study; Du QH, Han L, Li PT and Li WH designed the research; Du QH, Han L, Jiang JJ, Xu Y and Jia X performed the research; Wang XY contributed new analytic tools; Du QH, Han L and Li WH analyzed the data; and Du QH and Han L wrote the paper.

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β -arrestin 2 expression in the mesentery. The mRNA of ETAR and ETBR was determined using real-time polymerase chain reaction.

RESULTS: Treatment with Glytan reduced portal pressure (PP) and portal territory blood flow (PTBF) and increased both mean arterial pressure (MAP) and splanchnic vascular resistance (SVR). Especially at 4 wk, PP decreased by about 40%, while MAP increased by 13%, SVR increased by 12%, and PTBF decreased by about 21%. The effect of blood flow reduction was greatest in the mesentery (about 33%) at 4 wk. The mesenteric circulation ET-1 levels of BDL rats were lower and negatively correlated with PP at 4 wk. Glytan can increase mesenteric ET-1 content and inhibit ETBR, eNOS, GRK2, and β -arrestin 2 expression in the mesentery. Moreover, Glytan showed no effect on the expression of ETAR protein and mRNA.

CONCLUSION: The decreased PP and PTBF observed after Glytan treatment were related to increased mesenteric vasoconstriction and increased receptor sensitivity to vasoconstrictor.

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Abstract

AIM: To investigate the effects of Glytan on splanchnic hemodynamics and its reduction of portal pressure in portal hypertensive rats.

METHODS: Glytan (Ganluotong in Chinese), is composed of salvianolic acid B and diammonium glycyrrhizinate. Portal hypertension (PHT) was induced in the rats by common bile duct ligation (BDL). Hemodynamic studies were performed using the colored microsphere method. Radioimmunoassay (RIA) was used to determine endothelin (ET)-1 levels in the mesenteric circulation. Western blotting methods were used to investigate the effect of Glytan on ET A receptor (ETAR), ET B receptor (ETBR), endothelial NO synthase (eNOS), G-protein-coupled receptor kinase (GRK)2, and

Key words: Glytan; Portal hypertension; Hemodynamics; Mesentery; Endothelin-1; Receptor; Sensitivity

Core tip: The traditional Chinese medicine Glytan is composed of salvianolic acid B and diammonium glycyrrhizinate. Previous studies have shown that Glytan is a new preparation for portal hypertension. The present study indicated that decreases in portal pressure and portal territory blood flow observed after Glytan treatment in portal hypertensive rats were related to increased mesenteric endothelin-1 content and reduced endothelin B receptor, endothelial NO synthase, G-protein-coupled receptor kinase 2, and β -arrestin 2 expression, which may promote mesenteric vasoconstriction and increase receptor sensitivity to vasoconstrictors.

These results suggest the therapeutic potential of Glytan in portal hypertension induced by liver cirrhosis.

Du QH, Han L, Jiang JJ, Xu Y, Li WH, Li PT, Wang XY, Jia X. Glytan decreases portal pressure *via* mesentery vasoconstriction in portal hypertensive rats. *World J Gastroenterol* 2014; 20(44): 16674-16682 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16674.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16674>

INTRODUCTION

Since portal hypertension (PHT) was first proposed, finding a perfect medicine for PHT to reduce the risk of bleeding from esophageal varices has been the focus in this field. Non-selective β -receptor blockers have been considered the only drugs suitable for long-term administration. However, only 30%-40% of patients achieve a good therapeutic outcome with non-selective β -receptor blockers, because of their contraindications or side effects^[1].

Glytan, which is based on traditional Chinese medicine theory, is a new preparation for PHT. Glytan is composed of salvianolic acid B (SA-B) and diammonium glycyrrhizinate (DG). SA-B is one of the water-soluble compounds derived from *Salvia miltiorrhiza* Bunge (Danshen in Chinese), which is widely used for chronic liver diseases. DG is extracted and purified from liquorices (Gancao in Chinese). The liquorices exert an important function in the treatment of hepatitis because of their anti-inflammatory effects. Our previous work found that Glytan can reduce portal pressure (PP), improve liver function, and inhibit pseudobulb formation in rats with liver cirrhosis^[2]. Notably, the compatibility of SA-B and DG inhibits the steroid-like effects of DG^[2]. Based on its efficacy in decreasing PP, Glytan has been approved to begin stage II clinical trials for PHT treatment in China.

In accordance with Ohm's law, PP depends on intrahepatic resistance and portal inflow. In cases of cirrhosis, both intrahepatic resistance and splanchnic blood flow are increased. The initiating factor is an increase in intrahepatic vascular resistance, whereas the increase in splanchnic blood flow is a secondary phenomenon that maintains or worsens the increased PP and gives rise to hyperdynamic circulation^[3].

Hyperdynamic circulation is characteristic of progressive splanchnic vasodilatation^[4]. In previous work from our laboratory, we observed that treatment with Glytan resulted in reduced mesenteric vasodilatation. Thus, one aim of the present study was to verify whether this phenomenon was related to a decrease in PP.

A previous study demonstrated that endothelin (ET)-1 and ET B receptor (ETBR) may be one of the mechanisms of splanchnic vasodilatation^[5]. In addition, the effects of ET-1 receptors are also affected by their responsiveness. ET receptors could be desensitized by

phosphorylation through G-protein-coupled receptor kinases (GRKs) and binding to β -arrestin-2^[6]. Thus far, seven types of GRKs have been cloned. GRK2 is the most likely of the GRKs to initiate the desensitization of human ETAR and ETBR^[7]. ET signaling in arterial smooth muscle is tightly regulated by GRK2^[8]. ETAR-bound β -arrestin 2 demonstrates a higher affinity than β -arrestin 1 and does not interact with visual arrestin^[9]. Therefore, we investigated the effect of Glytan on ET-1 and its receptors to investigate its mechanism of decreasing PP.

MATERIALS AND METHODS

Animal models

Male Sprague-Dawley rats (about 250 g; Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) underwent sham surgery or common bile duct ligation (BDL). The common bile ducts were exposed after median laparotomy and ligated twice. In each animal, the segment between the two ligations was resected, and the abdomen of the animal was sutured closed. Sham-operated rats served as controls. In these rats, the common bile duct was exposed, but no ligation or resection was performed. Seven animals were used in each group. All experimental procedures were conducted in accordance with the guidelines for the use of experimental animals and were approved by the Institutional Review Committee on Animal Care and Use at the Experimental Animal Centre of Beijing University of Chinese Medicine [Certificate of Conformity: SCXK (jing) 2012-0001].

The rats were divided into the sham, BDL and Glytan groups. Each group was examined at 2 and 4 wk. Glytan was provided by Beijing Huaxin Wanbang Medicine Technology Limited Company. The proportion of SA-B to DG in Glytan was 1:1. Before use, Glytan was diluted with distilled water. After 1 wk BDL, the Glytan group was treated with Glytan (25 mg/kg per day) by gavage. The dose of Glytan depended on the pharmacodynamic experimental results^[2]. Rats in the sham and BDL groups were administered the same amount of distilled water by gavage.

Hemodynamic studies

After 2 or 4 wk following BDL, hemodynamic studies were performed under chloral hydrate anaesthesia (3.5 g/kg intraperitoneal injection). The left femoral artery and vein were cannulated with PE-50 catheters for the measurement of mean arterial pressure (MAP) and blood withdrawal. A PE-50 catheter was inserted into the portal vein to measure PP. Another PE-50 catheter was advanced *via* the right carotid artery into the left ventricle under pulse curve control. This catheter was used for microsphere application. The catheter in the femoral artery and portal vein was connected to a pressure transducer for blood pressure measurements.

A reference sample was obtained for 1 min at a rate of 0.65 mL/min using a continuous withdrawal pump;

300000 yellow microspheres (15 μm in diameter; Triton Technologies, San Diego, CA, United States) were suspended in 0.3 mL saline containing 0.05% Tween. Microspheres were injected into the left ventricle 10 s after the withdrawal pump had been started. Upon completion of the hemodynamic measurements, the animals were sacrificed and the stomach, intestine, colon, pancreas, mesentery, and spleen were resected. The tissues were weighed and minced with scissors. The tissue and blood samples were processed and the microspheres were recovered by sedimentation. The tissue samples and the blood reference were digested by the addition of alkaline digestion reagent containing 1 mol/L KOH. The tubes from the tissue and blood preparation steps were placed in a temperature-controlled laboratory oven, set to a maximum of 50 $^{\circ}\text{C}$, and the tissue and blood samples were allowed to digest overnight. The digested tissue and blood samples were centrifuged. The color from the filtered microspheres was dissolved in 0.2 mL dimethylformamide (DMF), and the absorption was measured by spectrophotometry.

Calculations

The hemodynamic parameters for portal circulation were calculated as follows. Portal territory blood flow (PTBF) was defined as the sum of the blood flow through the stomach, intestine, colon, pancreas, spleen, and mesentery. Splanchnic perfusion pressure was defined as the difference between MAP and PP. Splanchnic vascular resistance (SVR) was calculated from the ratio between splanchnic perfusion pressure and PTBF.

RIA of ET-1

Blood was drawn from the superior mesenteric artery (SMA) for ET-1 analysis. The level of ET-1 was measured in the mesenteric circulation using a commercial RIA kit (PLA Institute of RIA, Beijing, China) according to the manufacturer's protocol.

Real-time quantitative polymerase chain reaction (PCR)

The mesentery was isolated and stored in liquid nitrogen for RNA detection by real-time PCR. Total RNA was isolated according to the TRIzol reagent instructions (Invitrogen, Carlsbad, CA, United States). The primers of ETAR and ETBR were designed by Primer Premier 5.0 with amplification products of 205, 212, and 183 bp. For ETAR, the upstream primer was 5'-GGT TCC CTC TTC ACT TAA GC-3' and the downstream primer was 5'-GTG ACA ACA GCA ACA GAG G-3'. For ETBR, the upstream primer was 5'-GAG CAA TCC TCA GAG GTG T-3' and the downstream primer was 5'-GAC TGT TTT TCC TCA AAC GTT-3'. For β -actin, the upstream primer was 5'-AAC GAG CGG TTC CGA TGC CCT GAG-3' and the downstream primer was 5'-TGT CGC CTT CAC CGT TCC AGTT-3'. β -Actin, an internal housekeeping gene, was used to normalize the differences in RNA isolation and RNA degradation. The abundance of mRNA was determined by real-time PCR. Real-

time PCR was performed using the ABI 7700 sequence detector (Applied Biosystems, Foster City, CA, United States). PCR was performed in a volume of 25 μL SYBR Mix (Hangzhou Bioer Technology Co. Ltd.) containing 2 μL cDNA. The results are expressed as the number of cycles required to exceed a threshold (Ct value) at which the fluorescence signal exceeded a defined threshold. The difference in Ct values of the target gene and the endogenous control are expressed as negative ΔCt values. Therefore, higher $2^{-\Delta\text{Ct}}$ values denote higher mRNA levels.

Western blot analysis

For western blot analysis, samples of rat mesentery were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 20 mmol/L pepstatin A, 20 mmol/L leupeptin, and 1 mmol/L phenylmethanesulfonyl fluoride. The protein content of the cleared homogenates was assessed with a bicinchoninic acid (BCA) assay kit (Applygen, Beijing, China). After boiling with SDS sample buffer (Applygen), 50 μg protein per lane from each sample was subjected to SDS-PAGE (10% gels for GRK2 and 12.5% gels for ETAR, ETBR and β -arrestin 2). After blotting on polyvinylidene difluoride membrane (Millipore, Bedford, MA, United States), the membranes were probed with primary antibodies diluted in TBS containing blocking protein and 0.1% Tween, and left to incubate overnight at 4 $^{\circ}\text{C}$. The following primary antibodies in the indicated dilutions were used: mouse anti-GRK2, 1:500 (Abcam, Cambridge, MA, United States); and rabbit anti-ETAR/ETBR/endothelial NO synthase (eNOS) and mouse anti- β -arrestin 2, 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, United States). Thereafter, the membranes were washed and incubated with the appropriate peroxidase-coupled secondary antibodies diluted 1:5000 in TBS containing blocking protein and 0.1% Tween for 45 min (goat anti-rabbit or goat anti-mouse; Jackson, West Grove, PA, United States). Detection was performed with enhanced chemiluminescence (Applygen).

Statistical analysis

All data are presented as the mean \pm SD; statistical comparisons were performed using one-way analysis of variance. The results of the molecular assays represent the means of samples from at least five rats in each group. $P < 0.05$ was considered statistically significant.

RESULTS

Hemodynamic effects of Glytan

To assess whether Glytan can decrease PP and to determine its effects on splanchnic hemodynamics, we tested PP, MAP and various splanchnic hemodynamic parameters. The BDL rats showed increased PP and decreased MAP at 2 and 4 wk (Figure 1A and B). With the

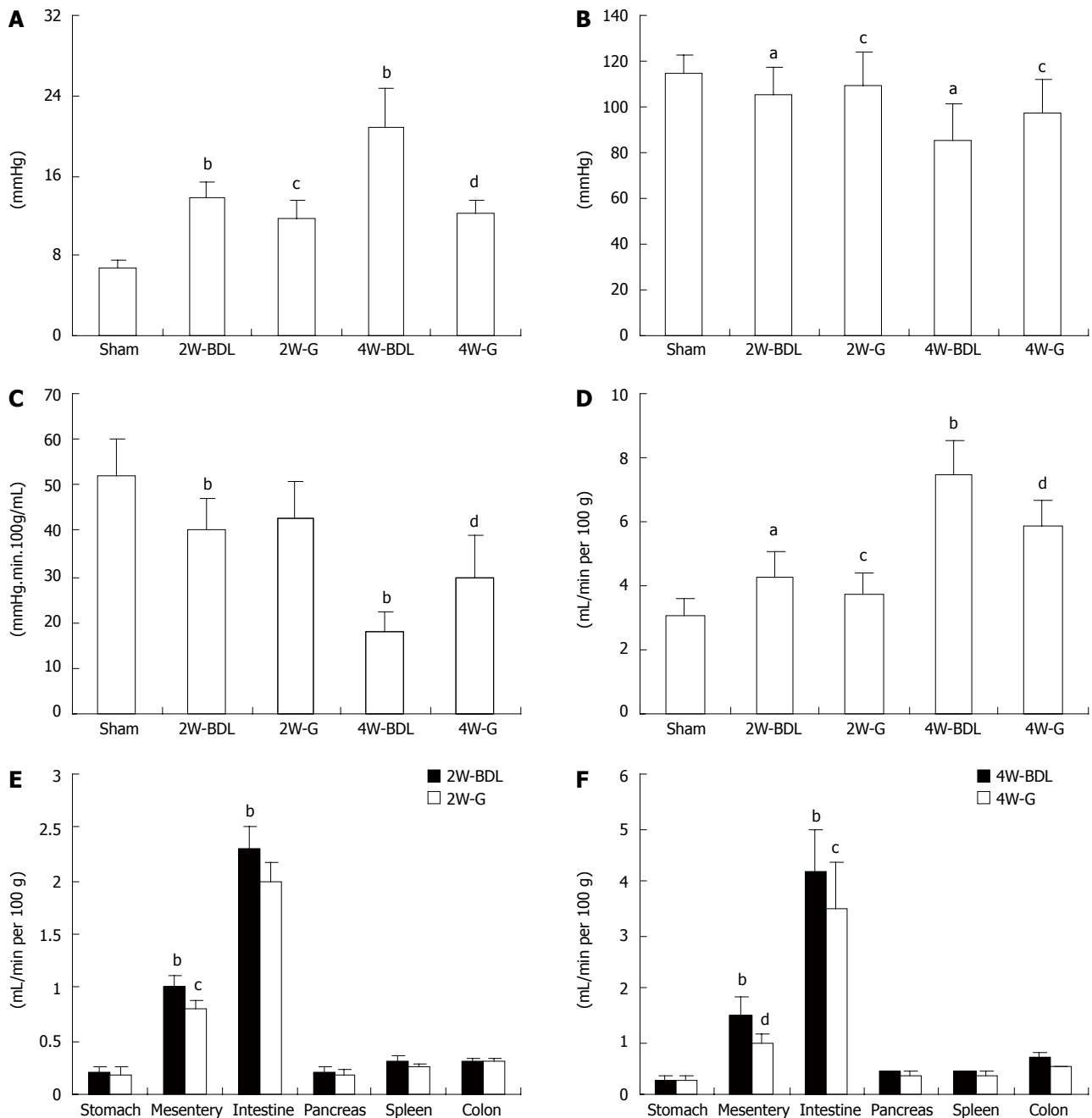


Figure 1 Effect of Glytan on splanchnic hemodynamics. A: Effect of Glytan on portal pressure; B: Effect of Glytan on mean arterial pressure; C: Effect of Glytan on splanchnic vascular resistance; D: Effect of Glytan on portal territory blood flow; E: Effect of Glytan on the blood flow of splanchnic organs at 2 wk; F: Effect of Glytan on the blood flow of splanchnic organs at 4 wk. Results are from experiments on seven sham-operated rats and seven bile duct ligation (BDL) rats. Mean \pm SEM values are shown. ^a $P < 0.05$, ^b $P < 0.01$, BDL rats vs sham-operated rats; ^c $P < 0.05$, ^d $P < 0.01$, rats in the Glytan group vs BDL rats. G: Glytan.

progression of PHT, characteristic splanchnic hemodynamic abnormalities appeared, including decreased SVR and increased PTBF (Figure 1C and D). Treatment with Glytan reduced PP and PTBF, while it increased MAP and SVR (Figure 1). Especially at 4 wk, PP was decreased by about 40%, while MAP increased by 13%, SVR increased by 12%, and PTBF decreased by about 21%. To determine which organs demonstrated decreased blood flow, we tested the blood flow of various organs, including the stomach, intestine, colon, pancreas, spleen, and mesentery. The blood flow of the intestine and mesentery decreased significantly at 4 wk, by 16% and 33%,

respectively (Figure 1F). We investigated the mechanism behind the mesenteric-blood-flow-reducing properties of Glytan.

Effect of Glytan on ET-1 levels in the mesenteric circulation

The concentration of ET-1 in the mesenteric circulation was measured by an RIA method (Figure 2). At 2 and 4 wk, ET-1 levels in the mesenteric circulation of BDL rats were significantly lower than those in sham group rats. After treatment with Glytan, ET-1 content was obviously increased at 4 wk.

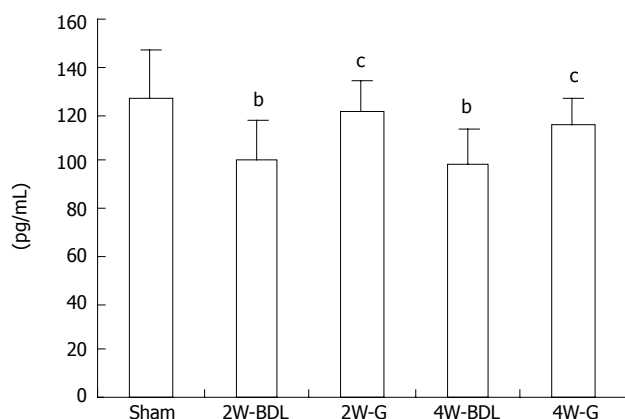


Figure 2 Effect of Glytan on ET-1 levels in the mesenteric circulation. The results are from experiments on seven sham-operated rats and seven bile duct ligation (BDL) rats. Mean \pm SEM values are shown. ^b $P < 0.01$, BDL rats vs sham-operated rats; ^c $P < 0.05$, rats in the Glytan group vs BDL rats.

Correlation analysis of PP and ET-1 levels in the mesenteric circulation

There was a significant negative correlation between PP and ET-1 levels in the mesenteric circulation at 4 wk (Figure 3). This correlation was not significant at 2 wk.

Effect of Glytan on ETAR and ETBR expression in the mesentery

The effect of ET-1 on vascular tone depends on the receptor type. The protein and mRNA trends of ETBR in BDL rats differed (Figure 4A and B). ETBR mRNA was higher at 2 wk than that at 4 wk, while ETBR protein expression increased constantly over the two time points. Treatment with Glytan resulted in post-transcriptional and mRNA downregulation of ETBR expression in the mesentery (Figure 4). However, treatment with Glytan had no effect on ETAR expression at protein and mRNA levels (Figure 4).

Effect of Glytan on eNOS, β -arrestin 2, and GRK2 expression in the mesentery

Expression of eNOS always implies the degree of vasodilation. β -arrestin 2 and GRK2 both affect ET receptor responsiveness. Treatment with Glytan significantly reduced eNOS and GRK2 expression at 2 and 4 wk. Glytan downregulated β -arrestin 2 expression at 4 wk (Figure 5).

DISCUSSION

We examined the effect of the Chinese medicine Glytan on splanchnic hemodynamics and the mechanisms of its effects. The results indicate that Glytan reduced PP and PTBF, while it increased MAP and SVR. The mesentery was the target organ of Glytan. On the one hand, Glytan increased ET-1 levels to promote vasoconstriction. On the other hand, Glytan inhibited mesenteric eNOS, ETBR and GRK2 expression to inhibit vasodilation.

In this study, the proportion of SA-B and DG was

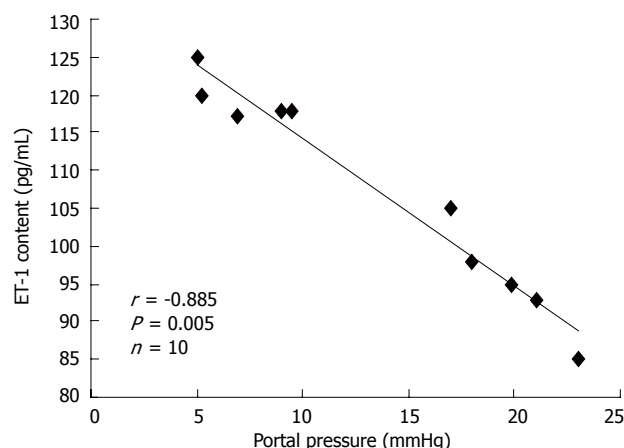


Figure 3 Correlation between portal pressure and ET-1 levels in the mesenteric circulation at 4 wk.

1:1, which was proven by a compatibility proportion test^[10]. Glytan was administered after 1 wk BDL. At 1 wk, the increased PP was not elevated to a level meeting the diagnostic criteria of PHT; therefore, this was a preventive study. Treatment with Glytan reduced PP and PTBF and increased SVR. Especially at 4 wk, PP was decreased by about 40% and PTBF was decreased by about 21%. Through analysis of blood flow of the splanchnic organs, we verified that the mesentery was the target organ of Glytan. Glytan reduced mesenteric blood flow by 33% at 4 wk, which is consistent with the decreased vasodilation of the mesenteric vascular bed observed in the present study. Next, we attempted to explain why Glytan reduced mesenteric blood flow and PP from the standpoint of ET-1 and its receptors.

ET-1 is one of the strongest vasoconstrictors. Changes in ET-1 levels in the splanchnic circulation are not consistent. Nagasue *et al.*^[11] found that ET-1 levels were increased in the mesenteric venous plasma. Vashist *et al.*^[12] found decreased SVR in PHT and that central hypovolemia stimulated sympathetic nerves, increasing vasoconstriction. However, Coll *et al.*^[13] found that gene and protein expression decreased in the SMA of rats with hepatic fibrosis and portal vein ligation. In our study, the ET-1 levels in the mesenteric circulation of BDL rats were significantly lower than those in sham group rats at 2 and 4 wk. ET-1 levels were negatively correlated with PP at 4 wk. Therefore, increased ET-1 levels resulting from Glytan administration may be a reason for the decrease observed in PP.

Excessive synthesis of NO in splanchnic tissue contributes to vasodilation in hyperdynamic circulation^[14]. eNOS is the main source of NO^[15]. Thus, inhibition of eNOS expression or activity in the visceral vascular system can effectively inhibit vascular dilatation. In addition, ET-1 binding to ETBR expressed in vascular endothelial cells activates the $G\alpha$ and $G\beta\gamma$ subunits. $G\beta\gamma$ activates the PI3-Akt-eNOS pathway and promotes NO synthesis^[16]. Therefore, either directly inhibiting eNOS expression or indirectly inhibiting ETBR expression can both

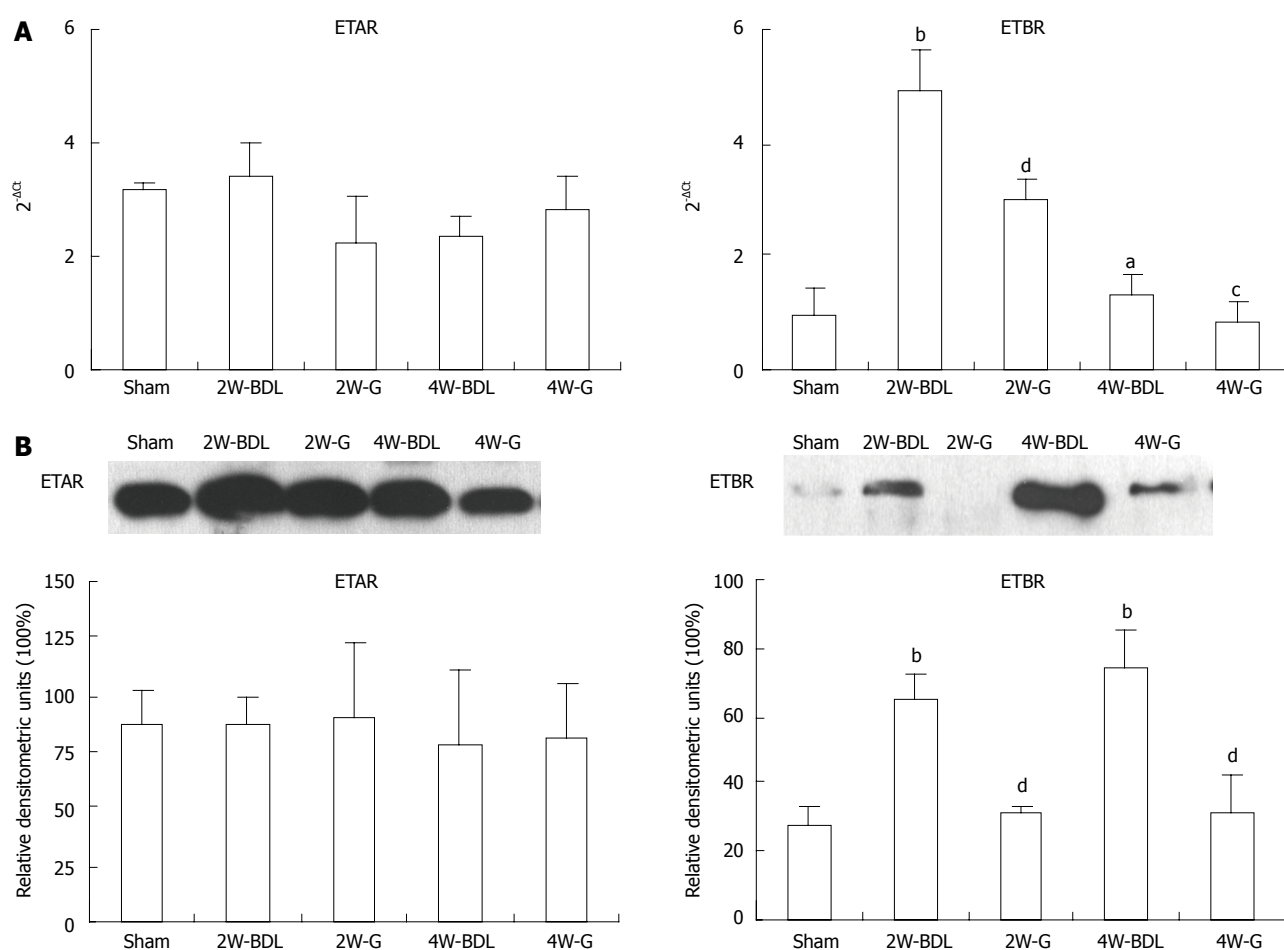


Figure 4 Effect of Glytan on ETAR and ETBR expression at mRNA and protein levels. A: ETAR and ETBR mRNA levels in the mesentery. Higher 2^{-ΔCt} values represent higher mRNA concentrations. Results from all experiments are shown (*n* = 7 per group); B: Western blot analysis for ETAR and ETBR protein levels (*n* = 8 per group). ^a*P* < 0.05, ^b*P* < 0.01, bile duct ligation (BDL) rats vs sham-operated rats; ^c*P* < 0.05, ^d*P* < 0.01, rats in the Glytan group vs BDL rats.

reduce NO synthesis, in theory. Our results showed that eNOS expression was significantly increased in the mesenteric tissue of BDL rats. Increased eNOS not only resulted in mesenteric vasodilation but also induced a lower reactivity of the vasculature to vasoconstrictors^[17-19]. Treatment with Glytan decreased eNOS significantly, which may be related to PP reductions.

The effect of ETBR in the mesentery is more complex. ET-1 binding to ETBR induces NO synthesis and thus vasodilation. ET-1 binding to ETAR causes vasoconstriction^[20]. ETBR also acts in the clearance of ET-1^[21]. With an increase in PP, ETBR expression increases in the mesenteric vasculature of BDL rats. It shows simultaneous stimulation of ETAR and ETBR in the rat renal microcirculation, and ETBR plays a major role in vasodilation^[22]. In our study, increased ETBR in the mesentery of BDL rats may have promoted vasodilation to accommodate increased blood flow. The fact that Glytan decreases ETBR expression could mean two things: (1) it may inhibit vasodilation directly or indirectly through an ETBR-eNOS pathway; and (2) it may promote vasoconstriction through decreased clearance of ET-1. Because of increased levels of ET-1, ETAR can bind to more ET-1 to induce vasoconstriction.

Although NO is an important factor in splanchnic vascular dilation, NOS-deficient mice still demonstrate progressive splanchnic vasodilation. This indicates that NO is not the only factor related to hyperdynamic circulation^[23,24]. Our study also found that eNOS expression was not increased with increases in PP. Therefore, a NO-independent pathway, such as hyporesponsiveness of the splanchnic vascular system to vasoconstrictors, cannot be ignored. In rats with portal vein ligation, the mesenteric artery and thoracic aorta show hyporesponsiveness to an adrenergic receptor^[25]. In the aortas of rats with liver fibrosis and the hepatic arteries of human patients with liver cirrhosis, the numbers of angiotensin receptors are unchanged. However, AT1-R is desensitized by GRK2 and β-arrestin 2^[26]. It is known that GRKs and arrestins are key participants in the canonical pathways leading to phosphorylation-dependent or independent G-protein-coupled receptor desensitization and endocytosis^[27,28]. The difference between the phosphorylation-dependent and -independent pathways is that the former depends on arrestins, while the latter does not. Our previous results showed that increased PP resulted in upregulation of mesenteric GRK2 expression but not of β-arrestin 2^[5]. In this study, treatment with Glytan resulted in de-

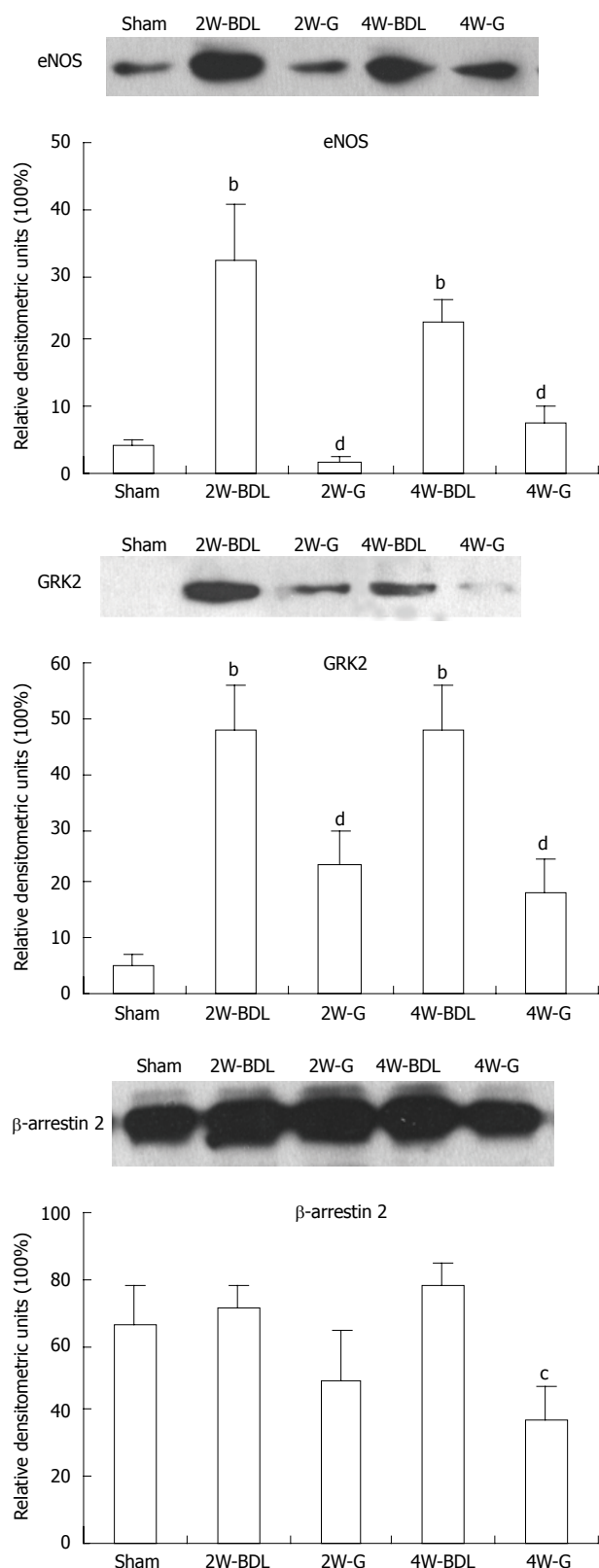


Figure 5 Effect of Glytan on eNOS, GRK2, and β-arrestin 2 expression. Western blot analysis for eNOS, GRK2 and β-arrestin 2 protein levels ($n = 5$ per group). ^b $P < 0.01$, bile duct ligation (BDL) rats vs sham-operated rats; ^c $P < 0.05$, ^d $P < 0.01$, rats in the Glytan group vs BDL rats.

creased GRK2 and β-arrestin 2 expression in BDL rats. These results imply that the sensitivity of ETAR or other

vasoconstrictor receptors to their agonists may increase. The exact sensitivity change in ET receptors by GRK2 and β-arrestin 2 after Glytan treatment needs to be verified through an immunoprecipitation test. In addition, Liu *et al.*^[29,30] have found that GRK2 expression increases in sinusoidal endothelial cells from portal hypertensive rats, while the knockout of GRK2 restores both Akt phosphorylation and NO production and normalizes PP. Therefore, in the liver, increased GRK2 promotes vasoconstriction. In the splanchnic organs, it remains unclear whether increased GRK2 has the same effect as in the liver. GRK2 promotes both vasoconstriction and vasodilation, therefore, the final effect of GRK2 is a balance of both functions.

In summary, treatment with Glytan reduced PP and PTBF, especially at 4 wk. The mesentery was the target organ of Glytan. Increased ET-1 levels and decreased ETBR and eNOS expression by Glytan may promote mesenteric vasoconstriction. Decreased GRK2 and β-arrestin 2 expression by Glytan may increase the sensitivity of vasoconstrictor receptors to their agonists and promote vasoconstriction. These findings suggest the therapeutic potential of Glytan for PHT.

COMMENTS

Background

Portal hypertension (PHT) is one of the most significant complications associated with liver cirrhosis, which can give rise to many other severe and often lethal conditions, such as bleeding esophageal varices, ascites, hepatic encephalopathy and hepatorenal syndrome. Non-selective β-receptor blockers have been considered the only drugs suitable for long-term administration. However, only 30%-40% of patients achieve a good therapeutic outcome with non-selective β-receptor blockers, because of their contraindications or side effects. Finding a perfect medicine for PHT to reduce the risk of bleeding from esophageal varices has been the focus in this field. Glytan is composed of salvianolic acid B (SA-B) and diammonium glycyrrhizinate (DG). SA-B is one of the water-soluble compounds derived from *Salvia miltiorrhiza* Bunge, which is widely used for chronic liver diseases. DG is extracted and purified from liquorices. The liquorices exert an important function in the treatment of hepatitis because of their anti-inflammatory effects. Our previous work found that Glytan can reduce portal pressure (PP), improve liver function, and inhibit pseudobulb formation in rats with liver cirrhosis. Notably, treatment with Glytan resulted in reduced mesenteric vasodilation. According to Ohm's law, PP depends on intrahepatic resistance and portal inflow. An increase in splanchnic blood flow worsens and maintains PHT. Thus, the aim of this study was to verify whether reduced mesenteric vasodilation is related to a decrease in PP and to investigate the mechanisms by which Glytan decreases PP in portal hypertensive rats.

Research frontiers

Bleeding from esophageal varices is the most serious complication of PHT. The most important goal of treating PHT is to reduce the risk of bleeding. It has been found that the incidence of varices at 5 years increased from 25% to 50% in patients with hepatic vein pressure gradient (HVPG) > 10 mmHg. Either non-selective β-receptor blockers or endoscopic band ligation can be used for primary prophylaxis in patients with cirrhosis and medium or large varices with high risk of bleeding. Both propranolol and carvedilol are non-selective β-receptor blockers. Carvedilol possesses both non-selective β_{1/2}-antagonist and α₁-receptor antagonist activity. Although carvedilol is more tolerated and highly effective than propranolol, it may cause arterial hypotension and worsen renal function. Traditional Chinese medicine is characteristic of multi-targets, which is consistent with the complicated pathophysiological features of PHT. In recent years, researchers have realized that it is feasible to develop drugs for PHT according to the progress of traditional Chinese medicine in the field of anti-fibrosis. Traditional Chinese medicine is the next battlefield for researchers

to develop drugs for PHT.

Innovations and breakthroughs

Based on its efficacy in decreasing PP, Glytan is the first herbal compound that has been approved to begin stage II clinical trials in China. This study is believed to be the first to show that the mesentery is a target organ of Glytan. The decreased PP and portal territory blood flow (PTBF) observed after Glytan treatment are related to increased mesenteric vasoconstriction and increased endothelin (ET) receptor sensitivity to vasoconstrictors. This is also the first study to demonstrate the effect of Glytan on hyperdynamic circulation.

Applications

Based on the effect of Glytan on splanchnic hemodynamics and mesenteric ET receptors, this study suggests the therapeutic potential of Glytan on liver cirrhosis-induced PHT.

Terminology

PHT is the main complication of cirrhosis and is defined as an HVPG > 5 mmHg. Clinically significant PHT is defined as HVPG \geq 10 mmHg. Hyperdynamic circulation is a state of portal hypertension that is characterized by splanchnic and peripheral vasodilation, increased plasma volume, and increased cardiac output. Glytan is a new preparation for PHT, which is based on traditional Chinese medicine theory. Glytan is composed of SA-B and DG.

Peer review

The manuscript discusses a very interesting topic. In addition, the manuscript is well written. It presents the results in a clear and well explained manner both in text and in figures.

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Azathioprine does not reduce adenoma formation in a mouse model of sporadic intestinal tumorigenesis

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Abstract

AIM: To investigate if azathioprine could reduce adenoma formation in *Apc^{Min/+}*, a mouse model of sporadic intestinal tumorigenesis.

METHODS: Azathioprine was administered *via* drinking water (estimated 6-20 mg/kg body weight per day) to *Apc^{Min/+}* and wildtype mice. Control animals received vehicle only (DMSO) dissolved in drinking water. At 15 wk of age all mice were sacrificed and intestines of *Apc^{Min/+}* were harvested for evaluation of polyp number. Azathioprine induced toxicity was investigated by immunohistochemical analysis on spleens.

RESULTS: All azathioprine treated mice showed signs of drug-associated toxicity such as weight loss and development of splenic T-cell lymphomas. Although this suggests that the thiopurine concentration was clearly in the therapeutic range, it did not reduce tumor formation (48 ± 3.1 adenomas *vs* 59 ± 5.7 adenomas, $P = 0.148$).

CONCLUSION: We conclude that in the absence of inflammation, azathioprine does not affect intestinal tumorigenesis.

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Key words: Azathioprine; Thiopurine; Intestinal adenoma; Polyp; ApcMin; Chemoprevention; Lymphoma; Colon cancer

Core tip: Treatment with thiopurines is associated with a reduced risk of developing colorectal cancer in patients with inflammatory bowel disease. The molecular target of azathioprine, Rac1 has recently been implicated as a critical player during sporadic intestinal tumorigenesis. Here, we investigated the potential preventive role of azathioprine in *Apc^{Min/+}*, a mouse model of sporadic intestinal tumorigenesis. Even though all azathioprine treated mice showed signs of drug-associated toxicity, it did not reduce tumor formation. We therefore conclude that in the absence of inflammation azathioprine does not affect intestinal tumorigenesis.

Wielenga MCB, van Lidth de Jeude JF, Rosekrans SL, Levin AD, Schukking M, D'Haens GRAM, Heijmans J, Jansen M, Muncan V, van den Brink GR. Azathioprine does not reduce adenoma formation in a mouse model of sporadic intestinal tumorigenesis. *World J Gastroenterol* 2014; 20(44): 16683-16689 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16683.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16683>

INTRODUCTION

The thiopurines azathioprine and 6-mercaptopurine are widely used to induce and maintain remission of inflammatory bowel disease (IBD)^[1-4]. In addition to their immunosuppressive effects, exposure to thiopurines is associated with a substantially reduced risk of developing colorectal cancer in patients with IBD^[2,5].

The presumed mechanism of action of thiopurines is through selective inhibition of the GTPase RAC1^[6,7]. This member of the Rho family of GTPases acts as a key-component in diverse signaling pathways and plays a critical role in many cellular processes such as proliferation, apoptosis and migration^[8]. It has recently been shown that Rac1 signaling plays a key role in intestinal adenoma formation downstream of the mutation in *Apc*^[9]. The *Apc* gene is frequently mutated in intestinal adenomas and carcinomas and patients with familial adenomatous polyposis (FAP) carry a germline mutation in one copy of the *APC* gene^[10]. *Apc*^{Min/+} mice carry a germline mutation in *Apc* similar to patients with FAP and can be used to model FAP specifically or *Apc* dependent adenoma development more in general^[11].

Since Rac1 signaling plays a critical role in intestinal tumorigenesis and thiopurines are widely used drugs that inhibit Rac1 activity, we reasoned that thiopurines may protect against the development of intestinal tumorigenesis. Here we tested this hypothesis by treating *Apc*^{Min/+} mice with azathioprine.

MATERIALS AND METHODS

Mouse experiments

The protocol of this study was approved by the animal ethics committee of the University of Amsterdam (permit number ALC102806). *Apc*^{Min/+}^[11] and littermate wild type C57B/6J mice of five weeks old (12 males and 12 females of each genotype) were ordered at the Jacksons Laboratory. Upon arrival animals were given drinking water in which azathioprine (Sigma Aldrich A4638) was dissolved at 0.04 mg/mL as previously described by others^[12]. The estimated dose was 6-20 mg/kg body weight per day, given that a mouse weighs approximately 20-30 g and drinks approximately 4-8 mL of water per day^[12]. Control animals received vehicle only (DMSO) dissolved in drinking water. We expected mice to develop a mean of 60 polyps (with 10% standard deviation) and reasoned that azathioprine could only be clinically applicable if the polyp number would be reduced by at least 25%. At a *P* of 0.05 (alpha 0.025) and power of 0.8, the sample size was calculated for 6 animals by group.

Histological analysis

After paraffin embedding, 4 µm sections were made and used for routine hematoxylin eosin staining. Immunohistochemistry was performed as described previously^[13] using the following antibodies: anti-Cd3 (Dako, rabbit polyclonal, A0452), B220. Anti-Cd45R (Biolegend/ITK, rat monoclonal, 103202), anti-Ki67 (Monosan, Rabbit

monoclonal, MONX10284), anti-β-catenin (transduction laboratories, mouse monoclonal, 610154). Histological evaluation of spleens was performed by an expert pathologist (MJ).

Statistical analysis

All data are presented as mean ± SE. For animal experiments, the Mann-Whitney test was used. For the analysis of adenoma size distribution, a two-way ANOVA was used.

RESULTS

Treatment with azathioprine results in severe toxicity in mice

Two weeks after the start of azathioprine administration (dissolved in the drinking water at 0.04 mg/mL), *Apc*^{Min/+} and wild type female mice started to lose weight and became moribund whereas female control mice treated with solvent only continued to gain weight (Figure 1A and B). According to local animal research guidelines we euthanized mice losing more than 15% of initial weight, resulting in termination of the entire group of 12 female mice receiving azathioprine by the end of the fourth week of treatment. In contrast, male mice did not show any symptoms of drug-associated toxicity at this time point. Post mortem investigation showed signs of profound anemia with discoloration of extremities and internal organs of all azathioprine treated female mice. Upon examination of the intestines, we did not identify polyps in these 9-wk-old animals.

In order to reduce potential drug toxicity in the remaining male animals, we lowered the azathioprine dose to 0.02 mg/mL at four weeks after the start of treatment. Although the body weight of male mice remained stable at first, we eventually observed weight loss in male animals. To prevent further deterioration of the mice, we terminated the experiment at fifteen weeks of age (Figure 1C).

Azathioprine treatment results in the development of splenic T-cell lymphomas

We observed enlargement of the spleen in all azathioprine treated mice (both controls and *Apc*^{Min/+} mice), but in none of the control animals (Figure 2A). The development of splenic lymphomas is a known adverse effect of azathioprine treatment in both humans and mice^[14]. We therefore performed further histological evaluation of samples of azathioprine treated control and *Apc*^{Min/+} mice. All spleens of azathioprine treated animals showed an expanded red pulp with a pleomorphic population of lymphocytic blasts that displayed prominent variation in nuclear size, contour and atypical mitoses (Figure 2B). This is diagnostic of a diffuse lymphoproliferative disease. We performed immunohistochemical staining to further analyze the composition of these infiltrates. This showed that the pre-existent peri-arteriolar B- and T-cell areas were preserved while the red pulp was diffusely infiltrated by Cd3 positive atypical lymphocytes (Figure

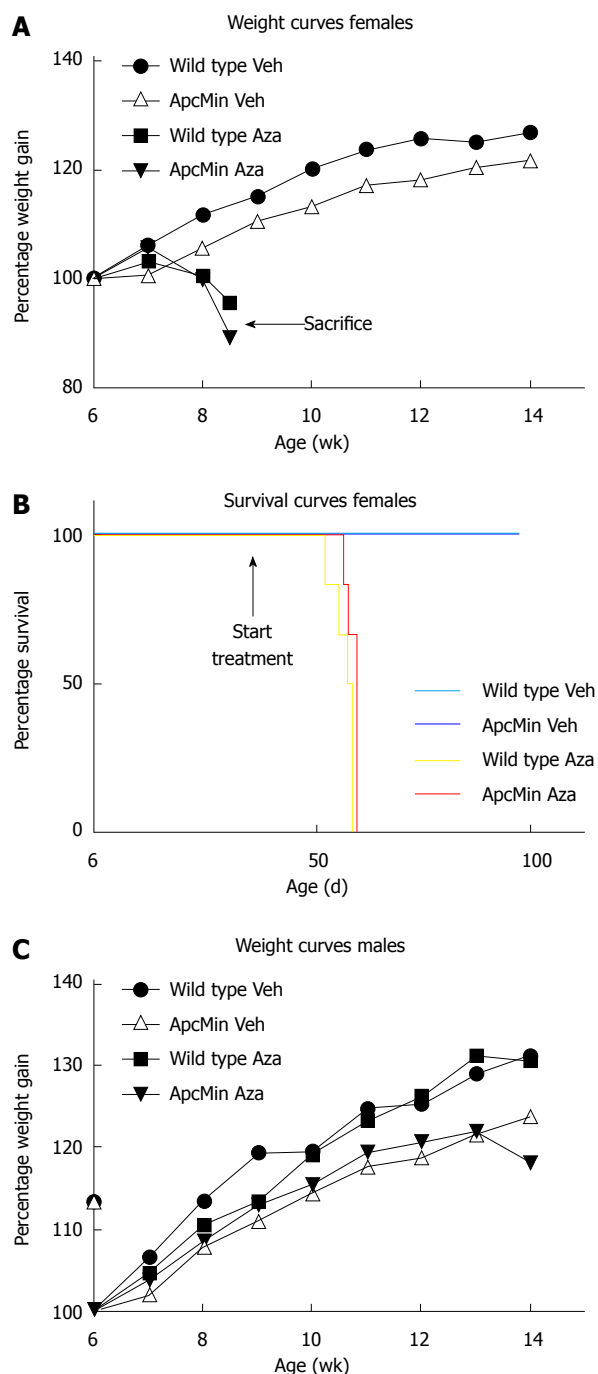


Figure 1 Treatment with azathioprine results in severe drug associated toxicity. A: Weight curve of female mice. All azathioprine treated female mice had to be sacrificed three to four weeks after start of the treatment due to progressive weight loss; B: Survival curve of female mice; C: Weight curve of male mice. After 9 to 10 wk of treatment male mice started to show signs of toxicity. Veh: Vehicle; Aza: Azathioprine.

2C). Analysis of proliferation using Ki67 showed that proliferative activity in germinal centers was retained as expected, whereas splenic T-cell lymphomas exhibited a near 100% proliferative index (Figure 2D). β -catenin did not show nuclear labeling in either $Apc^{Min/+}$ or wild type mice (Figure 2D). This suggests that the lymphomas developed in an Apc independent manner. This is consistent with the fact that no difference was observed in the

severity of lymphoma development between control and $Apc^{Min/+}$ mice.

Azathioprine treatment does not affect adenoma development in $Apc^{Min/+}$ mice

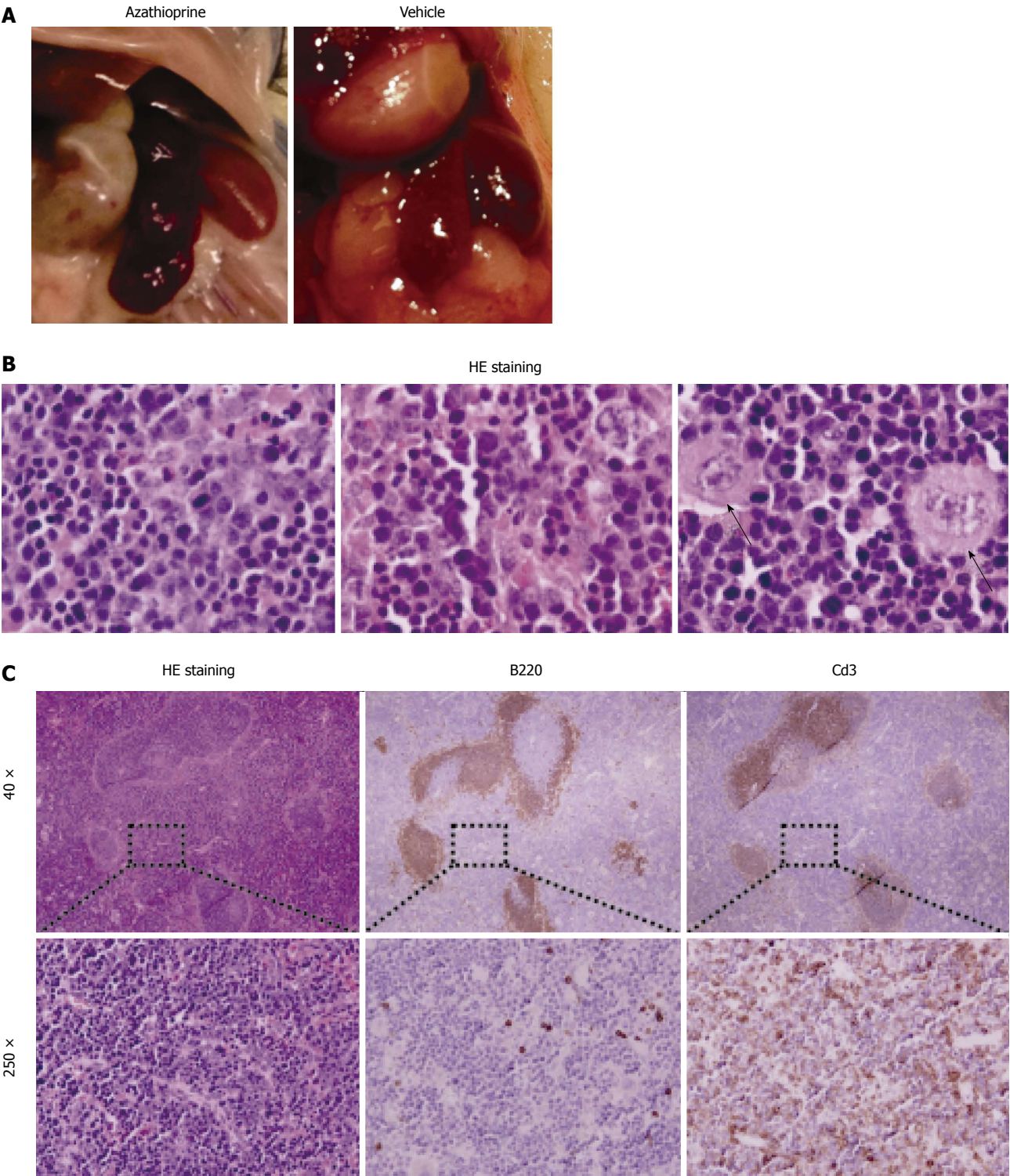
We next assessed if intestinal adenoma development was affected by the administration of azathioprine. Animals that had received azathioprine ($n = 6$) did not exhibit a significant decrease in polyp numbers compared to $Apc^{Min/+}$ mice that had received vehicle only ($n = 6$) (48 ± 3.1 adenomas vs 59 ± 5.7 adenomas respectively, $P = 0.148$, Figure 3A). Furthermore, analysis of adenoma localization and size did not show any difference between azathioprine and vehicle treated animals (Figure 3B and C). Based on these results we concluded that azathioprine treatment did not affect the incidence or progression of adenoma development in $Apc^{Min/+}$ animals.

DISCUSSION

Use of thiopurines is associated with a reduced risk of developing colorectal cancer in patients with IBD but their role in sporadic tumor formation has thus far not been investigated. In our study, treatment of mice with the highest tolerable azathioprine dose, results in severe drug associated toxicity both in $Apc^{Min/+}$ and wild type mice. Azathioprine treated animals suffered from profound weight loss and displayed development of splenic lymphomas. Nonetheless, even at this high dose, azathioprine treatment did not affect Apc dependent intestinal adenoma development in mice.

In humans, the major side effects of treatment with azathioprine are gastrointestinal complaints such as nausea and abdominal pain^[15], hepatotoxicity^[16], pancreatitis^[17] and bone marrow depression^[18]. We observed substantial toxicity in all azathioprine treated mice. Surprisingly, this resulted in significant more morbidity in female mice compared to males. Azathioprine induced toxicity is related to activity of the enzyme thiopurine S-methyltransferase (TPMT)^[19,20]. Therefore the deficiency of TPMT enzyme activity may cause increased sensitivity to azathioprine induced toxicity^[21,22]. In accordance to this it was recently shown that TPMT enzyme activity was lower in females than compared to males^[23]. Although we did not assess TPMT activity in our experiments, this may explain why female mice were more vulnerable to azathioprine than males.

Thiopurine use is also associated with an increased relative risk but small absolute risk in development of lymphomas^[24-26]. Also in our hands mice display a remarkable susceptibility to develop azathioprine-induced lymphomas. This suggests that the azathioprine may have been overdosed in our experiment or that mice have an increased susceptibility to lymphoma development. Unfortunately we have so far been unable to determine drug levels in mouse blood in our institute. However, since overdosing would more likely result in overestimation of the protective effect of azathioprine, it is unlikely to ex-



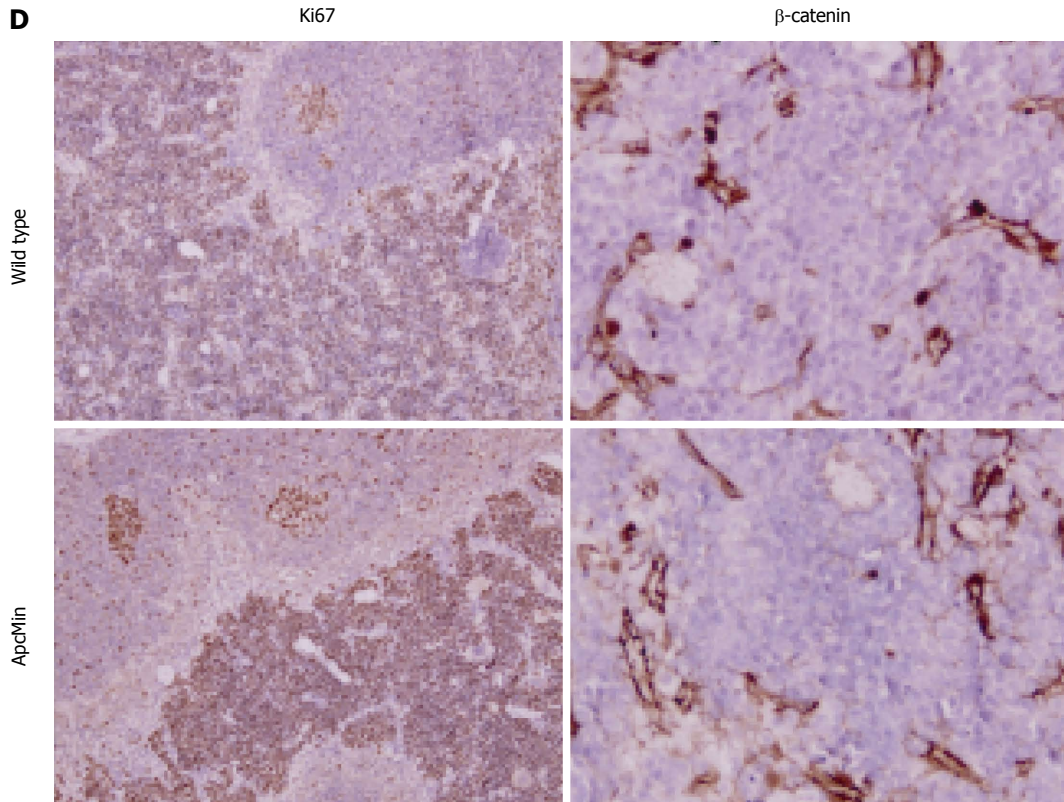


Figure 2 Azathioprine treatment results in the development of splenic T cell lymphomas. A: Representative image of vehicle and azathioprine treated mice showing splenic enlargement and discolored liver and kidneys in azathioprine treated animals; B: Representative photomicrographs of the morphology of the splenic infiltrates. The images show a highly atypical and pleomorphic population of lymphocytic blast-like cells with prominent variation in nuclear size and contour (left panel), atypical mitoses (middle panel, top right) and admixed giant cells (right panel); C: Splenic architecture. Peri-arteriolar B and T cell areas are preserved (B220 and Cd3 top panel), while the red pulpa is effaced by a Cd3 positive atypical infiltrate, diagnostic of T-cell lymphoma; D: Ki67 staining shows limited proliferative activity in pre-existent germinal centers; the surrounding atypical infiltrate demonstrates a nearly 100% labeling index. β-catenin does not show nuclear labeling in either genotype.

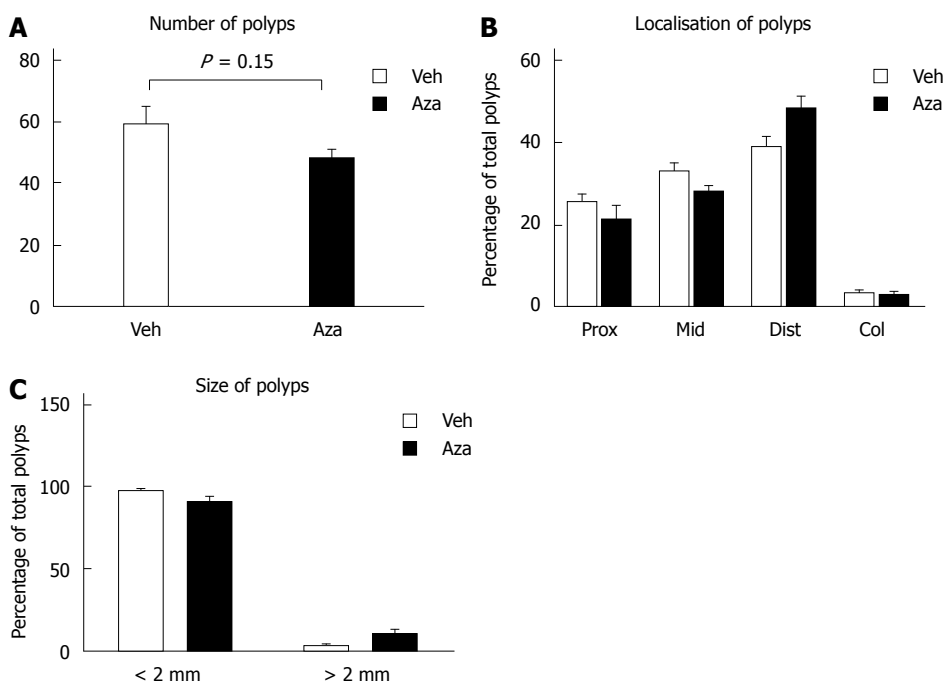


Figure 3 Azathioprine treatment does not affect adenoma development in *Apc^{Min/+}* mice. Total adenoma number in azathioprine ($n = 6$) and vehicle treated ($n = 6$) mice (A). Localization (B) and size (C) of adenomas did not differ between vehicle and azathioprine treated mice. Veh: Vehicle; Aza: Azathioprine.

plain the lack of effect on adenoma development.

The lack of effect of azathioprine on adenoma development in our experiments could be due to pleiotropic effects on cellular signaling by azathioprine. Another potential difference between the genetic loss of Rac1 signaling as investigated by Myant *et al.*^[9] and treatment with azathioprine is that genetic deletion of *Rac1* occurred concurrent with loss of *Apc* whereas the *Apc*^{Min/+} mice in our experiments carried a germline *Apc* mutation. The effect of Rac1 inhibition could be mediated at the earliest stages of adenoma development. Since we started azathioprine treatment at the age of five weeks, it may be that precursor lesions were already established before the initiation of azathioprine treatment. Although azathioprine is known to reduce Rac1 signaling in humans, we can not formally exclude that in mice Rac1 signaling remains unaltered during azathioprine treatment. The reduced risk of developing colorectal cancer in patients with IBD treated with thiopurines was observed in a retrospective analysis of 2578 patients in which 1% developed advanced neoplasia (high grade dysplasia or colorectal cancer). Whether this association is the result of a better control of inflammation in thiopurine treated patients, a direct effect on inflammation driven carcinogenesis or potential confounding factors will be hard to elucidate. The results from our current study suggest that azathioprine may not prevent *Apc* dependent sporadic intestinal tumorigenesis.

COMMENTS

Background

Treatment with thiopurines is associated with a reduced risk of developing colorectal cancer in patients with inflammatory bowel disease. If thiopurines can also reduce adenoma formation in the absence of inflammation remains thus far unknown.

Research frontiers

The presumed mechanism of action of thiopurines is through selective inhibition of the GTPase RAC1. It has recently been shown that Rac1 signaling also plays a key role in sporadic intestinal adenoma formation downstream of the mutation in *Apc*. The *Apc* gene is frequently mutated in intestinal adenomas and carcinomas and patients with familial adenomatous polyposis (FAP).

Innovations and breakthroughs

The role of azathioprine in inflammation and inflammation associated cancer has been studied extensively. In the current manuscript the authors report for the first time that in the absence of inflammation, azathioprine does not reduce adenoma formation in a mouse model of sporadic intestinal tumorigenesis.

Applications

The molecular target of azathioprine, RAC1 has recently been implicated as a critical player during sporadic intestinal tumorigenesis. Given the extensive clinical experience with thiopurines, this may thus be a candidate for chemoprevention of colorectal cancer in patients that have increased risk for developing this disease. Here the authors tested this hypothesis by treating *Apc*^{Min/+} mice with azathioprine and found that azathioprine does not reduce adenoma formation.

Terminology

Rac1 is a member of the Rho family of GTPases and acts as a key-component in diverse signaling pathways and plays a critical role in many cellular processes such as proliferation, apoptosis and migration. *Apc*^{Min/+} mice carry a germ line mutation in *Apc* similar to patients with FAP and can be used to model FAP specifically or *Apc* dependent adenoma development more in general.

Peer review

In this experimental study some evidence is given that azathioprine does not

reduce adenoma formation in the *Apc*^{Min/+} mouse model of sporadic intestinal tumorigenesis.

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Alleviated mucosal and neuronal damage in a rat model of Crohn's disease

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Abstract

AIM: To establish a rat model suitable to investigate the repetitive relapsing inflammations (RRI) characteristic to Crohn's disease.

METHODS: Colitis was induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). RRI were mimicked by repeating administrations of TNBS. Tissue samples were taken from control, once, twice and three times treated rats from the inflamed and adjacent non-inflamed colonic segments at different timepoints during the acute intestinal inflammation. The means of the ulcerated area were measured to evaluate the macroscopic mu-

cosal damage. The density of myenteric neurons was determined on whole mounts by HuC/HuD immunohistochemistry. Heme oxygenase-1 (HO-1) expression was evaluated by molecular biological techniques.

RESULTS: TNBS-treated rats displayed severe colitis, but the mortality was negligible, and an increase of body weight was characteristic throughout the experimental period. The widespread loss of myenteric neurons, and marked but transient HO-1 up-regulation were demonstrated after the first TNBS administration. After repeated doses the length of the recovery time and extent of the ulcerous colonic segments were markedly decreased, and the neuronal loss was on a smaller scale and was limited to the inflamed area. HO-1 mRNA level was notably greater than after a single dose and overexpression was sustained throughout the timepoints examined. Nevertheless, the HO-1 protein up-regulation after the second TNBS treatment proved to be transient. Following the third treatment HO-1 protein expression could not be detected.

CONCLUSION: Experimentally provoked RRI may exert a protective preconditioning effect against the mucosal and neuronal damage. The persistent up-regulation of HO-1 mRNA expression may correlate with this.

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Key words: Crohn's disease; Experimental rat model; Heme oxygenase-1; Myenteric neurons; Repetitive relapsing inflammation

Core tip: We report our first results derived from a newly developed rat model with chronic experimental colitis allowed us to modelling the recurring periods of recrudescence and remission in Crohn's disease. Colitis was induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). Repetitive recurrent inflammations (RRI) were

mimicked by repeated administrations of TNBS. This study demonstrates for the first time that experimentally provoked RRI develop preconditioning effect by speeding up mucosal healing and restoring myenteric neuronal injury. Decreased severity of gut inflammation after repeated TNBS treatments might be associated with the persistent up-regulation of heme-oxygenase 1 messenger RNA expression.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of chronic intestinal inflammatory conditions. The major types of IBDs are Crohn's disease (CD) and ulcerative colitis. CD is characterized by relapsing transmural inflammation that can affect any part of the gastrointestinal tract. Although the pathogenesis of CD is still unclear, the most widely accepted hypothesis is that an impairment of the mucosal barrier function due to a dysregulated immune response to an environmental factor can generate prolonged inflammation^[1,2]. The development of irreversible pathological alterations including stricturing and penetrating complications in response to the repetitive relapsing inflammations (RRI) characteristic of CD can indicate the transition from the early to the late disease which is dependent on the intensity rather than on the duration of the inflammation^[3].

The intestinal symptoms common among CD patients are often caused by intestinal motility abnormalities related to enteric neuropathy. The intestinal motor functions are regulated by the myenteric neurons. The evidence suggests that both the quantitative properties and function of the myenteric neurons are altered substantially by intestinal inflammation^[4]. In a previous study 40% loss of myenteric neurons was demonstrated in the inflamed segment of the colon four days following the induction of colitis in rats^[5]. Moreover, the complete loss of myenteric neurons was observed in the strictured region^[6]. Persistent alterations in neuronal signalling were documented even after the resolution of the colitis. The AH neurons, one electrophysiological type of enteric neurons that function as intrinsic afferent neurons, remained hyperexcitable eight weeks after the induction of colitis. In the same experiment larger amplitudes of fast excitatory postsynaptic potentials were measured in the S neurons as compared with the controls^[7]. Physiological disturbances are not restricted to the site of the inflammation. Suppression of noradrenaline release was observed in both the distal and the transverse colon and

in the terminal ileum in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis^[8].

During the past two decades, experimental animal models of IBD have proved to be important tools for the detection of potential therapeutic agents and for the investigation of the pathogenesis. One of the most widely used haptinizing agents TNBS is used to induce colitis, resulting in mucosal inflammation mediated by a Th1 response. Nevertheless, in most cases the induction of acute necrotizing enterocolitis with a single high dose of TNBS was not able to generate the pathological alterations characteristic of CD and was accompanied by a high mortality rate^[9]. The repeated administration of TNBS in increasing doses resulted in chronic colitis and fibrosis in mice^[10] and in rats^[11] that most likely reflects the characteristics of the chronic phase of CD. Nevertheless, considerable levels of mortality, 25% and 18% respectively were still demonstrated.

Our aim was to establish a rat model of chronic colitis with the possible lowest mortality rate, suitable for the investigations of the long-term consequences of acute inflammation. In the present study the extent of mucosal and myenteric neuronal injuries were investigated in the acute phase of the experimentally mimicked RRI. Since increased expression of heme oxygenase-1 (HO-1) has been described in patients with CD^[12,13], it has been suggested that the activation of HO-1 may act as a natural defence to alleviate inflammation and tissue damage^[14-16]. Therefore, the quantitative changes in HO-1 mRNA and protein expression were also evaluated here.

MATERIALS AND METHODS

Animal model

All experiments were approved by from the Local Ethics Committee for Animal Research Studies at the University of Szeged. Adult male Sprague-Dawley rats, weighing 200-220 g, kept on standard laboratory chow (Bioplan Kft., Hungary) and with free access to drinking water, were used throughout the experiments. They were housed in a restricted access room with controlled temperature (23 °C) and a light/dark (12 h:12 h) cycle. Colitis was induced locally with TNBS (Sigma-Aldrich, St. Louis, MO, United States; 10 mg) dissolved in 0.25 mL of 25% ethanol administered with a polyethylene cannula 8 cm proximal to the anus under pentobarbital anaesthesia (45 mg/kg, *ip*). The animals were deprived of food at 24 h before the induction of inflammation. Relapsing inflammations were mimicked by repeating the administration of TNBS with a two week-lag. The animals were randomly divided into control ($n = 18$), and once ($n = 13$), twice ($n = 14$) or three times ($n = 11$) TNBS-treated groups. The control animals received an enema of 0.25 mL of 0.9% saline. The rats were weighed weekly and monitored for activity, bloody diarrhoea and mortality.

Tissue handling

The animals were killed by cervical dislocation under

chloral hydrate anaesthesia (375 mg/kg *ip*) two ($n = 11$), four ($n = 13$) and eight ($n = 14$) days following the TNBS treatments. The final 8 cm region of the descending colon from the anus was dissected. Tissue samples were taken from the inflamed segment and also proximally and distally to the inflamed segment of the colon. The gut segments were cut along the mesentery and pinched flat. Digital photographs were taken to evaluate the macroscopic mucosal damage. The extent of the ulceration was measured two, four and eight days after the first and repeated TNBS treatments. The means of the ulcerated area (cm^2) per area of total colon segments (cm^2) were determined by Image J 1.44 (National Institute of Health, Bethesda, MD, United States) and the mean \pm SE was calculated with GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, United States). After cutting longitudinally, half of the colon samples were processed for immunohistochemistry. The other halves were further divided and processed either for qRT-PCR or Western blotting analysis. Tissue samples for qRT-PCR were incubated overnight at 4 °C in RNA Later (Qiagen, Venlo, The Netherlands). Those for Western blotting analysis were frozen immediately in liquid N₂ and stored at -80 °C until use.

Investigation of the quantitative properties of the myenteric neurons

Wholemout preparations were immunostained with the pan-neuronal marker HuC/HuD as described earlier^[17]. Briefly, wholemounts were incubated overnight with anti-human neuronal protein HuC/HuD developed in mouse (Sigma-Aldrich, St. Louis, MO, United States; final dilution 1:50). After washing in phosphate buffer (PB, 0.05 mol/L), tissue samples were incubated with biotinylated anti-mouse IgG (Amersham, Buckinghamshire, United Kingdom; final dilution 1:100) for 6 h, followed by overnight incubation in streptavidin-biotinylated horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom; final dilution 1:100). Peroxidase activity was revealed by using 3, 3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, United States) as substrate chromogen. Wholemounts were then mounted on gelatin-coated slides in glycerol-PB. Twenty digital photographs at magnification $\times 200$ were taken from each colonic segment from each experimental group with an Olympus BX51 light microscope equipped with an Olympus DP70 camera. The number of neurons was counted with Plexus Pattern Analysis software^[18]. Statistical analysis was performed by using one-way ANOVA and the Newman-Keuls test. The results were evaluated with GraphPad Prism 4.0, and a probability $P < 0.05$ was set as the level of significance. The results were expressed as mean \pm SE.

Quantification of HO-1 mRNA expression by qRT-PCR

Tissue samples were homogenized in AccuZol (Bioneer, Daejeon, South Korea) directly before qRT-PCR. Total RNA was prepared from tissue homogenates as sug-

gested by the manufacturer (Bioneer, Daejeon, South Korea). The reverse transcription was achieved by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, United States). 2 μg of total RNA from HO-1 was transcribed in 15 μL of reaction mixture (1.2 μL dNTPs, 1.5 μL of MultiScribe Reverse Transcriptase (50 U/ μL), 3 μL of RT Buffer, 3 μL of RT primer and 6.3 μL of nuclease-free water). All reactions were carried out for 10 min at 25 °C and then for 2 h at 37 °C in MyGenie32 Thermal Block (Bioneer, Daejeon, South Korea). qRT-PCR was performed in an Exicycler 96 (Bioneer, Daejeon, South Korea) in a total volume of 20 μL containing 10 μL of FastStart SYBR Green PCR Master Mix, 1 μL of specific primer (0.5 pmol/ μL) and 50 ng of cDNA template. The PCR program began with a 15-min initial step at 95 °C to activate the Taq DNA polymerase. This was followed by 45 cycles of 15 s at 95 °C for denaturation, 45 s at 60 °C for annealing and 25 s at 72 °C for extension. The sequences of primers were derived from NCBI RefSeq Database entry NM_012580.2 for HO-1 (forward: 5'-GTCAAGCACAGGGTGACAGA-3' and reverse: 5'-CTGCAGCTCCTCAAACAGC-3'). Every sample was measured three times and the comparative C_T ($\Delta\Delta\text{C}_T$) method was applied with the Exicycler 96 Analysis Software (Bioneer, Daejeon, South Korea) for the relative quantification of transcription levels. Hypoxanthine guanine phosphoribosyltransferase (NCBI RefSeq Database entry: NM_012583.2; forward: 5'-GACCGGTTCTGTCATGTTCG-3' and reverse 5'-ACCTGGTTCATCATCACTAATCAC-3') was used as a housekeeping gene to normalize expression data. The results were expressed as means \pm SD.

Western blotting analysis of the expression of HO-1 protein

Tissue samples were homogenized in TRIS-mannitol buffer and then total protein was denaturated from each sample as described earlier^[19]. 10 μg of total cellular protein was separated by SDS-PAGE and was transferred to nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom). The membrane was probed with anti-HO-1 monoclonal antibody (Enzo Life Sciences, Farmingdale, NY, United States; final dilution 1:1000) and then was incubated with horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States; final dilution 1:2000) at room temperature. Immunoreaction was visualized with an enhanced chemiluminescence system Immobilon Western HRP Substrate (Millipore Corporation, Billerica, MA, United States) and scanned with LI-COR C-DiGit™ Blot Scanner (Li-Cor Corporate, Lincoln, NE, United States).

RESULTS

Macroscopic observations

Consistent with the findings of previous studies, the

Table 1 Weight characteristics of the four experimental groups of rats before the TNBS (initial) and eight days after the TNBS treatments (final)

	Body weight (g) \pm SE			
	Initial		Final	
Control	201.5 \pm 1.6	<i>n</i> = 34	356 \pm 20.9 ^b	<i>n</i> = 10
1 \times TNBS	238.3 \pm 3.6	<i>n</i> = 42	239 \pm 10.9	<i>n</i> = 11
2 \times TNBS	242.7 \pm 3.3	<i>n</i> = 40	319.3 \pm 8.2 ^b	<i>n</i> = 10
3 \times TNBS	197.2 \pm 3.5	<i>n</i> = 50	376.5 \pm 11.3 ^b	<i>n</i> = 11

^b*P* < 0.01, initial *vs* final. TNBS; 2,4,6-trinitrobenzenesulfonic acid.

TNBS-treated rats already displayed severe ulcerative intestinal inflammation associated with weakness and bloody diarrhoea on the first day after the induction of colitis with either single or repeated doses of TNBS. The mortality rate was negligible: only two animals died during the experiments. Independently of how many doses were administered, the symptoms always presented with the same severity, but the length of the recovery time decreased spectacularly after repeated treatments. Except for the first week of acute inflammation, when a gain in weight was not detected, a gradual increase in body weight was characteristic in all the rats throughout the experimental period (Table 1). The severe symptoms, like bloody diarrhoea accompanied by acute inflammation lasted for seven or eight days after the administration of a single dose of TNBS but were already resolved four or five days after repeated treatments. The accelerated mucosal healing after the repeated TNBS administrations was clearly demonstrated by the significant differences in the mean ulcerated areas on days two, four and eight after the induction of colitis (Figure 1). The mean area of the ulcerated colonic segment decreased significantly in all TNBS-treated groups between days two and eight following induction. Whereas, the area was still noteworthy eight days after the single TNBS administration, it was significantly reduced and hardly detectable after the second (Figure 1) and even undetectable after the third treatment (not shown). However, long-range hyperaemia was always detected in all colonic samples even on day eight after colitis induction.

Quantitative changes of the myenteric neurons

Whole mounts of colonic segments after HuC/HuD immunohistochemistry were used to evaluate the density of the myenteric neurons (Figure 2). Data were always compared to the age-matched controls (Figure 3). Irrespective of the number of TNBS treatments, the colitis in the acute phase was always associated with a rapid and significant loss of HuC/HuD-immunoreactive myenteric neurons. Significant decrease in the number of neurons was first demonstrated four days after the administration of a single dose of TNBS, when 43% of the neurons were lost (Figure 3). Further significant decrease in neuronal density was demonstrated until day eight, when the number of neurons was 58% less relative to the age-matched

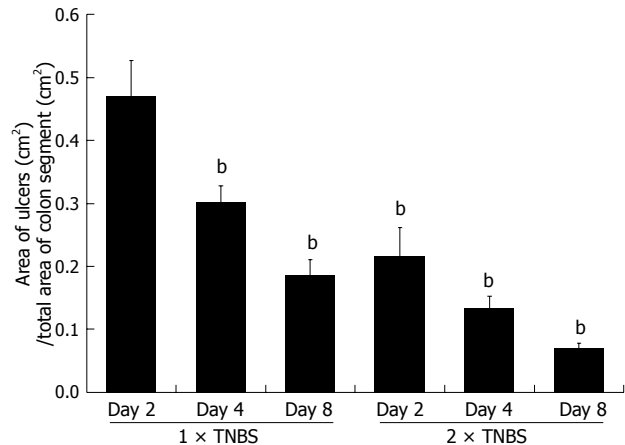


Figure 1 Means of the ulcerated area per total area of colonic segments in rat descending colon two (*n* = 8), four (*n* = 9) and eight (*n* = 10) days after the first and second TNBS treatments. The extension of the ulcerated area was reduced significantly after the second dose of TNBS compared to that in the animals treated only once. Data are expressed as mean \pm SE. ^b*P* < 0.01 *vs* once TNBS-treated group on day two. TNBS: 2,4,6-trinitrobenzenesulfonic acid.

controls (Figure 3). After the second and third TNBS treatments significant decreases in myenteric neuronal number were also observed first on day four after the induction of colitis, but in these cases the neuronal loss was less extensive (36% and 23%, respectively) and did not accelerate between days four and eight after the induction of colitis (Figure 3). Moreover, the decrease in myenteric neuronal density in the acute phase of the inflammation was not limited to the apparent inflamed area. Proximally and distally to the inflamed colonic segment a significant neuronal (25% and 34%, respectively) was noticed four days after the first TNBS administration. However following the repeated doses of TNBS a significant loss of neurons in the colonic segments adjacent proximally and distally to the inflamed area was not detected (Figure 3).

Quantitative changes in HO-1 mRNA expression

The HO-1 mRNA expression was evaluated by qRT-PCR in all three colonic segments after single and repeated TNBS treatments. The HO-1 gene expression was markedly induced in the inflamed segment on day four after the first TNBS administration (Figure 4). However four days after the second treatment an approximately 50% higher HO-1 mRNA level was detected (Figure 4). The HO-1 mRNA expression then declined until day eight in the samples from the once TNBS-treated rats, and to a lesser extent also in the twice TNBS-treated rats, but it has never returned to the baseline level (Figure 4). Nevertheless, after the third treatment a nearly 45% increase in gene expression was measured between days four and eight and this high level of HO-1 mRNA expression was sustained even in the chronic phase of inflammation (not shown). Proximally to the inflamed colonic segment a mild increase of HO-1 mRNA expression was observed in the acute phase of the inflammation four days after the single and repeated TNBS doses, but in the distal

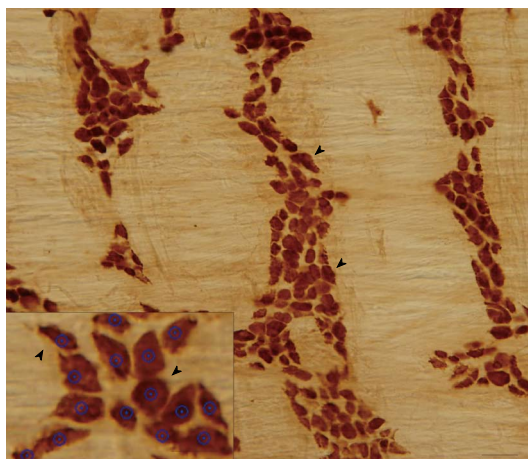


Figure 2 Representative light micrographs of whole mount preparations of the rat descending colon after HuC/HuD immunohistochemistry. Arrowheads point to stained myenteric neurons. Density of neurons per field was determined with Plexus Pattern Analysis software, where the soma of the neurons was encircled (insert). Bars: 50 μ m and 25 μ m (insert).

segment the HO-1 mRNA remained at the baseline level at this timepoint. Eight days after the TNBS treatments expression of HO-1 mRNA in the non-inflamed sites adjacent to the inflamed colonic segments was down-regulated (Figure 4).

Changes in HO-1 protein expression

The changes in HO-1 protein expression were evaluated by Western blotting analysis in the inflamed segments of the colon samples following single and repeated TNBS treatments. Four days after the first and second TNBS administration a notable up-regulation of HO-1 protein expression was demonstrated. Following treatment with a single dose, HO-1 protein expression declined fast to the control level, while after repeated TNBS doses it has not returned back to the baseline level until the eighth day. Nevertheless, after the third TNBS treatment, HO-1 protein expression could not be detected at all (Figure 5).

DISCUSSION

To mimic RRI characteristic of that in CD patients^[20], a rat model of chronic colitis was established with repeated administration of TNBS. In contrast with literature data^[11,21] the rats in this case were treated with a low dose of TNBS (10 mg) dissolved in 25% ethanol. Already on the first day post-induction, severe mucosal inflammation associated with bloody diarrhoea, significant myenteric neuronal loss and marked HO-1 up-regulation indicated the primary events of acute inflammation after the single and after each repeated TNBS treatment. Despite the severity of the initial symptoms the mortality rate was negligible, which makes this rat model more suitable for investigations of the long-term consequences of acute intestinal inflammation than other TNBS models published to^[9,11,21-25]. Besides the low mortality rate, the alleviated macroscopic mucosal damage and accelerated

mucosal healing were salient features in the acute phases of inflammation after repeated TNBS treatment. However, regardless of the number of treatments long-range hyperaemia was always detected several days after the period of acute inflammation. This observation indicates that the post-inflammation remodelling of the vascular pattern is delayed in these rats. The delay in returning to the normal vascular pattern well after the mucosal healing is otherwise a regular problem in clinical practice^[26-28]. We therefore consider that the rat model developed here will be suitable in future studies to reveal the molecular events behind vascular remodelling in acute intestinal inflammation and thereby help to open up new therapeutic avenues for the treatment of CD patients.

Although there are an appreciable number of unexplainable differences in the findings regarding CD-related alterations in the numbers of enteric nerve cells in human patients and animal models^[29-31], the quantitative alterations of the ENS are now considered a hallmark of bowel inflammation. We therefore set out to establish whether the experimentally provoked RRI also alleviates the inflammatory damage in the ENS and reduces the rapid, significant and widespread loss of myenteric neurons demonstrated four and even more eight days after the administration of a single dose of TNBS. After the second and third TNBS treatments significant but less extensive decreases in the number of myenteric neurons were also observed first on day four post-induction, but further neuronal loss was never demonstrated. Examination of the intestinal segments adjacent proximally and distally to the inflamed area likewise significant decreases in neuronal density after the single dose of TNBS indicating the spreading of the neuronal injury outside the inflamed area. Literature data from surgical practice which indicate a neuronal pathway for the spreading of intestinal inflammation at the resection margins^[32-34] are in accordance with these findings. However, after the administration of repeated doses of TNBS the neuronal loss was strictly limited to the inflamed segments of the colon: no proximal or distal spread of the neuronal injury was noticed. Further long-range studies with the present model are now in progress to elucidate the pathogenetic role of the myenteric plexus in the spreading of CD.

These observations of alleviated mucosal and neuronal injury, accelerated mucosal healing and reduced and restricted neuronal cell loss clearly indicate the preconditioning effect of the experimentally provoked RRI. However, the accelerated recovery in the acute phase of inflammation might be misleading in early diagnosis of CD, and thus in avoiding the development of chronic complications. Therefore, studies on the long-term structural and functional consequences of this protective effect are currently in progress in our laboratory.

Since an increased expression of HO-1 was previously described in patients with CD as a crucial mediator of the mucosal defence^[12,13], we expected changes in HO-1 gene expression behind the mucosal and neuronal defence demonstrated here. The HO-1 mRNA expression

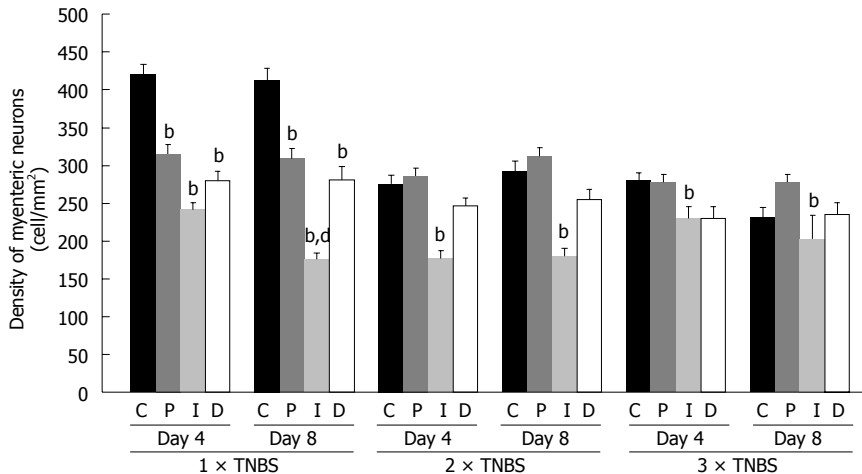


Figure 3 Density of HuC/HuD-immunoreactive myenteric neurons in the colonic segments of control (C) ($n = 12$) and TNBS-treated rats four ($n = 13$) and eight ($n = 14$) days after colitis induction. In the TNBS-treated groups the inflamed segment (I) and the adjacent proximal (P) and distal (D) colonic segments were examined. Significant decrease in myenteric neuronal density was first detected on day four after each TNBS administration. When the rats were treated only once ($1 \times$ TNBS) the number of myenteric neurons decreased significantly in all three colonic segments, while after repeated treatments ($2 \times$ TNBS, $3 \times$ TNBS), a significant decrease in neuronal number was demonstrated exclusively in the I segments. After the single dose of TNBS a further significant neuronal loss was detected until day eight post-induction, whereas after repeated treatments the number of myenteric neurons did not decrease further between days four and eight. Data are expressed as means \pm SE. ^b $P < 0.01$ vs age-matched control groups; ^d $P < 0.01$ vs once TNBS-treated group on day four. TNBS: 2,4,6-trinitrobenzenesulfonic acid.

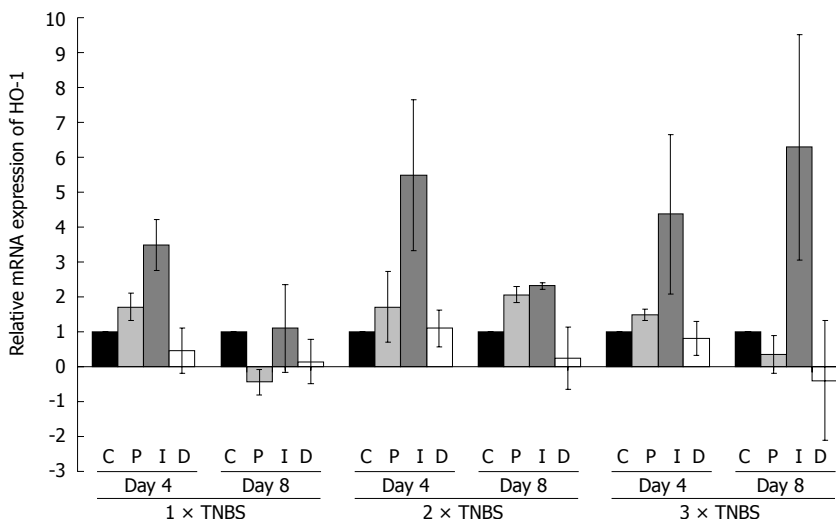


Figure 4 Relative mRNA expression of heme oxygenase-1 compared to the controls ($n = 12$) (C) in the inflamed segment (I) and the adjacent proximal (P) and distal (D) colonic segments four ($n = 13$) and eight ($n = 14$) days after TNBS treatments. Irrespectively of the number of treatments ($1 \times$ TNBS, $2 \times$ TNBS, $3 \times$ TNBS), the HO-1 expression was always marked on day four in the I segments and a more limited expression was also detected in the P but not in the D colonic segments. After the first and second TNBS administrations the rate of HO-1 gene expression decreased, but never returned to baseline level up to day eight post-induction. Nevertheless, after the third TNBS treatment the high level of HO-1 expression was not merely sustained: a pronounced further increase was demonstrated until day eight post-induction. Data are expressed as means \pm SD. TNBS: 2,4,6-trinitrobenzenesulfonic acid; HO-1: Heme oxygenase-1.

was therefore evaluated by qRT-PCR after single and repeated TNBS treatments. Marked increase in HO-1 gene expression was already demonstrated on day four after administration of the single dose of TNBS and the expression was enhanced to a great extent after the second and even more so after the third treatment. There was a decline in HO-1 expression between days four and eight in samples from the once and twice TNBS-treated rats, although it never returned to the baseline level. However after the third treatment there was no decline at all, but rather a more enhanced expression of HO-1 mRNA was detected until the post-induction day eight. HO-1 mRNA

down-regulation in adjacent non-inflamed sites at the same time led us to hypothesize that an early inactivation of the endogen antioxidant defence system at the border-line of inflammation might contribute to the recurrence of CD proximal to the inflamed gut segments observed regularly after surgical resection^[27,33,34].

In parallel with the sustained up-regulation of HO-1 mRNA expression the increase in HO-1 protein level proved to be transient here and after the third treatment HO-1 protein expression could not be detected at all. Therefore, we suggest a post-transcriptional control mechanism for HO-1 expression in RRI. Recent stud-

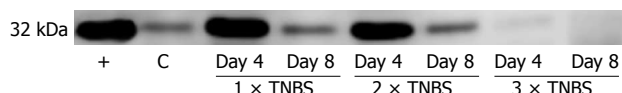


Figure 5 Protein expression of heme oxygenase-1 (32 kDa) in the inflamed segments of the colon four ($n = 4$) and eight ($n = 5$) days after TNBS treatments, compared to the controls ($n = 4$) (C). Four days after the first and second treatment (1 \times TNBS, 2 \times TNBS) elevated heme oxygenase-1 (HO-1) protein level was detected. Then the amounts of protein in both samples declined and eight days after the first treatment it reached already the baseline level. Following the third treatment (3 \times TNBS) HO-1 protein expression could not be detected at all. TNBS: 2,4,6-trinitrobenzenesulfonic acid; HO-1: Heme oxygenase-1.

ies^[35-37] indicate that a regulatory feedback network may exist between HO-1 and microRNAs for controlling gene expression at the post-transcriptional level in response to oxidative damage^[38]. However, the details of this feedback mechanism needs to be explored, we hypothesize that HO-1 up-regulation under RRI resulted in elevated amount of microRNAs, which in turn could lead to the inhibition of HO-1 protein expression after the third TNBS treatment.

In conclusion, we assumed that the alleviated mucosal and neuronal damage in the acute phase of RRI may be associated with the posttranscriptional regulation of HO-1 mRNA expression. Thus a better understanding of the mechanisms and regulatory factors involved in these regulatory processes might be of therapeutic interest.

COMMENTS

Background

Crohn's disease (CD) is a chronic relapsing inflammatory bowel disease associated with marked abnormalities in intestinal motility suggesting that impairment in the enteric nervous system underlines some of the functional abnormalities observed in patients with inflammatory bowel disease. Although there have been numerous studies of the enteric nervous system in inflammation, the structural and molecular changes to the enteric nervous system under the repetitive relapsing inflammations characteristic to CD has not been studied yet.

Research frontiers

The author aimed to establish a chronic experimental rat model which allow to modelling the recurring periods of recrudescence and remission in CD and to investigate the enteric nervous system and its intestinal microenvironment under repetitive relapsing inflammations.

Innovations and breakthroughs

This study demonstrates for the first time that experimentally provoked repetitive relapsing inflammations develop preconditioning effect by speeding up mucosal healing and restoring myenteric neuronal injury. Decreased severity of gut inflammation might be associated with the persistent up-regulation of heme oxygenase-1 messenger RNA expression.

Applications

The authors hypothesize that inactivation of the endogen antioxidant defence system at the borderline of inflammation might contribute to the recurrence of CD proximal to the inflamed gut segments observed regularly after surgical resection. Although distal spreading of inflammation post-surgically has not been reported, our results do not exclude its possibility. Thus a better understanding of the heme oxygenase-1 regulatory processes might be of therapeutic interest.

Terminology

The intestinal motor functions are regulated by the myenteric neurons. The evidence suggests that both the quantitative properties and function of the myenteric neurons are altered substantially by intestinal inflammation. The increased expression of heme oxygenase-1 was previously described in patients

with CD as a crucial mediator of the mucosal defence.

Peer review

The authors reported a modified model for CD that used repeated administrations of TNBS with a two-week-lag and concluded that experimentally provoked recurrent inflammation may exert a protective preconditioning effect against the mucosal and neuronal damage. The study is interesting, the experiments were well described and the results were clearly presented. In general the paper is well written.

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Splanchnic vein thrombosis in necrotizing acute pancreatitis: Detection by computed tomographic venography

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Core tip: Computed tomographic venography (CTV) is an effective examination for splanchnic vein thrombosis (SVT) detection in necrotizing acute pancreatitis (AP) with high positive and negative predictive values. As a non-invasive and quick procedure, CTV might effectively replace digital subtraction angiography and be the routine imaging method for screening and assessing SVT in necrotizing AP patients.

Abstract

AIM: To assess the diagnostic accuracy of computed tomographic venography (CTV) for splanchnic vein thrombosis (SVT) detection in necrotizing acute pancreatitis (AP) patients.

METHODS: Forty-three patients with necrotizing AP who underwent both CTV and digital subtraction angiography (DSA) within 3 d were analyzed in this retrospective comparative study. All CTV procedures were performed with a dual-source CT scanner. The presence and location of SVT were determined *via* blinded imaging data analyses.

RESULTS: According to the DSA results, 17 (39.5%) of the total 43 patients had SVT. The sensitivity, specificity, positive and negative predictive values of CTV for SVT detection were 100% (95%CI: 77.1%-100%), 92.3% (95%CI: 73.4%-98.7%), 89.5% (95%CI: 65.5%-98.2%) and 100% (95%CI: 82.8%-100%), respectively.

CONCLUSION: CTV is an effective examination for SVT detection in patients with necrotizing AP with high positive and negative predictive values.

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INTRODUCTION

As a well-recognized acute pancreatitis (AP) complication, splanchnic vein thrombosis (SVT) may lead to severe clinical consequences such as hemorrhage due to local portal hypertension and the formation of gastric varices, small bowel ischemia due to superior mesenteric vein (SMV) occlusion, and hepatic failure due to portal vein (PV) occlusion^[1-3]. In previous studies, the incidence of SVT varied from 1% to 24% depending on the severity of the study population and the detecting technique that was utilized^[1]. SVT is more often associated with necrotizing acute pancreatitis and a recent study showed that SVT was associated with the presence, location, and extent of pancreatic necrosis^[4].

With the development of computed tomography (CT) techniques as non-invasive and quick examinations, computed tomographic venography (CTV) has become

a routine procedure for assessing the abdominal vascular system, such as collateral vessels^[5,6]. Few studies, however, have evaluated the effectiveness of this technique for SVT detection in necrotizing AP patients. In our study, we aimed to assess the diagnostic accuracy of CTV for SVT detection in necrotizing AP as compared with digital subtraction angiography (DSA).

MATERIALS AND METHODS

We reviewed data from patients diagnosed with necrotizing AP in our center from July 1, 2011 to June 30, 2013. Those who underwent both CTV and DSA within 3 d were included in this study. AP was diagnosed according to clinical presentation (typically abdominal pain), laboratory parameters (serum amylase or lipase levels that exceeded three times the normal upper limit) and abdominal imaging by contrast-enhanced CT (CECT). The severity and pancreatic or peripancreatic necrosis were defined according to the Determinant-Based Classification of Acute Pancreatitis Severity^[7]. Patients with chronic pancreatitis, malignancy and cirrhosis were excluded. The study center was a tertiary referral pancreatic critical center at Jinling Hospital, Nanjing, and the local research ethics committee approved the study.

Imaging protocols

All CTV procedures were performed with a dual-source CT scanner (Somatom Definition, Siemens Medical Solutions). A non-contrast CT scan of the entire abdomen was initially performed. Then, 70 mL of iopromide (Ultravist; 300 mg I/mL, Bayer Schering Pharma, Berlin, Germany) was injected with a power injector at a rate of 3 mL/s *via* an 18 gauge catheter that was typically positioned in the antecubital vein. Arterial and portal venous phase images were acquired at a 25 s and 60 s delay from the start of the intravenous contrast injection, respectively. Axial images were reconstructed with a slice thickness of 1.25 mm at an interval of 0.625 mm and were stored for analysis.

Patients underwent DSA for a continuous regional arterial infusion, for hemorrhage spot detection or when there was a significant clinical SVT manifestation such as ascites. DSA was performed using Seldinger technique through the femoral artery with a biplane digital subtraction angiography unit (Axiom Artis dTA; Siemens Healthcare). After selective catheterization of the splenic, superior mesenteric and inferior mesenteric arteries, 25–40 mL of iopromide (Ultravist; 300 mg I/mL, Bayer Schering Pharma) was injected with a power injector at a rate of 5–10 mL/s *via* a 5 F catheter. The views were acquired during the hepatic arterial and portal venous phases for analysis.

Image analysis

All image resources were stored on a commercial workstation (Syngo VE32E, Siemens Medical Solutions). Two experienced gastrointestinal radiologists who were blind-



Figure 1 Splanchnic vein thrombosis in necrotizing acute pancreatitis detected by computed tomographic venography. The arrow denotes the filling defects suggesting the formation of splenic vein thrombosis. SVT: Splanchnic vein thrombosis; AP: Acute pancreatitis; CTV: Computed tomographic venography.

ed to the clinical and DSA data, reviewed all of the CT data separately. An experienced interventional radiologist and an experienced endovascular gastrointestinal surgeon who were blinded to the clinical and CT data, reviewed all of the DSA data separately. The presence and location of the SVT were assessed, and any diagnostic differences between the two doctors were resolved by a discussion after which a consensus on the results was reached.

Statistical analysis

The data analyses were performed using SPSS 20.0 (IBM SPSS Statistics; IBM Corporation). In our study, with DSA as the comparison, the sensitivity, specificity, positive predictive value, and negative predictive values of CTV were determined, and 95% confidence intervals (CIs) were calculated according to the efficient-score model. A kappa value was used to quantify the inter-reader agreement for detecting the presence of SVT by CTV and DSA. A *P* value < 0.05 was regarded as statistically significant (two-tailed test).

RESULTS

Between July 1, 2011 and June 30, 2013, of all the 358 patients with clinical, laboratory, or radiographic AP evidence, 43 with pancreatic or peripancreatic necrosis underwent CTV and DSA within 3 d. Their mean age was 44.6 ± 13.3 years, and 30 (69.8%) of them were male. Twenty-seven (62.8%) patients had critical AP, 5 (11.6%) had severe AP, and 11 (25.6%) had moderate AP.

According to the DSA results, 17 (39.5%) of the 43 total patients had SVT (the SVT locations and numbers are presented in Table 1), while according to the CTV results, 19 (44.2%) patients had SVT. CTV identified all of the DSA-positive cases, but it also resulted in 2 false-positive diagnoses. Figure 1 shows a typical example of SVT identified by CTV. One was diagnosed as a multiple filling defect, and the other was not clearly displayed by CTV. Thus, the CTV positive predictive and negative predictive values for SVT detection were 89.5% (95%CI:

Table 1 Distribution of splanchnic vein thrombosis

Location of SVT	No. of patients
PV isolated	1
SplV isolated	7
SMV isolated	5
SMV + PV	1
PV + SplV	0
SplV + SMV	2
SMV + PV + SplV	1

SVT: Splanchnic vein thrombosis; PV: Portal vein; SplV: Splenic vein; SMV: Superior mesenteric vein.

65.5%-98.2%), and 100% (95%CI: 82.8%-100%), respectively (Table 2). The κ value was 0.91, which indicated an excellent inter-reader agreement.

DISCUSSION

In our study, we observed that SVT has a high incidence in patients with necrotizing AP, and CTV has high positive and negative predictive values for detecting SVT. That finding suggests that CTV could serve as an alternative examination for screening and assessing SVT in this entity.

The incidence of SVT for all AP patients is relatively low, and the complications directly related to SVT are rare^[4,8]. However, perivascular inflammation and compression by peripancreatic collections or pancreatic necrosis increase the frequency of SVT^[1,2]. A retrospective study recently showed that 53% of patients with necrosis developed SVT^[4]. Our study suggested that approximately 4 out of 10 patients with necrotizing AP have SVT.

It has been reported that SVT can lead to hemorrhage, bowel ischemia, and liver failure, but the signs and symptoms may overlap with those of pancreatitis^[2,9]. Additionally, collaterals and varices caused by SVT increase the risk of hemorrhage during minimally invasive approaches^[3]. Thus, it is important to establish a protocol for screening SVT in patients with necrotizing AP.

DSA is an invasive and time-consuming examination that usually is performed only when the thrombosis causes clinical conditions or is significantly suspected. In contrast, CTV is a non-invasive and quick procedure that can be easily added after performing CECT^[10,11]. Additionally, CTV also reveals extravascular abnormalities, mesenteric edema, and the relationship between peripancreatic necrosis and blood vessels^[1]. Thus, CTV serves as a more suitable screening tool for patients with necrotizing AP. In addition to the technical matters, CTV results are more likely influenced by extravascular abnormalities. In our study, CTV made 2 diagnoses that were different from the DSA diagnoses.

One limitation of our study is that during the interval between CTV and DSA, the splanchnic system status may have changed. Additionally, the sample size was not large enough and it was a single-center retrospective study.

Although more experience should be gained to deter-

Table 2 Diagnostic accuracy of computed tomographic venography in splanchnic vein thrombosis in patients with necrotising acute pancreatitis

Findings	Positive (DSA)	Negative (DSA)	Total
Positive (CTV)	17	2	19
Negative (CTV)	0	24	24
Total	17	26	43
Sensitivity	100% (95%CI: 77.1%-100%)		
Specificity	92.3% (95%CI: 73.4%-98.7%)		
Positive predictive value	89.5% (95%CI: 65.5%-98.2%)		
Negative predictive value	100% (95%CI: 82.8%-100%)		

CTV: Computed tomographic venography; SVT: Splanchnic vein thrombosis; AP: Acute pancreatitis; DSA: Digital subtraction angiography.

mine the role of CTV for detecting SVT in necrotizing AP patients, our study findings suggest that CTV might effectively replace DSA and be the routine imaging method.

COMMENTS

Background

Splanchnic vein thrombosis (SVT) may cause severe clinical conditions like hemorrhage, small bowel ischemia and hepatic failure. Its incidence in acute pancreatitis (AP) patients varied widely in different studies. In necrotizing AP patients, the perivascular inflammation and compression by peripancreatic collections or pancreatic necrosis increase the frequency of SVT.

Research frontiers

SVT is a well-recognized acute pancreatitis complication and more often occurs in severe patients, especially those with necrosis collections. Few studies have evaluated the effectiveness of computed tomographic venography (CTV) for SVT detection in necrotizing AP patients. In this study, the authors show that CTV could serve as an alternative examination for SVT screening with high accuracy.

Innovations and breakthroughs

Recent studies have suggested that it is important to establish a protocol for screening SVT in patients with necrotizing AP. This is the first article which shows that CTV is an effective examination for SVT detection in patients with necrotizing acute pancreatitis.

Applications

By showing that SVT has a high incidence in patients with necrotizing AP and CTV has high accuracy for detecting it, this study suggests that CTV might effectively replace DSA and be the routine imaging method.

Peer review

This paper is well written and very interesting. It indicates that CTV is an effective examination for SVT detection in patients with necrotizing AP with high positive and negative predictive values. It has great clinical significance.

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Decreased expression of gastrophilin 1 in gastric mucosa of gastric cancer patients

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Abstract

AIM: To investigate the expression of gastrophilin 1 (GKN1) in normal gastric mucosa, precancerous lesions and gastric cancer tissues, and to analyse its correlations with tumour site and pathological pattern.

METHODS: Thirty gastric cancer patients (12 cases of diffuse type and 18 cases of intestinal type), 13 atrophic gastritis patients and 15 healthy volunteers with almost normal gastric mucosa (superficial gastritis) were enrolled in this study. *Helicobacter pylori* (*H. pylori*) infection was examined in all subjects. All gastric mucosa biopsy specimens were obtained. Cancer-adjacent specimens were taken from corresponding gastric cancer patients. Immunohistochemistry and real-time PCR were performed to determine the expressions of the GKN1 protein and mRNA, respectively.

RESULTS: *H. pylori* infection had no significant association with age, gender, tumour site or pathological pattern in all subjects. Compared with the superficial gastritis and atrophic gastritis groups, the expression of GKN1 protein ($P = 0.011$) and mRNA ($P < 0.001$) in gastric cancer was significantly decreased. The *GKN1*

mRNA level in diffuse type gastric cancer was significantly lower than in intestinal type gastric cancer (0.296 ± 0.076 vs 0.525 ± 0.164 , $P < 0.001$).

CONCLUSION: Compared with almost normal gastric mucosa, *GKN1* expression in the gastric mucosa of gastric cancer patients is decreased; this is associated with progression and prognosis of gastric cancer.

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Key words: Gastrophilin 1; Gastric cancer; Gastric mucosa; Expression

Core tip: The current study evaluated the expression of gastrophilin 1 (GKN1) in normal gastric mucosa, gastric cancer tissues and gastric lesions. We found that *GKN1* expression was decreased in gastric cancer tissues. Furthermore, low expression of *GKN1* was particularly associated with diffuse types of gastric cancer patients. The role of GKN1 in gastric carcinogenesis requires further investigation.

Guo XY, Dong L, Qin B, Jiang J, Shi AM. Decreased expression of gastrophilin 1 in gastric mucosa of gastric cancer patients. *World J Gastroenterol* 2014; 20(44): 16702-16706 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16702.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16702>

INTRODUCTION

Gastric cancer is the fourth most common cancer and second leading cause of cancer death worldwide^[1,2], with a high incidence in Asia^[3]. In China, the incidence of gastric cancer ranks third among all malignant tumours^[4]. According to a study conducted by Sun *et al*^[5], the mortality rate of gastric cancer in China is 26.3 per 100000. The risk factors for gastric cancer include *Helicobacter pylori* (*H. pylori*) infection, geographical location, diet,

Table 1 Clinical characteristics and pathological data of the four groups

Group	Superficial gastritis	Gastric cancer		Cancer-adjacent	Atrophic gastritis
		Diffuse type	Intestinal type		
Case (n)	15	12	18	30	13
Age (yr)	54.3 ± 12.4	57.6 ± 10.9	59.8 ± 11.2	58.9 ± 11.08	60.5 ± 10.98
Gender (male/female)	10/5	8/4	12/6	20/10	8/5
<i>H. pylori</i> infection (positive/negative)	9/5	7/5	10/8	17/13	7/6

H. pylori: *Helicobacter pylori*.

and the population's genetic background^[2,6]. However, the aetiology of gastric cancer is unclear. Identifying the prevalent risk factors of gastric cancer may contribute to preventing tumour growth and spread, and could reduce the incidence and mortality. According to the classification criteria developed by Lauren in 1965^[7], gastric cancer is frequently divided into the intestinal type and the diffuse type. The intestinal type is more common in males and older age groups, while the diffuse type is more common in younger age groups; the incidence has no association with sex difference^[8]. The different histological types may have different molecular changes and aetiologies.

Gastroke 1 (GKN1), also known as AMP18 or CA11, has been detected recently in normal gastric mucosa, but not in other regions of the gastrointestinal tract^[9]. Decreased GKN1 expression is found in gastric cancer tissues and precancerous lesions^[10,11]. In *H. pylori* infection-induced lesions, GKN1 is also downregulated^[12,13]. Some studies have reported that GKN1 is involved in the protection of gastric mucosal integrity, has roles in mucosal healing after lesions, and that the overexpression of GKN1 in gastric cancer cells can induce apoptosis^[14]. Although the function of GKN1 has not been clearly defined in previous studies, it is strongly suggested that GKN1 may play a role as a tumour suppressor in, and is a biomarker for, gastric cancer^[15].

In the present study, we examined the expression of GKN1 in normal gastric mucosa, precancerous lesions and gastric cancer tissues. The relationships between GKN1 expression with gastric cancer histological type and *H. pylori* infection were investigated.

MATERIALS AND METHODS

Subjects

Patients with gastric cancer and precancerous lesions in the Second Affiliated Hospital of Xi'an Jiao Tong University of Medicine School (Xi'an, China) and People's Hospital of Yan'an (Yan'an, China) were enrolled in this study from July 2010 to July 2012. There were 30 gastric cancer patients (12 cases of diffuse type and 18 cases of intestinal type) and 13 atrophic gastritis patients, who were confirmed through pathological diagnosis by at least two different specialists. No patient received pre-operative chemotherapy or radiotherapy. Fifteen healthy volunteers with almost normal gastric mucosa (superficial gastritis), and without any history of gastric cancer, were

selected as controls. Patients with other types of tumours were excluded. All subjects were examined for *H. pylori* infection using the rapid urea test, C¹³ urea breath test and serum antibody test. A positive diagnosis was defined when the results of two or more methods were positive. The clinical and pathological data are described in Table 1.

All subjects received gastroscopy and all gastric mucosa biopsy specimens were obtained. Cancer-adjacent biopsy specimens were taken from corresponding gastric cancer patients, with a distance of 5 cm to the visible edge of the tumour tissue. Each specimen was immediately frozen in liquid nitrogen for 1 h and subsequently stored at -80 °C for subsequent analysis. At least three slides were made for different areas of specimens in each group. The Ethics Committee of the Second Affiliated Hospital of Xi'an Jiao Tong University of Medicine College approved this study. Informed consent was obtained from all subjects.

Immunohistochemistry method

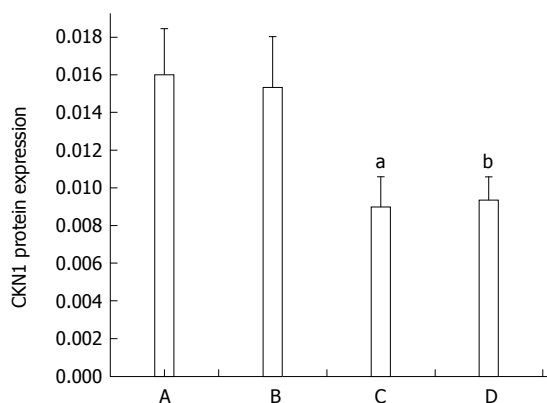
After being fixed in 10% formaldehyde, the tissues were embedded in paraffin and then cut into slices, before being placed onto slides. Antigen retrieval was performed in citrate buffer (pH = 6.0 ± 0.1) using the microwave method. The slides with the primary antibody, which were diluted 1:100 (Abcam Inc., Cambridge, MA, United States), were left overnight at 4 °C. On the second day, after incubation with the second antibody for 25 min and streptavidin/peroxidase for 30 min, colouration analysis was performed. The results of staining were assessed quantitatively using IPP 6.0 software (Media Cybernetics, Silver Springs, MD, United States).

Real-time PCR analysis

A Trizol reagent kit (Takara, Tokyo, Japan) was used to extract total cellular RNA from snap-frozen samples, according to the manufacturer's protocol. Absorbance at 260 nm using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States) determined the concentration of the RNA. In addition, electrophoresis on an ethidium bromide-stained 1% agarose gel was used to assess RNA integrity. The PrimeScript™ RT reagent kit (Takara) was used to reverse transcribe the total cellular RNA at 37 °C for 15 min; the reaction was stopped by heat inactivation for 5 s at 85 °C. Primer sequences used for amplification were as follows: GKN1 upstream primer, 5'-GCTT-

Table 2 Tumour site and pathological pattern in the gastric cancer group

Index		Case (n)	P value
Tumour site	Upper and middle	20	> 0.05
	Lower	10	
Pathological pattern	Diffusion	11	> 0.05
	Intestines	19	

**Figure 1** Expressions of Gastrokine 1 protein in the four groups. A: superficial gastritis; B: atrophic gastritis; C: gastric cancer; D: Cancer-adjacent. ^a*P* < 0.05 vs superficial gastritis group; ^b*P* < 0.05 vs the atrophic gastritis group.

GCCTACTCCTCTGTCCACTG-3', downstream primer, 5'-CTCACTGACTGCTGCCACTTCC-3'; β -actin upstream primer, 5'-AGGAAGGAAGGCTGGAA-GAGTG-3', downstream primer, 5'-AGGAAGGAAG-GCTGGAAGAGTG-3'. An iCycler iQ system (Bio-Rad Laboratories Inc., Hercules, CA, United States) was used for mRNA expression analysis using quantitative real-time PCR. Real-time PCR was performed using an ABI PRISM 7500 sequence detector and SYBR green I chemistry (Applied Biosystems, Foster City, CA, United States). PCR conditions were as follows: 3 min denaturation at 94 °C in a final volume of 25 μ L, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The PCR amplification products were determined using agarose gel electrophoresis. Melt curve analysis was performed to determine the specificity of PCR products. All cDNA samples were analysed in duplicate. The expression of β -actin was used as the internal control. Expression levels in various biopsy specimens were quantified by calculating the initial target concentrations using the obtained threshold cycle values. Calculation of the fold-change in mRNA expression was performed relative to the β -actin endogenous control using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were expressed as means \pm SD. Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., IL, United States). Tukey's *t*-test or ANOVA were used to analyse the differences between the groups. *P* < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics and pathological data

The clinical characteristics and pathological data of the subjects are shown in Tables 1 and 2. The relationship between *H. pylori* infection and clinical pathological factors was analysed using Logistic regression. *H. pylori* infection had no significant association with age, gender, tumour site or pathological pattern in all subjects.

Expression of GKN1 protein

Expression of the GKN1 protein in gastric cancer was significantly decreased compared with the superficial gastritis group (0.009 ± 0.001 vs 0.016 ± 0.002 , *P* = 0.011) and atrophic gastritis group (0.009 ± 0.001 vs 0.015 ± 0.002 , *P* = 0.044, Figure 1). Expression of the GKN1 protein was also decreased in the cancer-adjacent group (0.009 ± 0.001). There was no significant difference between the atrophic gastritis and superficial gastritis groups (*P* = 0.803), or between the gastric cancer and cancer-adjacent groups (*P* = 0.354). The immunoreactions mainly appeared in the nucleus, with hardly any staining in the cytoplasm, of normal gastric epithelial cells, cancer cells, inflammatory cells and gland cells (Figure 2).

Expression of GKN1 mRNA

GKN1 mRNA was weakly expressed in the gastric cancer group (Figure 3). By contrast, *GKN1* mRNA expression was abundant in the superficial gastritis group (0.989 ± 0.181), and was significantly higher than in the gastric cancer group (0.433 ± 0.176 , *P* < 0.001) and cancer-adjacent group (0.626 ± 0.167 , *P* < 0.001). In the atrophic gastritis group (0.819 ± 0.123), the expression level of *GKN1* was significantly lower than in the superficial gastritis group (*P* = 0.011). The mRNA level of *GKN1* in diffuse type gastric cancer patients was lower than in intestinal type gastric cancer patients (Figure 4) (0.296 ± 0.076 vs 0.525 ± 0.164 , *P* < 0.001). Compared with the superficial gastritis group, the *GKN1* mRNA levels in the two types of gastric cancer were both decreased (*P* < 0.001).

DISCUSSION

GKN1 is a novel protein that is exclusively expressed in normal gastric tissue, but absent in gastric cancer tissues. GKN1 is located in the superficial gastric epithelium, playing a significant role in protecting and maintaining gastric epithelium integrity after injury. If the protein is downregulated, the repair process may be impaired. Recently, a number of studies have found that deficiency of GKN1 can result in instability of the gastric epithelium. Invasive factors such as *H. pylori* contribute to the downregulation of GKN1, whilst inducing ulceration and cancer. GKN1 may play a key role in the progression of gastric cancer, and may be a potential biomarker for the early detection of gastric cancer.

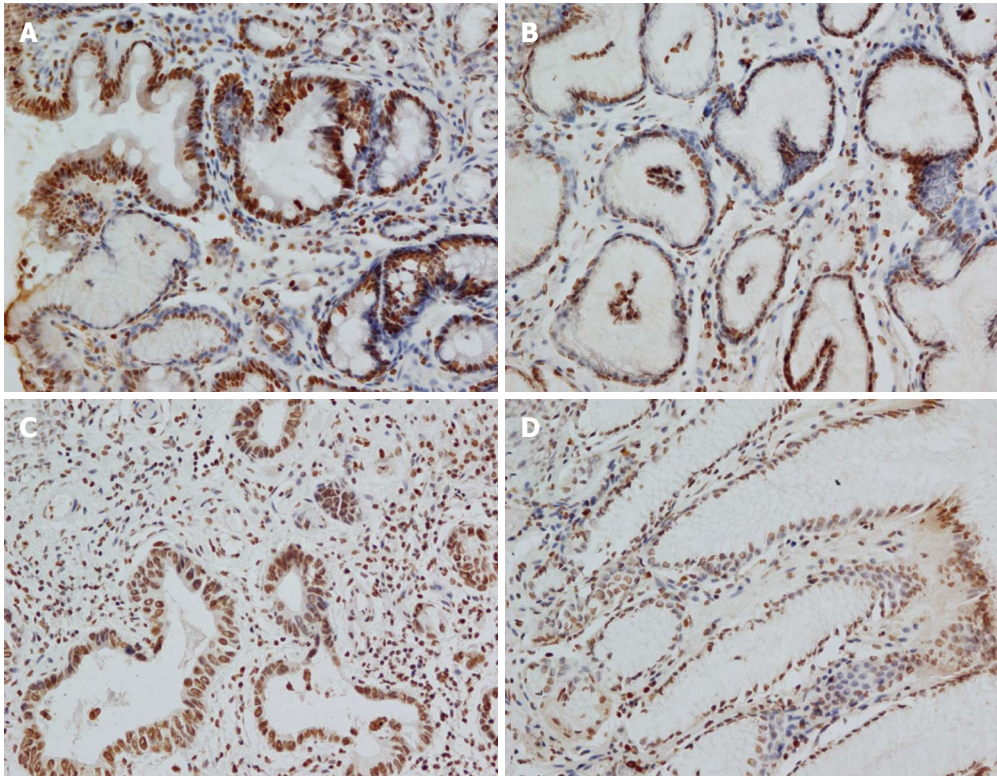


Figure 2 Immunohistochemical staining results in the four groups. A: Superficial gastritis; B: Atrophic gastritis; C: Gastric cancer; D: Cancer-adjacent.

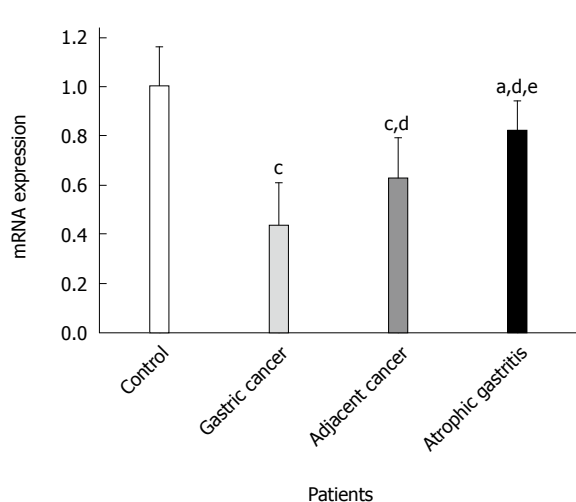


Figure 3 Expressions of Gastrakine 1 mRNA in the four groups. ^a $P < 0.05$ vs superficial gastritis group; ^c $P < 0.01$ vs superficial gastritis group; ^d $P < 0.01$ vs gastric cancer group; ^e $P < 0.01$ vs cancer-adjacent group.

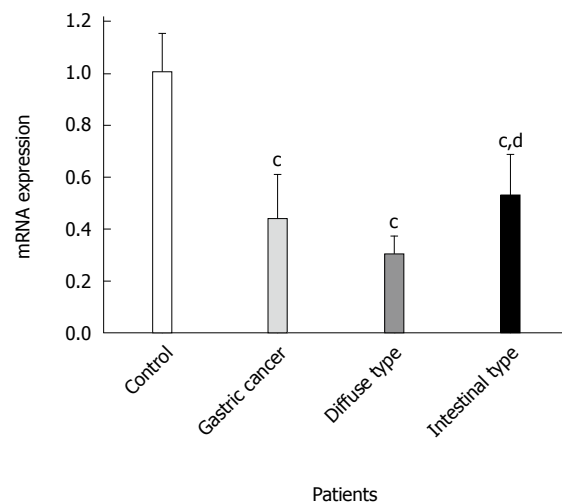


Figure 4 Expressions of Gastrakine 1 mRNA in diffuse type and intestinal type gastric cancer. ^c $P < 0.01$ vs superficial gastritis group; ^d $P < 0.01$ vs diffuse type gastric cancer.

The present study investigated GKN1 expression in different mucosa biopsy specimens from gastric cancer, cancer-adjacent lesions, atrophic gastritis and normal control subjects (superficial gastritis patients). We found that *GKN1* mRNA expression was progressively downregulated from corresponding distant non-tumour tissues to tumour tissues, and was lower than in the control tissues of both groups. This suggested that low or absent expression of *GKN1* may contribute to gastric carcinogenesis. This is consistent with a previous study that dem-

onstrated decreased GKN1 expression in gastric cancer tissues^[16]. In addition, we analysed *GKN1* expression in two types of gastric cancer and found that the mRNA level of *GKN1* was lower in patients with diffuse type gastric cancer. This indicates that GKN1 may be related to tumour classification.

In conclusion, we found differential expression of *GKN1* in gastric cancer tissue, gastric lesions and normal gastric tissue. Low expression of GKN1 was especially detected in diffuse type gastric cancer patients. However,

the role of GKN1 in gastric carcinogenesis requires further studies.

COMMENTS

Background

Gastric cancer remains the fourth most common cancer and second leading cause of cancer death worldwide. Gastrokeine 1 (GKN1), detected recently in normal gastric mucosa, is involved in the development of gastric lesions and gastric cancer.

Research frontiers

GKN1 has been found to be downregulated in gastric cancer, but the relationship between GKN1 expression and clinical characteristics is unclear. The current study aimed to evaluate the expression of GKN1 in normal gastric mucosa, gastric cancer tissues and matched tumour-adjacent tissues, and to investigate its relationship with *Helicobacter pylori* (*H. pylori*) infection and histological classification.

Innovations and breakthroughs

This study reported that GKN1 expression is decreased in gastric cancer tissue. Furthermore, low expression of GKN1 is especially found in diffuse type gastric cancer patients.

Applications

The authors found GKN1 was expressed differently in gastric cancer and normal gastric tissue, and it may be related to the tumour classification. The results provide a better understanding of the role of GKN1 in the development of gastric cancer.

Peer review

The authors examined the expression of GKN1 in normal gastric mucosa, pre-cancerous lesions and gastric cancer tissues. The relationships between GKN1 expression and gastric cancer histological type and *H. pylori* infection were also investigated. The results are interesting and may supply a new direction for GKN1 in gastric carcinogenesis.

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Complete laparoscopic resection of the rectum using natural orifice specimen extraction

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Author contributions: Hisada M and Katsumata K contributed equally to this work; Kasuya K and Tsuchida A designed the research; Hisada M, Ishizaki T, Enomoto M and Matsudo T performed the research; Hisada M and Katsumata K analyzed the data; Hisada M and Katsumata K wrote the paper.

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Abstract

AIM: To investigate how complete laparoscopic anterior resection with natural orifice specimen extraction (NOSE), as a novel minimally invasive surgery, compares to conventional laparoscopic surgery.

METHODS: Twenty patients who underwent complete laparoscopic anterior resection with NOSE and 50 patients who underwent laparoscopic assisted anterior resection by the conventional method between 2011 and 2012 were studied. Selection for complete laparoscopic anterior resection with NOSE was decided on the basis of tumor size, localization of the tumor, and body mass index. Outcomes related to surgery, including operation time, postoperative wound pain, hospital stay after surgery, the number of totally dissected lymph nodes, postoperative complications (suture failure and wound infection), and anal function, were reviewed retrospectively. Anal function was assessed at 3 and 6 mo after surgery using the Wexner fecal incontinence scoring system.

RESULTS: Complete laparoscopic resection with NOSE was performed to completion in all 20 patients. There was no patient emergency that required conversion to conventional laparoscopic surgery or open surgery. The comparison between complete laparoscopic resection with NOSE and conventional laparoscopic surgery showed no significant differences in the maximal diameter of the tumor, number of totally dissected lymph nodes, bleeding volume, mean operation time, time to start of oral ingestion, postoperative hospital stay, and postoperative complications. On the other hand, with regard to pain after epidural anesthesia, the total usage of analgesia in this novel surgical technique was 1.85 ± 1.8 times, whereas it was 5.89 ± 2.86 in conventional laparoscopic surgery ($P < 0.001$). The postoperative pain period was 1.9 ± 1.9 d in this novel surgical technique, whereas it was 3.43 ± 1.41 d in conventional laparoscopic surgery ($P < 0.004$). In complete laparoscopic surgery with NOSE, the mean postoperative follow-up period was 20 mo (range: 12-30 mo). Neither local recurrence nor remote metastasis was observed during the follow-up period.

CONCLUSION: Complete laparoscopic anterior resection using NOSE does not require any incision and has excellent cosmetic properties, with mitigated postoperative pain.

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Key words: Complete laparoscopic surgery; Incisionless surgery; Natural orifice specimen extraction; Transanal specimen extraction; Less invasive surgery

Core tip: Natural orifice specimen extraction (NOSE) has been reported as a less invasive surgery to avoid the problems arising from small incisions. In this study, we present details of a surgical technique for NOSE and the outcomes of complete laparoscopic anterior resection using NOSE are compared with conventional

laparoscopic anterior resection. Complete laparoscopic anterior resection using NOSE has more advantages in terms of cosmetic outcomes and mitigating postoperative pain compared with conventional laparoscopic anterior resection. Based on our study, we consider complete laparoscopic anterior resection using NOSE as an acceptable and novel minimally invasive surgery.

Hisada M, Katsumata K, Ishizaki T, Enomoto M, Matsudo T, Kasuya K, Tsuchida A. Complete laparoscopic resection of the rectum using natural orifice specimen extraction. *World J Gastroenterol* 2014; 20(44): 16707-16713 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16707.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16707>

INTRODUCTION

With the recent advances in minimally invasive surgery, laparoscopic anterior resection for rectal cancer has become a common practice. However, an incision of about 5-6 cm is still made in the abdomen for resection of the lesion or insertion of the anvil head of the automatic anastomosis device. This incision, though small, carries risks of postoperative wound pain, infections, adhesions after surgery, or abdominal incisional hernia. For this reason, an even less minimally invasive surgical technique is required. In such a situation, natural orifice specimen extraction (NOSE) has been introduced as a less invasive surgery to solve the problems arising from small incisions. However, these surgical techniques have not yet become widespread.

Therefore, we have performed complete laparoscopic anterior resection in 20 patients through transanal extraction of the lesion without making any incision in the abdomen, and herein describe the surgical techniques and outcomes of this treatment.

MATERIALS AND METHODS

The ethics committee of Tokyo Medical University Hospital approved the study and written informed consent was obtained from patients who would receive complete laparoscopic anterior resection with NOSE. Patients diagnosed with rectal cancer were selected for complete laparoscopic anterior resection with NOSE using several criteria. The indications for complete laparoscopic anterior resection with NOSE were as follows: (1) tumor located at the distal side of the sigmoid colon to the upper side of the rectum; (2) a tumor diameter less than 5 cm and no serosal exposure, as evaluated by computed tomography; (3) lymph node metastasis less than cN1; and (4) no bulky mesorectum, as evaluated by a body mass index less than 30 kg/m². A massive tumor, surrounding lymph nodes depicted on CT, or obese patients were excluded. A team that was proficient in various laparoscopic colorectal procedures at our hospital since 2004 performed all the operations. NOSE surgery was

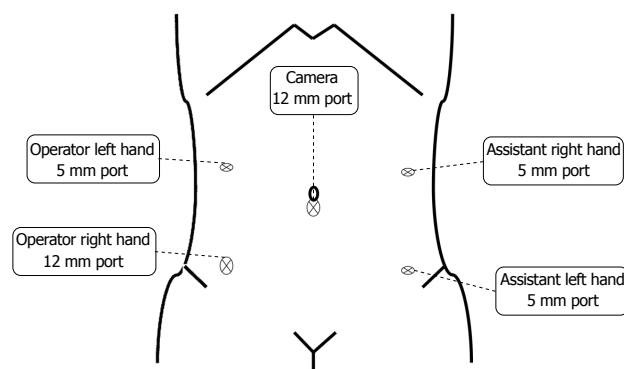


Figure 1 Port sites.

performed since 2010. If drawing the resected bowel intracorporeally was impossible or critical damage to the residual rectum occurred during the drawing, the operative procedure was converted to conventional laparoscopic surgery using the double stapling technique or to open surgery. All patients underwent oral magnesium citrate bowel preparation twice, 2 d before the operation. Twenty patients who underwent complete laparoscopic anterior resection with NOSE and 50 patients who underwent laparoscopic assisted anterior resection by the conventional method between 2011 and 2012 were studied. Parameters including the operation time, postoperative wound pain, hospital stay after surgery, the number of totally dissected lymph nodes, postoperative complications, and anal function were evaluated. The anal function was assessed at 3 and 6 mo after surgery using the Wexner fecal incontinence scoring system. Statistical differences were examined using the *t*-test and Fisher's χ^2 test. A *P* value < 0.05 indicated a statistically significant difference. The statistical analysis software used was Dr. SPSS II for Windows.

Surgical techniques

Five ports were created (Figure 1), and a pneumoperitoneum was created at 8-10 mmHg. One 12 mm camera port was inserted in the umbilical region or its adjacent area, another 12 mm port was used for the lower right abdomen, and 5 mm ports for the right and left upper abdomen and lower left abdomen were created. The mesosigmoid adjacent to the right common iliac artery was exfoliated from the inside. Exfoliation was continued cephalad, and the inferior mesenteric root was identified. To treat the inferior mesenteric artery, high ligation was performed; however, in some cases, the left colic artery was preserved by low ligation, depending on the stage of progression. The back side of the mesentery was exfoliated toward the outside as usual, and the lateral side of the sigmoid colon was exfoliated and communicated with the medial exfoliated layer. The descending colon should be manipulated as much as possible; otherwise the transanal extraction of the bowel becomes difficult or the tension after anastomosis may cause the risk of suture failure. At this time, the oral dissection line at 10 cm or more from the tumor is determined, as the guidelines indicate, to

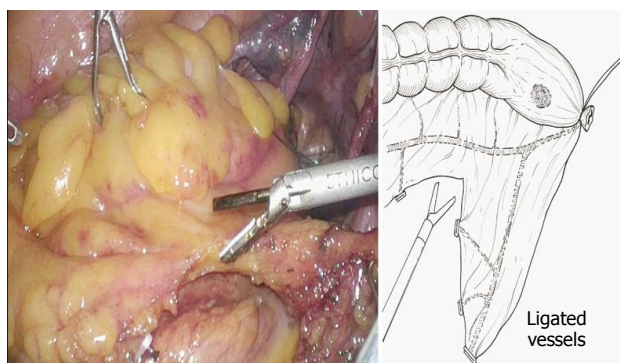


Figure 2 Dissecting the sigmoid colon mesentery.

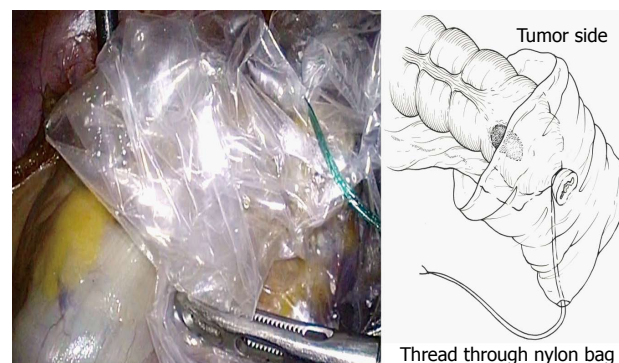


Figure 5 Protecting the tumor using a nylon bag.

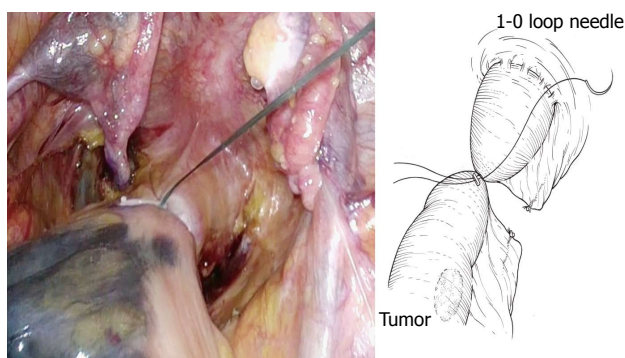


Figure 3 Bowel closure.

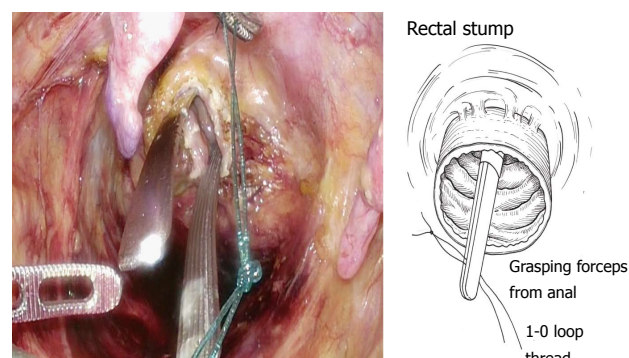


Figure 6 Grasping a 1-0 loop thread for drawing.

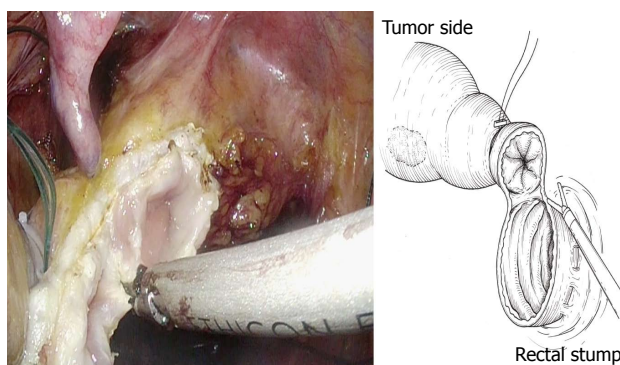


Figure 4 Opening the rectum using laparoscopic coagulating shears.

facilitate transanal extraction, and the marginal artery is totally dissected in a preserving manner, confirming the blood flow in the reconstruction bowel. The mesentery is dissected along the superior rectal artery and vein (Figure 2), and the oral dissection line is confirmed to sufficiently reach the pelvic floor.

Although it depends on the position of the tumor, the rectum is exfoliated sufficiently measuring 5 cm or longer from the tumor toward the anus. The dissection line of the bowel towards the anal side is determined and a trimming treatment of the mesorectum is performed.

The bowel should be closed at the tumor towards the anal side with a 1-0 loop needle. First, the needle is inserted in the anterior wall of the bowel up to the se-

romuscular level, and turned to the posterior wall of the bowel. Seromuscular suturing is performed in the contralateral bowel, and the closing ligation is made through the loop. After tightening the loop, a Hem-o-lok clip is attached to the thread on the needle side and the bowel side is tightened for bondage (Figure 3). Rectal irrigation is performed from the anus with 500 mL of physiological saline containing povidone-iodine, and then the rectum is opened by laparoscopic coagulating shears (LCS) (Figure 4). A nylon bag is then inserted to protect the tumor from the 12 mm port in terms of implantation or infections, and a 1-0 loop needle thread is pierced through it (Figure 5). To exteriorize the resected bowel, the straight grasping forceps are inserted transanally, followed by grasping a 1-0 loop thread, and drawing it into the residual rectum (Figure 6). In doing this, butyl scopolamine is administered intravenously, if necessary, to prevent the residual rectum from developing a spasm. The tumor is removed and the bowel is reconstructed from the body transanally. The tumor is dissected towards the oral side and the marginal artery is treated by inserting the anvil head of the automatic anastomosis device, repositioning the reconstructed bowel inside the body cavity (Figure 7). After sufficient irrigation of the rectal stump and pelvic cavity, purse-string suturing of the rectal stump with a 2-0 monofilament is performed laparoscopically, and then the anvil from the rectum is inserted and ligated for closure, keeping the central rod out (Figure 8A and B). When reefing of the rectal stump seems insufficient, oc-

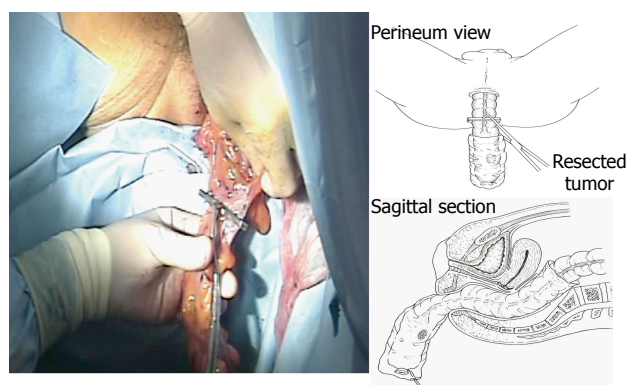


Figure 7 Removing the tumor transanally.

casionally additional reefing may be performed with an end loop. Finally, after sufficient irrigation of the pelvic cavity, anastomosis is performed with the single staple technique (SST), using an automatic anastomosis device.

RESULTS

Information concerning the 20 patients undergoing this surgical technique is shown in Table 1. Patients comprised 12 men and 8 women. No patient required conversion to conventional laparoscopic surgery or open surgery. The maximal diameter of the tumor ranged from 10 mm to 50 mm, with a mean of 27 ± 9 mm. The comparison with conventional laparoscopic surgery is shown in Table 2. In laparoscopic surgery, the tumor diameter ranged from 10 mm to 90 mm, with a mean of 38.5 ± 18 mm. Although there was no significant difference between the new surgical technique and laparoscopic surgery, the tumor diameter was slightly smaller. The number of totally dissected lymph nodes was 17.7 ± 7.7 in the new surgical technique, whereas it was 17.5 ± 8.8 in conventional laparoscopic surgery, showing no significant difference.

The bleeding volume ranged from 10 mL to 245 mL with a mean of 114 ± 72 mL. The bleeding volume in conventional laparoscopic surgery was 120 ± 56 mL, denoting no significant difference between the new technique and conventional laparoscopic surgery. The mean operation time was 278 ± 39 min; the time required for purse-string suturing was 15 ± 4 min; the mean operation time in conventional laparoscopic surgery was 240 ± 77 min. There were no statistically significant differences between the techniques in procedure times.

The time to start oral ingestion after surgery was 4 ± 1.4 d for this surgical technique, which was not significantly different to the 4.3 ± 0.9 d for the conventional technique. Postoperative hospital stay was 11.8 ± 1.6 d for this surgical technique, which was not significantly different to the 11 ± 3.2 d for conventional laparoscopic surgery.

Regarding postoperative analgesia, in both groups, 0.75% ropivacaine was used for epidural anesthesia, and when pain occurred, 15 mg pentazocine mixed in 100 mL of physiological saline was administered intravenously

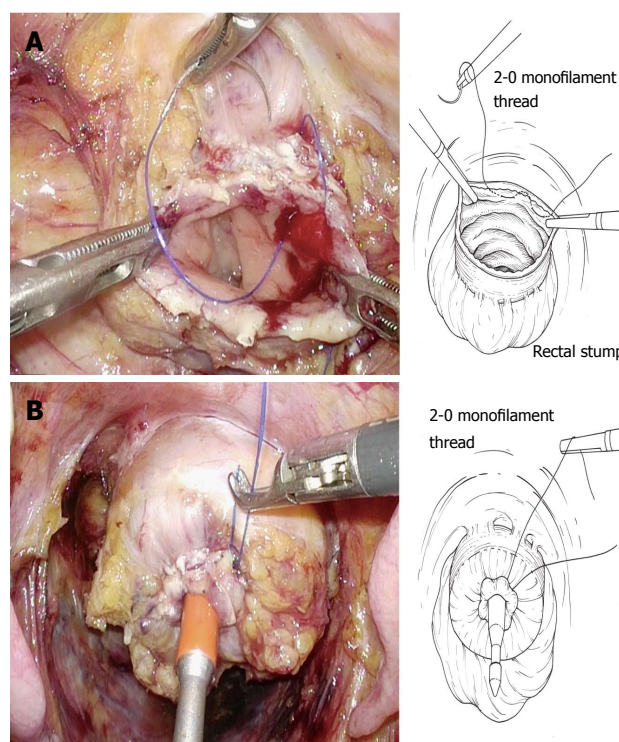


Figure 8 Purse string suture of the rectal stump (A) and ligation for closure of the rectal stump (B).

after 1 h. With regard to pain after epidural anesthesia, the total postoperative usage of analgesia in this surgical technique was 1.85 ± 1.8 times, whereas it was 5.89 ± 2.86 in conventional laparoscopic surgery, showing a significant decrease ($P = 0.001$). The postoperative pain period was 1.9 ± 1.9 d for this surgical technique, whereas it was 3.43 ± 1.41 d in conventional laparoscopic surgery, showing a significant decrease ($P = 0.004$).

Postoperative complications in this surgical technique included suture failure in one patient, which was conservatively mitigated, and one patient each with ischemic enteritis in the anastomotic part and anal pain, which were observed early after the surgery and were conservatively mitigated, without occurrence of surgical site infection (SSI). In the conventional laparoscopic surgery cases, suture failure was found in four patients, and one of them underwent colostomy. SSI was observed in 8 patients. The postoperative follow-up period for this surgical technique ranged from 12 to 30 mo, with a mean of 20 mo. Patients at stage 3a and above underwent postoperative chemotherapy. Neither local recurrence nor remote metastasis was observed during the follow-up period. In conventional laparoscopic surgery, the postoperative follow-up period ranged from 13 to 32 mo, during which time anastomotic recurrence and remote metastasis were found in one patient each. This surgical technique draws the tumor from the anus; therefore, those with a low T factor were selected, and there may be no difference in terms of radical cure between these surgical techniques, although the background factors may vary with the patients.

Anal function was evaluated using the Wexner fecal

Table 1 Patient information

Case	Age	Gender	Tumor size (mm)	TNM stage	Operation time (min)	Suturing time (min)	Blood loss	Complication	Wexner incontinence score	
									3 mo	6 mo
1	63	F	30	T2N0	214	13	10	-	0	0
2	72	F	22	T1N0	252	20	90	-	0	0
3	47	F	35	T3N0	315	24	10	Anal pain	0	0
4	70	M	38	T3N0	343	20	150	Ischemic colitis	6	1
5	62	M	20	T1N0	312	23	228	-	0	0
6	77	M	35	T3N0	256	17	200	-	1	0
7	65	M	38	T3N1	260	13	35	Leakage	1	0
8	72	F	15	T1N0	345	15	203	-	0	0
9	65	M	26	T2N0	262	13	27	-	0	0
10	66	M	23	T2N0	280	18	120	-	0	0
11	70	F	20	T1N0	247	13	118	-	0	0
12	46	M	18	T1N0	277	18	92	-	0	0
13	56	F	22	T1N0	250	14	30	-	0	0
14	68	F	18	T1N0	291	11	145	Anastomotic ulcer	0	0
15	57	F	33	T2N0	267	8	95	-	0	0
16	40	M	15	T1N0	281	10	30	-	0	0
17	65	M	22	T1N1	355	9	245	-	0	0
18	77	M	50	T3N0	277	15	150	-	0	0
19	63	M	35	T3N0	215	13	192	-	0	0
20	60	M	25	T2N0	271	11	116	-	0	0

Table 2 Comparison with conventional laparoscopic surgery

	Conventional LAP	Complete LAP
Age	66.3 ± 11	63.7 ± 9
Tumor size (mm)	38.5 ± 18	27 ± 9
Dissected lymph node (count)	17.5 ± 8.8	17.7 ± 7.7
Blood loss (mL)	120 ± 56	114 ± 72
Operation time (min)	240 ± 77	278 ± 39
Count of usage of analgesic (times)	5.89 ± 2.86	1.85 ± 1.8 ^a
Term of pain (d)	3.43 ± 1.41	1.9 ± 1.9 ^a
Orally take (d)	4.3 ± 0.9	4 ± 1.4
Hospital stay (d)	11.2 ± 3.2	11.0 ± 3
Suture failure	4 cases	1 case
SSI	8 cases	None

^aP < 0.05, complete laparoscopic surgery *vs* conventional laparoscopic surgery. SSI: Surgical site infection; LAP: Laparoscopic.

incontinence scoring system. Evaluations at 3 mo after surgery were Score 6 in one patient, Score 1 in two patients, and Score 0 in the remaining seven patients. The patient with Score 6 developed postoperative ischemic change in the anastomotic part, and was improved to Score 1 about 6 mo after surgery. At 6 mo post surgery, only one patient had Score 1, and the other nine had Score 0.

All patients underwent intraoperative cytodiagnosis and culture: no floating cancer cells or bacteria were detected.

DISCUSSION

Laparoscopic surgery has become widely accepted as a minimally invasive surgery. With the occasional adoption

of surgical methods such as single port surgery, natural orifice transluminal endoscopic surgery, or NOSE, the latter two of which do not involve making an incision in the abdomen, minimally invasive surgery is expected to be further developed in the future. For NOSE, the transvaginal technique has been described in terms of problems such as elasticity of the tissue or wound healing^[1-5]. However, there are few reports on the transanal method. The reasons include a cumbersome procedure to transanally draw the lesion and to reconstruct it. Wolthuis *et al*^[6] reported that they inserted the separation bag transanally and drew the lesion for left colon cancer. However, it is also reported that the resected specimen gets folded in the bag using this method, making the diameter of the specimen larger than that of the rectum, leading to damage to the residual rectum or the anus, which is the route by which the tumor is removed. To solve this problem, there is a report of a procedure to insert the wound retractor transanally and facilitate the extraction of the lesion^[7]. The surgical technique that we have devised and reported uses a 1-0 loop thread for closure of the tumor towards the anal side, forming the supporting thread, and the tumor is drawn into the rectum, thereby allowing the tumor to be removed in the longitudinal direction against the rectal stump, leading to easy extraction. This method is contrary to the method of resecting the bowel after inversion, as reported by Hara *et al*^[8]. According to Katsuno *et al*^[9], oral dissection is done after the tumor is extracted; thus it becomes possible to dissect directly after confirming the positional relation between the tumor and the vessels in the mesentery. After tumor resection, the anvil head is mounted in the reconstructed bowel extracorporeally, and is repositioned in the body cavity. Purse-string suturing of the dissected rectum is then performed lapa-

roscopically, and reconstruction using SST is performed. SST was reported to have a reduced risk of suture failure compared with the double staple technique (DST) in some of the literature; however, other reports described no difference between them. Even though there is no established opinion^[10,11] on this matter, it was considered advantageous for wound healing because no staple-on-staple anastomosis was involved. The distal margin was also considered advantageous because of homogeneity around the entire circumference.

This method necessitates bowel incision within the peritoneal cavity; therefore, intraoperative infections may occur. However, there is a report stating that bowel irrigation before opening the bowel can decrease the infection risk and intraoperative opening of the bowel does not lead to the risk of SSI^[12,13].

To prevent local recurrence from opening the bowel, we performed rectal irrigation from the anus with 500 mL of physiological saline containing povidone-iodine and used intraoperative cytodiagnostic procedures to confirm that there were no cancer cells in the irrigation outflow from the residual rectum.

We also covered the resected bowel with a nylon bag to reduce the risk further. In fact, in patients that we have treated, pathogenic bacteria were not detected from the intraoperative irrigation fluid. With regard to intraoperative floating cancer cells, McKenzie *et al.*^[2] reported that transvaginal NOSE does not pose a risk for tumor implantation, and Ooi *et al.*^[14] stated that the protective barrier and specimen bag can reduce the risk of tumor implantation or local recurrence. We also performed extraction by covering the tumor with a nylon bag to completely prevent the risk of local recurrence, and the intraoperative cytodiagnostic procedures performed in all the patients indicated that no cancer cells were observed. Operation time, bleeding volume, postoperative wound pain, postoperative hospital stay, the number of totally dissected lymph nodes, and postoperative complications were compared between this method and conventional laparoscopic surgery. There were no significant differences in bleeding volume, postoperative hospital stay, the number of totally dissected lymph nodes, and suture failure between both groups, indicating similar results to conventional laparoscopic surgery. On the other hand, in conventional laparoscopic surgery, the mean operation time was 38 min shorter, although this was not a statistically significant difference. This may reflect the time required for purse-string suturing of the rectal stump and the time to adequately manipulate the descending colon. However, the mean operating time gradually decreased with the increase in the number of treated cases, suggesting the existence of a learning curve. The postoperative complications of this surgical technique included ischemic enteritis of the anastomosis part and postoperative anal pain in one patient each, which were conservatively mitigated. SSI was observed in eight patients in conventional laparoscopic surgery, whereas there were no occurrences in this surgical technique. Although no significant differ-

ence was observed in the incidence of SSI, it may become evident in the future with the accumulation of cases.

The indications for this method may require several conditions, as described below, to perform the extraction transanally.

Indications for transanal extraction include: the location of the primary lesion is at the distal side of the sigmoid colon to the upper side of the rectum; the tumor diameter is less than 5 cm; there is no serosal exposure, as evaluated by CT; there is little metastasis of the lymph nodes; and there is no bulky mesorectum, as evaluated by a body mass index less than 30 kg/m². Those with a massive tumor or surrounding lymph nodes depicted on CT were excluded because of the difficulty of transanal extraction. Sufficient exfoliation and manipulation up to the splenic flexure are necessary to remove the tumor and to reconstruct the bowel transanally out of the body. If the sigmoid colon has sufficient length, the surgery becomes much easier. If these conditions are not fulfilled, insufficient resection of the proximal margin or damage to the mesentery of the reconstructed bowl from excessive traction of the resected bowel may occur. If the resection line towards the anal side is ≥ 2 cm lower than the peritoneal reflection, laparoscopic purse-string suturing may be technically difficult; in such cases, sufficient exfoliation of the rectum towards the anal side is necessary.

For these reasons, the difficulty level and the pros and cons of the surgery tend to be dependent on the location of the tumor and the degree of progression. The best indication is for a patient with a tumor of less volume, located in the vicinity of the rectosigmoid segment, and length of the sigmoid colon has sufficient margins.

Despite the existence of the above conditions, this method, compared with the conventional method, had a significantly lower frequency of postoperative analgesic usage and shorter postoperative pain period because it does not involve the creation of an incision. Even though no significant difference could be established because of the small number of patients and no complications of SSI were observed, it can be inferred that there may be a sufficient advantage in not making any incision. With regard to the number of totally dissected lymph nodes or postoperative recurrence, no significant differences were observed, and with regard to radical curation and safety, this method was equivalent to the conventional method in these 20 patients. Therefore, we concluded that this method can be accepted as a minimally invasive surgery for rectal cancer or sigmoid colon cancer.

In conclusion, we have performed complete laparoscopic anterior resection using the NOSE method in 20 patients with rectal cancer. This method does not require any incision in the abdomen, and has excellent cosmetic properties with mitigated postoperative pain. Therefore, we this technique should be accepted as a novel minimally invasive surgery. This surgical technique may require several conditions, and it will be necessary to establish this technique and indications based on further examination and accumulation of more cases.

COMMENTS

Background

Recently, laparoscopic anterior resection for rectal cancer has become a common practice. However, a small incision is still made in the abdomen to resect the tumor. This small incision causes postoperative wound pain, and has risks of infections, adhesions after surgery, or incisional herniation.

Research frontiers

At present, natural orifice specimen extraction (NOSE) has been reported as a less invasive surgery to solve complications caused by creating incisions.

Innovations and breakthroughs

The present study showed that complete laparoscopic anterior resection with NOSE is the same as conventional laparoscopic anterior resection in terms of safety and oncological outcome, does not require any incision in the abdomen, and has excellent cosmetic properties.

Applications

NOSE for colorectal cancer can avoid making any incision to extract the specimen. The method of extracting the specimen is the most important process in NOSE surgery. We describe an easy way to extract the specimen without causing residual rectal injury. This should allow NOSE surgery to be applied in treating various diseases.

Terminology

NOSE for colorectal cancer can avoid making incisions to extract the specimen. Complete laparoscopic anterior resection with NOSE is the same as conventional laparoscopic anterior resection in terms of surgical outcomes, and had a significantly lower frequency of postoperative analgesic usage and shorter postoperative pain period, as it does not involve making any incision.

Peer review

This study reported the detailed surgical procedures and benefits of laparoscopic rectal surgery with the NOSE technique. This method, as an advanced minimally invasive surgery, seems to be quite attractive and may hold a high position among previously reported NOSE techniques.

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Hepatic clearance measured with ^{99m}Tc -GSA single-photon emission computed tomography to estimate liver fibrosis

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Abstract

AIM: To evaluate the clinical utility of hepatic clearance (HC) measured with technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl human serum albumin (^{99m}Tc -GSA) single-photon emission computed tomography (SPECT) to estimate the degree of liver fibrosis.

METHODS: Seventy-eight consecutive patients who underwent initial hepatectomy due to hepatocellular carcinoma were enrolled in this study. Indocyanine green clearance (ICG R15), quantitative indices estimated by ^{99m}Tc -GSA [the receptor index (LHL15 and HH15) and HC *via* SPECT analysis], and conventional liver function tests were performed before hepatectomy. Correlations among the quantitative indices for liver functional reserve, conventional liver function tests, and

the degree of liver fibrosis were evaluated.

RESULTS: The degree of liver fibrosis was correlated with ICG R15, HH15, LHL15, and HC. HC showed the best correlation with conventional liver function tests. According to multivariate analysis, HC and LHL15 were significant independent predictors of severe fibrosis. HC was the most valuable index for predicting severe fibrosis.

CONCLUSION: HC measured with ^{99m}Tc -GSA SPECT is a reliable index for assessing liver fibrosis before hepatectomy.

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Key words: Fibrosis; Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl human serum albumin; Single-photon emission computed tomography; Hepatic clearance; Liver resection

Core tip: This retrospective study evaluated the clinical utility of hepatic clearance measured with technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl human serum albumin (^{99m}Tc -GSA) single-photon emission computed tomography for estimating the degree of liver fibrosis. We demonstrated that ^{99m}Tc -GSA hepatic clearance showed strong correlations with the degree of liver fibrosis and conventional liver function tests. It is a reliable index for assessing severe liver fibrosis. We believe that this quantitative index can yield a more accurate estimation of liver fibrosis compared with currently used measures before hepatectomy for hepatobiliary surgeons.

Taniguchi M, Okizaki A, Watanabe K, Imai K, Uchida K, Einama T, Shuke N, Miyokawa N, Furukawa H. Hepatic clearance measured with ^{99m}Tc -GSA single-photon emission computed tomography to estimate liver fibrosis. *World J Gastroenterol* 2014; 20(44): 16714-16720 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Liver fibrosis is a negative predictive factor for postoperative hepatic failure^[1-3]. Cirrhosis is a well-known risk factor for postoperative hepatic failure^[1,3,4]. Moreover, morbidity and mortality are high for patients with severe liver fibrosis undergoing liver resection^[2,5,6]. Therefore, the accurate preoperative estimation of the extent of hepatic fibrosis is essential for successful liver surgery. Although many liver fibrosis indicators have been proposed for preoperative evaluation^[7-10], the best indicator for evaluating liver fibrosis has not yet been established.

Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl human serum albumin (^{99m}Tc -GSA) liver scintigraphy reflects the liver functional reserve and is reported to correlate with several hepatic function tests^[11,12]. However, few available analyses can determine the degree of liver fibrosis. Single-photon emission computed tomography (SPECT) analysis in ^{99m}Tc -GSA liver scintigraphy, which can evaluate GSA accumulation in the liver, was also developed to investigate liver function^[13]. These analyses calculate hepatic clearance (HC) with the outline extraction method, using a program based on a radio-pharmacokinetic model, as described by Shuke *et al.*^[14,15].

In this study, we investigate the contribution of HC measured with ^{99m}Tc -GSA SPECT to assess liver fibrosis.

MATERIALS AND METHODS

Patients

Between January 2011 and March 2014, 78 consecutive patients who underwent an initial hepatectomy due to hepatocellular carcinoma were enrolled in this study. The surgery was performed within 1 wk after ^{99m}Tc -GSA liver scintigraphy examination, and conventional tests were performed. All procedures were performed after informed consent was received from the patients and after approval from the Ethics Committee of Asahikawa Medical University Hospital was obtained. This study was performed in accordance with the ethical standards established in the 1964 Declaration of Helsinki.

^{99m}Tc -GSA liver scintigraphy and the receptor index

^{99m}Tc -GSA liver scintigraphy was scheduled for the patients on the day before their hepatectomy. ^{99m}Tc -GSA was supplied by Nihon Medi-Physics (Nishinomiya, Japan). After the intravenous injection of 185 MBq ^{99m}Tc -GSA, dynamic imaging was performed with the patient in the supine position. LHL15 was calculated by dividing the radioactivity of the region of interest (ROI) of the liver by the radioactivity of the ROI of the liver and the heart 15 min after injection. HH15 was calculated by dividing the radioactivity of the ROI of the heart 15 min after injection by the radioactivity of the ROI of the

heart 3 min after injection^[16,17].

SPECT analysis in ^{99m}Tc -GSA liver scintigraphy

Dynamic SPECT was performed using a dual-head gamma camera system equipped with low-energy, general-purpose collimators and a dedicated data processing unit (Millennium VG, GE, Tokyo, Japan). The in-plane spatial resolution of this system was 14 mm full width at half-maximum. After fasting overnight, the patient was placed in a supine position to ensure that the liver and lower part of the heart were within the detectors' field of view. ^{99m}Tc -GSA (185 MBq) was injected intravenously as a bolus. After it was confirmed that the entire liver was covered by the detector's view, dynamic SPECT data acquisition was started 1 min after injection and continued for 20 rotations in a 180° continuous rotation mode with an acquisition time of 1 min per rotation. In each rotation, the data from 60 projections were recorded in a 64 × 64 matrix (pixel size = 68.84 mm × 8.84 mm). SPECT images were reconstructed with a filtered back-projection method using a ramp filter after preprocessing with a Butterworth filter (cutoff frequency = 0.40 cycle per centimeter; order of 8) to obtain 8.84-mm-thick transaxial SPECT images. HC was determined from the SPECT data and was calculated with the outline extraction method using a program based on a radio pharmacokinetic model, as described by Shuke *et al.*^[14,15].

Conventional liver function tests

The serum albumin (Alb), total bilirubin (T-bil), and cholinesterase (Ch-E) levels; prothrombin time international normalized ratio (PT-INR); and platelet count (Plt) were measured in the peripheral blood before hepatectomy. The indocyanine green (ICG) test was conducted preoperatively, and the ICG clearance (ICG R15) was calculated using standard methods. The model for end-stage liver disease (MELD) score^[18] and the Child-Turcotte-Pugh (CTP) score^[19] were used as indices of liver dysfunctions.

Histopathological features of liver specimens

Liver fibrosis was diagnosed using surgical specimens, which were resected at a distance from the tumors. The degree of hepatic fibrosis was assessed and graded 0-6 according to the Ishak classification for chronic hepatitis^[20]: 0: no fibrosis; 1: fibrous expansion of some portal areas, with or without short fibrous septa; 2: fibrous expansion of most portal areas, with or without short fibrous septa; 3: fibrous expansion of most portal areas with occasional portal-to-portal bridging; 4: fibrous expansion of portal areas with marked bridging (portal to portal as well as portal to central); 5: marked bridging (portal to portal and/or portal to central) with occasional nodules; and 6, cirrhosis, probable or definite. Scores of 0, 1, 2, and 3 were considered to reflect nonsevere fibrosis. Scores of 4, 5, and 6 were recorded as severe fibrosis. Tumor size, tumor number, and tumor vascular invasion (portal vein, hepatic artery, and hepatic vein) were evaluated using surgical specimens.

Table 1 Patient characteristics

Variables	<i>n</i> = 78
Age (yr)	66.7 ± 10.3
Gender (male/female)	63/15
HBs-Ag (+/-)	26/52
HCV-Ab (+/-)	21/57
Alcohol abuse (+/-)	10/68
NASH (+/-)	14/64
Diabetes mellitus (+/-)	25/73
Hyperlipidemia (+/-)	18/60
Platelets ($\times 10^3/\text{mm}^3$)	16.6 ± 7.0
Prothrombin time (INR)	1.05 ± 0.11
Albumin (g/dL)	4.0 ± 0.6
Total bilirubin (mg/dL)	0.8 ± 0.3
Cholinesterase (U/L)	248 ± 70
Tumor size (cm)	49.6 ± 36.9
Tumor number	1.2 ± 0.5
Tumor vascular invasion (+/-)	21/57
Ishak classification 0/1/2/3/4/5/6	14/11/8/18/4/13/10
MELD score	5.3 ± 1.3
CTP score	5.2 ± 0.2
ICG R15 (%)	11.6 ± 6.0

HBs-Ag: Hepatitis B surface antigen; HCV-Ab: Hepatitis C virus antibody; NASH: Nonalcoholic steatohepatitis; MELD score: Model for end-stage liver disease score; CTP score: Child-Turcotte-Pugh score; ICG R15: Indocyanine green dye retention at 15 min.

Statistical analysis

The data are expressed as the mean ± SD unless otherwise stated. The data were analyzed using the Mann-Whitney *U* test, Pearson's correlation coefficient, and linear regression. These statistical analyses were performed using SPSS 11.0 for Windows (SPSS, Chicago, IL, United States). The receiver operating characteristic (ROC) curve for calculating the area under the ROC curve (AUC) and interactive dot diagrams were created using MedCalc (software, 12.7.4; Ostend, Belgium).

RESULTS

Patient characteristics

The clinical characteristics of all participating patients are listed in Table 1. The mean age of the 78 patients was 66.7 ± 10.3 years, and there were 63 men. Of the 78 patients, 71 had chronic liver disease (chronic hepatitis B, *n* = 26; chronic hepatitis C, *n* = 21; non-alcoholic steatohepatitis, *n* = 14; and alcoholic hepatitis, *n* = 10). The remaining patients were diagnosed with normal livers. Concerning the degree of hepatic fibrosis, 10 patients were graded 6, 13 were graded 5, 4 were graded 4, 18 were graded 3, 8 were graded 2, 11 were graded 1, and 14 were graded 0. The mean ICG R15 was 11.6 ± 6.0.

Correlations between the degree of liver fibrosis and quantitative indices of liver functional reserve

Table 2 shows the correlations between the degree of liver fibrosis and preoperative liver function parameters. The degree of liver fibrosis was positively linearly correlated with ICG R15 and HH15 and negatively linearly

Table 2 Correlations between the degree of liver fibrosis and quantitative indices for liver functional reserve

	<i>r</i>	<i>P</i> value
ICG R15	0.330	0.003
HH15	0.272	0.016
LHL15	-0.198	0.083
HC	-0.598	< 0.00001

The degree of liver fibrosis was correlated with ICG R15, HH15, and HC. ICG R15: Indocyanine green dye retention at 15 min; HC: Hepatic clearance.

correlated with HC.

Correlations between quantitative indices for liver functional reserve and conventional liver function tests

As Table 3 shows, we evaluated the correlations between the preoperative parameters for liver function and conventional liver function tests. LHL15 was correlated with platelet count (*r* = 0.235, *P* = 0.038) and albumin level (*r* = 0.263, *P* = 0.020), and HH15 was correlated with total bilirubin level (*r* = 0.289, *P* = 0.010) and cholinesterase level (*r* = -0.263, *P* = 0.020). HC was correlated with all conventional liver function tests after liver resection: platelet count (*r* = 0.348, *P* = 0.002), prothrombin time (*r* = -0.287, *P* = 0.011), albumin level (*r* = 0.233, *P* = 0.040), total bilirubin level (*r* = -0.345, *P* = 0.002), and cholinesterase level (*r* = -0.419, *P* = 0.0001).

Univariate and multivariate stepwise regression analysis of various factors affecting liver fibrosis

Univariate analysis showed that platelet count (*P* < 0.001), prothrombin time (*P* = 0.032), total bilirubin level (*P* = 0.001), tumor size (*P* = 0.042), MELD score (*P* = 0.009), ICG R15 (*P* = 0.019), LHL15 (*P* = 0.042), HH15 (*P* = 0.0004), and HC (*P* < 0.0001) were significant predictors of severe cirrhosis. When we entered platelet count, prothrombin time, total bilirubin level, tumor size, MELD score, ICG R15, LHL15, HH15, and HC into a multivariate logistic regression model to identify variables with independent predictive value for severe fibrosis, we found that HC and LHL15 were the significant independent predictors (Table 4).

ROC curve and interactive dot diagrams of HC and LHL15 for the diagnosis of severe fibrosis

In Figure 1, we present the ROC curves for each of the 2 variables, HC and LHL15, that were identified as the significant independent predictors of severe fibrosis. The AUC of the ROC curves for HC and LHL15 were 0.826 and 0.641, respectively. There was a significant difference between the two values (*P* = 0.0146). Based on the analysis employing interactive dot diagrams, the cutoff values for predicting severe cirrhosis with the highest sensitivity and specificity were 298 (sensitivity, 77.8%; specificity, 84.3%) for HC and 0.926 (sensitivity, 74.1%; specificity, 60.8%) for LHL15.

Table 3 Correlations between quantitative indices for liver functional reserve and conventional liver function tests

	ICG R15		LHL 15		HH 15		HC	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Platelets ($\times 10^4/\text{mm}^3$)	-0.160	0.161	0.235	0.038	-0.185	0.105	0.348	0.002
Prothrombin time (INR)	0.082	0.473	-0.122	0.289	-0.016	0.888	-0.287	0.011
Albumin (g/dL)	-0.044	0.703	0.263	0.020	-0.123	0.285	0.233	0.040
Total bilirubin (mg/dL)	0.204	0.073	-0.217	0.057	0.289	0.010	-0.345	0.002
Cholinesterase (U/L)	-0.113	0.324	0.221	0.052	-0.263	0.020	0.419	0.0001

LHL15 was correlated with platelet count and albumin level. HH15 was correlated with total bilirubin level and cholinesterase level. HC was correlated with all conventional liver function tests. ICG R15: Indocyanine green dye retention at 15 min; HC: Hepatic clearance.

Table 4 Univariate and multivariate analyses of variables predictive of severe fibrosis

Variable	Severe fibrosis		<i>P</i> value	
	Yes (<i>n</i> = 27)	No (<i>n</i> = 51)	Univariate analysis	Multivariate analysis
Gender (male/female)	23/4	Nov-40	0.474	
Age (yr)	66.5 \pm 10.0	66.8 \pm 10.5	0.950	
HBs-Ag (+/-)	10/17	16/35	0.616	
HCV-Ab (+/-)	10/17	11/40	0.145	
Alcohol abuse (+/-)	2/25	8/43	0.301	
NASH (+/-)	4/23	10/41	0.602	
Platelets ($\times 10^4/\text{mm}^3$)	12.7 \pm 3.9	18.7 \pm 7.4	< 0.001	0.096
Prothrombin time (INR)	1.09 \pm 0.12	1.03 \pm 0.10	0.032	0.223
Albumin (g/dL)	4.0 \pm 0.6	4.1 \pm 0.6	0.388	
Total bilirubin (mg/dL)	0.9 \pm 0.3	0.7 \pm 0.3	0.001	0.354
Cholinesterase (U/L)	234 \pm 75	255 \pm 68	0.229	
Tumor size (cm)	3.5 \pm 1.6	5.7 \pm 4.2	0.042	0.137
Tumor number	1.1 \pm 0.4	1.2 \pm 0.6	0.543	
Tumor vascular invasion (+/-)	5/22	16/35	0.226	
MELD score	5.8 \pm 1.1	5.1 \pm 1.2	0.009	
CTP score	5.3 \pm 0.6	5.2 \pm 0.4	0.685	
ICG R15 (%)	14.3 \pm 6.1	10.2 \pm 5.5	0.019	0.183
LHL 15	0.901 \pm 0.044	0.935 \pm 0.024	0.042	0.041
HH 15	0.648 \pm 0.068	0.556 \pm 0.067	0.004	0.053
HC	263.3 \pm 90.4	381.1 \pm 96.7	< 0.001	0.030

Platelet count, prothrombin time, total bilirubin level, tumor size, MELD score, ICG R15, LHL15, HH15, and HC were significant predictors of severe cirrhosis in the univariate analysis. In the multivariate analysis, HC and LHL15 were the significant independent predictors. HBs-Ag: Hepatitis B surface antigen; HCV-Ab: Hepatitis C virus antibody; NASH: Nonalcoholic steatohepatitis; MELD score: Model for end-stage liver disease score; CTP score: Child-Turcotte-Pugh score; ICG R15: Indocyanine green dye retention at 15 min; HC: Hepatic clearance.

DISCUSSION

In the current study, we demonstrated correlations between the degree of liver fibrosis and ICG R15, HH15, LHL15, and HC. Among these indicators, HC showed the best correlation with conventional liver function tests. HC was the most valuable index for predicting severe cirrhosis. An HC of 298 could be used to predict severe cirrhosis.

The degree of liver fibrosis is a negative predictor of liver regeneration and the restoration of liver function after liver resection^[9]. Therefore, estimating the liver functional reserve, which is a reflection of liver fibrosis, is important. Several laboratory variables, such as prothrombin time and cholinesterase, have prognostic value in chronic liver disease^[21]. In addition, the Alb level, T-bil level, and prothrombin time are the most useful routine laboratory tests for establishing a prognosis for hepatitis patients^[22]. However, none of these laboratory variables reflects liver fibrosis directly. As a result, these variables

cannot be used as indices for determining the extent of liver resection for patients with liver tumors. In contrast, several studies have evaluated the liver functional reserve before hepatectomy^[23-25]. In particular, the indocyanine green (ICG) clearance test has been widely used to evaluate liver functional reserve^[25,26] for liver resection. However, it does not provide quantitative parameters. Moreover, there are occasional discrepancies between the ICG clearance values and histologic findings in the liver because of the imbalance of portal inflow or portasystemic shunts. Such discrepancies make direct assessments of the extent of liver fibrosis difficult. Therefore, a new method to estimate the liver functional reserve that accurately reflects the degree of fibrosis is required.

The asialoglycoprotein receptor (ASGPR) is localized on hepatocytes and is involved in the clearance of glycoproteins containing terminal galactose residues from the circulation^[27,28]. The expression of this receptor decreases according to the number of functional hepatocytes. Therefore, liver scintigraphy with ^{99m}Tc-GSA,

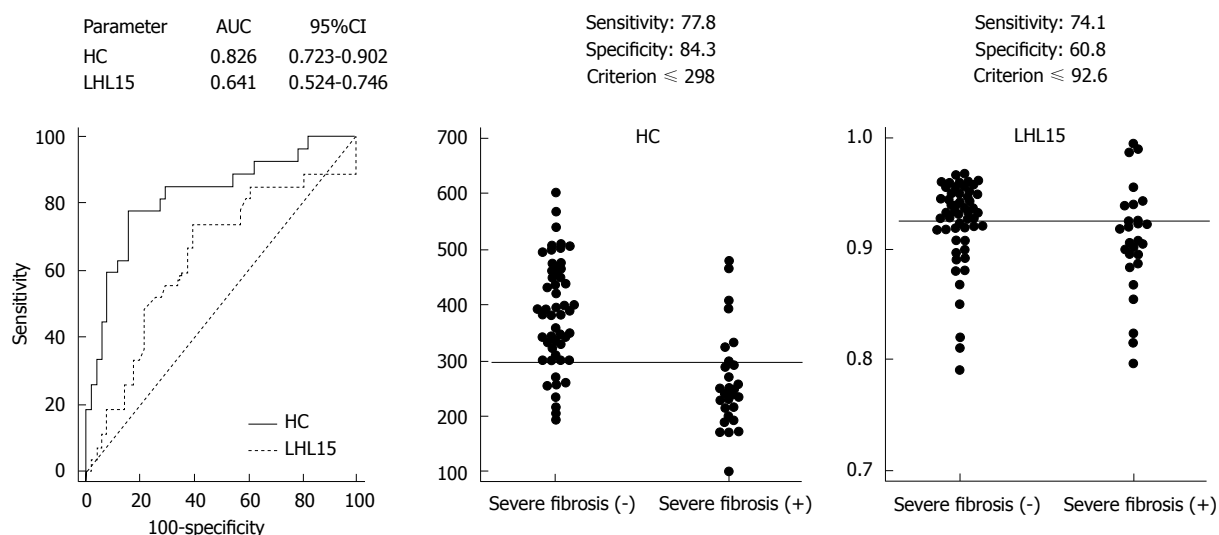


Figure 1 Receiver operating characteristic curve and interactive dot diagrams of hepatic clearance and LHL15 for the diagnosis of severe fibrosis. A: ROC analysis for HC and LHL15. There was a significant difference between the two values ($P = 0.0146$); B: Interactive dot diagrams showing HC predicts severe cirrhosis. The cutoff value for predicting severe cirrhosis with the highest sensitivity and specificity was 298 (sensitivity, 77.8%; specificity, 84.3%) for HC. The horizontal line indicates the cutoff point with the best separation between the 2 groups (severe fibrosis+, severe fibrosis-); C: Interactive dot diagrams showing LHL15 predicts severe cirrhosis. The cutoff value for predicting severe cirrhosis with the highest sensitivity and specificity was 0.926 (sensitivity, 74.1%; specificity, 60.8%) for LHL15. The horizontal line indicates the cutoff point with the best separation between the 2 groups (severe fibrosis+, severe fibrosis-). AUC: Area under the ROC curve; ROC: Receiver operating characteristic; HC: Hepatic clearance.

an analog of asialoglycoprotein, enables the quantitative evaluation of liver functional reserve. SPECT analysis in ^{99m}Tc -GSA liver scintigraphy, which allows the evaluation of GSA accumulation in the liver, was also developed to investigate liver function^[13]. ^{99m}Tc -GSA HC, which is determined based on SPECT data, demonstrates the precise distribution of ASGPR in the liver, thereby providing an accurate calculation of liver functional reserve^[29]. In this study, ^{99m}Tc -GSA HC showed a correlation with conventional liver function tests and the extent of liver fibrosis that was better than that of LHL15 or HH15. LHL15 and HH15, which are hepatic uptake and blood clearance ratios in ^{99m}Tc -GSA liver scintigraphy, are the simplest and most commonly used variables. However, they may be insufficient for accurately estimating the degree of liver fibrosis because these indices are calculated from planar scintigraphic images, which do not correctly reflect hepatocyte volume. In contrast, ^{99m}Tc -GSA HC measured by SPECT analysis contains volumetric information and may correctly estimate the hepatocyte volume, thus reflecting the degree of liver fibrosis.

In liver surgery, the risk of perioperative complications is generally believed to increase when the remnant liver volume (RLV) is excessively small^[30]. Therefore, reports have advocated preoperatively assessing RLV with CT volumetry^[31]. However, CT volumetry can never reflect the function of the remnant liver, especially in patients with parenchymal disease^[30,32], such as chronic hepatitis or cirrhosis. Additionally, several reports concerning ^{99m}Tc -GSA SPECT findings have indicated that regional function is not necessarily uniform throughout the liver^[33,34], suggesting that an accurate estimation of regional liver function is more important for predicting

postoperative liver functional reserve. In this study, ^{99m}Tc -GSA HC strongly reflected the degree of liver fibrosis. Therefore, we believe that using the combined ^{99m}Tc -GSA HC and CT volumetric measurements of the remnant liver can evaluate remnant liver functional reserve after hepatectomy^[35]. Further studies are needed to test this hypothesis.

In conclusion, we demonstrated that HC measured with ^{99m}Tc -GSA SPECT showed correlations with the degree of liver fibrosis and conventional liver function tests. ^{99m}Tc -GSA HC was the most valuable index for predicting severe fibrosis. It could yield a more accurate estimation of liver fibrosis compared with currently used measures before hepatectomy for hepatobiliary surgeons.

COMMENTS

Background

Liver fibrosis is a negative predictive factor for postoperative hepatic failure. Therefore, the accurate preoperative estimation of the extent of hepatic fibrosis is essential for successful liver surgery. Although many liver fibrosis indicators have been proposed for preoperative evaluation, the best indicator for evaluating liver fibrosis has not yet been established.

Research frontiers

Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl human serum albumin (^{99m}Tc -GSA) liver scintigraphy reflects the liver functional reserve and is reported to correlate with several hepatic function tests. In addition, single-photon emission computed tomography analysis in ^{99m}Tc -GSA liver scintigraphy, which can evaluate GSA accumulation in the liver, was also developed to investigate liver function.

Innovations and breakthroughs

Hepatic clearance which was measured with ^{99m}Tc -GSA single-photon emission computed tomography (SPECT) is a reliable index for assessing liver fibrosis.

Applications

Hepatic clearance which was measured with ^{99m}Tc -GSA SPECT could yield a

more accurate estimation of liver fibrosis compared with currently used measures before hepatectomy for hepatobiliary surgeons.

Terminology

^{99m}Tc-GSA liver scintigraphy: Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl human serum albumin liver scintigraphy. SPECT analysis: Single-photon emission computed tomography analysis.

Peer review

The manuscript evaluates the utility of ^{99m}Tc-GSA SPECT to reliably predict the degree of liver fibrosis in patients for liver resection is planned. Comparisons are made to particularly state that hepatic clearance is superior to other measurements (LHL15 and HH15), other techniques (ICGR15), and clinical parameters of liver function when predicting fibrosis. The study has relevance and is interesting in its concept; however some conclusions are made that need to be justified by more rigorous data analysis.

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Safety and efficacy of a partially covered self-expandable metal stent in benign pyloric obstruction

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Abstract

AIM: To evaluate the safety and efficacy of partially covered self-expandable metallic stents (SEMSs) in benign pyloric obstruction.

METHODS: We retrospectively analyzed data from 10 consecutive patients with peptic ulcer-related pyloric obstructive symptoms (gastric outlet obstruction scoring system (GOOSS) score of 1) between March 2012 and September 2013. The patients were referred to and managed by partially covered SEMS insertion in our tertiary academic center. We assessed the technical success, symptom improvement, and adverse events after stenting.

RESULTS: Early symptoms were improved just 3 d after SEMS placement in all 10 patients. The GOOSS score of all patients improved from 1 to 3. There were no serious immediate adverse events. The overall rate of being symptom free was 90% at a median of 11 mo of follow-up (range: 4-43 mo). Five patients were managed by a rescue SEMS because of failure of previous endoscopic balloon dilatation. Among them, four patients had sustained symptom improvement after the SEMS procedure. During the follow-up period, migra-

tion of the SEMS was observed in two patients (20.0%), both of whom had previous endoscopic balloon dilatation before SEMS insertion.

CONCLUSION: Despite the small number in this study, partially covered SEMSs showed a favorable and safe outcome in the treatment of naïve benign pyloric obstruction and in salvage treatment after balloon dilatation failure.

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Key words: Benign pyloric obstruction; Balloon dilatation; Self-expandable metallic stent; Gastric outlet obstruction scoring system

Core tip: Partially covered self-expandable metallic stents had a safe and favorable outcome in the treatment of naïve benign pyloric obstruction and in salvage treatment after balloon dilatation failure.

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INTRODUCTION

The causes of benign pyloric obstruction are peptic ulcer, anastomotic structures after gastric surgery, corrosive injury, and stricture secondary to intervention. Among these, peptic ulcer disease is the most common etiology of benign pyloric obstruction^[1]. Patients with pyloric obstruction have discomfort with dyspepsia, abdominal bloating, nausea, and vomiting, which results in weight loss and a poor quality of life.

Surgery has been the conventional treatment for the

benign pyloric obstruction^[2]. However, it carries a significant risk of postoperative comorbidity and is not always suitable for patients in a poor condition, or for elderly people. Endoscopic balloon dilatation was first conducted by Benjamin *et al*^[3]. This procedure has the advantage of being relatively simple for both patients and endoscopists in the treatment of pyloric obstruction. However, the efficacy of balloon dilatation is controversial^[4-7]. The self-expandable metal stent (SEMS) was originally developed for treatment of malignant obstruction of the esophagus, colon, and gastric outlet. This treatment showed favorable results comparable to those of surgery for palliation and as a bridge to surgery^[8,9]. However, there are few reports on SEMS in benign pyloric obstruction^[10,11]. In addition, the partially covered SEMS, which was developed for overcoming the disadvantage of covered or uncovered SEMS, has not been validated for the treatment of benign pyloric obstruction. The aim of this study was to evaluate the safety and efficacy of partially covered SEMSs in benign pyloric obstruction.

MATERIALS AND METHODS

Patients

We retrospectively analyzed data from 10 consecutive patients with peptic ulcer and outlet obstruction referred to and managed by SEMS insertion in our tertiary academic center between March 2012 and September 2013. These patients had a common obstructive symptom of frequent vomiting even with a liquid diet. The benign pyloric obstruction was shown by endoscopic biopsy and imaging study. In all patients, the endoscope could not be passed through the obstructed lumen. All the patients were recommended to undergo surgical treatment initially. However, these patients wanted to undergo endoscopic treatment rather than surgical treatment. Some patients had undergone prior endoscopic balloon dilatation with poor results. This study was approved by the ethics committee of Kyungpook National University Hospital.

SEMS procedure

After the patient was sedated, an endoscope (CF-Q160J; Olympus Optical Co.) was inserted through the stomach with fluoroscopic guidance. After identifying the obstructive pyloric lesion, a biliary guidewire (Jagwire, Boston Scientific Co.) was passed through the working channel of the endoscope. A water-soluble contrast medium (Gastrografin, Bracco Co.) was then injected through the obstructed lumen and the length of the obstruction was measured directly using the guidewire by fluoroscopy. The heavy wire was placed and the delivery system advanced into position under fluoroscopy and endoscopy. A partially covered SEMS was used for all cases. After ascertaining that the position of the delivery system under fluoroscopy and endoscopy was correct, the stent was released from the distal end toward the stricture. After placing the stent, a water-soluble contrast was injected through the stent to check its passage through the stent under fluoroscopy.

Good expansion and position of the stent were confirmed by serial abdominal plain radiography.

Evaluation of subjective symptoms after SEMS

The subjective obstructive symptoms of the patient were evaluated with the gastric outlet obstruction scoring system (GOOSS)^[12]. The GOOSS value was assigned on a 4-point scale: 0, no oral intake; 1, liquids only; 2, soft solids only; 3, low residue or full diet. The GOOSS score was assessed before and 3 d after the procedure. After discharge, subjective symptoms including GOOSS score and position of the stent by abdominal plain radiography were evaluated at the outpatient department at 1, 2, and 3 mo after the SEMS procedure. If the patients had good subjective symptoms with a GOOSS score of 3, the SEMS was planned to be removed under endoscopy and fluoroscopic guidance at 3-6 mo after insertion.

RESULTS

Baseline characteristics of the patients

Nine of the 10 patients who underwent SEMS insertion were men. The median age at index endoscopy was 56 years (range: 40-71 years). The causes of benign pyloric obstruction were duodenal ulcer in four patients (40.0%) and both gastric and duodenal ulcers in six patients. Five patients underwent endoscopic balloon dilation prior to SEMS insertion (Table 1).

Clinical outcomes and complications

Technical success was achieved in all the 10 patients. The total procedure time was 20.5 ± 11.7 (mean \pm SD) minutes. Early symptom improvement at 3 d after SEMS was excellent with a GOOSS score of 3 in all 10 patients. There were no immediate complications such as serious bleeding, bowel perforation, or procedure-related mortality during the SEMS insertion. During follow-up, migrations of the SEMS were observed in two patients (20.0%) (Table 1). In one patient (case number 10), the SEMS migrated 1 day after the procedure. An additional secondary SEMS was inserted at 5 d after the migration of initial SEMS. However, the secondary SEMS also migrated 10 d later. In another patient (case number 8), the SEMS migrated 1 mo after the procedure. However, the symptoms in these two patients were not aggravated after migration of the stent after 4 and 10 mo of follow-up. The overall rate of being symptom free was 90% at a median of 11 mo of follow-up (range: 4-43 mo).

Removal of the SEMS

The removal of the SEMS was performed 3-6 mo after insertion. However, in one patient (case number 2), removal of the SEMS was impossible because the SEMS adhered to adjacent duodenal mucosa. This patient was carefully observed without complications or symptom aggravation during 17 mo of follow-up. The symptoms in another patient (case number 6) decreased to a GOOSS score of 2 after removal of the stent. One patient

Table 1 Patient characteristics and results of partially covered self-expandable metal stent

Case No.	Sex/age (yr)	Etiology	Number of prior endoscopic balloon dilatation	Stent name, company	Stent diameter (mm)	Stent length (mm)	Symptom change ¹	Adverse event	Duration of stenting (mo)	Removal of stent	Follow up duration (mo)
1	M/64	DU	No	Niti-S	20	120	1 - > 3	No	6	Yes	22
2	M/49	DU + GU	No	Hanaro	20	70	1 - > 3	No	17	No ²	17
3	M/68	DU + GU	No	Hanaro	20	130	1 - > 3	No	6	Yes	12
4	M/51	DU	No	Hanaro	20	90	1 - > 3	No	6	Yes	8
5	M/52	DU + GU	No	Hanaro	20	110	1 - > 3	No	6	No	5
6	M/40	DU + GU	2	Niti-S	20	120	1 - > 3 - > 2 ³	No	4	Yes	43
7	F/71	DU	1	Bona	22	120	1 - > 3	No	19	No	19
8	M/44	DU + GU	1	Niti-S	20	120	1 - > 3	Migration	1	NA	10
9	M/71	DU + GU	1	Hanaro	20	130	1 - > 3 - > 1 - > 3 ⁴	No	4	No ⁴	4
10	M/59	DU	1	Hanaro	20	90	1 - > 3	Migration	10 d	NA	4

¹Evaluated by gastric outlet obstruction scoring system (GOOSS) score; ²Failure of removal of stent; ³Aggravated symptoms by GOOSS score 3 to 2 after removal of stent; ⁴Symptoms were aggravated by GOOSS score 3 to 1 after removal of stent. After stent was reinserted 2 mo later, the symptoms were improved by GOOSS score 1 to 3. DU: Duodenal ulcer; GU: Gastric ulcer; NA: Not available.

(case number 9) had a GOOSS score of 1 after removal of the stent. This patient underwent SEMS reinsertion and had improved symptoms with a GOOSS score of 3. The other seven patients were maintained without recurrence of obstructive symptoms regardless of removal of stent during the follow-up.

DISCUSSION

Following the advance of through-the-scope techniques, endoscopic therapies were developed for the treatment of benign pyloric obstruction. Among them, endoscopic balloon dilatation is regarded as the first-line option with favorable relief of obstructive symptoms^[13]. In a recent study, 21 patients with benign pyloric obstruction were managed by endoscopic balloon dilatation with medication. All patients remained in symptomatic remission during a median follow-up period of 43 mo (range: 5-90 mo)^[5]. However, in another study, 84% of patients (16/19) had recurrence of symptoms during a follow-up period of 45 mo (range: 25-96 mo)^[14]. In addition, in another study that reported the prospective results of 42 patients with balloon dilatation for benign pyloric obstruction, 14 patients (33%) had surgical intervention for perforation ($n = 4$) and the overall symptom-free rates declined with the duration of follow-up (85.3% at 12 mo and 68.8% at 48 mo)^[15]. In addition, more than two courses of balloon dilatation for symptom relief was the only significant prognostic factor. Recurrent obstruction after balloon dilatation is thought to be related to relatively short dilatation time. When we apply the balloon dilatation into the narrow lumen through the endoscope, the real dilatation time against the radical vector force of obstructed lumen is estimated about a few minutes. The dilated lumen tends to return to the original status of the stricture over the course of time after balloon dilatation. Therefore, another treatment option with long term effect, such as stenting, is needed for the treatment of benign pyloric

obstruction.

In a recent meta-analysis, SEMSs for malignant pyloric obstruction have been shown to have significant clinical success, with a short time from the procedure to the start of oral intake, and lower incidence of morbidity compared with surgery^[8]. Although the numbers of patients have been small, previous studies have validated SEMSs in benign pyloric obstruction and found them to be effective^[10,16]. In this study of 10 patients with benign pyloric obstruction, SEMSs had excellent results with 100% technical success and immediate symptom improvement. In addition, the overall symptom free rate was 90% after a median of 11 mo of follow-up (range: 4-43 mo). Partially covered SEMSs improved obstructive symptoms for 1 year after 5 times-failed balloon dilatation procedures for benign pyloric obstruction in a recent case study^[17]. In our study, five patients had experienced failed balloon dilatation. Among them, four patients had sustained symptom improvement after the SEMS procedure. The other patient (case number 6) also showed moderate symptom improvement of the GOOSS score from 1 to 2. Therefore, SEMSs also could be an alternative treatment for patients who are poor candidates for surgery after failed endoscopic balloon dilatation.

In another recent study, the authors reported on 22 patients who were treated with covered SEMSs for benign pyloric obstruction. During the mean follow-up period of 10.2 mo, 15 patients (62.5%) had stent migration with seven (46.6%) patients showing continued symptom improvement^[16]. In malignant pyloric obstruction, migration of the SEMS is one of the major complications. It is more likely to occur with the covered type of SEMS than the uncovered. To reduce the migration rate, an anchoring technique or a long-length SEMS might be considered^[18]. In this study, we used a partially covered SEMS and observed migration of the SEMS in two patients (20%). After successful placement of SEMS, retrieval of the SEMS was possible in all but one case. In addition,

the two patients with stent migration had previous balloon dilatation before SEMS insertion. Previous balloon dilatation can stretch the stricture tissue in the pylorus and thereby enhance the rate of SEMS migration. In summary, partial SEMSs showed a lower migration rate than covered SEMSs and may be more effective in naïve benign pyloric obstruction.

There is a major concern regarding tissue ingrowth into the stent wall. This makes removal of the stent difficult for not only the uncovered stent but also the covered stent. Removal of the stent is required after improvement of obstructive symptoms. However, there is no guideline about the timing of stent removal. In our study, two patients had an aggravated GOOSS score after stent removal. These patients had a stent duration of 4 mo. Excepting for these two patients and two patients with migrated stents, the other six patients had stent durations over 6 mo and showed no aggravation of symptoms regardless of removal of the stent. Therefore, for SEMSs in benign pyloric obstruction, stent duration over 6 mo may be needed for prolonged symptom improvement.

This study has several limitations. First, the retrospective design with a small number of cases limits our ability to assess the effectiveness of the SEMS in benign pyloric obstruction. Another limitation is that the long-term effectiveness of the SEMS in benign pyloric obstruction has not been evaluated. Third, although the partially covered SEMS showed good results in this study, we did not confirm that the SEMS is better than endoscopic balloon dilatation. Further large, prospective studies comparing the SEMS with endoscopic balloon dilatation or with specific treatment methods according to the site of the benign pyloric obstruction are warranted. We expect our study results could provide the basis for further studies.

In conclusion, partially covered SEMSs had a safe and favorable outcome in the treatment of naïve benign pyloric obstruction and in salvage treatment after balloon dilatation failure. Further prospective, large-scale studies with a longer follow-up period are needed to confirm these results.

COMMENTS

Background

Endoscopic balloon dilatation has the advantage of being relatively simple for both patients and endoscopists in the treatment of benign pyloric obstruction. However, the efficacy of balloon dilatation is controversial, especially long term effectiveness.

Research frontiers

The self-expandable metal stent (SEMS) was originally developed for treatment of malignant obstruction of the esophagus, colon, and gastric outlet. However, there are few reports on SEMS in benign pyloric obstruction.

Innovations and breakthroughs

In addition, the partially covered SEMS, which was developed for overcoming the disadvantage of covered or uncovered SEMS, has not been validated for the treatment of benign pyloric obstruction. The aim of this study was to evaluate the safety and efficacy of partially covered SEMS in benign pyloric obstruction.

Applications

Partially covered SEMSs had a safe and favorable outcome in the treatment of naïve benign pyloric obstruction and in salvage treatment after balloon dilata-

tion failure.

Peer review

The authors present their experience of using partially covered SEMS in the treatment of benign pyloric obstruction. Since there are already similar reports in the literature, a comparative trial would have been more interesting.

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Boceprevir is highly effective in treatment-experienced hepatitis C virus-positive genotype-1 menopausal women

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Abstract

AIM: To investigate the safety/efficacy of Boceprevir-based triple therapy in hepatitis C virus (HCV)-G1 menopausal women who were historic relapsers, partial-responders and null-responders.

METHODS: In this single-assignment, unblinded study, we treated fifty-six menopausal women with

HCV-G1, 46% F3-F4, and previous PEG- α /RBV failure (7% null, 41% non-responder, and 52% relapser) with 4 wk lead-in with PEG-IFN α 2b/RBV followed by PEG-IFN α 2b/RBV+Boceprevir for 32 wk, with an additional 12 wk of PEG-IFN- α 2b/RBV if patients were HCV-RNA-positive by week 8. In previous null-responders, 44 wk of triple therapy was used. The primary objective of retreatment was to verify whether a sustained virological response (SVR) (HCV RNA undetectable at 24 wk of follow-up) rate of at least 20% could be obtained. The secondary objective was the evaluation of the percent of patients with negative HCV RNA at week 4 (RVR), 8 (RVR BOC), 12 (EVR), or at the end-of-treatment (ETR) that reached SVR. To assess the relationship between SVR and clinical and biochemical parameters, multiple logistic regression analysis was used.

RESULTS: After lead-in, only two patients had RVR; HCV-RNA was unchanged in all but 62% who had $\leq 1 \log_{10}$ decrease. After Boceprevir, HCV RNA became undetectable at week 8 in 32/56 (57.1%) and at week 12 in 41/56 (73.2%). Of these, 53.8% and 52.0%, respectively, achieved SVR. Overall, SVR was obtained in 25/56 (44.6%). SVR was achieved in 55% previous relapsers vs. 41% non-responders ($P = 0.250$), in 44% F0-F2 vs 54% F3-F4 ($P = 0.488$), and in 11/19 (57.9%) of patients with cirrhosis. At univariate analysis for baseline predictors of SVR, only previous response to antiviral therapy (OR = 2.662, 95%CI: 0.957-6.881, $P = 0.043$), was related with SVR. When considering "on treatment" factors, 1 \log_{10} HCV RNA decline at week 4 (3.733, 95%CI: 1.676-12.658, $P = 0.034$) and achievement of RVR BOC (7.347, 95%CI: 2.156-25.035, $P = 0.001$) were significantly related with the SVR, although RVR BOC only (6.794, 95%CI: 1.596-21.644, $P = 0.010$) maintained significance at multivariate logistic regression analysis. Anemia and neutropenia were managed with Erythropoietin and Filgrastim supplementation, respectively. Only six patients discontinued therapy.

CONCLUSION: Boceprevir obtained high SVR response independent of previous response, RVR or baseline fibrosis or cirrhosis. RVR BOC was the only independent predictor of SVR.

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Key words: Hepatitis C virus treatment; Pegylated Interferon; Viral Hepatitis; Menopause; Genotype 1

Core tip: After menopause liver disease in hepatitis C virus-positive women becomes rapidly progressive, severe fibrosis develops, and response to antiviral therapy becomes very low. Re-treatment with standard dual therapy in previous failures of Peginterferon- α + Ribavirin (PEG-IFN α /RBV) treatments does not achieve more than 5%-10% sustained virological response (SVR). The addition of Boceprevir to PEG-IFN α /RBV in menopausal women with HCV-1 genotype infection, who had previously failed dual antiviral therapy, determined a striking improvement of SVR. More than 45% of women re-treated with triple therapy achieved SVR, with few side effects and good tolerability. Response after 4 wk of Boceprevir was the only independent factor predicting SVR.

Bernabucci V, Ciancio A, Petta S, Karampatou A, Turco L, Strona S, Critelli R, Todesca P, Cerami C, Sagnelli C, Rizzetto M, Cammà C, Villa E. Boceprevir is highly effective in treatment-experienced hepatitis C virus-positive genotype-1 menopausal women. *World J Gastroenterol* 2014; 20(44): 16726-16733 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16726.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16726>

INTRODUCTION

Menopausal women with chronic hepatitis C are a group of patients with remarkably distinctive characteristics when compared with women of reproductive age or with males of similar age^[1,2]. Acceleration of fibrosis in the past was attributed to several different factors: length of hepatitis C virus (HCV) infection^[3], alcohol and smoking^[4], genetic characteristics of patients^[5]. Lately, the distinctive role of menopause became apparent^[2,6-8]. Soon after menopause liver disease becomes rapidly progressive and severe hepatic fibrosis develops^[6-9], likely as a consequence of the rapid increase of inflammation as a direct consequence of estrogen deprivation^[8,10]. In menopausal HCV-positive women there is a striking up-regulation of hepatic tumor necrosis factor- α (TNF- α), suppressor of cytokine signaling-3 (SOCS3), interleukin-6 (IL-6), whose levels correlate with higher necro-inflammation and with faster progression of liver fibrosis^[9]. Not surprisingly, hormone replacement therapy (HRT) was shown to exert a positive effect slowing down fibrosis progression^[6]. Even more disappointing is the fact that menopausal HCV-positive women become

also resistant to conventional antiviral therapy with Peg-Interferon- α + Ribavirin (PEG-IFN α /RBV): in our study of women with HCV-1 genotype menopause was the only independent factor predicting failure of dual antiviral therapy^[9]. This finding was confirmed in a prospective cohort study aimed to evaluate viral and host factors influencing antiviral therapy: genotype 1 females over 50 years, had greatly reduced efficacy of interferon-based therapy^[11]. Furthermore, the evaluation of our database for Hepatitis C showed that SVR rate after retreatment of menopausal women with HCV-1 genotype was very low, ranging from 5% after retreatment with dual PEG-IFN α /RBV in those having other unfavorable predictive factors like IL 28B (rs12979860) other than CC or high BMI^[11-13] to 15% in those who had only menopause as risk factor (personal data).

We decided, therefore, to perform an exploratory study using the results from the previous retreatment studies as historical control to evaluate whether the addition of Boceprevir to standard PEG-IFN- α 2b/RBV was able to increase the SVR rate in a difficult-to-treat cohort of menopausal HCV genotype 1 women with a previous PEG-IFN- α /RBV failure.

MATERIALS AND METHODS

All patients gave written informed consent before starting the study. The study was approved by the Institutional Review Board of the Azienda Ospedaliero-Universitaria of Modena (EudraCT 2011-002459-33) and by the appropriate Institutional Review Boards of the other Institutions and was conducted in accordance with provisions of the Declaration of Helsinki and Good Clinical Practice guidelines (Clinical Trials ID: NCT01457937).

Study patients

From December 2011 to June 2012 we screened 87 consecutive menopausal women with HCV genotype 1 infection and a documentation of a failed prior course of PEG-IFN- α /RBV for at least 12 wk, followed up in the out patients clinics of the Liver Units of Modena, Turin, Palermo and Naples.

Inclusion criteria included patients with relapse (undetectable HCV RNA level at the end of treatment, without subsequent attainment of a sustained virological response), prior partial-response (defined as decrease of HCV-RNA $\geq 2 \log_{10}$ by week 12 of prior therapy but with detectable HCV-RNA throughout the course of therapy), or null-response (decrease of HCV-RNA $\leq 2 \log_{10}$ by week 12 of prior therapy).

A liver biopsy within the last 2 years with histology consistent with chronic hepatitis C (CHC) and no other etiology was required. In subjects with bridging fibrosis or cirrhosis, an ultrasound within 6 mo of the Screening Visit (or between Screening and Day 1) with no findings suspect for hepatocellular carcinoma (HCC) was mandatory.

Exclusion criteria included co-infection with the hu-

man immunodeficiency virus (HIV) or hepatitis B virus (HBsAg positive), treatment with any investigational drug within 30 d of the randomization visit in this study, evidence of decompensated liver disease including history or presence of clinical ascites, bleeding varices, or hepatic encephalopathy, diabetic and/or hypertensive subjects with clinically significant ocular examination findings (like retinopathy, cotton wool spots, optic nerve disorder, retinal hemorrhage, or any other clinically significant abnormality), pre-existing psychiatric conditions, clinical diagnosis of substance abuse of the specified drugs within the specified timeframes, any known pre-existing medical condition that could interfere with the subject's participation in and completion of the study, evidence of active or suspected malignancy, or a history of malignancy, within the last 5 years (except adequately treated carcinoma in situ and basal cell carcinoma of the skin). Further exclusion criteria included protocol-specified hematologic, biochemical, and serologic criteria: hemoglobin < 12 g/dL; neutrophils < 1500/mm³; platelets < 100000/mm³, direct bilirubin > 1.5 × upper limit of normal (ULN), serum albumin < lower limit of normal (LLN), thyroid-stimulating hormone (TSH) > 1.2 × ULN or < 0.8 × LLN of laboratory, serum creatinine > ULN of the laboratory reference, protocol-specified serum glucose concentrations, prothrombin time/partial thromboplastin time (PT/PTT) values > 10% above laboratory reference range, anti-nuclear antibodies > 1:320.

Study design

In this single-assignment, unblinded study we treated menopausal women with previous treatment failure to a prior course of PEG-IFN/RBV. The primary objective of retreatment was to verify whether a sustained virological response (SVR) (HCV RNA undetectable at 24 wk of follow-up) rate of at least 20% could be achieved with Boceprevir in menopausal women with chronic HCV genotype 1 with a previous failure of PEG IFN/Ribavirin. Secondary objective of this study was the evaluation of the percent of patients with negative HCV RNA at week 4 (rapid virological response, RVR), 8 (rapid virological response after BOC addition, RVR BOC), 12 (early virological response, EVR), or at the end-of-treatment (ETR) that reached SVR.

All patients received 4-wk lead-in with with PEG-IFN- α -2b at 1.5 μ g/kg subcutaneously weekly, in combination with weight-based oral RBV at a total dose of 800 to 1400 mg per day according to body weight. In relapse and partial responder patients after 4 wk of lead-in, all patients received 32 wk of BOC 800 mg administered orally three times a day, in combination with PEG-IFN and RBV. These patients then received an additional 12 wk of PR only if HCV-RNA was positive by week 8 yet. In previous null-responders, after the lead-in phase, triple therapy with PEG-IFN/Ribavirin and Boceprevir was continued until week 48. All drugs were self-administered by the patients.

In all patients, if HCV RNA was detectable at week

12, treatment was stopped.

IL28 genotyping

IL28B rs12979860 genotype was tested as already described^[13].

Safety assessment

Adverse events (AEs) were graded according to WHO grading system. The safety analyses included all subjects who receive at least one dose of study medication. Non severe hematological adverse events were managed by pharmacological dose reduction. In case of neutrophil count < 0.75 × 10⁹/L, granulocyte colony stimulating factor (GCS-F) was also used. In case of hemoglobin decrease < 10 g/dL, Ribavirin dose was reduced and/or erythropoietin administered.

Subjects having AEs were monitored with appropriate clinical assessments and laboratory tests, as determined by the investigator.

Statistical analysis

Analysis regarding the primary end-point included all patients who had received at least one dose of any study medication (intention-to-treat analysis). Our primary end-point was attainment of sustained virological response, defined as undetectable circulating HCV RNA at week 24 of follow-up. Secondary end point was the identification of independent baseline and on-treatment SVR predictors.

To calculate sample size, we estimated to obtain at least a 20% increase in SVR *vs* historical controls with the same characteristic. Thus, with 80% power and a type 1 error set to 0.05, 49 subjects were required. A 15% excess of inclusions was allowed in order to compensate for withdrawals.

Dichotomous or continuous variables were compared with the Fisher exact test with mid-p correction or the nonparametric Mann-Whitney rank-sum test, respectively.

To assess the relationship between SVR and clinical and biochemical parameters, two multiple logistic regression models were used; the first assessed baseline variables only and the second assessed both baseline and on-treatment parameters. In the statistical model, the dependent variable was coded as 1 (present) *vs* 0 (absent). In all analyses, partial and null responders were considered together and referred to as non-responders. Variables associated with the dependent variable in univariate analyses (probability threshold, *P* < 0.10) were included in the multivariate regression model. The PASW Statistics 20 program (SPSS, Inc, Chicago, IL) was used for the analysis.

RESULTS

Study patients

A total of 87 menopausal women with chronic Hepatitis C, genotype 1, with previous treatment failure to standard antiviral therapy, were evaluated (Figure 1). Baseline characteristics of the 56 enrolled patients are shown in Table

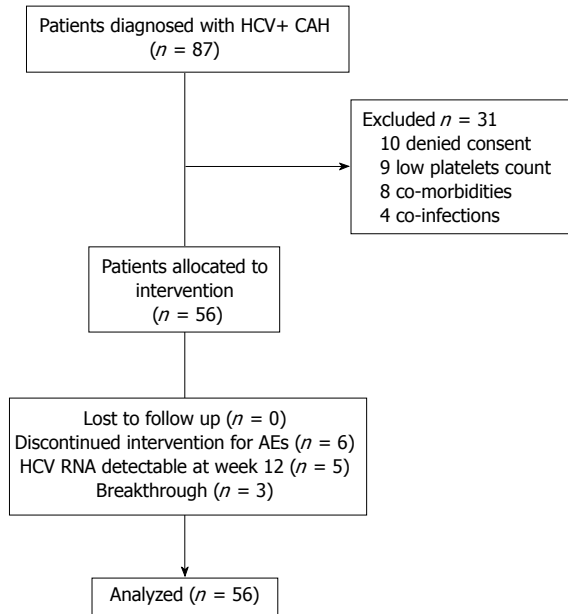


Figure 1 Trend statement flow diagram. HCV: Hepatitis C virus; AE: Adverse event.

1. Thirty-eight women were from Northern Italy while 18 were from Southern Italy. There were no significant differences between them regarding previous response to antiviral therapy, percentage of patients with cirrhosis, IL28B genotype, basal BMI, duration of menopause, basal viral load. All patients completed follow up and were included in the analysis.

Mean age was 56.8 ± 6.1 years; mean menopausal duration was 11.4 ± 6.1 years (median 12.5 years). Mean BMI was 26.2 ± 4.0 (median 24.8). Four patients (7.1%) were previous null responders, 23 (41.1%) partial-responders and 29 (51.8%) relapsers. According to Metavir fibrosis score, 30/56 (54.0%) women had a score of 1 or 2, while 26/56 (46.0%) had a score of 3 or 4; of these, 21 (37.5%) had cirrhosis. Approximately 60% of patients had a high viral load (an HCV RNA level > 800000 IU per milliliter). Genotype 1b infection was predominant (52/56, 92.9%). IL28B rs12979860 genotype was available for 43 patients (9 CC, 26 TC, and 8 TT).

Efficacy

In the entire population, SVR was obtained in 25/56 (44.6%) patients. There was no significant difference between women of Northern and Southern origin in the SVR rate (44.7% *vs* 44.4%, respectively, $P = 0.964$).

After 4 wk of lead-in, none but two patients achieved RVR, while the HCV RNA drop of at least 1 \log_{10} from baseline (IFN sensitivity) was obtained in 62% of patients. After Boceprevir addition, RVR BOC, EVR and ETR were obtained in 32/56 (57.1%), 41/56 (73.2%), and 42 (75.0%) respectively. Of these, 53.8%, 52.0%, and 52.0%, respectively, achieved SVR.

Fourteen patients (25.0%) stopped therapy [6/14 (43%) for intolerance, 5/14 (36%) for HCV RNA detect-

Table 1 Demographic characteristics of the 56 patients at enrollment

Variables	
Mean age, mean \pm SD (yr)	56.8 ± 6.1
Mean age at menopause, mean \pm SD (yr)	49.3 ± 4.3
Time from menopause, mean \pm SD (yr)	11.4 ± 6.1
Previous response <i>n</i> (%)	
Relapse	29 (51.8)
Non response	23 (41.1)
Null response	4 (7.1)
Grading	5.1 ± 2.4
Staging	2.3 ± 1.3
Cirrhosis	21 (37.5)
Stiffness (kPa)	10.3 ± 7.1
BMI, mean \pm SD	26.2 ± 4.0
Blood glucose, mean \pm SD (mg/dL)	101 ± 22
Insulin_base (U/mL)	9.9 ± 7.9
HOMA score	2.5 ± 1.5
Blood Iron (μ g/dL)	128 ± 87
Hb (g%)	13.0 ± 2.2
WBC (K/ μ L)	4.9 ± 1.4
Neutrophils (K/ μ L)	2.5 ± 0.9
Platelets ($10^3/\text{mm}^3$)	206 ± 78
BUN (mg/dL)	36 ± 11
Creatinine (mg/dL)	0.7 ± 0.1
AST (IU/L)	58 ± 24
ALT (IU/L)	66 ± 27
ALP (IU/L)	95 ± 28
Gamma-GT (IU/L)	45 ± 15
HCV RNA (IU/mL, 10^3)	1.440 ± 1.178

BMI: Body mass index; ALT: Alanine aminotransferase; WBC: White blood cell; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

ability at week 12; 3/14 (21%) for viral breakthrough]. All experienced a virological relapse.

The rate of sustained virological response among patients with prior relapse or non-response was 55% and 41% respectively ($P = 0.250$). No significant correlation was present between SVR and high viral load (HCV RNA > 800.000 IU/mL: $P = 0.597$) or IL28 status ($P = 0.333$). Patients with IFN sensitivity had significantly higher SVR rates compared patients with IFN insensitivity (57.1% *vs* 26.3%, $P = 0.030$). Level of fibrosis did not negatively influence the SVR rate, which occurred in 44% of women with F0-F2 *vs* 54% of those with F3-F4 fibrosis ($P = 0.488$) nor was the presence of cirrhosis (presence of cirrhosis *vs.* absence: $P = 0.485$). Figure 2 depicts SVR rates according to the pattern of prior response, severity of fibrosis and baseline viral load.

At univariate analysis for baseline predictors of SVR, none of the clinical and biochemical parameters but previous response to antiviral therapy (OR = 2.662, 95%CI: 0.957-6.881, $P = 0.043$) was related with SVR. When taking into consideration also "on treatment" factors, 1 \log_{10} HCV RNA decline at week 4 (3.733, 95%CI: 1.676-12.658, $P = 0.034$) and achievement of RVR BOC (7.347, 95%CI: 2.156-25.035, $P = 0.001$) were significantly related with SVR, although RVR BOC only (6.794, 95%CI: 1.596-21.644, $P = 0.010$) maintained significance at multivariate logistic regression analysis (Table 2).

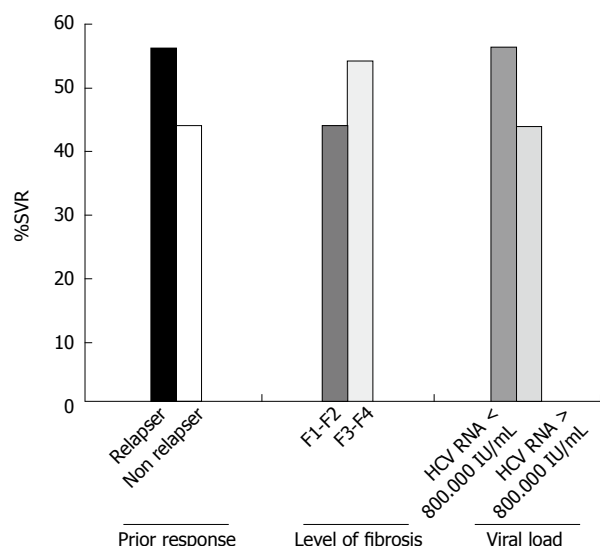


Figure 2 Percentage of patients with a sustained virologic response according to prior response to Peginterferon- α + Ribavirin treatment, level of fibrosis and viral load. HCV: Hepatitis C virus; SVR: Sustained virological response.

Safety

Anaemia and neutropenia were managed with Erythropoietin and Granulokine supplementation, respectively: only six patients discontinued therapy.

The treatment was well tolerated, with low rates of severe adverse events. Fatigue and nausea were the most common adverse events. Hemoglobin levels decreased significantly at all-time points, without requiring the introduction of erythropoietin before week 6 (*i.e.*, 2 wk following the addition of boceprevir). Conversely, the neutrophil count reduction required Granulokine supplementation already at week 2. Anemia was successfully managed with erythropoietin supplementation, as neutropenia with Granulokine supplementation. The supplementation of both Erythropoietin and Granulokine was necessary to preserve the full treatment doses. None of the patients experienced skin problems; dysgeusia was frequent, but no patient discontinued therapy due to this event (Table 3).

DISCUSSION

In this study on a cohort of difficult-to-treat genotype 1 CHC patients (menopausal females, failure to a previous course of PEG-IFN/RBV, high prevalence of F3-F4 fibrosis) we showed that retreatment with a BOC-based TT regimen leads to SVR in about 45% of cases, RVR BOC being the only independent predictor of SVR.

The achievement of SVR in a great proportion of patients gives BOC-based therapy a relevant option for this group of difficult-to-treat patients, where a deferral strategy towards IFN-free regimens should be carefully evaluated due to the rapid progression of the liver diseases related to the menopausal status.

Menopausal women with chronic HCV genotype 1

infection are patients experiencing an utmost resistance to antiviral therapy with PEG-IFN- α and Ribavirin occurring at and soon after the onset of menopause^[9]. This issue could be probably further amplified in patients who failed a previous treatment with PEG-IFN and Ribavirin. In this clinical context several studies, not stratified for gender and menopausal status, were not able to obtain more than a 3%-21% SVR despite employing PEG-IFN/RBV-based treatment schedules with higher PEG-IFN- α dosages or longer period of treatment than routinely used^[14-18]. As in all the above studies, our cohort of menopausal patients with a previous failure to dual antiviral therapy exhibited a marked lack of sensitivity toward interferon, *i.e.*, none but two patients achieved RVR after the 4-wk lead-in, and a substantial proportion displayed less than 1 log decline of viral load. Despite this occurrence, the addition of Boceprevir determined a striking effect: viral load became undetectable in almost 60% of patients after 4 wk and in more than 70% at week 12, and finally SVR was obtained in 45% of women. The SVR we obtained is slightly lower than that reported in the study by Bacon *et al.*^[17] who, with a similar treatment regimen, obtained a 59% and 66% depending on the treatment schedule. It should, however, be underlined that the population undergoing treatment in the Bacon study was characterized by much more favorable characteristics in term of response to antiviral therapy like younger age and less advanced disease than ours. A recent paper by Vierling *et al.*^[19] reported higher SVR rates with BOC re-treatment. Enrolled patients belonged to control arm of 4 Boceprevir studies (SPRINT-2, RESPOND-2, SPRINT-1, or Protocol 05685)^[17,20-22] and had not achieved SVR. They were enrolled soon (< 2 wk) after a failed PEG-IFN- α /RBV course at least 12 wk that however, in about 40% of patients, had lasted 48 wk. This makes the results scarcely comparable with the other re-treatment studies and ours as the patients were exposed in a restricted period of time to PEG-IFN- α /RBV for more than double the usual duration of therapy. The results reported by Flamm *et al.*^[23] of retreatment of patients previously treated with chronic hepatitis C genotype 1 infection are consistent with ours: subjects who had had poor response to interferon therapy (< 1 log₁₀ decline in HCV RNA at week 4), had only a 39% SVR. Interestingly, none of the patients with less than 1 log decline had SVR when retreated with dual PEG-IFN/RBV. As a general remark, in none of the previously cited retreatment studies was the stratification by menopausal status available.

Another relevant finding of our study lies in the identification of RVR BOC as the only independent predictor of SVR. This result raises attention for relevant practical issues in the management of this difficult-to-treat group of patients, when RVR BOC occurs, the pattern of previous failure to dual therapy does not significantly affect the likelihood of SVR to BOC-based TT. Second, although 1 log₁₀ decline of viral load after 4 wk of dual therapy identified patients at higher SVR likelihood, it

Table 2 Univariate and multivariate analysis for factors predicting sustained virological response

Variables	Univariate analysis OR (95% CI)	P value	Multivariate analysis OR (95% CI)	P value
Age (yr)	1.006 (0.916-1.105)	0.900		
Age at menopause	1.150 (0.931-1.450)	0.195		
Previous response	2.662 (0.957-6.881)	0.043	2.927 (0.931-9.206)	0.066
Histological grading	0.894 (0.605-1.322)	0.576		
Histological staging	0.982 (0.586-1.645)	0.946		
Fibrosis	1.667 (0.528-5.265)	0.384		
Liver stiffness	0.986 (0.895-1.086)	0.774		
Cirrhosis	1.111 (0.155-7.974)	0.917		
BMI	1.000 (0.825-1.212)	1.000		
HCV RNA > 800.000 IU/mL	0.519 (0.120-2.248)	0.381		
RVR	1.200 (0.070-20.429)	0.900		
1 log decline at week 4	3.733 (1.676-12.658)	0.034	0.961 (0.194-4.757)	0.961
RVR BOC	7.347 (2.156-25.035)	0.001	6.794 (1.596-21.644)	0.010
ALT (IU/mL)	0.996 (0.977-1.014)	0.645		
Platelets ($\times 10^3/\text{mm}^3$)	1.000 (1.000-1.000)	0.165		
HOMA	0.907 (0.533-1.544)	0.719		

BMI: Body mass index; HCV: Hepatitis C virus; ALT: Alanine aminotransferase.

Table 3 Adverse events *n* (%)

Event	
Death, <i>n</i>	0
Drug Discontinuation due to AE	6 (10)
Dose Modification due to AE	8 (14)
Any life-threatening adverse event, <i>n</i>	0
Any serious adverse event	2 (4)
Hematologic event	
Reduced neutrophil count	
< 750 per mm^3	14 (25)
< 500 per mm^3	8 (15)
Mean change in hemoglobin from baseline (g/dL)	
At wk 12	-1.2
At wk 24	-2.2
At wk 48	-3.3
Erythropoietin use	20 (35)
Transfusion	1 (1.8)
Common adverse event	
Nausea	24 (43)
Anemia	23 (41)
Dysgeusia	20 (36)
Fatigue	18 (32)
Rash	6 (11)

AE: Adverse event.

was not confirmed as independently associated with SVR at multivariate analysis. Third, none of the other factors that are traditionally associated with achievement of a sustained virological response (*i.e.*, low viral load at baseline, absence of fibrosis or cirrhosis, IL 28B genotype, > 1 log₁₀ decrease in HCV RNA)^[24] was independently related with SVR. It is of note that the relevance of RVR BOC as predictor of SVR has been identified in other studies of difficult-to-treat population like patients with cirrhosis^[25,26].

The main limitation of this study lies in the potentially limited external validity of the results for different populations and settings. Our study included a cohort of Italian patients enrolled at tertiary care centers, who may be different from general population, in terms of both

metabolic features and severity of liver disease, limiting the broad application of the results.

In conclusion, Boceprevir addition to standard PEG-IFN- α /RBV therapy was effective in achieving SVR in about 50% of menopausal women with chronic hepatitis C, genotype 1, who had failed SVR with prior PEG-IFN- α /RBV treatment. Most importantly, Boceprevir-based triple therapy success rate was not influenced by pattern of previous response to DT or by the severity of liver fibrosis while RVR BOC was the only independent predictor of SVR. This opens a relevant possibility for patients who are at high risk of a rapid progression toward cirrhosis and/or decompensation.

COMMENTS

Background

Hepatitis C virus (HCV) infection is a leading cause of chronic liver disease. In menopausal women, the disease becomes much more rapidly progressing than during reproductive age, severe fibrosis develops, and response to antiviral therapy becomes low.

Research frontiers

The therapeutic options for HCV are rapidly growing. New drugs will be, however, extremely expensive and the use of the best cost-effective options should be pursued in order to offer treatment to the largest number of patients.

Innovations and breakthroughs

The results support the concept that triple antiviral treatment with PEG IFN/Ribavirin and Boceprevir is able to obtain a striking improvement in the SVR rate of menopausal women with HCV with previous treatment failure.

Applications

The management of patients who do not achieve a viral response has always been challenging. The results of this study support the efficacy, safety and tolerability of PEG IFN/Ribavirin and Boceprevir in a high percentage of previous non responders to PEG IFN/Ribavirin thus offering immediate cure to patients otherwise likely to rapidly progress toward severe fibrosis.

Terminology

SVR indicates sustained virological response, *i.e.*, viral clearance for more than 24 wk after treatment.

Peer review

In the manuscript the authors present data showing that the difficult to treat population of menopausal women display improved treatment results by the inclusion of boceprevir. The manuscript is clearly presented and generally well-

written, although it should be edited once more by the authors before publishing. HCV triple therapy including either telaprevir or boceprevir is now the current standard of care therapy for HCV infection, though this will soon change to "next generation" antivirals. The population numbers of the current study are limited to 56, and would be strengthened by expanding the study to a larger population.

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Splenic artery ligation associated with endoscopic banding for schistosomiasis portal hypertension

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Abstract

AIM: To propose a less invasive surgical treatment for schistosomiasis portal hypertension.

METHODS: Ten consecutive patients with hepatosplenic schistosomiasis and portal hypertension with a history of upper gastrointestinal hemorrhage from esophageal varices rupture were evaluated in this study. Patients were subjected to a small supraumbilical laparotomy with the ligation of the splenic artery and left gastric vein. During the procedure, direct portal vein pressure before and after the ligation was measured. Upper gastrointestinal endoscopy was performed at the 30th postoperative day, when esophageal varices diameter were measured and band ligation performed. During follow-up, other endoscopic procedures were performed according to endoscopy findings.

RESULTS: There was no intra-operative mortality and all patients had confirmed histologic diagnoses of

schistosomiasis portal hypertension. During the immediate postoperative period, two of the ten patients had complications, one characterized by a splenic infarction, and the other by an incision hematoma. Mean hospitalization time was 4.1 d (range: 2-7 d). Pre- and post-operative liver function tests did not show any significant changes. During endoscopy thirty days after surgery, a decrease in variceal diameters was observed in seven patients. During the follow-up period (57-72 mo), endoscopic therapy was performed and seven patients had their varices eradicated. Considering the late postoperative evaluation, nine patients had a decrease in variceal diameters. A mean of 3.9 endoscopic banding sessions were performed per patient. Two patients presented bleeding recurrence at the late postoperative period, which was controlled with endoscopic banding in one patient due to variceal rupture and presented as secondary to congestive gastropathy in the other patient. Both bleeding episodes were of minor degree with no hemodynamic consequences or need for blood transfusion.

CONCLUSION: Ligation of the splenic artery and left gastric vein with supraumbilical laparotomy is a promising and less invasive method for treating presinusoidal schistosomiasis portal hypertension.

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Key words: Endoscopic banding; Esophageal varices; Portal hypertension; Schistosomiasis; Variceal bleeding

Core tip: In a recent study from our group assessing systemic and portal hemodynamic changes in schistosomiasis patients undergoing esophagogastric devascularization and splenectomy, we showed that the splenic artery ligation alone promotes correction of the systemic hyper-dynamic state and significantly decreases portal pressure. The objective of the present study was to propose a less invasive surgical treatment for portal hypertension in schistosomiasis, which consists of splenic

artery ligation, followed by endoscopic variceal treatment. This study showed that this new technique is a promising method in the treatment of presinusoidal portal hypertension due to its less invasive characteristic.

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INTRODUCTION

Portal hypertension is a pathologic increase in pressure in the portal venous system that leads to portosystemic collateral circulation. Moreover, portal hypertension is frequently associated with digestive hemorrhage due to the rupture of gastroesophageal varicose veins, independent of hepatocellular function. Portal vein pressure is directly related to intrahepatic vascular resistance and portal blood flow. In most patients, portal hypertension results from both increased intrahepatic resistance, due to the architectural distortion of liver parenchyma secondary to fibrosis, and to splanchnic hyperflow^[1,2].

Schistosomiasis is an endemic disease in many countries and represents one of the main causes of portal hypertension worldwide. In the hepatosplenic subtype, the most severe form of the disease, liver fibrosis, hepatomegaly (mostly of the left lobe), presinusoidal portal hypertension, preserved hepatic function and substantial splenomegaly are observed^[2-7]. Esophageal varices rupture and bleeding is the most feared complication of the disease, observed in up to 52% of the patients, with a mortality rate of 11.7% for the first episode^[8-10].

Since upper gastrointestinal hemorrhage is the main cause of death in patients with portal hypertension and preserved liver function, surgical treatment is considered the best therapeutic alternative, mainly for those with hepatosplenic schistosomiasis^[6,10-12]. However, there is no agreement on which surgical technique is the most appropriate: esophagogastric devascularization and splenectomy (EGDS) or distal splenorenal shunt? Distal splenorenal shunt had been employed for the treatment of presinusoidal portal hypertension, however, due to high rates of late postoperative portosystemic encephalopathy and long-term worsening of liver function, this procedure is less frequently used^[13]. EGDS is the treatment of choice for the majority of cases, as it is a relatively simple technique with good results and no postoperative encephalopathy^[11,14-16]. The disadvantage of EGDS is bleeding recurrence, observed in 6%-29% of the patients, and postoperative endoscopic therapy is therefore necessary^[16,17].

In a recent study from our group, systemic and portal hemodynamic changes were assessed in schistosomal pa-

tients during EGDS and measurements were taken after every surgical step: ligation of splenic artery, splenectomy, and esophagogastric devascularization^[18]. The hyperdynamic state, characterized by cardiac output increase and peripheral vascular resistance, which was observed preoperatively in all patients, returned to normal values after EGDS. The intraoperative hemodynamic monitoring showed that within all surgery steps, the splenic artery ligation alone promotes the correction of the hyperdynamic state, thus leading to the conclusion that the systemic hemodynamic changes were related to splenic hyperflow.

The objective of the present study was to propose a new, less invasive surgical treatment for presinusoidal portal hypertension in patients with schistosomiasis, supported by the knowledge of the physiopathology of the disease based on hemodynamic behavior. The technique involves ligation of the splenic artery followed by postoperative endoscopic treatment (variceal band ligation). This is a pilot study involving ten patients that were subjected to conventional surgery with intra-operative measurement of portal pressure and evaluation of long-term results before continuing with a minimally invasive laparoscopic approach.

MATERIALS AND METHODS

The study was approved by the University Hospital Ethics Committee and all patients provided written informed consent before the operation. Ten consecutive patients with hepatosplenic schistosomiasis and portal hypertension with a history of upper gastrointestinal hemorrhage from rupture of esophageal varices were evaluated. Exclusion criteria included other liver diseases, such as hepatitis caused by alcohol or virus, and patients with portal or mesenteric venous system thrombosis. After admission, patients underwent laboratory and liver function tests evaluation, chest X-ray (anterior-posterior and lateral view), abdominal ultrasound with portal system Doppler evaluation, and upper gastrointestinal endoscopy with esophageal varices diameter measurement.

All cases were discussed in a multidisciplinary meeting before surgery and were electively operated on at least 30 d after the bleeding episode. For the operation, patients received a small (10 cm) supraumbilical, midline incision, ligation of gastroepiploic vessels leading to the exposure of the retroperitoneum, followed by ligation of the splenic artery (as close as possible from celiac trunk) and the left gastric vein. At the beginning of the procedure, a small (6 Fr) catheter was inserted through a jejunal venous branch, locating its extremity inside the portal vein, allowing a direct portal vein pressure measurement before and after the ligation of the splenic artery. At the end of the procedure, the jejunal vein catheter was removed and the vein ligated. To confirm the etiology of liver disease, liver biopsy was performed with a Tru-Cut needle in all patients. An upper gastrointestinal endoscopy was performed on the 30th postoperative day, at which time the

diameters of esophageal varices were measured and band ligation was performed. Patients were followed at the Liver Surgery unit and at the Endoscopy clinics, where other endoscopic procedures were made according to endoscopy findings.

RESULTS

Of the ten patients included in our study, seven were male and three were female with a mean age of 41.9 years (range: 26-66 years). All patients had normal liver function and diagnosis of hypersplenism, characterized by low white blood cell and platelet counts, under 140000 and 4000, respectively. There was no intra-operative mortality and all patients had confirmed histologic diagnosis of schistosomal portal hypertension. During the immediate postoperative period, two patients (2/10; 20%) had complications; one patient had a splenic infarction, which was conservatively treated with painkillers and did not need re-operation, with rapid improvement, and the other patient had an incision hematoma, which was re-operated and drained on the second postoperative day. Both immediate postoperative complications were easy to solve and patients' evolution was uneventful. No complications related to the jejunal vein catheterization were observed. The mean hospitalization time was 4.1 d (range: 2-7 d), during which, none of the patients presented any change in liver function. On the other hand, an increase in platelet and white blood cell counts was observed in nine patients during the immediate postoperative period, and an improvement in the red blood cell count was observed in six patients.

Pre- and postoperative liver function tests did not show any significant changes. Concerning the hypersplenism, nine patients presented a transient increase of approximately 14.5% in leukocyte and platelet levels. However, low platelet and white blood cell counts persisted throughout the late postoperative period. Thirty days after surgery, we observed a decrease in varices diameter during endoscopy in seven patients.

The mean follow-up period was 67.2 mo (range: 57-72 mo). During follow-up, endoscopic therapy was performed and seven patients had their varices eradicated; varices recurrence was observed in four patients who then underwent endoscopic re-treatment. Considering the late postoperative evaluation, nine patients had a decrease in varices diameter. A mean of 3.9 endoscopic banding sessions were performed per patient. Two patients presented bleeding recurrence during the late postoperative period. However, bleeding was controlled with endoscopic banding in only one patient due to variceal rupture. The other patient presented with bleeding secondary to congestive gastropathy. Both bleeding episodes were of a minor degree with no hemodynamic consequences or need for blood transfusion.

DISCUSSION

It has been shown that surgical treatment is the best

therapy for schistosomal patients with previous digestive hemorrhage due to esophageal varices rupture, though there is still no agreement on which is the best technique^[6,11]. Distal splenorenal shunt and EGDS were the most commonly performed operations during the last 20 years, with arguments in favor of and significant post-operative complications for both^[6,11]. Distal splenorenal shunts have excellent results considering hemorrhage relapse, with less than 5% bleeding recurrence^[15,19-21], however, it can lead to postoperative portosystemic encephalopathy in 3.3%-14.8% of patients^[15,16], and taking into account portal hypertension of schistosomal origin, where liver function is preserved and encephalopathy is not part of the disease clinical presentation, this procedure is not considered ideal. With this in mind, EGDS is the first choice due to its simplicity, good results, and lack of postoperative encephalopathy^[11,14,15]. A disadvantage of this technique is bleeding recurrence, which can occur in 6%-29% of patients, making the association with post-operative endoscopic therapy necessary^[22].

A previous study from our group showed that schistosomal patients subjected to EGDS present a hyper-dynamic circulation characterized by cardiac output increase, low peripheral resistance and an increase in portal flow^[18]. Hemodynamic measurements (portal and systemic) were taken after each step of the operation (splenic artery ligation, splenectomy, and esophagogastric devascularization), and it was shown that immediately after splenic artery ligation, the hyper-dynamic circulation normalized in all patients. Moreover, a 28% decrease in portal flow and a 30% decrease in portal pressure were also observed. No other surgical step changed the hemodynamic parameters, which remained stable after splenic artery ligation through the end of the procedure^[18]. Therefore, it became clear that splenomegaly and splenic overflow are important factors in the generation of hyper-dynamic circulation in the hepatosplenic form of schistosomiasis. In addition, Sakai *et al*^[22] showed that endoscopic sclerotherapy was more effective for schistosomal patients who had undergone EGDS compared to those without previous surgery, as varices have a smaller diameter, making the endoscopy easier and leading to significantly better results. The decrease in varices diameter may be related to portal pressure decrease after EGDS with consequent pressure decrease in esophageal vessels, as changes in portal pressure have a direct impact on esophageal varices^[23]. Lacerda *et al*^[24] measured the pressure in esophageal varices during splenectomy and left gastric vein ligation in schistosomal patients and found a 28.5% decrease in varices pressure after the procedure.

Based on the demonstration that splenic artery ligation alone leads to the normalization of cardiac output and peripheral vascular resistance and to a significant decrease in portal flow and pressure, and that splenectomy leads to a decrease in esophageal varices diameters, we proposed a new and less invasive treatment for patients with presinusoidal portal hypertension due to hepatosplenic schistosomiasis involving a simple splenic artery ligation with postoperative endoscopic treatment (esoph-

ageal variceal band ligation). Intraoperative mortality was not observed and the hospitalization period was short due to the low rate of complications. Spleen infarction was observed in one patient, possibly because the splenic artery ligation was performed in a distal portion of the artery due to technical issues.

We observed a decrease in the diameter of varices in 70% of the patients 30 d after surgery. During follow-up, seven patients had their varices eradicated, but four of them had recurrence. Ferraz *et al*^[11] obtained esophageal varices eradication in 18.2% patients with the EGDS operation alone, and in 52.7% with postoperative endoscopic sclerotherapy. We have previously shown that endoscopic exams performed after EGDS with postoperative varices banding program, led to varices eradication in 85.7% of patients, though recurrence was observed in 56.6% of the cases^[17]. In the last endoscopic evaluation, 90% of our patients had a decrease in varices diameter when compared with the preoperative period, which can be considered as an excellent result. Finally, two of our patients evolved with bleeding recurrence, but only one due to variceal rupture. In our experience, after long term follow-up, bleeding recurrence occurred in 24.7% of patients submitted to EGDS, half of which (14.6%) were due to varices rupture^[17].

CONCLUSION

The present pilot study shows that this new surgical technique is a promising treatment for presinusoidal schistosomiasis portal hypertension due to its less invasive characteristic and low complication rate. Further studies will utilize a minimally invasive laparoscopic approach.

COMMENTS

Background

In a recent study from our group, systemic and portal hemodynamic changes were assessed in schistosomal patients at every step during esophagogastric devascularization and splenectomy. The intraoperative hemodynamic monitoring showed the splenic artery ligation alone promotes the correction of the hyper-dynamic state, indicating that the systemic hemodynamic changes were related to splenic hyperflow.

Research frontiers

This study proposes a new, less invasive surgical treatment for portal hypertension in patients with schistosomiasis, supported by the knowledge of the physiopathology of the disease based on hemodynamic behavior.

Innovations and breakthroughs

All patients were submitted to conventional surgery with intra-operative measurement of portal pressure with the ligation of the splenic artery and left gastric vein.

Applications

The new surgical technique is a promising treatment for presinusoidal schistosomiasis portal hypertension due to its less invasive characteristic and low complication rate. This initial series is a pilot study and the surgical procedures were made through a small laparotomy. Future studies will use a minimally invasive laparoscopic approach to this technique.

Terminology

Portal hypertension is the pathologic increase in pressure within the portal system, leading to portosystemic collateral circulation. It is frequently associated with digestive hemorrhage due to the rupture of gastroesophageal varicose veins, independent of hepatocellular function. Schistosomiasis is an endemic

disease in many countries and represents one of the main causes of portal hypertension worldwide. Esophageal varices rupture and bleeding is the most feared complication of the disease.

Peer review

This article "Splenic artery ligation associated to endoscopic banding for schistosomal portal hypertension" is very interesting.

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Xiaoyao pill for treatment of functional dyspepsia in perimenopausal women with depression

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Abstract

AIM: To evaluate the efficacy and safety of the Xiaoyao pill for treatment of functional dyspepsia (FD) associated with perimenopausal depression.

METHODS: This was a double-blind, randomized, controlled trial including 180 patients with FD accompanied by depression that were divided into two groups of 90. Patients in the treatment group received oral administration of the Xiaoyao pill for soothing the liver and activating the spleen, and patients in the control group received a placebo. This trial included an 8-wk therapy period with a follow-up period of 6 mo. The total efficacy and degree of depression, as assessed by the Hamilton Rating Scale for Depression (HRSD), were evaluated. Plasma levels of motilin and gastrin were measured and a gastric emptying test was conducted in each participant.

RESULTS: The Xiaoyao pill had a good therapeutic effect and improved the symptoms in patients with perimenopausal FD as assessed by the HRSD score,

motilin and gastrin levels, and rate of gastric emptying. The total effective rate of the Xiaoyao pill in the treatment group was significantly superior to that of the placebo in the control group. In the control group, the initial HRSD score was 12.12 ± 2.29 and decreased to 7.14 ± 1.67 after therapy ($P < 0.01$). In the treatment group, the initial HRSD score was 11.44 ± 2.15 , which significantly decreased to 6.20 ± 2.08 after therapy ($P < 0.01$). Moreover, the HRSD score in the treatment group was significantly lower than in control group after 8 wk ($P < 0.01$). Motilin and gastrin levels in both groups were significantly increased after the 8-wk therapy ($P < 0.05$). The gastric emptying rate was also improved in both groups after therapy ($P < 0.05$), and the improvement was significantly better in the treatment group compared to the controls ($P < 0.05$). These results confirm the therapeutic effects of the Xiaoyao pill in perimenopausal FD patients and indicate that it is worthy of clinical promotion.

CONCLUSION: The Xiaoyao pill is effective and safe for the treatment of perimenopausal women with FD associated with depression.

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Key words: Chinese herbal medicine; Functional dyspepsia; Perimenopausal women; Xiaoyao pill

Core tip: This study observed the clinical effects of the Xiaoyao pill for treatment of perimenopausal women with functional dyspepsia (FD) and depression. The Xiaoyao pill improved patient symptoms as assessed by the Hamilton rating scale for depression and gastric emptying rate. The mechanism of these effects may be related to the observed increases in plasma motilin and gastrin levels, which can accelerate gastric emptying and improve propulsion through the small intestine.

Du HG, Ming L, Chen SJ, Li CD. Xiaoyao pill for treatment of

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INTRODUCTION

The etiology and pathogenesis of functional dyspepsia (FD) are not clear. Many researchers consider that it is caused by several different pathogenic factors. At present, it is thought to be mainly related to gastrointestinal motility disorder, increased visceral sensitivity, and psychological abnormality. However, perimenopausal FD (PMFD) is a manifestation of perimenopausal syndrome in the digestive system. At present, it is thought to be mainly caused by endocrine hypofunction, gastrointestinal motility functional disorder, and psychological factors. Gastrointestinal motility functional disorder is the main pathologic basis of FD, including proximal gastric accommodation abnormality, gastric emptying delay, gastroduodenal motility coordination abnormality, and inter-digestive phase III gastrointestinal motility abnormality. Recent research shows that if abnormalities arise in some parts of the brain-gut axis, digestive tract motility disorder or reduced visceral sense threshold occurs; both of which may become important factors in PMFD pathogenesis^[1]. Other research shows that digestive tract motility is related to estrogen, which has inhibitory effects on two aspects of stomach physiological functions: (1) gastric emptying, motility and rhythm; and (2) gastric secretion^[2].

Estrogen can also affect many neurotransmitters, including promoting the production of 5-hydroxytryptamine (5-HT), and increasing intracephalic 5-HT receptors^[3]. However, insufficiency of 5-HT function gives rise to depressive symptoms. Therefore, a decrease in estrogen may cause depressive symptoms and increase the occurrence of FD. Some Chinese researchers use low doses of conjugated estrogens to treat PMFD, which improves FD and psychiatric symptoms. In particular, nausea, abdominal distension, and epigastric discomfort are obviously improved. However, hormones are not used for replacement therapy in the control groups. Instead, patients are provided with oral gastric motility stimulants, without obvious improvement to FD and psychiatric symptoms. It is inferred from this that estrogen is related to, and can improve, the FD symptoms of perimenopausal women. Due to the obvious decrease in estrogen level, the occurrence of emotional disorders (such as depression, anxiety, and sleep disorders) in perimenopausal women is increased among FD patients^[4]. Moreover, such factors often exist at the same time and affect each other.

At present, there are no effective Western medicines for PMFD. Most researchers have attempted to use psychotherapy for patients with FD, and have found that it has better effects when compared with simple drug

therapy, especially for FD patients with serious symptoms or treatment resistance^[5]. Frequently used drugs include antacids, prokinetic agents, anxiolytics and antidepressants, digestants, and anti-*Helicobacter pylori* treatment^[6,7]. Standardized treatment for FD includes combination, comprehensive and individualized treatment. In China, however, there is considerable experience in treatment of PMFD with traditional Chinese medicine (TCM) and other methods^[8-11]. TCM considers that this disease is related to emotional repression and weak spleen and stomach. The disease is located in the stomach, and is closely related to the liver and spleen, that is, liver depression and spleen deficiency syndrome. It refers to the fact that the syndrome is dominated by epigastric pain due to liver qi stasis, stomach invasion and stomach imbalance.

In this study, we investigated the curative effect of the TCM Xiaoyao pill in women with PMFD and depression, by observation of motilin, gastrin and rate of gastric emptying, as well as the Hamilton Rating Scale for Depression (HRSD). We also investigated the possible pathogenesis of PMFD.

MATERIALS AND METHODS

Patient selection and diagnostic criteria

One hundred eighty patients with FD accompanied by depression were selected from the Department of Internal Medicine, The Second People's Hospital Affiliated with Fujian University of Traditional Chinese Medicine from December 2012 to December 2013. The patients were randomly divided into a treatment or control group by a random number table method.

The diagnostic criteria were based on the Rome III criteria^[12]. All subjects 41-52 years of age, who complained of at least one of the symptoms (early satiety, epigastric pain, epigastric burning, and postprandial fullness), which had lasted > 6 mo and had become more severe in the past 3 mo, were enrolled.

The TCM standard for diagnosing syndromes was worked out with reference to the standard for diagnosing the type of liver depression and spleen deficiency in the guidelines of diagnosing and treating FD. Major symptoms are stomach pain or discomfort and anorexia and loose stools. Minor symptoms include: (1) abdominal distention and pain; (2) impatience; (3) insomnia and dreamful sleep; (4) belching and acid reflux; (5) physical and mental fatigue; and (6) abdominal distention after eating. Patients with all the major symptoms and two or more minor symptoms were diagnosed as suffering from the syndrome of liver depression and spleen deficiency.

Inclusion and exclusion criteria

To be included in the study, patients had to meet the following inclusion criteria: (1) perimenopausal women 41-52 years of age who met the Rome III criteria for FD; (2) had liver depression and spleen deficiency syndrome; (3) ability to cease all medical treatment that could influence gastrointestinal motility at least one week prior to

Table 1 Curative effect of the Xiaoyao pill

Group	<i>n</i>	Cure	Obvious effect	Effectiveness	Ineffectiveness	Effective rate
Control	90	5	18	16	51	43.33%
Treatment	90	20	16	42	12	86.67% ^b

^b*P* < 0.01 *vs* control.

the test; and (4) agree to participate and give signed informed consent. Patients were excluded if they: (1) had structural diseases, such as esophagitis, erosive gastroduodenal lesions or ulcers that could explain symptoms; (2) had systemic diseases; (3) were pregnant or breast-feeding; (4) were receiving hormone replacement therapy; or (5) had a mental disease.

Therapy

Randomization was performed by opening a sealed envelope that contained a preassigned randomized treatment generated by computer on entry to the study. Both the investigators and patients were blinded to the assigned treatment throughout the study. The Xiaoyao and placebo pills were identical in appearance. In the treatment group, the patients were treated with the Xiaoyao pill, consisting of *chai hu* (*radix bupleuri*), *dang gui* (*Angelica sinensis*), *bai shao* (*radix paeoniae alba*), *chao bai zhu* (roasted *rhizoma atractylodis macrocephalae*), *fu ling* (*Wolfiporia extensa*), *zhi gan cao* (*radix glycyrrhizae*), *bo he* (mint), and *sheng jiang* (*rhizoma zingiberis recens*). In the control group, the patients were given a placebo, *chaoguya* (*fructus setariae germinatus*). Drugs were produced by Fuzhou Jinxiang Co. Ltd. They were administered at 3 g each time, 30 min before breakfast and supper, for 8 wk. During treatment, patients stopped taking other drugs.

Standard for evaluating curative effect

According to the TCM Diagnosis and Treatment Norms on Functional Dyspepsia Approved by the China Association of Chinese Medicine, Professional Committee of Spleen and Stomach Diseases, all symptoms are divided into three grades, mild, medium and severe, with a score of 1, 2 and 3, respectively, or 0 for no symptoms. The grading of symptoms is as follows: mild, symptoms do not affect work and life, and are bearable; medium, symptoms affect work and life, but are bearable; severe, symptoms hinder work and life, and are unbearable.

With reference to the Guideline for Directing Clinical Research into Treatment of Distention and Fullness with New Chinese Drugs, clinical control means that clinical symptoms and signs disappear and the accumulated score of syndromes is reduced by $\geq 95\%$. Obvious effect means that clinical symptoms and signs are obviously improved and the accumulated score of syndromes is reduced by $\geq 70\%$. Effectiveness means that clinical symptoms and signs are improved and the accumulated score of syndromes is reduced by $\geq 30\%$. Ineffectiveness means that clinical symptoms and signs are not improved

or aggravated, and the accumulated score of syndromes reduces by < 30%.

The formula for assessing the curative effect was: (accumulated score before treatment - accumulated score after treatment)/accumulated score before treatment $\times 100$. The HRSD was categorized according to Davis *et al.*^[13]: > 24 points, the patient may be suffering from severe depression; 17-24 points, the patient may be suffering from medium depression; 8-16 points, the patient may be suffering from mild depression; ≤ 7 points, the patient is without depressive symptoms.

Testing index and method

Before and after the therapy period, the two groups were tested for plasma levels of motilin and gastrin, evaluated with the HRSD, and gastric emptying rate (total number of barium $\times 100\%$ ^[14]) was determined. Tests were repeated 6 mo later to determine the recurrence.

Statistical analysis

All data are presented as mean \pm standard deviation and tested using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). A Student's *t*-test was used to compare the differences between the two sample means, χ^2 tests were used for numerical data, and the Ridit test was used to compare the clinical efficacy between the groups. *P* < 0.05 was considered to represent statistical significance.

RESULTS

The 90 patients in the treatment group were 41-49 years of age (average: 45.30 ± 2.81 years) and their disease course was 1-8 years (average: 4.20 ± 2.30 years). The 90 patients in the control group were 42-52 years of age (average: 46.09 ± 2.45 years) and their disease course was 1-9 years (average: 3.96 ± 2.43 years). There were no statistical differences between the two groups regarding age, illness course, or symptom distribution.

Comparison of curative effect of treatment

The curative effect was significantly higher in the treatment group compared to the control group (86.67 *vs* 43.33%, *P* < 0.01) (Table 1).

Comparison of HRSD scores

HRSD scores in the two groups were significantly lower after 8 wk (*P* < 0.01). HRSD scores in the treatment group were significantly lower than in the control group (*P* < 0.01) (Table 2).

Table 2 Hamilton rating scale for depression

Group	n	Before	After 8 wk
Control	90	12.12 ± 2.29	9.14 ± 1.67 ^a
Treatment	90	11.44 ± 2.15	6.20 ± 2.08 ^{b,d}

^b*P* < 0.01 *vs* the control group; ^d*P* < 0.01 *vs* before.

Comparison of motilin and gastrin levels and gastric emptying rate

Motilin and gastrin levels in the two groups were significantly increased after therapy (*P* < 0.05), and the levels in the treatment group were significantly higher than in the control group (*P* < 0.05). The gastric emptying rate was significantly improved after 8 wk (*P* < 0.05), and the improvement in the treatment group was significantly greater than in the control group (*P* < 0.05) (Table 3).

Safety assessment

There were no abnormalities in routine blood and urine examinations, or in renal or hepatic functions. None of patients in the two groups had any adverse drug reactions. According to the follow-up visit 6 mo after therapy, there were five relapses in the control group and none in the treatment group.

Comparison of symptoms in the follow-up period

The patients were subject to drug withdrawal 8 wk after treatment as well as a follow-up visit at 6 mo, after which, their symptoms were recorded. There was no significant difference between symptoms in PMFD patients before and after drug withdrawal, which indicates that there was no symptom relapse after 6 mo.

DISCUSSION

The prevalence of FD in Western countries is 20%-25%, compared with 8%-23% in Asia^[15,16]. In China, dyspepsia patients account for approximately 10% of the general medicine outpatient service, and 50% of the cases at digestive internal medicine clinics^[9]. Although there are numerous FD patients, rigorous clinical research rarely shows that therapeutic methods are more effective than placebo^[17]. As a result, treatment efficacy is not ideal, and FD can easily recur, which has a serious effect on quality of life.

TCM proposes liver controlling dispersion and spleen governing transportation and transformation. Liver controlling dispersion refers to comprehensive physiologic functions of unchoking, smoothing, ascending, dredging, and discharging, which mainly reflects regulation of spiritual emotion, and promotion of digestion and absorption. Liver qi refers to a manifestation of the physiologic function of the liver, mainly reflected in adjustment of spirit and emotion and promotion of digestion and absorption^[18-20]. If the discharging function of the liver is normal, the body is better able to coordinate its spiritual and emotional activities. Besides, it is

conducive to the order of the spleen, stomach and bile secretion, for maintaining normal digestion and absorption. Liver dysfunction may affect this order, resulting in abnormal digestive function, such as appetite disorder, dyspepsia, belching pantothenic acid, abdominal distension, and diarrhea. Spleen governing transportation and transformation means that the spleen is able to transform water and cereal into refined nutritious substances, and transport these into various organs and tissues throughout the body. Dysfunction of spleen transportation may cause abnormal digestion and absorption, resulting in pathologic changes in abdominal distension, loose stools, appetite disorder, and lassitude.

The Xiaoyao pill is made from eight types of TCM: *radix bupleuri*, Chinese angelica, *radix paeoniae alba*, parched white *atractylodes rhizome*, *poria cocos*, honey-fried licorice root, mint, and fresh ginger. The monarch drug, *radix bupleuri* is used to smooth the liver, dispel melancholy, and soothe liver-qi stagnation. Chinese angelica is bitter and is used to nourish and activate the blood, and *radix paeoniae alba* is used to nourish the blood and liver; the drugs are adjuvant drugs. Liver dysfunction may cause spleen deficiency, so we use white *atractylodes rhizome*, licorice root, and *poria cocos* to invigorate the spleen and supplement qi. Modern pharmacologic research shows that saikoside has anti-inflammatory, immunoregulatory and liver protective functions, but can also inhibit cholinesterase, act as a quasi-choline sample and adjust the digestive and nervous systems^[21,22]. This consequently cures liver stagnation and soothes liver-qi stagnation. White *atractylodes rhizome* can activate the muscarinic receptors of the gastrointestinal tract and acetylcholine receptors, and accelerate gastrointestinal motility and evacuation^[23]. *Poria cocos* can increase 5-HT levels^[24], indicating that the Xiaoyao pill can alter central monoamine neurotransmitter and hormone levels, thereby improving the clinical symptoms of stagnation of liver qi and spleen deficiency.

Motilin is mainly expressed in the gastrointestinal tract and strongly stimulates mechanical and electrical activity of the upper gastrointestinal tract. It can give rise to intense shrinkage of the stomach and obvious segmentation movement of the small intestine. Gastrin is a gastrointestinal hormone that is secreted by the ventricular sinuses and duodenum. It mainly promotes shrinkage of the gastroesophageal sphincter and smooth muscle of the digestive tract, and stimulates secretion of gastric acid, pancreatic enzymes, bile and small intestinal juice. Hyposecretion can relax smooth muscle in the stomach and intestine, relieving stomach tension and peristalsis, extending gastric emptying time and weakening movement of the small intestine^[25-27].

In conclusion, the results of this study show that the Xiaoyao pill is effective for improving symptoms in patients with PMFD. Its mechanism of action may be related to boosting plasma gastrin and motilin levels, accelerating gastric emptying, and improving propulsion in the small intestine. The relationship among improvement in depression, serum estrogen level, and 5-HT content

Table 3 Levels of motilin, gastrin and gastric emptying rate

Group	Time	MOT (ng/L)	GAS (ng/L)	Gastric emptying rate (%)
Control (n = 90)	Before	86.32 ± 21.89	62.18 ± 13.52	39.23 ± 9.42
	After	100.56 ± 23.34 ^c	76.33 ± 15.27 ^c	46.77 ± 8.52 ^c
Treatment (n = 90)	Before	87.67 ± 23.50	60.25 ± 12.59	41.26 ± 8.38
	After	197.46 ± 26.37 ^{a,c}	110.43 ± 17.47 ^{a,c}	73.35 ± 9.89 ^{a,c}

^aP < 0.05 *vs* control; ^cP < 0.05 *vs* before. MOT: Motilin; GAS: Gastrin.

requires further study. Whether serum estrogen levels in perimenopausal women and 5-HT content influence FD occurrence also requires further study.

COMMENTS

Background

Functional dyspepsia (FD) is a common gastrointestinal disease with high morbidity. Due to the drop in estrogen level, perimenopausal women with FD (PMFD) have an increase in emotional disorders such as depression, anxiety and sleep disorder. As there are no effective treatments currently available, the aim of the present study was to evaluate the clinical effects of the Xiaoyao pill for treatment of PMFD with depression. The authors discuss possible pathogenesis of PMFD through observing overall effects of traditional Chinese medicine, motilin and gastrin levels, gastric emptying rate, and Hamilton Rating Scale for Depression (HRSD). The results provide a basis for clinical application.

Research frontiers

FD etiology and pathogenesis are still not clear and may be related to multiple factors. It is generally acknowledged that gastrointestinal obstruction is the main pathophysiologic basis of FD. Meanwhile, mood, stress and *Helicobacter pylori* infection are thought to be closely related to FD morbidity. There is still no drug treatment with specific effects. Most researchers have attempted psychotherapy for patients with FD. In China, much experience has been gained with traditional Chinese medicine for treating FD. However, research on PMFD is rare.

Innovations and breakthroughs

This was a randomized, double-blind, placebo-controlled study. The authors observed the clinical effects of the Xiaoyao pill in perimenopausal women with FD and depression. This research provides a basis for further clinical applications. It can be inferred that PMFD pathogenesis is related to liver qi stagnation, according to PMFD symptom improvement, gastric emptying and changes in gastrointestinal hormones. The mechanism of action of the Xiaoyao pill may be related to boosting plasma gastrin and motilin levels, accelerating gastric emptying, and improving small intestinal propulsion.

Applications

The Xiaoyao pill was effective in improving symptoms in patients with PMFD, as assessed by HRSD scores, motilin and gastrin levels, and gastric emptying rate. Therefore, Xiaoyao merits consideration for clinical application.

Terminology

PMFD refers to the condition after entering the perimenopausal period, when ovarian functions weaken, estrogen levels drop, gastrointestinal mucosal barrier function reduces, neurologic function of the stomach and intestine is disordered, and is described by symptoms such as epigastric pain, gasteremphraxis, abdominal discomfort, belching, nausea, vomiting, and stomach discomfort.

Peer review

The authors studied the effect of the Xiaoyao pill on PMFD symptoms in a randomized, double-blind, placebo-controlled trial. The study was unique and well organized. The Xiaoyao pill was effective in improving symptoms of patients with PMFD, as assessed by HRSD scores, motilin and gastrin levels, and gastric emptying rate, which may help to describe the mechanism for improving propulsion in the small intestine.

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Temporal trends in inflammatory bowel disease publications over a 19-years period

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Abstract

AIM: To determine whether temporal changes occurred in the pediatric *vs* adult inflammatory bowel disease (IBD), both in terms of number and type of yearly published articles.

METHODS: We aimed to evaluate all PubMed-registered articles related to the field of IBD from January 1, 1993 and until December 31, 2011. We searched for articles using the key words "inflammatory bowel disease" or "Crohn's disease" or "ulcerative colitis" or "undetermined colitis", using the age filters of "child" or "adult". We repeated the search according to the total number per year of articles per type of article, for each year of the specified period. We studied randomized controlled trials, clinical trials, case reports, meta-anal-

yses, letters to the editor, reviews, systematic reviews, practice guidelines, and editorials.

RESULTS: We identified 44645 articles over the 19 year-period. There were 8687 pediatric-tagged articles *vs* 19750 adult-tagged articles. Thus 16208 articles were unaccounted and not assigned a "pediatric" or "adult" tag by PubMed. There was an approximately 3-fold significant increase in all articles recorded both in pediatric and adult articles. This significant increase was true for nearly every category of article but the number of clinical trials, meta-analysis, and randomized controlled trials increased proportionally more than the number of "lower quality" articles such as editorials or letters to the editor. Very few guidelines were published every year.

CONCLUSION: There is a yearly linear increase in publications related to IBD. Relatively, there are more and more clinical trials and higher quality articles.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Randomized clinical trial; Meta-analysis

Core tip: Since the first description of inflammatory bowel disease (IBD) in the 1700's, thousand of articles have been published on the topic. This study aimed to determine whether temporal changes occurred in IBD literature. We identified 44645 articles over the 19 year-period starting in 1993. There was an approximately 3-fold increase in all pediatric and adults articles recorded. This significant increase was true for nearly every category of articles but clinical trials, meta-analysis, and randomized controlled trials increased proportionally more than the number of "lower quality" articles such as editorials or letters to the editor. Very few guidelines were published every year.

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INTRODUCTION

In a 2005 article it was noted that a total of 8.1 million journal articles were recorded by MEDLINE between 1978 and 2001^[1]. During that period, the annual number of MEDLINE articles increased by a factor of 1.46, from an average of 272344 to 442756 per year^[1]. Such numbers indicate the enormous burden placed upon physicians and scientists in their attempt to stay up to date with their professional literature^[1-3]. This appears to be a universal phenomenon, already demonstrated in various fields of medicine^[4-9].

Inflammatory bowel diseases (IBDs) were described as early as in the 1700's by Giovanni Battista Morgagni^[10]. They were however recognized as a distinct pathological entity only after the description by Crohn, who reported in 1932 a case series of 14 patients with what he called "terminal ileitis"^[11]. Since then, thousands of articles have been published on IBD, and the recent development of biological, anti-inflammatory drugs has created a huge field of basic and clinical science trials^[12-21].

The aim of the current study was to determine whether temporal changes occurred in pediatric *vs* adult IBD literature, both in terms of number and type of yearly published articles.

MATERIALS AND METHODS

We used the Internet address: <http://www.ncbi.nlm.nih.gov/entrez> in order to evaluate PubMed articles registered from January 1, 1993 and until December 31, 2012 (a 20 year period). It became obvious at the time of the search on August 13-14, 2013 that not all 2012 articles were recorded in PubMed, thus we elected to remove 2012 and concentrated our search on a 19 years period. We focused upon articles in the field of IBD. In order to do so, we searched for articles using the key words "inflammatory bowel disease" or "Crohn's disease" or "ulcerative colitis" or "undetermined colitis", without species limitations, and using the filters of "ages: child-birth-18 years" (pediatric studies) or "adult: +19 years" (adult studies). We repeated the search year by year according to the total number per year of articles per type of article, for the 19 years of the specified period, analyzed year by year. The type of article was defined according to PubMed own filter. In particular, we studied randomized controlled trials (RCT), clinical trials, case reports, meta-analysis, letters to the editor, reviews, systematic reviews, practice guidelines, and editorials. In order to verify that the categorization and tagging offered automatically by PubMed was accurate, we used a random

sample of 10 studies each year, and in 100% of the cases, PubMed's categorization was found to be accurate.

Statistical analysis

The Minitab version 16 (Minitab Inc., State College, PA) was used for statistical analyses. We used linear regression to study trends over time. A *P*-value of < 0.05 was considered significant.

RESULTS

Over the 19 year-period, when we used no age filter, we identified 44645 articles. When we used pediatric *vs* adult filters, we identified 8687 pediatric-tagged articles *vs* 19750 adult-tagged articles. Thus 16208 articles were unaccounted for, and represent articles that were not assigned a "pediatric" or "adult" tag by PubMed. By category studied, when the age filter was used, there were 976 pediatric and 2242 adult clinical trials, 348 pediatrics and 968 adult RCTs, 41 pediatric and 70 adult meta-analysis, 838 pediatric and 1359 adult reviews, 26 pediatric and 32 adult guidelines, 1248 pediatric and 6121 adult case reports, 80 pediatric and 47 adult editorials, and 384 pediatric and 1531 adult Letters. The total number of articles per year as defined exceeds that provided by PubMed, because of overlap among certain categories of articles (for instance all RCTs are recorded also within clinical trials).

When pediatric and adult articles trends over the years were compared by linear regression, there was a significant increase in all articles recorded, from approximately 292 pediatric articles/year in 1993 to 917 in 2011 (*i.e.*, approximately a 3 fold increase, $r^2 = 0.86$, $P = 0.001$), and from 633 adult articles/year in 1993 to 1,939 in 2011 (also approximately a 3 fold increase, $r^2 = 0.91$, $P = 0.001$) (Figure 1A). There was a significant increase in clinical trials recorded, from approximately 16 pediatric articles/year in 1993 to 105 in 2011 (*i.e.*, approximately a 6.5 fold increase, $r^2 = 0.79$, $P = 0.001$), and from 42 adult articles/year in 1993 to 215 in 2011 (approximately a 5 fold increase, $r^2 = 0.88$, $P = 0.001$). There was a significant increase in RCT recorded, from approximately 12 pediatric articles/year in 1993 to 33 in 2011 (*i.e.*, approximately a 3 fold increase, $r^2 = 0.70$, $P = 0.001$), and from 26 adult articles/year in 1993 to 80 in 2011 (approximately a 3 fold increase, $r^2 = 0.75$, $P = 0.001$). There was a significant increase in meta-analysis recorded, from 0 pediatric articles/year in 1993 to 5 in 2011 (*i.e.*, $r^2 = 0.45$, $P = 0.002$), and from 0 adult articles/year in 1993 to 12 in 2011 ($r^2 = 0.76$, $P = 0.001$) (Figure 1B). There was no significant increase in guidelines recorded, from approximately 0 pediatric articles/year in 1993 to 2 in 2011 ($r^2 = 0.19$, $P = 0.06$), while the rise in the number of adult articles (0 in 1993 to 3 in 2011) reached statistical significance ($r^2 = 0.22$, $P < 0.04$). There was a significant increase in reviews recorded, from approximately 34 pediatric articles/year in 1993 to 67 in 2011 (*i.e.*, approximately a 2 fold increase, $r^2 = 0.69$, $P = 0.001$), and from 57 adult articles/year in 1993 to 79 in 2011 (approx-

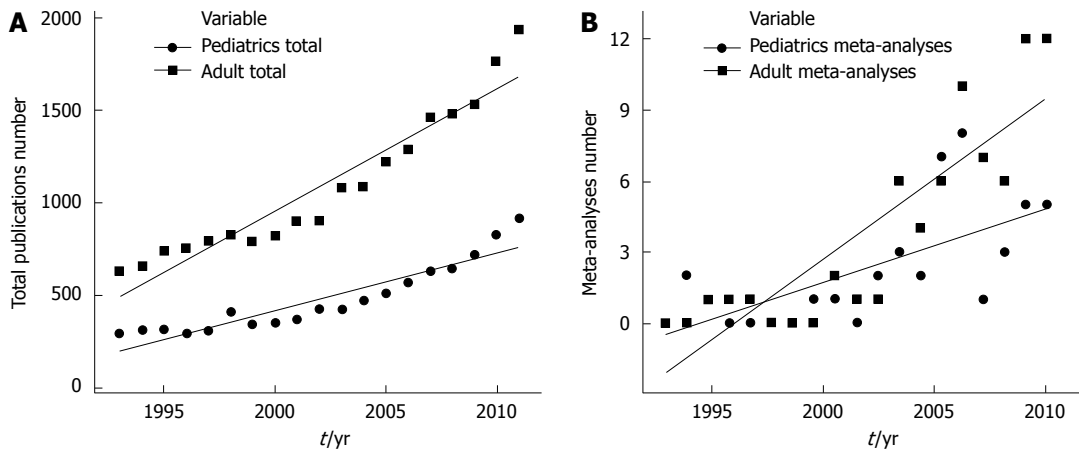


Figure 1 Total number of pediatric and adult articles vs year of publication (A) and number of pediatric and adult meta-analysis vs year of publication (B).

mately a 1.4 fold increase, $r^2 = 0.42$, $P = 0.003$). There was a significant increase in case reports recorded, from approximately 47 pediatric articles/year in 1993 to 95 in 2011 (*i.e.*, approximately a 2 fold increase, $r^2 = 0.79$, $P = 0.001$), and from 205 adult articles/year in 1993 to 448 in 2011 (approximately a 2.2 fold increase, $r^2 = 0.94$, $P = 0.001$). There was a significant increase in RCT recorded, from approximately 12 pediatric articles/year in 1993 to 33 in 2011 (*i.e.*, approximately a 3 fold increase, $r^2 = 0.70$, $P = 0.001$), and from 26 adult articles/year in 1993 to 80 in 2011 (approximately a 3 fold increase, $r^2 = 0.75$, $P = 0.001$). There was a significant increase in letters recorded, from approximately 13 pediatric articles/year in 1993 to 38 in 2011 (*i.e.*, approximately a 3 fold increase, $r^2 = 0.60$, $P = 0.001$), and from 42 adult articles/year in 1993 to 179 in 2011 (approximately a 4.3 fold increase, $r^2 = 0.82$, $P = 0.001$). There was no significant increase in editorials recorded, from approximately 3 pediatric articles/year in 1993 to 7 in 2010 (with a drop to 1 in 2011), and no significant change in adult articles 2/year in 1993 and 1 in 2011.

DISCUSSION

As hypothesized, we found a significant increase in the IBD-related yearly number of publications. Overall, the yearly number of both pediatric and adult-related articles increased in a similar manner (an approximate 3-fold increase) during this 19 years period, while a little less than a third of the articles were solely related to children. The number of clinical trials increased disproportionately more than other types of articles (a 6.5-fold increase in pediatric literature and a 5-fold increase in adult literature), however the rise in yearly number of RCTs was similar in children and adults, and similar to the overall trend (a 3-fold increase). Since in terms of strength of evidence, RCTs are considered as “stronger” than non-randomized clinical trials^[22-25], we speculate that the quality of the articles published in the field of IBD may not have increased more than what was expected from the overall increase. However, due to the large number of articles that

we retrieved, we were not able to determine what kind of papers (*i.e.*, clinical trial for biological therapy, *etc.*) in each category was increased, which is a limitation of this study. Meta-analysis also considered as very high quality in terms of evidence-based-medicine^[22], increased apparently dramatically, but this increase is somewhat artificially inflated from the fact that prior to 2000 these articles were practically inexistent, rising from nearly 0 to 5 per year in pediatric literature and 12 per year in the adult literature. Meta-analysis in general cannot be conducted without a sufficient cumulative sample size, often reached only by combining many studies, thus we speculate that in the future, we will see even more of such articles published.

At the other end of the spectrum in terms of strength of evidence, case reports increased by a factor of 2 both in pediatric and adult articles, and editorials did not increase at all. Case reports represent a low level of evidence^[22] and bring very little academic credit to their authors, which may explain why they did not increase in numbers proportionally to the rest of the IBD literature. This is even truer for Editorials, and we suspect that editors of medical journals are less likely than in the past to seek for the publication such articles, which often represent only the opinion of their author.

In conclusion, there is a linear increase in the number of yearly publications related to the field of IBD. It was not in the scope of this article to compare the rate of increase to that of articles in other fields of gastroenterology, or other fields of medicine. Nevertheless, the increase was significant in terms of the amount of time that a clinician may invest in his/her continuing education through the reading of IBD-specific literature. However, it appears that there are more and more of clinical trials and higher quality articles. We suggest that professional societies related to IBD, such as European Society for Paediatric Gastroenterology Hepatology and Nutrition, European Crohn's and Colitis Organisation, North American Society for Pediatric Gastroenterology, Hepatology and Nutrition, Crohn's and Colitis Foundation of America invest more time in such an endeavour, which might require improving their level of organization

and coordination.

COMMENTS

Background

An enormous burden is placed upon physicians and scientists in their attempt to stay up to date with their professional literature. Since the initial description of inflammatory bowel diseases (IBD) in the 1700's, thousand of articles have been published on the topic, and the recent development of biological, anti-inflammatory drugs has created a huge field of basic and clinical science trials. Thus, authors aimed to determine whether temporal changes occurred in pediatric and adult IBD literature over the past 2 decades.

Research frontiers

In this study, they aimed to verify whether the yearly number of high quality articles (such as clinical trials, meta-analysis, and randomized controlled trials) increased relatively more than that of lower quality articles (such as editorials or letters to the editor).

Innovations and breakthroughs

This article is the first that attempted to critically review trends of the IBD-related medical literature.

Applications

This article points out to the fact that overall, the quality of IBD-related publications is increasing. However, authors noted that few guidelines are issued every year, which emphasizes the need for more investment in this endeavour by professional societies related to IBD.

Terminology

The analysis of literature trends is an important tool for the global comprehension of a given medical field in terms of research activity and quality.

Peer review

Closer collaboration between experts and experts societies is necessary to enable the publication of up-to-date, evidence-based guidelines.

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Laparoscopic vs open D2 gastrectomy for locally advanced gastric cancer: A meta-analysis

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Abstract

AIM: To conduct a meta-analysis comparing laparoscopic (LGD2) and open D2 gastrectomies (OGD2) for the treatment of advanced gastric cancer (AGC).

METHODS: Randomized controlled trials (RCTs) and non-RCTs comparing LGD2 with OGD2 for AGC treatment, published between 1 January 2000 and 12 January 2013, were identified in the PubMed, Embase, and Cochrane Library databases. Primary endpoints included operative outcomes (operative time, intraoperative blood loss, and conversion rate), postoperative outcomes (postoperative analgesic consumption, time to first ambulation, time to first flatus, time to first oral

intake, postoperative hospital stay length, postoperative morbidity, incidence of reoperation, and postoperative mortality), and oncologic outcomes (the number of lymph nodes harvested, tumor recurrence and metastasis, disease-free rates, and overall survival rates). The Cochrane Collaboration tools and the modified Newcastle-Ottawa scale were used to assess the quality and risk of bias of RCTs and non-RCTs in the study. Subgroup analyses were conducted to explore the incidence rate of various postoperative morbidities as well as recurrence and metastasis patterns. A Begg's test was used to evaluate the publication bias.

RESULTS: One RCT and 13 non-RCTs totaling 2596 patients were included in the meta-analysis. LGD2 in comparison to OGD2 showed lower intraoperative blood loss [weighted mean difference (WMD) = -137.87 mL, 95%CI: -164.41--111.33; $P < 0.01$], lower analgesic consumption (WMD = -1.94, 95%CI: -2.50--1.38; $P < 0.01$), shorter times to first ambulation (WMD = -1.03 d, 95%CI: -1.90--0.16; $P < 0.05$), flatus (WMD = -0.98 d, 95%CI: -1.30--0.66; $P < 0.01$), and oral intake (WMD = -0.85 d, 95%CI: -1.67--0.03; $P < 0.05$), shorter hospitalization (WMD = -3.08 d, 95%CI: -4.38--1.78; $P < 0.01$), and lower postoperative morbidity (odds ratio = 0.78, 95%CI: 0.61-0.99; $P < 0.05$). No significant differences were observed between LGD2 and OGD2 for the following criteria: reoperation incidence, postoperative mortality, number of harvested lymph nodes, tumor recurrence/metastasis, or three- or five-year disease-free and overall survival rates. However, LGD2 had longer operative times (WMD = 57.06 min, 95%CI: 41.87-72.25; $P < 0.01$).

CONCLUSION: Although a technically demanding and time-consuming procedure, LGD2 may be safe and effective, and offer some advantages over OGD2 for treatment of locally AGC.

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Key words: D2 lymph node dissection; Gastrectomy; Gastric cancer; Laparoscopy; Meta-analysis

Core tip: The Japanese Gastric Cancer Association guidelines stipulate that D2 gastrectomy is required for the treatment of advanced gastric cancer. Due to its technical difficulty and the lack of long-term results, the application of laparoscopic D2 gastrectomy (LGD2) remains questionable. Based on the results of this study, LGD2 had similar reoperation incidence, mortality, and oncologic outcomes compared with the open D2 gastrectomy for locally advanced gastric cancer treatment. Furthermore, LGD2 was associated with lower intraoperative blood loss, lower analgesic consumption, quicker recovery, shorter hospitalization, and lower morbidity, albeit with longer operative time.

Zou ZH, Zhao LY, Mou TY, Hu YF, Yu J, Liu H, Chen H, Wu JM, An SL, Li GX. Laparoscopic vs open D2 gastrectomy for locally advanced gastric cancer: A meta-analysis. *World J Gastroenterol* 2014; 20(44): 16750-16764 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16750.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16750>

INTRODUCTION

Gastric cancer is the third most common cancer and the second leading cause of cancer-related deaths in the world^[1]. Radical gastrectomy, with lymph node dissection, is essential to cure this type of cancer^[2]. The first reported usage of laparoscopic gastrectomy (LG) for early gastric cancer (EGC) came from Kitano *et al.*^[3]. Currently, LG is the accepted treatment of choice for EGC due to low postoperative pain, faster recovery, shorter hospital stay, and a better cosmetic outcome compared with open gastrectomy (OG)^[4-7]. Three non-randomized clinical trials (non-RCTs) reported comparable five-year long-term oncologic outcomes using this type of treatment^[8-10].

Uyama *et al.*^[11] were the first to report the use of LG with D2-extended lymph node dissection (LGD2) for the treatment of advanced gastric cancer (AGC) in 2000. The Japanese Gastric Cancer Association (JGCA) guidelines stipulate that D2 gastrectomy is required for treating AGC^[12,13]. In the last decade, only a few surgeons worldwide, particularly in East Asia, have performed LGD2 to treat AGC^[14-32]. However, the application of this treatment remains dubious due to its technical difficulty and the lack of long-term results^[19,23,27,29,31,32].

According to the JGCA guidelines, D2 dissection of stations 12a or 10 can be technically demanding due to the serious risks of organ injury, bleeding, and/or bile and pancreatic leakage from a major vessel^[29,32]. Nodal dissection can increase morbidity and mortality rates similar to those of open resections^[33-35]. The laparoscopic approach for treatment of tumors with serosal invasion also risks the peritoneal seeding of malignant cells dur-

ing the procedure. Several theories regarding the etiology of port-site recurrence, associated with pneumoperitoneum and visceral manipulation, have been proposed^[36]. Another concern is the lack of long-term oncologic outcomes^[31,32]. A meta-analysis of seven case-control studies comparing laparoscopy-assisted distal gastrectomy with OG for AGC revealed that LG was associated with better short-term outcomes and comparable three-year overall survival rates. However, these studies were comprised of only 1271 cases, as well as D1, D1+, and D2 lymph node dissections^[37]. Consequently, we performed meta-analyses to evaluate whether LGD2 is an acceptable alternative to OGD2 for AGC treatment.

MATERIALS AND METHODS

Literature search

All RCTs and non-RCTs comparing LGD2 with OGD2 for AGC were identified by searching the PubMed, EMBASE, and Cochrane Library databases for studies published between 1 January 2000 and 12 January 2013. Only articles published in English or Chinese were included in this study. The following medical subject headings and free-text terms were used: stomach neoplasms; stomach cancer; gastric carcinoma; gastric cancer; laparoscopy; laparoscopic; minimally invasive; laparotomy; conventional gastrectomy; OG; D2 lymph node dissection; extended; radical. Additional relevant articles were identified using references of relevant articles and previous meta-analyses. The PubMed database was used to search for additional studies and trials using authors' names and the "related articles" function. The World Health Organization International Clinical Trials Registry Platform, Clinical Trials, Cochrane Central Register of Controlled Trials, and Chinese Clinical Trial Register were used to identify any ongoing RCTs.

Definitions

Based on the preoperative clinical assessment or postoperative pathologic examination, AGC was defined as cancerous growth invading beyond the submucosal layer of the stomach. Locally AGC is the subgroup of AGC excluding stage IV. LG was defined as total LG or laparoscopy-assisted gastrectomy. In all included studies, D2 lymph node dissection was performed according to the JGCA lymph node classification^[38]. The evaluated endpoints were classified as operative outcomes (operative time, intraoperative blood loss, and conversion rate), postoperative outcomes (postoperative analgesic consumption, time to first ambulation, time to first flatus, time to first oral intake, length of postoperative hospital stay, postoperative morbidity, incidence of reoperation, and postoperative mortality), and oncologic outcomes (number of lymph nodes harvested, tumor recurrence and metastasis, and disease-free and overall survival rates). The primary endpoints were postoperative morbidity and mortality as well as disease-free and overall survival rates. Other variables were considered as secondary endpoints.

Inclusion and exclusion criteria

The analyses included studies comparing LGD2 with OGD2 in patients with AGC. In cases when more than one publication reported on a single trial, only the most recent data were included, unless relevant outcomes were reported only in earlier publications. The following criteria were applied to exclude a study: < 40 cases; combined examination of AGC and EGC cases and/or D1-D3 lymphadenectomy, which prevented extraction of relevant or the authors' provision of such data by email; malignant stromal tumors, benign disease, or emergency operations; use of hand-assisted LG, gasless laparoscopic surgery, or robotic surgery.

Method of review

The meta-analyses were performed in accordance with the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-analysis statement^[39]. Two reviewers (Zou ZH and Zhao LY) independently evaluated all retrieved studies to determine if they met the criteria, to assess study quality, and extract data. The study team resolved all of their disagreements through discussion to reach a consensus.

Methodological quality assessment

The Cochrane Collaboration Handbook 5.1.0 was used to independently determine the quality and risk of bias of RCTs^[40]. Following domains were assessed: sequence generation; allocation concealment; completeness of outcome data; selective outcome reporting; baseline comparability of groups; dropout rates. The risk of bias in each domain was assessed and classified as low, high, or unclear. Blinding methods were not examined in this review because both LGD2 and OGD2 are invasive, and the patients were informed preoperatively about the planned procedures.

The methodological quality of non-RCTs was assessed using the modified Newcastle-Ottawa scale^[41]. Patient selection, comparability of LGD2 and OGD2 groups, and assessment of measured outcomes were examined. In assessing comparability between groups, focus was on the variables that might affect primary endpoints such as, patient age and sex, pathologic tumor-node-metastasis stage, type of gastrectomy, resection margin, tumor size, histologic type, reconstruction, and adjuvant treatment.

Studies were scored using an ordinal star scale, with higher scores representing higher quality. A maximum of one star was awarded to a study for each numbered item within the selection and outcome assessment. A maximum of two stars was awarded for the comparability of the two groups. The quality of each study was graded as level 1 (0-5 stars) or level 2 (6-9 stars).

Statistical analysis

Review Manager (RevMan, version 5.0; The Cochrane Collaboration, Oxford, United Kingdom) and STATA (version 11.2; STATA Corporation, College Station, TX,

United States) software were used for statistical analyses. Weighted mean differences (WMDs) with 95% CIs were calculated for continuous variables, including operative time, intraoperative blood loss, postoperative analgesic consumption, time to first ambulation, time to first flatus, time to first oral intake; length of postoperative hospital stay, and number of harvested lymph nodes. The odds ratios (ORs) with 95% CIs were calculated for dichotomous variables, including postoperative morbidity and mortality rates, incidence of reoperation, tumor recurrence, and metastasis. The hazard ratios (HRs) with 95% CIs extracted from Kaplan-Meier curves were used for disease-free and overall survival rates^[42,43]. A random effects model was used to pool studies with significant heterogeneity, as determined by the χ^2 test ($P \leq 0.10$) and the inconsistency index ($I^2 \geq 50\%$)^[44,45].

An alternative statistical effect model was used to re-analyze the data for the sensitivity analysis (*e.g.*, a random effects model instead of a fixed effects model or *vice versa*). The incidences of various postoperative morbidities and recurrence, and metastasis patterns were determined using subgroup analyses. The Begg's test was used to assess the presence of publication bias. Publication bias was present when the continuity-corrected $Pr > |\chi|$ value was ≤ 0.1 ^[46].

RESULTS

Descriptive assessment and study characteristics

Of the 493 publications identified in the initial literature search, 14 trials (1 RCT, 13 non-RCTs) were included in the analyses^[19,32]. A total of 2596 participants (1328 in the LGD2 group and 1268 in the OGD2 group) were included in the study (Figure 1, Table 1).

Study quality

A methodological quality assessment revealed that the RCT had unclear random sequence generation, satisfactory allocation concealment, adequately addressed incomplete outcome data, and had no selective outcome reporting^[23]. The quality of all 13 non-RCTs was level 2 (6-9 stars) on the Newcastle-Ottawa scale (Table 1).

Meta-analyses of operative outcomes

Thirteen studies provided operative time data^[19-25,27-32]. The LGD2 group's weighted mean operative time was 57.06 min longer than in the OGD2 group (95%CI: 41.87-72.25; $P < 0.01$), with significant heterogeneity among studies ($I^2 = 90\%$; $P < 0.01$) (Table 2, Figure 2A).

Blood loss data was found in 11 studies^[19-23,25,27-30,32], revealing a significantly lower blood loss in the LGD2 compared to the OGD2 groups (WMD = -137.87 mL, 95%CI: -164.41--111.33; $P < 0.01$), with significant heterogeneity among studies ($I^2 = 90\%$; $P < 0.01$) (Figure 2B).

Laparoscopic procedure conversion rates were documented in eight studies, ranging from 0.00 to 6.67%, with a weighted average of 1.68%^[19,21-24,28,30,32]. Four articles

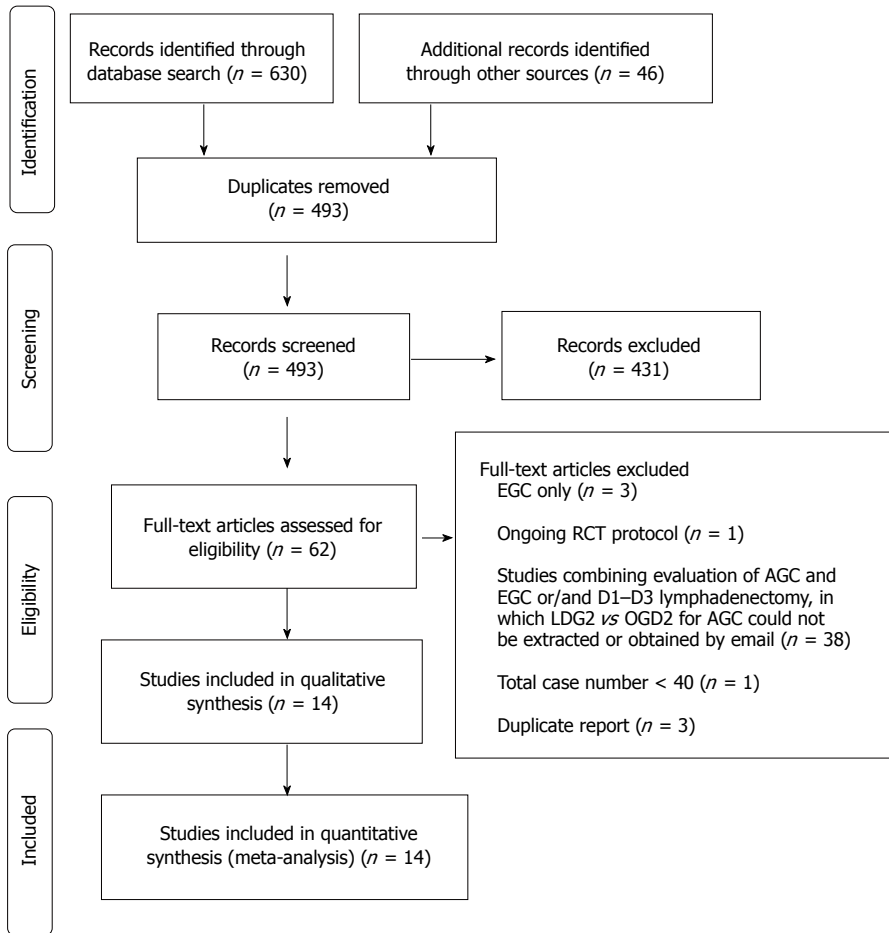


Figure 1 Flow chart of the identification and inclusion of studies. Studies evaluating laparoscopic gastrectomy with D2 (LGD2) were identified to evaluate the procedure as an acceptable alternative to open gastrectomy with D2 (OGD2) for locally advanced gastric cancer (AGC); EGC: Early gastric cancer; RCT: Randomized controlled trial.

Table 1 Characteristics and quality of studies included in the meta-analysis

Publication	Study design	Cases (L/O)	Type of gastrectomy	Type of laparoscopy	Mean follow-up (mo)	Matching criteria ¹	Quality score
Shinohara <i>et al</i> ^[32]	Non-RCT	186/123	DG, TG, PG	TLG	48.8	1, 2, 3, 4, 7, 8, 9	8
Kim <i>et al</i> ^[31]	Non-RCT	88/88	DG, TG, PG	LAG	L: 53.7; O: 58.1	1, 2, 3, 4, 5, 7	8
Wang <i>et al</i> ^[30]	Non-RCT	210/180	DG, TG, PG	LAG	L: median 24; O: median 26	1, 2, 3, 4, 5	7
Sato <i>et al</i> ^[29]	Non-RCT	32/118	DG, TG, PG	LAG	43	1	7
Hamabe <i>et al</i> ^[28]	Non-RCT	66/101	DG, TG	LAG	L: 30.4; O: 53.5	1, 2, 3, 4, 7, 9	6
Chen <i>et al</i> ^[27]	Non-RCT	224/112	DG, TG	LAG	NS	1, 2, 3, 4, 6, 7, 8	7
Zang <i>et al</i> ^[26]	Non-RCT	156/156	TG	LAG	NS	1, 2, 3, 4, 6, 8	6
Shuang <i>et al</i> ^[25]	Non-RCT	35/35	DG	LAG	L: 36.5; O: 38.5	5, 8	6
Scatizzi <i>et al</i> ^[24]	Non-RCT	30/30	DG	TLG	18	1, 2, 3, 4, 6	7
Cai <i>et al</i> ^[23]	RCT	49/47	DG, TG, PG	LAG	22.1	1, 2, 3, 4, 5, 6, 7	RCT
Huang <i>et al</i> ^[22]	Non-RCT	66/69	DG	LAG	Range: 1-19	1, 2, 3, 4, 6, 7, 8	7
Du <i>et al</i> ^[21]	Non-RCT	82/94	TG	LAG	2.5	1, 2, 3, 4, 5, 7	7
Du <i>et al</i> ^[20]	Non-RCT	78/90	DG	LAG	25.2	1, 2, 3, 4, 5, 6, 7	7
Hur <i>et al</i> ^[19]	Non-RCT	26/25	DG	LAG	29.0	1, 2, 3, 4, 5, 7, 8, 9	7

¹Matching criteria: 1 = age; 2 = sex; 3 = pathologic tumor-node-metastasis stage; 4 = type of gastrectomy; 5 = resection margin; 6 = tumor size; 7 = histologic type; 8 = reconstruction; 9 = adjuvant treatment. DG: Distal gastrectomy; L: Laparoscopic gastrectomy; LAG: Laparoscopy-assisted gastrectomy; NS: Not stated; O: Open gastrectomy; PG: Proximal gastrectomy; RCT: Randomized controlled trial; TG: Total gastrectomy; TLG: Total laparoscopic gastrectomy.

reported the following reasons for converting to open procedures: hemorrhage ($n = 2$); overlarge tumor ($n = 2$); common bile duct injury ($n = 1$); obesity ($n = 1$); techni-

cal difficulty ($n = 1$); lack of pneumoperitoneum ($n = 1$); failure of the linear stapler ($n = 1$); dense adhesion after open sigmoidectomy ($n = 1$); relatively fixed tumor ($n = 1$);

Table 2 Meta-analysis results of endpoints from all available studies

Measured outcome	Studies (<i>n</i>)	Patients (<i>n</i>)	OR, WMD, or HR	95%CI		<i>P</i>	Heterogeneity test		<i>Pr</i> > <i>z</i>
							<i>I</i> ²	<i>P</i>	
Operative outcomes									
Operative time	13	2300	57.06	41.87	72.25	< 0.00001	90%	< 0.00001	0.502
Intraoperative blood loss	11	2064	-137.87	-164.41	-111.33	< 0.00001	90%	< 0.00001	0.533
PO outcomes									
PO analgesic consumption	4	441	-1.94	-2.50	-1.38	< 0.00001	77%	0.005	0.308
Time to first ambulation	5	977	-1.03	-1.90	-0.16	0.02	97%	< 0.00001	1.000
Time to first flatus	9	1588	-0.98	-1.30	-0.66	< 0.00001	89%	< 0.00001	0.536
Time to first oral intake	6	987	-0.85	-1.67	-0.03	0.04	86%	< 0.00001	1.000
Length of PO hospital day	10	1782	-3.08	-4.38	-1.78	< 0.00001	88%	< 0.00001	0.721
Overall morbidity	13	2284	0.78	0.61	0.99	0.04	14%	0.30	0.161
Anastomotic stenosis	12	2108	0.89	0.36	2.16	0.79	0%	0.74	0.308
Anastomotic leakage	13	2284	0.74	0.36	1.50	0.40	0%	0.80	1.000
Duodenal stump leakage	13	2284	1.12	0.42	3.01	0.82	0%	0.83	1.000
Pancreatic fistula/ pancreatitis	13	2284	0.75	0.37	1.52	0.42	0%	0.91	0.308
Intra-abdominal bleeding	13	2284	0.99	0.41	2.38	0.98	0%	0.83	1.000
Ileus	12	2108	0.56	0.21	1.46	0.23	0%	0.73	1.000
Wound problems	13	2284	0.56	0.34	0.93	0.03	0%	0.66	0.152
Pneumonia	13	2284	0.38	0.21	0.71	0.002	17%	0.29	1.000
Reoperation	7	1289	1.58	0.58	4.31	0.37	0%	0.63	1.000
Mortality	13	2284	0.69	0.21	2.26	0.54	0%	0.64	-
Oncologic outcomes									
Lymph nodes harvested (<i>n</i>)	13	2526	-0.11	-2.72	2.50	0.94	95%	< 0.00001	0.537
Tumor recurrence/metastasis	8	1587	0.79	0.60	1.04	0.09	20%	0.27	0.035
Local/lymphatic recurrence	5	853	0.79	0.46	1.34	0.38	0%	0.41	0.296
Peritoneal recurrence	5	853	1.20	0.70	2.07	0.50	0%	0.50	0.296
Distant metastasis	5	853	0.67	0.42	1.07	0.09	45%	0.12	0.089
Three-year DFS	4	703	1.02	0.64	1.61	0.94	0%	0.88	-
Three-year overall survival	8	1363	0.87	0.59	1.27	0.46	0%	0.99	-
Five-year DFS	3	652	1.02	0.66	1.57	0.92	0%	0.67	-
Five-year overall survival	3	652	0.79	0.46	1.34	0.38	0%	0.90	-

DFS: Disease-free survival; HR: Hazard ratio; OR: Odds ratio; PO: Postoperative; WMD: Weighted mean difference.

small incision metastasis (*n* = 1).**Meta-analyses of postoperative outcomes**

Analgesic administration was reported by only four articles included in this study^[21,22,24,25]. Meta-analysis revealed a significantly lower frequency of analgesic administration in the LGD2 group than in the OGD2 group (WMD = -1.94, 95%CI: -2.50--1.38; *P* < 0.01), with significant heterogeneity among studies (*I*² = 77%; *P* < 0.01) (Table 2, Figure 3A).

The time to first ambulation was reported in five papers^[21,23,24,27,32]. This time was significantly shorter in the LGD2 group than in the OGD2 group (WMD = -1.03 d, 95%CI: -1.90--0.16; *P* < 0.05), with significant heterogeneity among studies (*I*² = 97%; *P* < 0.01) (Figure 3B).

The time to first flatus was reported in nine articles^[19-24,27,30,31]. The time was significantly shorter in the LGD2 group than in the OGD2 group (WMD = -0.98 d, 95%CI: -1.30--0.66; *P* < 0.01), with significant heterogeneity among studies (*I*² = 89%; *P* < 0.01) (Figure 3C).

The time to first oral intake was reported in six papers^[19-22-24,27,32]. Meta-analysis demonstrated this time was significantly shorter in the LGD2 group than in the OGD2 group (WMD = -0.85 d, 95%CI: -1.67--0.03; *P* < 0.05), with significant heterogeneity among studies (*I*² =

86%; *P* < 0.01) (Figure 3D).

The length of postoperative hospitalization was reported in 10 articles^[19,20,22-25,27,28,30,32]. The LGD2 group had significantly shorter postoperative hospitalization than the OGD2 group (WMD = -3.08 d, 95%CI: -4.38--1.78; *P* < 0.01). There was a significant heterogeneity among studies (*I*² = 88%; *P* < 0.01) (Figure 4A).

The postoperative morbidity rates were reported in 13 studies^[19-25,27-32]. Meta-analysis demonstrated a significantly lower overall postoperative morbidity after LGD2 than after OGD2 (OR = 0.78, 95%CI: 0.61-0.99; *P* < 0.05), with no significant heterogeneity among studies (*I*² = 14%) (Figure 4B).

The subgroup analyses showed significantly lower incidence rates of wound problems (wound infection and dehiscence) and pneumonia in the LGD2 group. No difference in the incidence rate of major surgical site complications, such as anastomotic stenosis, anastomotic leakage, duodenal stump leakage, pancreatic fistula or pancreatitis, and intra-abdominal bleeding, was found between the two groups (Table 2). Subgroup analyses demonstrated no significant differences between groups in major surgical site complications with regard to surgical extensions (distal gastrectomy/proximal gastrectomy/total gastrectomy). This includes anastomotic stenosis,

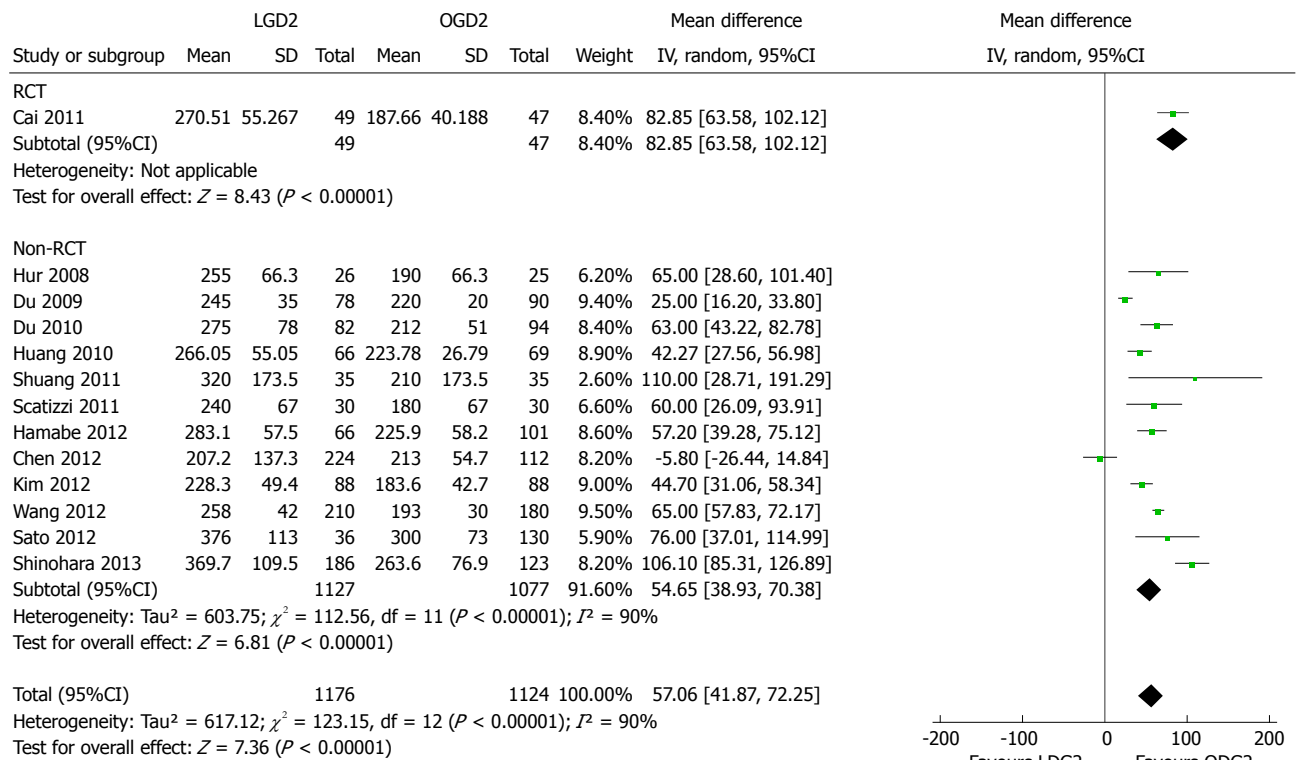
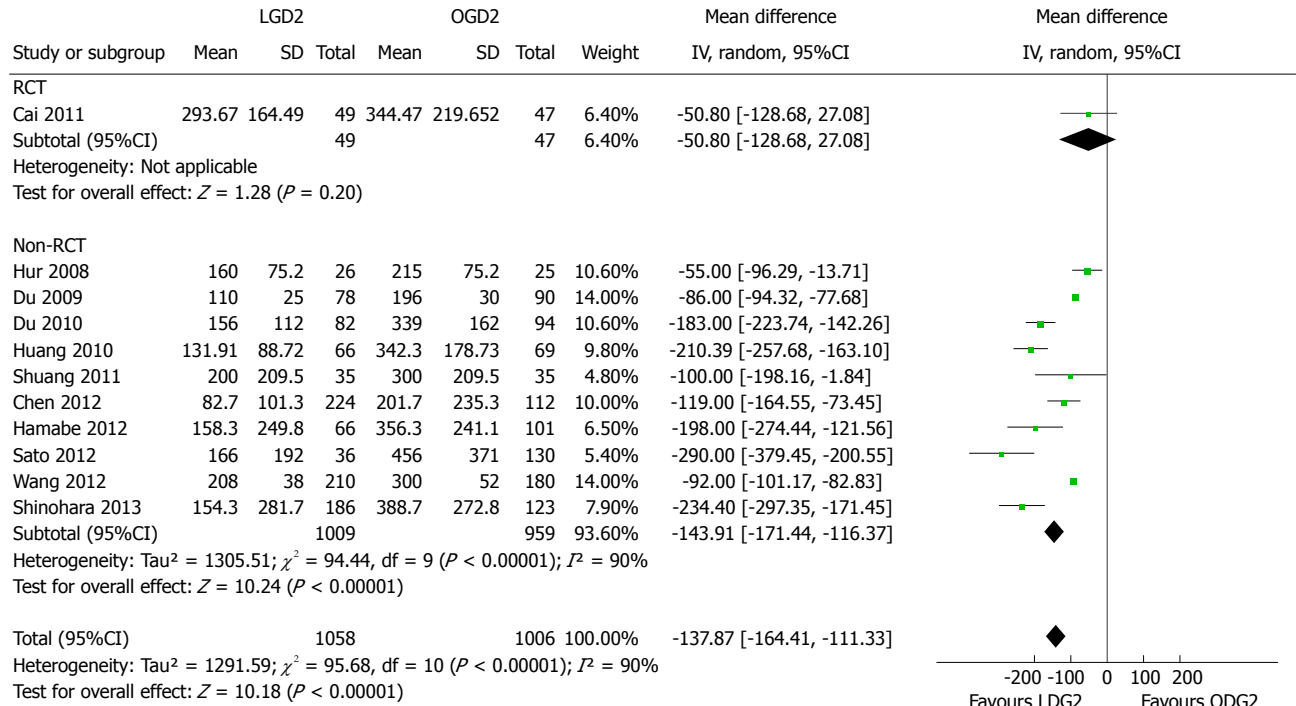
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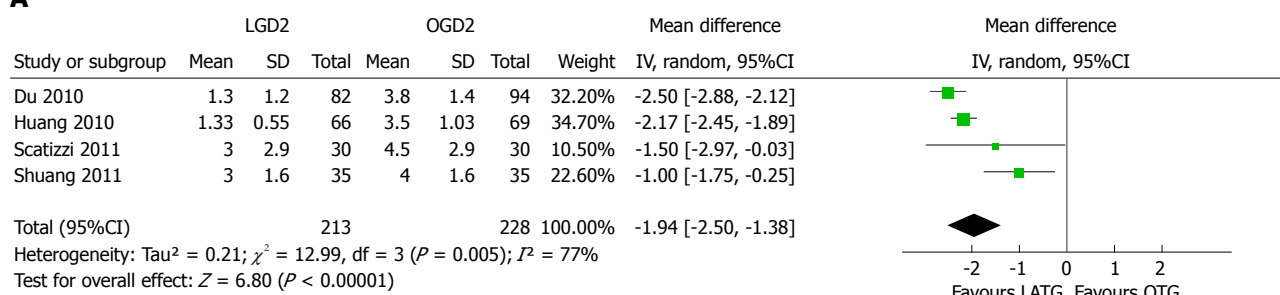
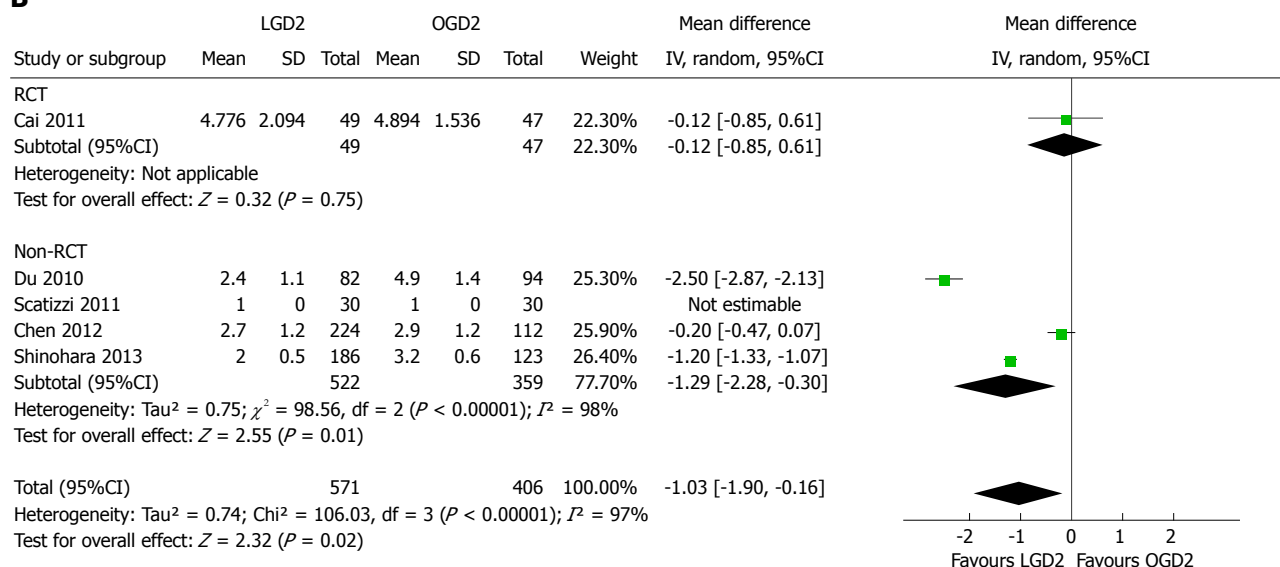
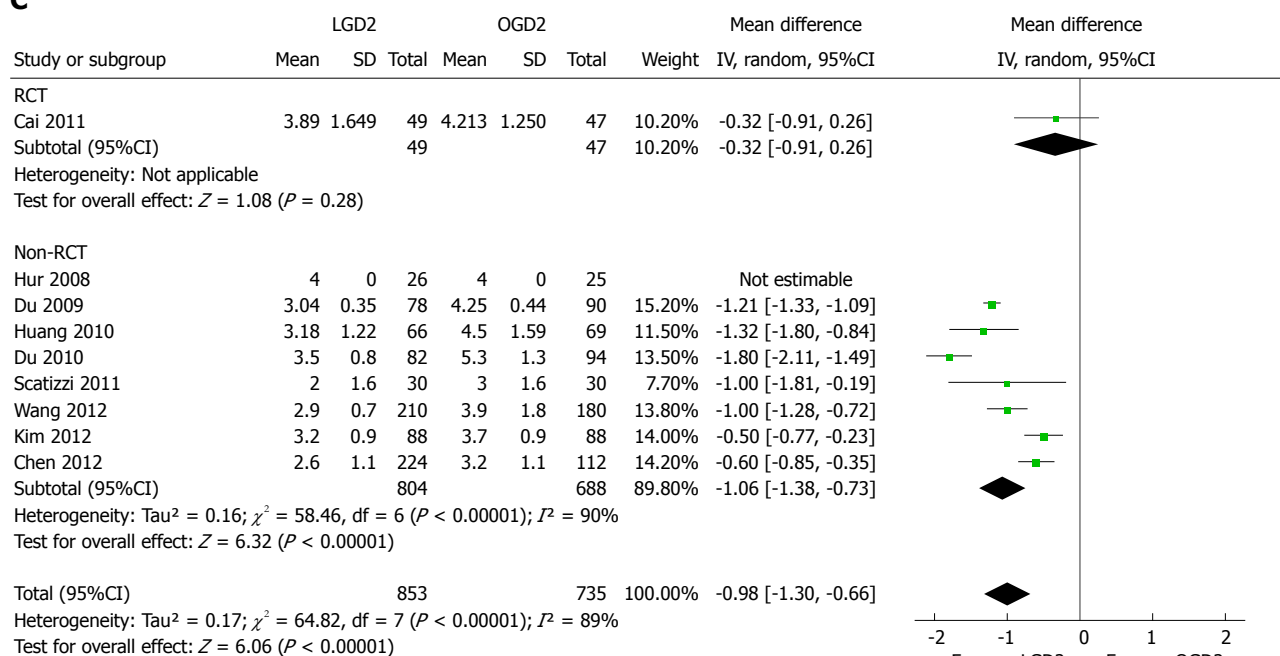
Figure 2 Meta-analyses of procedure characteristics. A: Weighted mean operative time; B: Intraoperative blood loss. LGD2: Laparoscopic gastrectomy with D2 extended lymph node dissection; OGD2: Open gastrectomy with D2 extended lymph node dissection; RCT: Randomized controlled trial.

anastomotic leakage, duodenal stump leakage, pancreatic fistula or pancreatitis, and intra-abdominal bleeding.

The reoperation incidence rate was reported in seven articles^[19,20,22,24,30-32]. No significant difference in this parameter was found between the LGD2 and OGD2

groups (OR = 1.58, 95%CI: 0.58-4.31) with no significant heterogeneity among studies ($I^2 = 0\%$) (Figure 4C).

The postoperative mortality rates were reported in 13 studies^[19-25,27-32] with no significant difference in the rate between the LGD2 and OGD2 groups (OR = 0.69,

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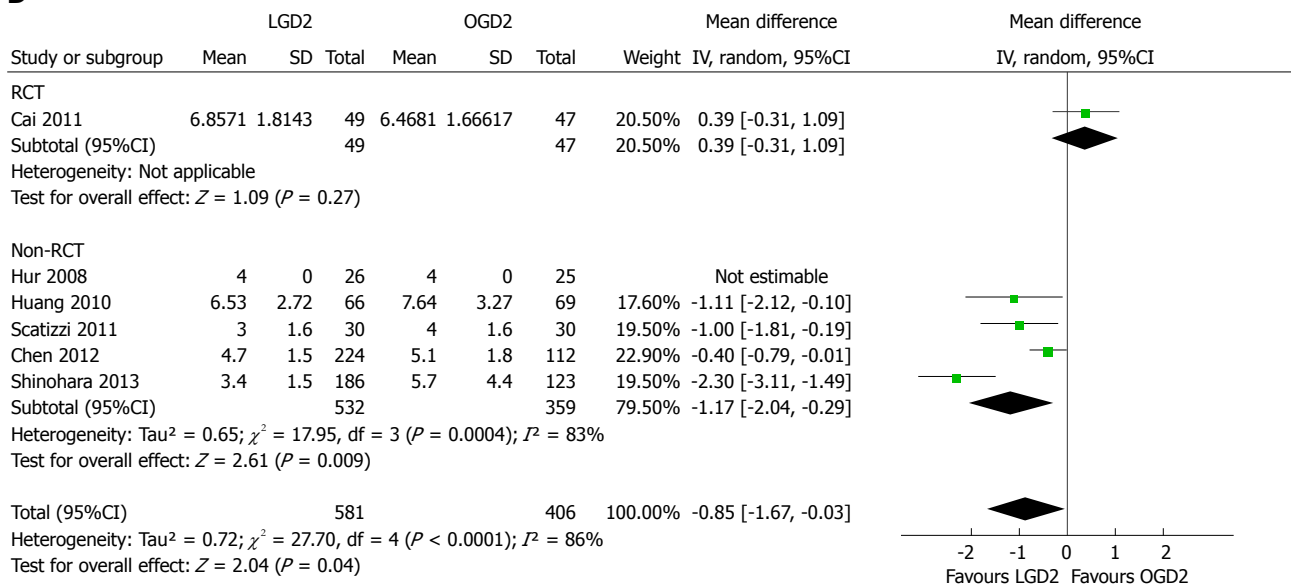
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Figure 3 Meta-analyses of patient characteristics. A: Analgesic consumption; B: Time to first ambulation; C: Time to first flatus; D: Time to first oral intake. LGD2: Laparoscopic gastrectomy with D2 extended lymph node dissection; OGD2: Open gastrectomy with D2 extended lymph node dissection; RCT: Randomized controlled trial.

95%CI: 0.21-2.26) with no significant heterogeneity among studies ($I^2 = 0\%$) (Figure 4D).

Meta-analyses of oncologic outcomes

The number of lymph nodes harvested was reported in 13 studies^[19-24,26-32]. Although meta-analysis showed no significant difference in this parameter between the two groups (WMD = -0.11, 95%CI: -2.72-2.50), there was significant heterogeneity among the studies ($I^2 = 95\%$; $P < 0.01$) (Table 2, Figure 5A).

Tumor recurrence and metastasis were recorded in eight studies^[19-21,28-32]. The meta-analysis showed no statistical difference between the LGD2 and OGD2 groups (OR = 0.79, 95%CI: 0.60-1.04), as well as no significant heterogeneity among studies ($I^2 = 20\%$) (Figure 5B). Subgroup analyses showed no significant difference in recurrence and metastasis patterns between the groups (Table 2).

Four trials involving 703 patients provided three-year disease-free survival rates^[19,28,31,32]. Three trials involving 652 patients provided five-year disease-free survival rates^[28,31,32]. The two groups showed no significant difference in three-year (HR = 1.02, 95%CI: 0.64-1.61) (Figure 6A) or five-year (HR = 1.02, 95%CI: 0.66-1.57) (Figure 6B) disease-free survival rates (Figure 6). There was no significant heterogeneity among studies ($I^2 = 0\%$ for both rates) (Table 2).

Eight trials involving 1363 patients provided three-year overall survival rates^[19,23-25,27,28,31,32], and three trials involving 652 patients provided five-year overall survival rates^[28,31,32]. The two groups showed no significant differences in three-year (HR = 0.87, 95%CI: 0.59-1.27) (Figure 6C) or five-year (HR = 0.79, 95%CI: 0.46-1.34) (Figure 6D) overall survival rates, accompanied with no

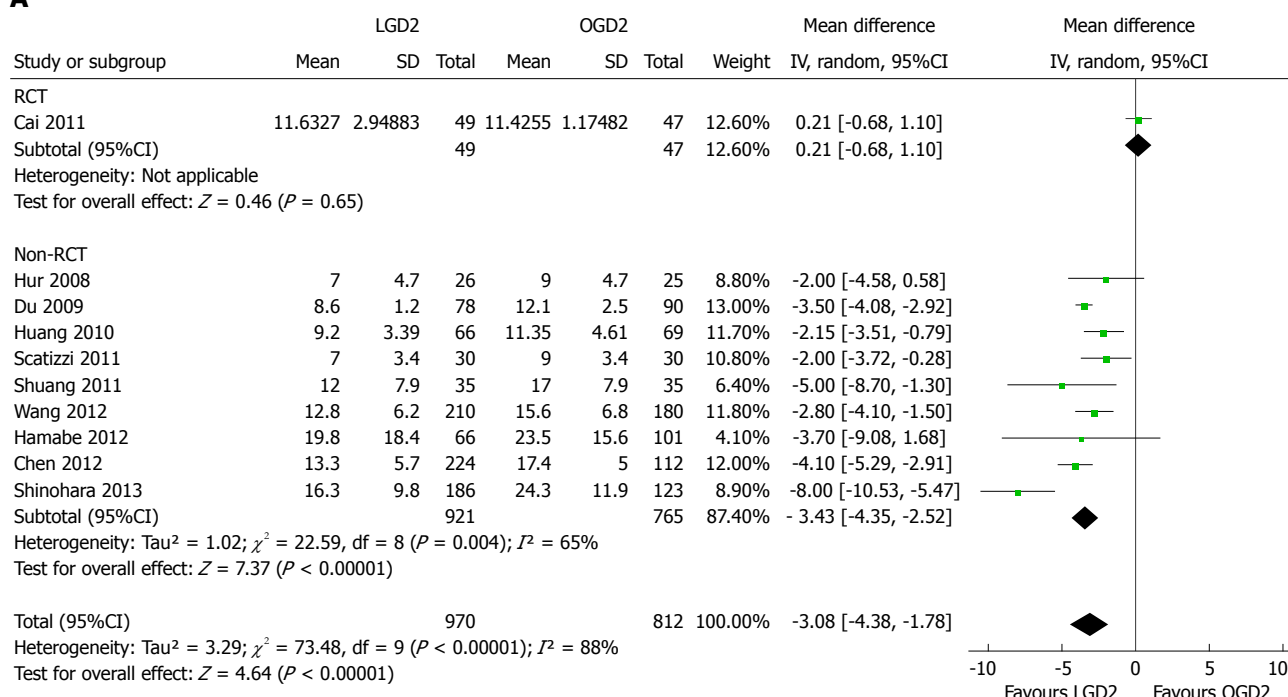
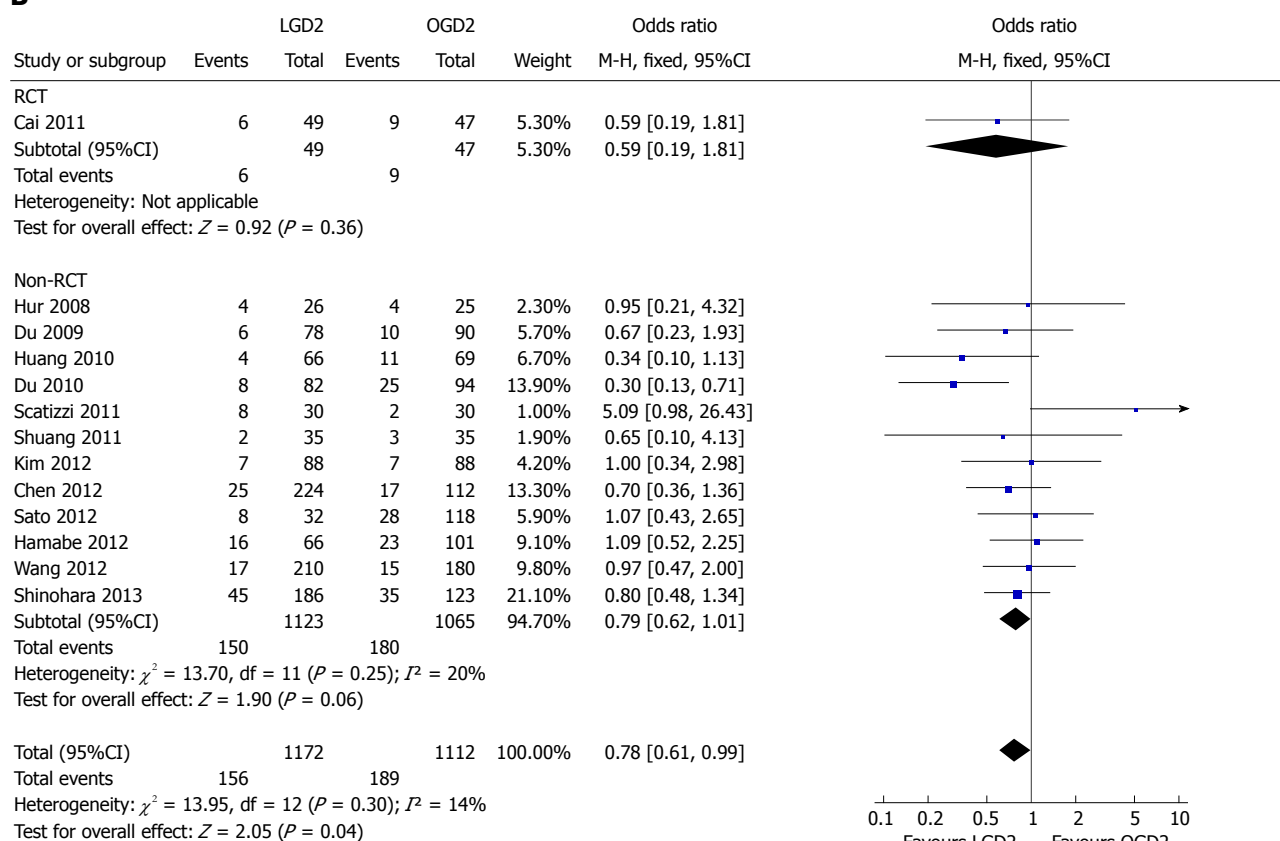
significant heterogeneity among studies ($I^2 = 0\%$ for both). Among the studies included, only Shinohara *et al.*^[32] presented calculated disease-free and overall survival rates after LGD2 and OGD2 with regard to tumor stage, with no significant differences observed between the two groups.

Sensitivity analysis and publication bias

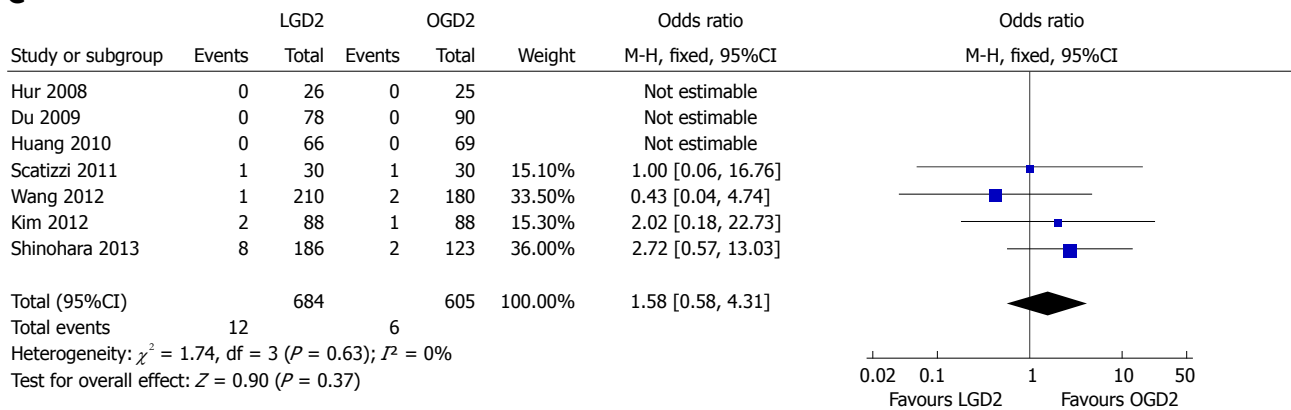
Study results were reanalyzed using alternative (random or fixed effects) models showing no significant difference in pooled effects, except comparable incidences of overall morbidity in the two groups (OR = 0.78, 95%CI: 0.59-1.02). Furthermore, the studies showed no significant heterogeneity ($I^2 = 14\%$). Endpoint analysis revealed no strong evidence of bias (Begg's rank correlation test, continuity-corrected $Pr > |\hat{z}| > 0.1$), except for tumor recurrence/metastasis ($Pr > |\hat{z}| = 0.035$) and distant metastasis ($Pr > |\hat{z}| = 0.089$).

DISCUSSION

This meta-analysis examined whether LGD2 is an acceptable alternative to OGD2 for AGC from a clinical perspective. The results suggest that despite LGD2 being a technically demanding and time-consuming procedure with longer operative times and acceptable conversion rates, it can be used to achieve long-term prognoses. Comparison between LGD2 and OGD2 showed similar numbers of harvested lymph nodes, tumor recurrence and metastasis rates, and disease-free and overall survival rates. Furthermore, LGD2 provides better short-term prognoses with lower postoperative pain, faster recovery, and shorter hospital stays. There was a lower postoperative morbidity associated with LGD2, which may have

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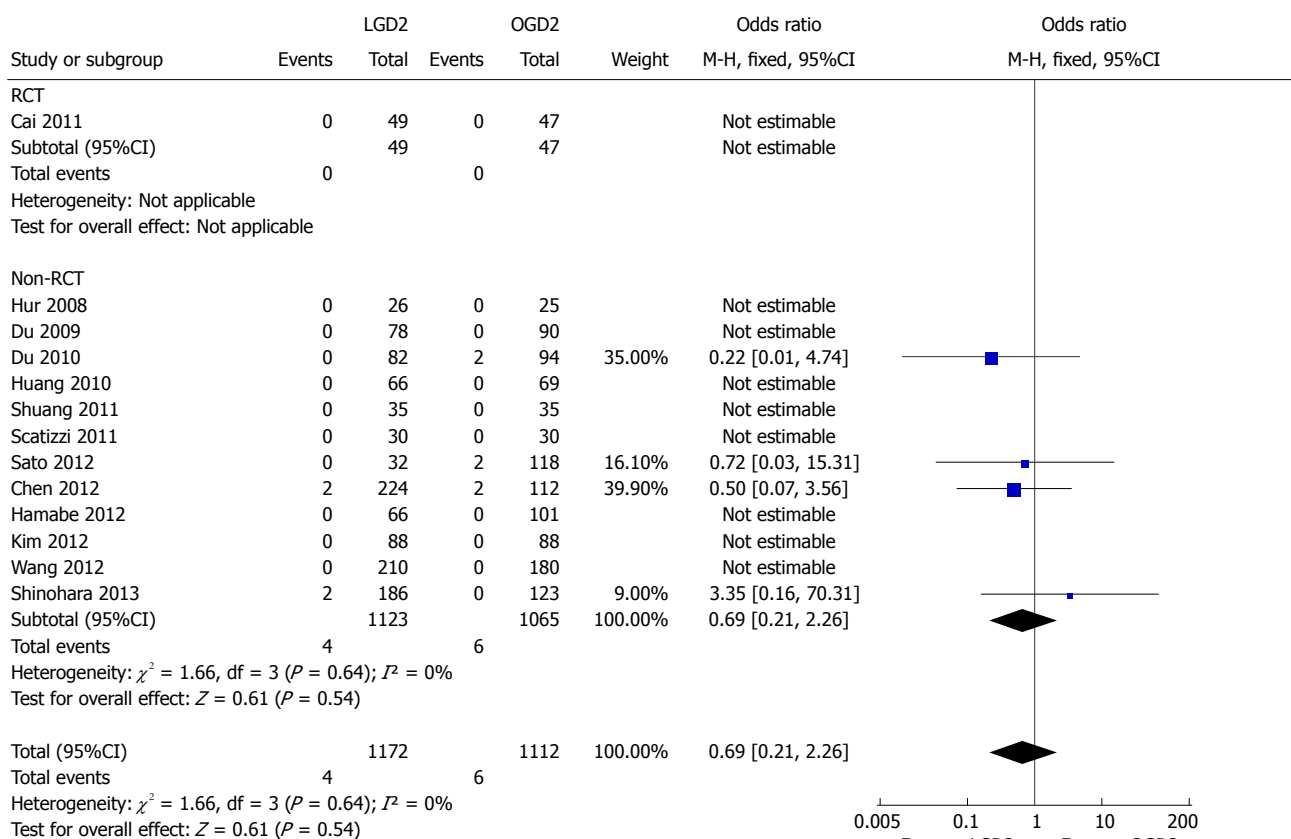


Figure 4 Meta-analyses of postoperative events. A: Postoperative hospitalization; B: Morbidity; C: Reoperation; D: Mortality. LGD2: Laparoscopic gastrectomy with D2 extended lymph node dissection; OGD2: Open gastrectomy with D2 extended lymph node dissection; RCT: Randomized controlled trial.

been due to the minimal invasiveness, reduced postoperative pain, earlier ambulation, and fewer pulmonary complications associated with the LGD2 procedure, though some comparable major surgical-site complications and postoperative mortality remained. Hence, LGD2 may provide better short-term prognoses than OGD2.

The results of the present study suggest equivalent long-term oncologic results can be obtained with LGD2 as with an open radical surgery. This finding mainly reflects similar pathologic tumor-node-metastasis stages in the two groups and the prioritization of and adherence to oncologic principles, such as *en bloc* resection, the no-

touch technique, and systemic lymphadenectomy^[22]. However, there are still challenges associated with the LGD2 procedure, including a learning curve for training and the mastery of essential techniques of distal LG with systemic lymphadenectomy for treating major EGC, which requires experience from 60-90 cases^[47]. Thus, LGD2 is not recommended in small-volume centers.

The present meta-analysis has several limitations. First, all but one of the included studies were observational. Second, most of the included studies were conducted at tertiary centers and major institutions in East Asia (eight in China, three in Japan, two in South Korea,

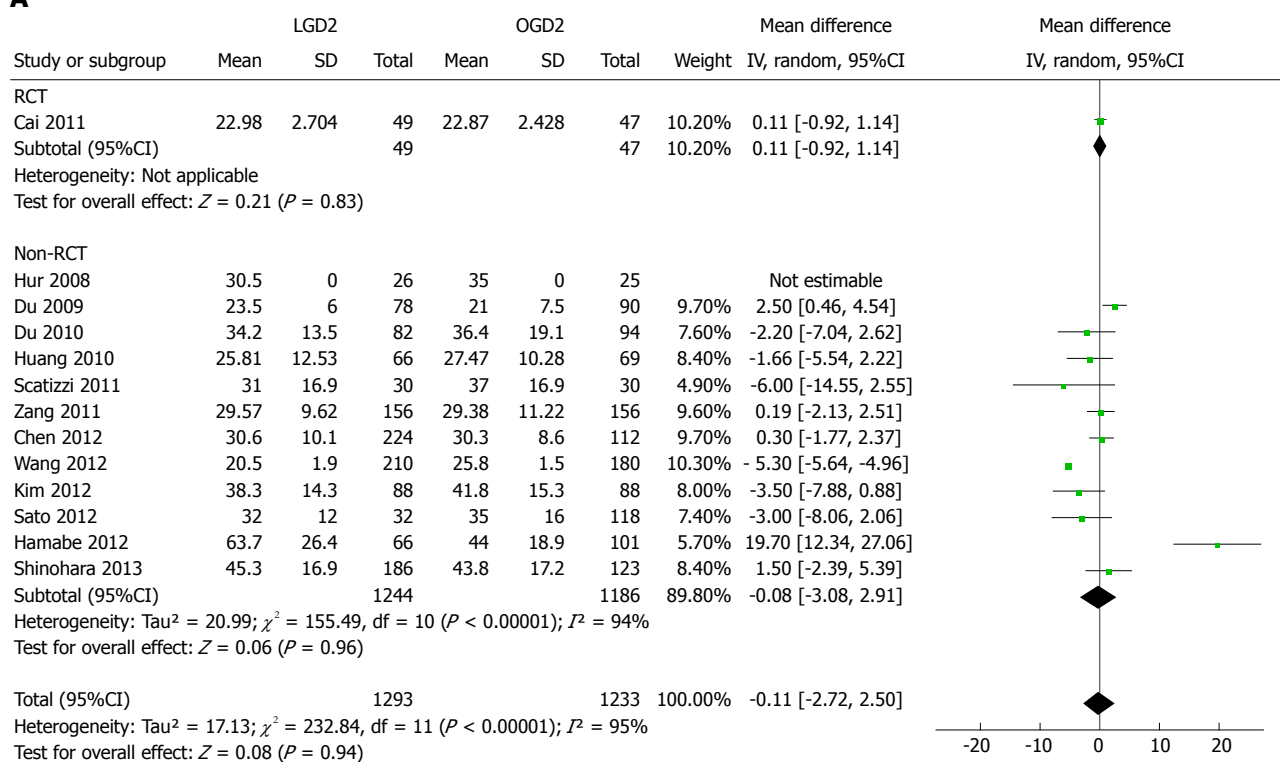
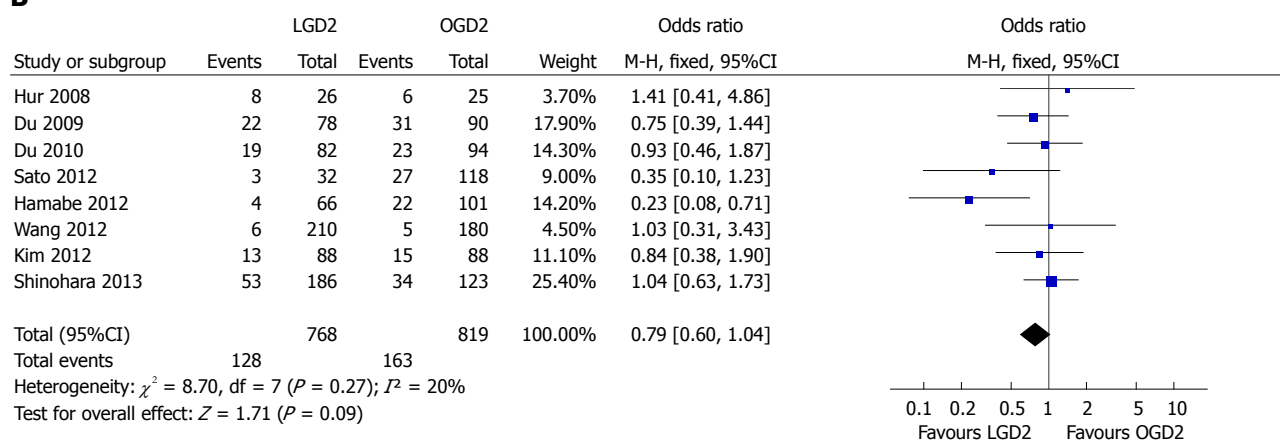
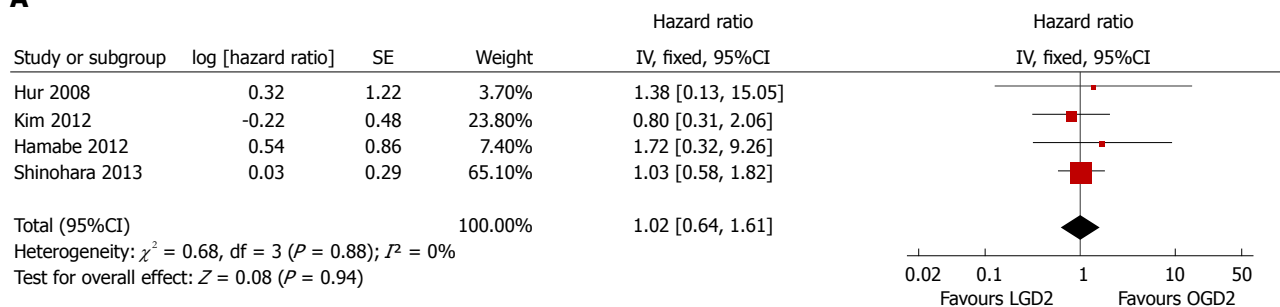
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Figure 5 Meta-analyses of lymph node harvest and tumor recurrence. A: Lymph nodes harvested; B: Tumor recurrence and metastasis. LGD2: Laparoscopic gastrectomy with D2 extended lymph node dissection; OGD2: Open gastrectomy with D2 extended lymph node dissection; RCT: Randomized controlled trial.

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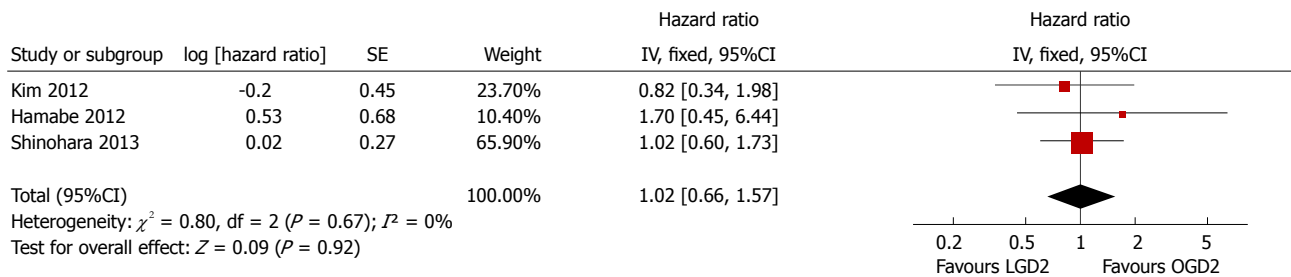
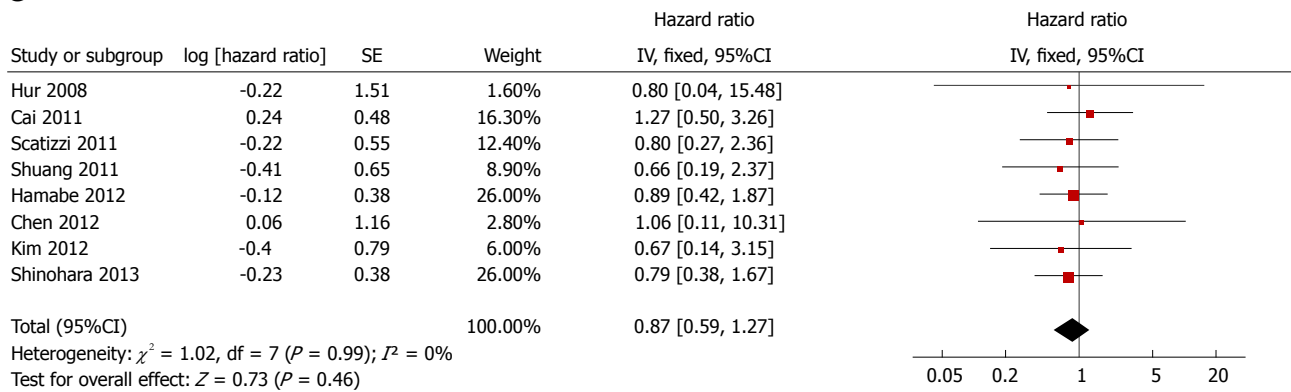
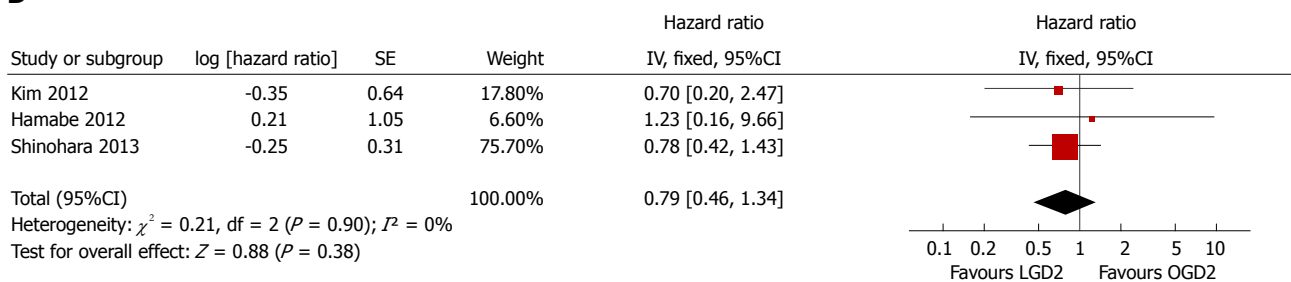
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Figure 6 Meta-analyses of treatment outcomes between laparoscopic and open D2 gastrectomy. A: Three-year disease-free survival; B: Five-year disease-free survival; C: Three-year overall survival; D: Five-year overall survival. LGD2: Laparoscopic gastrectomy with D2 extended lymph node dissection; OGD2: Open gastrectomy with D2 extended lymph node dissection.

Table 3 Ongoing randomized controlled trials comparing laparoscopic and open D2 gastrectomy for advanced gastric cancer

Contact	Country	Sample size	Type of cancer	Start date	Completion date
Li <i>et al</i> ^[48]	China	1056	Locally AGC	2012/9/1	2018/6/1
Shi <i>et al</i> ^[49]	China	328	Locally AGC	2010/2/1	2015/2/1
Huang <i>et al</i> ^[50]	China	111	AGC	2011/11/1	Not stated
Han <i>et al</i> ^[51]	South Korea	1050	Locally AGC	2011/10/1	2016/9/1
Kim <i>et al</i> ^[52]	South Korea	204	Locally AGC	2010/6/1	2016/12/1
Kim <i>et al</i> ^[53]	South Korea	124	Locally AGC	2008/8/1	2013/7/1
Tsuyoshi <i>et al</i> ^[54]	Japan	500	Locally AGC	2009/11/1	Not stated

and one in Italy). Hence, the included patients might not reflect general patient populations. Furthermore, any application of the conclusions to Western patients should be performed cautiously. Third, because > 95% of patients had locally AGC with stages ranging from I B to III, the conclusions should be applied only to similar cases. Fourth, the studies showed significant heterogeneity in operative time, intraoperative blood loss, postoperative

analgesic consumption, time to first ambulation, time to first flatus, time to first oral intake, length of postoperative hospital stay, and number of lymph nodes harvested. Differences in study design, sample size, adjuvant treatment, and other factors might explain this heterogeneity. Additionally, calculations using the random effects model yielded more conservative estimates of statistical significance. Finally, this meta-analysis was performed at the

study level and did not address or incorporate individual factors at the patient level.

In conclusion, although LGD2 is a technically demanding and time-consuming procedure, the results of this meta-analysis suggest it may be an acceptable alternative to OGD2 for locally AGC. The procedure may yield comparable oncologic results and better short-term prognoses than OGD2. However, additional clinical trials are needed for further evaluation of this procedure. We identified seven ongoing RCTs comparing the use of LGD2 and OGD2 to treat AGC in East Asia (three in China, three in South Korea, and one in Japan) (Table 3)^[48-54]. The results of these trials will help researchers address this question in the future.

COMMENTS

Background

Laparoscopic gastrectomy is gaining popularity worldwide as a minimally invasive alternative treatment to traditional open surgery in treating gastric cancer. The Japanese Gastric Cancer Association guidelines stipulate that D2 gastrectomy is required to cure advanced gastric cancer. However, the application of laparoscopic D2 gastrectomy (LGD2) remains questionable due to its technical difficulty and the lack of long-term results.

Research frontiers

The authors performed a meta-analysis comparing LGD2 with open D2 gastrectomy (OGD2) in patients with advanced gastric cancer, evaluating endpoints of operative, postoperative, and oncological outcomes.

Innovations and breakthroughs

Compared with OGD2, LGD2 is a safer and more effective method, with lower overall morbidity, enhanced postoperative recovery, and comparable oncologic outcomes.

Applications

LGD2 is safe and effective, and offers some advantages over OGD2 in treatment of locally advanced gastric cancer. However, well-designed, prospective, multicenter, randomized controlled trials comparing LGD2 with OGD2 for treatment of advanced gastric cancer are warranted before recommending LGD2 for wider use in surgical practice.

Peer review

The paper is a well-organized and structured meta-analysis of currently available data on the benefits of using LGD2 over OGD2 for the treatment of advanced gastric cancer. The authors concluded that LGD2 is safe and effective in treating locally advanced gastric cancer. This meta-analysis is well written and an important addition of knowledge to successful treatment of locally advanced gastric cancer.

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Xeroderma pigmentosum group D polymorphisms and esophageal cancer susceptibility: A meta-analysis based on case-control studies

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Abstract

AIM: To clarify the effects of the xeroderma pigmentosum group D (XPD) Asp312Asn and Lys751Gln gene polymorphisms on the risk of esophageal cancer (EC).

METHODS: A computerised literature search was conducted to identify the relevant studies from the PUBMED and EMBASE databases, reviews, and reference lists of relevant articles. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to assess the associations between the XPD Asp312Asn and/or Lys751Gln polymorphisms and EC susceptibility. Statistical analyses were performed using the software Stata 12.0. A fixed or random effects model was selected based on a heterogeneity test. Publication bias was estimated using funnel plots and Egger's linear regression method. Subgroup analyses were performed based on histological type and ethnicity.

RESULTS: Thirteen case-control studies with a total of 10 comparisons for the Asp312Asn polymorphism, including 2373 cases and 3175 controls, and 15 comparisons for the Lys751Gln polymorphism, including 3226 cases and 5237 controls, were recruited for the meta-analysis. In terms of the XPD Asp312Asn polymorphism, significantly increased EC risks were identified in the Asp/Asn vs Asp/Asp comparison (OR = 1.17, 95%CI: 1.02-1.33, $P = 0.03$) and in the dominant-model comparison (Asn/Asn+Asp/Asn vs Asp/Asp: OR = 1.18, 95%CI: 1.04-1.34, $P = 0.01$). However, no significant associations were found in the Asn/Asn vs Asp/Asp comparison (OR = 1.30, 95%CI: 1.00-1.70, $P = 0.05$) or in the recessive-model comparison (Asn/Asn vs Asp/Asn + Asp/Asp: OR = 1.17, 95%CI: 0.91-1.50, $P = 0.22$). In terms of the XPD Lys751Gln polymorphism, a significant association with EC susceptibility was found under the recessive model (Gln/Gln vs Lys/Gln+Lys/Lys: OR = 1.21, 95%CI: 1.02-1.43, $P = 0.03$). However, no associations were identified in the other comparisons (co-dominant model: Lys/Gln vs Lys/Lys: OR = 1.11, 95%CI: 0.94-1.31, $P = 0.20$; Gln/Gln vs Lys/Lys: OR = 1.31, 95%CI: 0.98-1.75, $P = 0.07$; dominant model: OR = 1.14, 95%CI: 0.96-1.35, $P = 0.14$).

CONCLUSION: The results of this meta-analysis suggest that the XPD Asp312Asn and Lys751Gln gene polymorphisms are associated with a significantly increased risk for EC.

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Key words: Esophageal cancer; Xeroderma pigmentosum group D; Polymorphism; Meta-analysis

Core tip: To clarify the effects of xeroderma pigmentosum group D (XPD) gene polymorphisms on the risk of esophageal cancer (EC), we performed a meta-analysis of all of the case-control studies that evaluated the association between the genetic polymorphisms of

XPD (Asp312Asn and Lys751Gln) and EC susceptibility. Thirteen case-control studies were recruited in the meta-analysis. For the XPD Asp312Asn polymorphism, significantly increased EC risks were found in the Asp/Asn *vs* Asp/Asp comparison and in the dominant model comparison. For the XPD Lys751Gln polymorphism, a significant association between the XPD Lys751Gln polymorphism and EC susceptibility was found under the recessive model.

Yang R, Zhang C, Malik A, Shen ZD, Hu J, Wu YH. Xeroderma pigmentosum group D polymorphisms and esophageal cancer susceptibility: A meta-analysis based on case-control studies. *World J Gastroenterol* 2014; 20(44): 16765-16773 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16765.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16765>

INTRODUCTION

Esophageal cancer (EC) is the sixth most frequently diagnosed cancer and the fifth most common cause of cancer death among males^[1]. The major risk factors for EC are not well understood but are thought to include smoking, excessive alcohol consumption, poor nutritional status, low intake of fruits and vegetables, *etc.*^[2-5]. Several studies have suggested that the genes involved in the DNA repair system play a crucial role in protecting against mutations, while a decreased DNA repair capacity is viewed as a crucial event in carcinogenesis^[6]. The xeroderma pigmentosum group D (XPD) enzyme, an evolutionarily conserved ATP-dependent helicase, plays an important role in the repair of bulky DNA adducts, such as pyrimidine dimers, photoproducts and cross-links^[7,8]. Mutations at different sites in the XPD gene can give rise to repair and transcription defects, and altered DNA repair capacity can render a higher risk of developing different types of cancer^[9-11]. Several single nucleotide polymorphisms (SNPs) have been identified in the XPD gene. Among them, Asp312Asn (rs1799793 G>A) and Lys751Gln (rs13181 T>G) are commonly identified and result in amino acid changes.

Currently, there are many molecular epidemiological studies exploring the associations between the genetic polymorphisms of XPD, particularly Asp312Asn and Lys751Gln, and EC susceptibility, but the results remain controversial rather than conclusive. To address the inconsistencies in the findings of these studies, we performed a meta-analysis, based on published case-control studies, to derive a more precise estimation of the association between these two XPD polymorphisms and EC susceptibility.

MATERIALS AND METHODS

Search strategy

We systematically searched PubMed, Embase, previous reviews and the reference lists from identified articles

published up to January 1, 2014 for studies related to EC and genetic polymorphisms^[12,13]. We used the following search terms: “ERCC2” or “XPD” or “xeroderma pigmentosum group D” or “excision repair cross-complementing group 2” or “DNA repair gene”, “polymorphism” or “variant”, “esophageal” or “esophagus”, and “cancer” or “carcinoma” or “squamous cell” or “adenocarcinoma”, of which the exploration was limited to human studies. No language restrictions were imposed, and all of the eligible studies were examined carefully, and their references were checked for other relevant publications. All of the literature findings were independently reviewed by two professional co-workers (Yang R. and Wu Y.) to identify the studies that met the following criteria: (1) case-control study design; (2) evaluating the associations between XPD polymorphisms (Asp312Asn and/or Lys751Gln) and EC susceptibility; and (3) reporting the odds ratio (OR) and the corresponding 95% confidence intervals (CIs), or the size of the sample. Any differences were resolved by consensus. The major excluding criteria included the following: (1) not a case-control study; (2) review publications; or (3) overlapping data.

Data extraction

We used a standardised data extraction method to extract the data from the included papers^[14]. Information was collected from each article, including the first author, year of publication, country, journal, racial descent of the study population, demographics, number of cases and controls for each genotype, genotyping method, histological type and confirmation of diagnosis. While the allele frequencies were not given, they were calculated from the corresponding genotype frequencies of the case and control groups.

Statistical analysis

The ORs were employed to evaluate the associations between the XPD Asp312Asn and/or Lys751Gln polymorphisms and EC susceptibility^[15]. For Asp312Asn, the pooled ORs were calculated for a co-dominant model (Asp/Asn *vs* Asp/Asp, Asn/Asn *vs* Asp/Asp), a dominant model (Asn/Asn+Asp/Asn *vs* Asp/Asp), a recessive model (Asn/Asn *vs* Asp/Asn+Asp/Asp) and an additive model [(2Asn/Asn+Asp/Asn) *vs* 2(Asp/Asn+Asn/Asn+Asp/Asp)]. We evaluated the risks of the same four models for the Lys751Gln genotype as well. The χ^2 goodness-of-fit test was used to evaluate whether the genotypes among the control subjects conformed to the Hardy-Weinberg equilibrium (HWE). We applied two models of meta-analysis for any dichotomous outcomes according to the results of heterogeneity tests among the individual studies, using the software Stata 12.0 (Stata Corp., College Station, TX, United States): the fixed-effects model (the Mantel-Haenszel method) and the random-effects model (the DerSimonian and Laird method)^[15]. Subgroup analyses were performed based on histological type and ethnicity. The publication bias was investigated with a funnel plot, in which the standard error (SE) of log (OR) for each study was plotted against

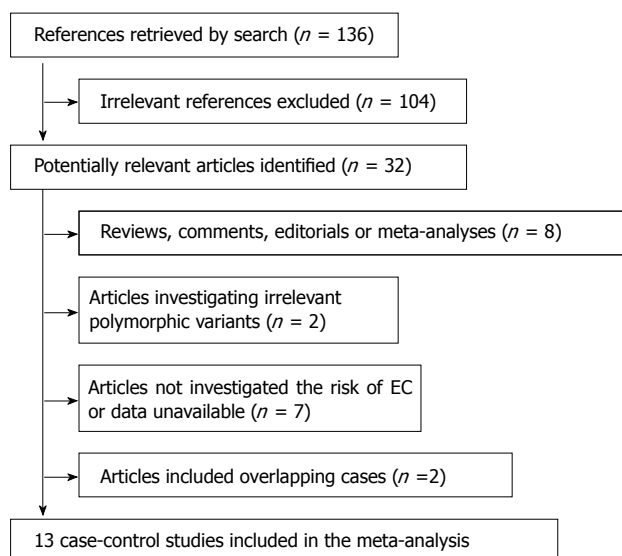


Figure 1 Flow diagram of the study selection process.

the respective log (OR). The funnel plot asymmetry was assessed using Egger's linear regression method^[15]. The significance of the intercept was determined by the *t*-test, and $P < 0.05$ was considered statistically significant^[16]. All of the statistical tests were performed using Stata version 12.0. All of the *P*-values were two-sided.

RESULTS

Eligible studies

A total of 136 articles were identified by the combined database search (PubMed and Embase) and manual approach (searching the previous studies cited in previous reviews and use of the reference lists from identified articles) of case-control studies, of which 15 case-control studies satisfied the inclusion criteria. After reading the full texts, two studies by Liu *et al.*^[17] and Huang *et al.*^[18] were excluded because the subjects had also been included in the studies by Tse *et al.*^[19] or Huang *et al.*^[20]. Therefore, 13 case-control studies were eventually included in the meta-analysis^[19-31]. Figure 1 presents a flowchart of the retrieved and excluded studies with a specification of reasons. Table 1 shows the characteristics of the included studies. Overall, of the 13 included studies, a total of 10 comparisons, including 2373 cases and 3175 controls, for the Asp312Asn polymorphism, and 15 comparisons, including 3226 cases and 5237 controls, for the Lys751Gln polymorphism were reviewed. The distribution of genotypes in the controls of all of the included studies was in accordance with the HWE.

Meta-analysis

XPD Asp312Asn: Table 2 indicates the associations between the XPD Asp312Asn polymorphism and EC susceptibility. Significantly increased risks were found in the Asp/Asn *vs* Asp/Asp comparison (OR = 1.17, 95%CI: 1.02-1.33, $P = 0.03$, Table 2) and in the dominant model comparison (Asn/Asn + Asp/Asn *vs* Asp/Asp: OR =

1.18, 95%CI: 1.04-1.34, $P = 0.01$, Figure 2, Table 2). However, no significant associations were found in the Asn/Asn *vs* Asp/Asp comparison (OR = 1.30, 95%CI: 1.00-1.70, $P = 0.05$, Table 2) or in the recessive model comparison (Asn/Asn *vs* Asp/Asn + Asp/Asp: OR = 1.17, 95%CI: 0.91-1.50, $P = 0.22$, Table 2). In the subgroup analysis according to cancer type [esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma (EADC)], significant associations between the XPD Asp312Asn polymorphism and EC susceptibility were detected in the EADC subgroup in the co-dominant model (Asp/Asn *vs* Asp/Asp: OR = 1.26, 95%CI: 1.03-1.53, $P = 0.02$; Asn/Asn *vs* Asp/Asp: OR = 1.40, 95%CI: 1.04-1.89, $P = 0.03$, Table 2) and the dominant model (OR = 1.29, 95%CI: 1.07-1.55, $P = 0.01$, Figure 2, Table 2). Further analysis by ethnicity revealed significant associations of the XPD Asp312Asn polymorphism with EC susceptibility in non-Chinese populations in the Asp/Asn *vs* Asp/Asp comparison (OR = 1.23, 95%CI: 1.03-1.47, $P = 0.02$, Table 2) and in the dominant model comparison (OR = 1.24, 95%CI: 1.05-1.47, $P = 0.01$, Table 2, Figure 3), but the same associations were not seen in Chinese populations. Finally, for the additive model (Table 2), individuals carrying the 312Asn allele were not significantly associated with an increased risk for EC (OR = 1.10, 95%CI: 1.00-1.21, $P = 0.06$).

XPD Lys751Gln: Table 3 lists the overall results of the meta-analysis for the associations between the XPD Lys751Gln polymorphism and EC susceptibility. There was a significant association with EC susceptibility for the recessive model comparison (Gln/Gln *vs* Lys/Gln + Lys/Lys: OR = 1.21, 95%CI: 1.02-1.43, $P = 0.03$, Figure 4, Table 3). However, such associations were not found in the other comparisons (co-dominant model: Lys/Gln *vs* Lys/Lys: OR = 1.11, 95%CI: 0.94-1.31, $P = 0.20$; Gln/Gln *vs* Lys/Lys: OR = 1.31, 95%CI: 0.98-1.75, $P = 0.07$; dominant model: OR = 1.14, 95%CI: 0.96-1.35, $P = 0.14$, Table 3). In the stratified analysis based on cancer type (ESCC or EADC), we observed an OR of 1.44 (95%CI: 1.01-2.06, $P = 0.05$, Table 3) for ESCC risk and an OR of 1.26 (95%CI: 1.02-1.56, $P = 0.03$, Table 3) for EADC risk, when comparing the Gln/Gln type to the wild type Lys/Lys (Table 3). When stratified by ethnicity, statistically significantly elevated risks were found in Chinese populations in the Gln/Gln *vs* Lys/Lys comparison (OR = 2.49, 95%CI: 1.44-4.29, $P = 0.001$, Table 3) and in the recessive model comparison (OR = 2.37, 95%CI: 1.38-4.10, $P = 0.002$, Figure 5, Table 3), but the same associations were not identified in non-Chinese populations. Finally, for the additive model (Table 3), individuals carrying the 751Gln allele were not significantly associated with an increased risk for EC (OR = 1.10, 95%CI: 0.99-1.22, $P = 0.10$, Table 3).

Heterogeneity and sensitivity analysis

There was moderate heterogeneity among the studies that described the XPD Asp312Asn polymorphism (co-dominant model: Asp/Asn *vs* Asp/Asp, $P = 0.97$; Asn/Asn *vs*

Table 1 Characteristics of the studies included in the meta-analysis

Ref.	Country	Ethnicity	Control source	Cancer type	Genotype distribution (case/control)						P for HWE	
					Asp312Asn			Lys751Gln			Asp312Asn	Lys751Gln
					Asp/Asp	Asp/Asn	Asn/Asn	Lys/Lys	Lys/Gln	Gln/Gln		
Xing <i>et al</i> ^[21] 2002	China	Chinese	PB	ESCC	381/461	49/62	3/1	367/451	63/70	3/3	0.47	0.87
Xing <i>et al</i> ^[22] 2003	China	Chinese	PB	ESCC	286/338	38/45	1/0	278/331	44/49	3/3	0.22	0.43
Yu <i>et al</i> ^[23] 2004	China	Chinese	HB	ESCC	121/136	14/16	0/0	108/133	16/17	11/2	0.49	0.11
Casson <i>et al</i> ^[24] 2005	Canada	Caucasian	HB	EADC	-	-	-	31/34	21/46	4/15	-	0.93
Ye <i>et al</i> ^[25] 2006	Sweden	Swedish	PB	EADC	31/176	51/237	14/57	27/198	51/203	18/71	0.09	0.11
				ESCC	30/176	41/237	10/57	23/198	44/203	14/71	0.09	0.11
Sobti <i>et al</i> ^[26] 2007	India	Indian	HB	ESCC	-	-	-	52/63	61/77	7/20	-	0.64
Doecke <i>et al</i> ^[27] 2008	Australia	Mixed	PB	EADC	-	-	-	108/575	123/588	32/174	-	0.22
Ferguson <i>et al</i> ^[28] 2008	Ireland	Caucasian	PB	EADC	-	-	-	80/91	94/121	34/35	-	0.61
Tse <i>et al</i> ^[19] 2008	United States	Mixed	HB	EADC	117/199	150/206	43/49	104/193	159/208	49/52	0.69	0.72
Pan <i>et al</i> ^[29] 2009	United States	Caucasian	HB	ESCC	16/201	20/185	1/48	17/187	18/216	3/53	0.58	0.43
				EADC	137/201	163/185	43/48	137/187	153/216	56/53	0.58	0.43
Zhai <i>et al</i> ^[30] 2009	China	Chinese	HB	ESCC	-	-	-	167/148	31/51	2/1	-	0.12
Huang <i>et al</i> ^[29] 2012	China	Chinese	HB	ESCC	171/298	42/60	0/0	150/274	55/79	8/5	0.08	0.80
Li <i>et al</i> ^[31] 2013	China	Chinese	PB	ESCC	342/351	56/47	2/2	283/321	105/73	12/6	0.75	0.43

PB: Population-based study; HB: Hospital-based study; ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma; HWE: Hardy-Weinberg equilibrium.

Table 2 Results of the meta-analysis for the xeroderma pigmentosum group D Asp312Asn polymorphism and esophageal cancer susceptibility

Study group	Co-dominant model						Dominant model			Recessive model			Additive model		
	Asp/Asn vs Asp/Asp			Asn/Asn vs Asp/Asp			Asn/Asn + Asp/Asn vs Asp/Asp			Asn/Asn vs Asp/Asn + Asp/Asp			(2Asn/Asn + Asp/Asn) vs 2(Asp/Asn + Asn/Asn + Asp/Asp)		
	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph
Total	1.17 (1.02, 1.33)	0.03	0.97	1.30 (1.00, 1.70)	0.05	0.75	1.18 (1.04, 1.34)	0.01	0.98	1.17 (0.91, 1.50)	0.22	0.71	1.10 (1.00, 1.21)	0.06	1.00
Cancer type															
ESCC	1.09 (0.91, 1.31)	0.35	0.95	0.99 (0.54, 1.79)	0.96	0.48	1.09 (0.91, 1.30)	0.35	0.99	0.93 (0.53, 1.63)	0.79	0.40	1.06 (0.90, 1.24)	0.49	0.99
EADC	1.26 (1.03, 1.53)	0.02	0.97	1.40 (1.04, 1.89)	0.03	0.93	1.29 (1.07, 1.55)	0.01	0.99	1.24 (0.94, 1.64)	0.13	0.90	1.12 (0.99, 1.28)	0.07	0.98
Ethnicity															
Chinese	1.08 (0.88, 1.33)	0.45	0.88	2.08 (0.57, 7.60)	0.27	0.66	1.10 (0.90, 1.35)	0.36	0.93	2.06 (0.57, 7.51)	0.27	0.65	1.10 (0.91, 1.33)	0.34	0.98
Non-Chinese	1.23 (1.03, 1.47)	0.02	0.95	1.27 (0.97, 1.67)	0.08	0.54	1.24 (1.05, 1.47)	0.01	0.93	1.14 (0.89, 1.47)	0.31	0.53	1.10 (0.98, 1.23)	0.11	0.92

ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma; Ph: P value of the Q-test for heterogeneity.

Asp/Asp, $P = 0.75$; dominant model: $P = 0.98$; recessive model: $P = 0.71$; additive model: $P = 1.00$), but this was not observed in the Lys751Gln polymorphism (co-dominant model: Lys/Gln vs Lys/Lys, $P = 0.01$; Gln/Gln vs Lys/Lys, $P = 0.03$; dominant model: $P = 0.001$; recessive model: $P = 0.11$; additive model: $P = 0.02$). The details are shown in Tables 2 and 3.

A sensitivity analysis was carried out by individually omitting each study included in the meta-analysis, and the subsequent results of each genetic model were not materially altered (data not shown), indicating that the results

were statistically robust.

Publication bias

Begg's funnel plot and Egger's test were performed to assess any possible publication bias. The shape of the funnel plots did not reveal any obvious asymmetry. We have presented the funnel plots of XPD Asp312Asn for the dominant model (Asn/Asn + Asp/Asn vs Asp/Asp) and XPD Lys751Gln for the recessive model (Gln/Gln vs Lys/Gln + Lys/Lys) in Figure 6. The statistical evidence from the results of Egger's test confirmed the funnel

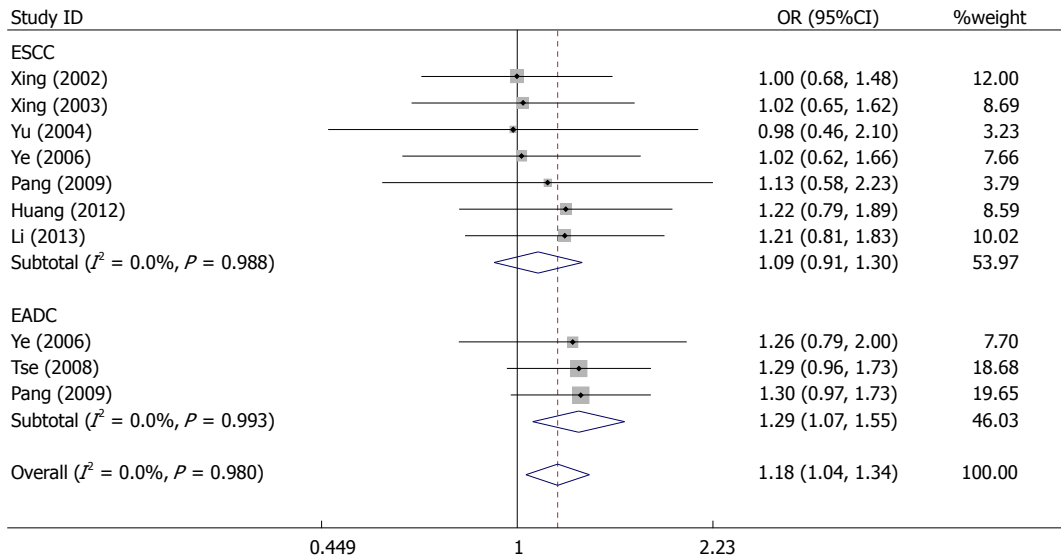


Figure 2 Forest plot for the xeroderma pigmentosum group D Asp312Asn polymorphism when stratified by cancer type in a dominant model comparison. Dominant model: Asn/Asn + Asp/Asn vs Asp/Asp; ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma.

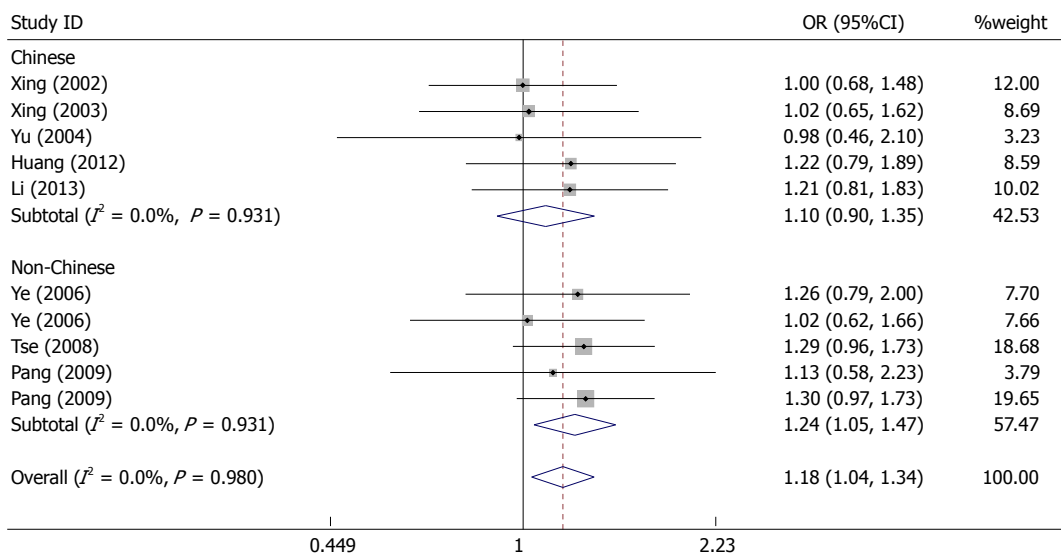


Figure 3 Forest plot for the xeroderma pigmentosum group D Asp312Asn polymorphism when stratified by ethnicity in a dominant model comparison. Dominant model: Asn/Asn + Asp/Asn vs Asp/Asp; ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma.

plot symmetry (XPD Asp312Asn: $P = 0.31$ for Asp/Asn vs Asp/Asp, $P = 0.77$ for Asn/Asn vs Asp/Asp, $P = 0.06$ for the dominant model, $P = 0.89$ for the recessive model, and $P = 0.11$ for the additive model; XPD Lys751Gln: $P = 0.38$ for Lys/Gln vs Lys/Lys, $P = 0.99$ for Gln/Gln vs Lys/Lys, $P = 0.40$ for the dominant model, $P = 0.86$ for the recessive model, and $P = 0.69$ for the additive model).

DISCUSSION

DNA repair enzyme gene polymorphisms that are capable of altering the function or efficiency of damaged DNA repair can lead to genetic instability and carcinogenesis^[32]. A small proportion of published studies have

explored the relationship between XPD polymorphisms and EC risk and have yielded inconsistent results^[17-31]. In order to derive a more precise estimation of the relationship, we performed a meta-analysis of 13 case-control studies, including 10 comparisons for the Asp312Asn polymorphism (2373 cases and 3175 controls) and 15 comparisons for the Lys751Gln polymorphism (3226 cases and 5237 controls).

In the case of the XPD Asp312Asn polymorphism, our results indicated that individuals carrying the variant heterozygous Asp/Asn showed an increased risk for EC compared to those with the wild-type homozygous Asp/Asp (OR = 1.17, 95%CI: 1.02-1.33). Similarly, a significant association between the XPD Asp312Asn polymorphism and EC was found under the dominant model (OR

Table 3 Results of the meta-analysis for the xeroderma pigmentosum group D Lys751Gln polymorphism and esophageal cancer susceptibility

Study group	Co-dominant model						Dominant model			Recessive model			Additive model		
	Lys/Gln <i>vs</i> Lys/Lys			Gln/Gln <i>vs</i> Lys/Lys			Gln/Gln + LysGln <i>vs</i> Lys/Lys			Gln/Gln <i>vs</i> Lys/Gln + Lys/Lys			(2Gln/Gln + Lys/Gln) <i>vs</i> 2(Lys/Gln + Gln/Gln + Lys/Lys)		
	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph	OR (95%CI)	P	Ph
Total	1.11 (0.94, 1.31)	0.20	0.01	1.31 (0.98, 1.75)	0.07	0.03	1.14 (0.96, 1.35)	0.14	0.001	1.21 (1.02, 1.43)	0.03	0.11	1.10 (0.99, 1.22)	0.10	0.02
Cancer type															
ESCC	1.13 (0.89, 1.42)	0.31	0.03	1.44 (1.01, 2.06)	0.05	0.06	1.16 (0.91, 1.49)	0.23	0.01	1.26 (0.90, 1.77)	0.17	0.08	1.13 (0.94, 1.36)	0.21	0.02
EADC	1.09 (0.85, 1.40)	0.51	0.02	1.26 (1.02, 1.56)	0.03	0.05	1.11 (0.85, 1.44)	0.45	0.01	1.19 (0.98, 1.45)	0.08	0.25	1.07 (0.98, 1.18)	0.13	0.22
Ethnicity															
Chinese	1.10 (0.82, 1.47)	0.53	0.02	2.49 (1.44, 4.29)	0.001	0.64	1.18 (0.88, 1.60)	0.27	0.01	2.37 (1.38, 4.10)	0.002	0.65	1.21 (0.94, 1.56)	0.15	0.02
Non-Chinese	1.12 (0.91, 1.38)	0.30	0.03	1.13 (0.82, 1.56)	0.45	0.02	1.11 (0.89, 1.39)	0.35	0.01	1.12 (0.93, 1.34)	0.23	0.14	1.06 (0.98, 1.15)	0.16	0.27

ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma; Ph: *P* value of the *Q*-test for heterogeneity.

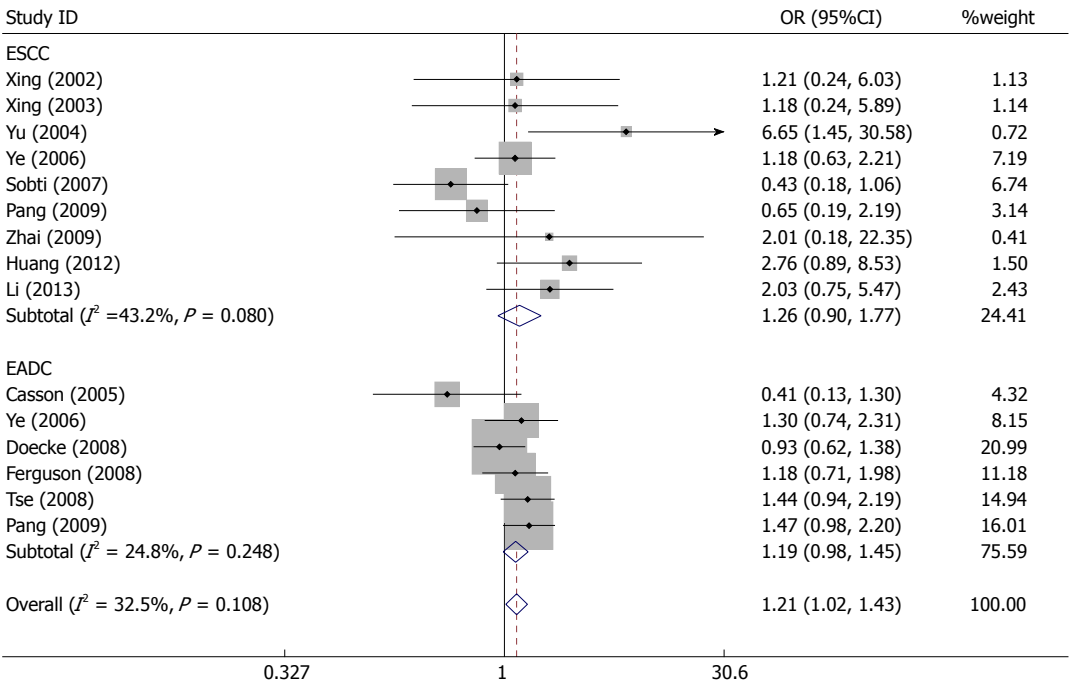


Figure 4 Forest plot for the xeroderma pigmentosum group D Lys751Gln polymorphism when stratified by cancer type in a recessive model comparison. Recessive model: Gln/Gln *vs* Lys/Gln+Lys/Lys; ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma.

= 1.18, 95%CI: 1.04-1.34). After stratifying based on cancer type, an association was found in both the co-dominant model and the dominant model for EADC (Asp/Asn *vs* Asp/Asp: OR = 1.26, 95%CI: 1.03-1.53; Asn/Asn *vs* Asp/Asp: OR = 1.40, 95%CI: 1.04-1.89; dominant model: OR = 1.29, 95%CI: 1.07-1.55) but not for ESCC. This was opposite to the results of the meta-analysis performed by Duan *et al*^[33], which showed a borderline association with the dominant model for ESCC but not for EADC. Our meta-analysis excluded the study by Liu *et al*^[17], because the subjects had also been included in the study by Tse *et al*^[19], but this exclusion was not performed by Duan *et al*^[33]. In addition, our meta-analysis included a new study by Li *et al*^[31]. Therefore, the present meta-analysis provides more reliable evidence in regards to the importance of the XPD Asp312Asn polymorphism in

relation to EC. When stratified by ethnicity, a significant association was found in non-Chinese populations for the Asp/Asn *vs* Asp/Asp comparison (OR = 1.23, 95%CI: 1.03-1.47) and under the dominant model (OR = 1.24, 95%CI: 1.05-1.47), but the same association was not observed in Chinese populations, indicating that ethnic differences in the genetic background and the environment they live in may play a possible role in EC susceptibility. Therefore, the same XPD Asp312Asn polymorphism plays different roles in EC susceptibility among Chinese and non-Chinese populations, because cancer is a complicated multifactorial disease, and different genetic backgrounds may contribute to the discrepancy^[34].

In the case of the XPD Lys751Gln polymorphism, our meta-analysis showed that there was a significant association with EC susceptibility under the recessive

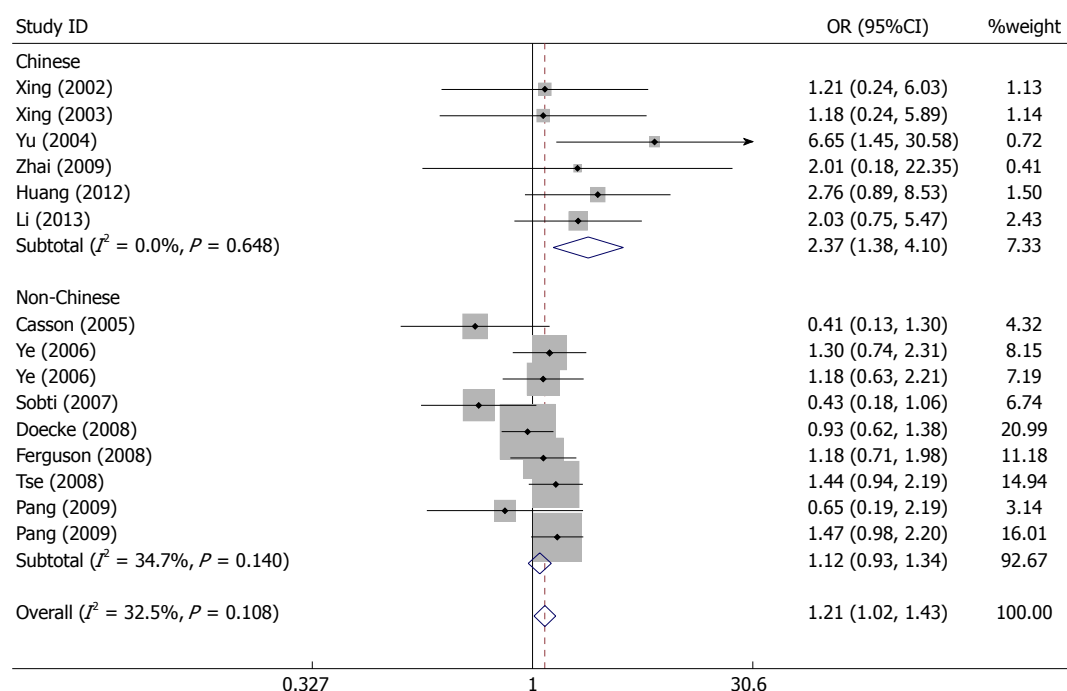


Figure 5 Forest plot for the xeroderma pigmentosum group D Lys751Gln polymorphism when stratified by ethnicity in a recessive model comparison. Recessive model: Gln/Gln vs Lys/Gln+Lys/Lys; ESCC: Esophageal squamous cell carcinoma; EADC: Esophageal adenocarcinoma.

sive model (OR = 1.21, 95%CI: 1.02-1.43). Our results were inconsistent with two previously published meta-analyses by Ding *et al.*^[35] and by Yuan *et al.*^[36], both of which showed a lack of association between the XPD Lys751Gln polymorphism and EC in total populations. Our meta-analysis included a larger number of studies and more EC cases when compared with these two earlier studies. Therefore, the present meta-analysis provides more reliable evidence about the importance of the XPD Lys751Gln polymorphism in terms of EC. In the analysis stratified according to histological type, a positive association was observed between the XPD Lys751Gln polymorphism and an elevated susceptibility to both ESCC and EADC (ESCC: OR = 1.44, 95%CI: 1.01-2.06; EADC: OR = 1.26, 95%CI: 1.02-1.56), when comparing the Gln/Gln type to the wild type Lys/Lys. When stratified by ethnicity, a significant association was found in Chinese populations for the Gln/Gln *vs* Lys/Lys comparison (OR = 2.49, 95%CI: 1.44-4.29) and under the recessive model (OR = 2.37, 95%CI: 1.38-4.10), suggesting that the XPD Lys751Gln polymorphism plays a greater role in Chinese populations. It is worth noting that this observation is opposite to that seen in the XPD Asp312Asn polymorphism.

The associations between the XPD Asp312Asn polymorphism and EC susceptibility have been researched in very few studies. Only one study by Huang *et al.*^[20] reported that XPD Asp312Asn was associated with a borderline decrease for the risk of ESCC in the Han and Uygur populations. The majority of the studies^[19,21-23,25,29,31] reported that there were no statistically significant associations between the XPD Asp312Asn polymorphism and the risk for EC, which is opposite to the results of

our meta-analysis, which shows a significant association between the XPD Asp312Asn polymorphism and EC susceptibility. A reason may be that the sample sizes of those studies were too small to explore the subtle association between the XPD Asp312Asn polymorphism and EC susceptibility, but the pool of ORs generated from 10 comparisons significantly increases the statistical power.

Many epidemiological studies have also investigated the association between the XPD Lys751Gln polymorphism and EC susceptibility. Xing *et al.*^[21], Pan *et al.*^[29] and Ferguson *et al.*^[28] reported that the Lys751Gln polymorphism in the XPD gene did not influence the risk for ESCC and/or EADC. However, Yu *et al.*^[23], Huang *et al.*^[20], Li *et al.*^[31], Ye *et al.*^[25] and Tse *et al.*^[19] revealed a contradictory result, which suggested an increased risk for ESCC and/or EADC in association with the XPD Lys751Gln polymorphism. A more interesting finding revealed by Zhai *et al.*^[30] and Casson *et al.*^[24] suggested an inverse association, which indicated that the XPD Lys751Gln polymorphism is a protective factor rather than a risk factor for ESCC or EADC. The differences in risk observed in different studies could be partially attributable to the small sample sizes and inappropriate study design. More importantly, the interaction with other polymorphisms and/or particular environmental exposures may also influence the genetic effects of a single polymorphism^[35].

There are some limitations to our meta-analysis that should be acknowledged. First, though it is known that the XPD gene has more polymorphisms than just Asp312Asn and Lys751Gln, we focused our meta-analysis on the two most studied polymorphisms due to limited research on other polymorphisms. Second, the studies investigating genetic associations should be based on a

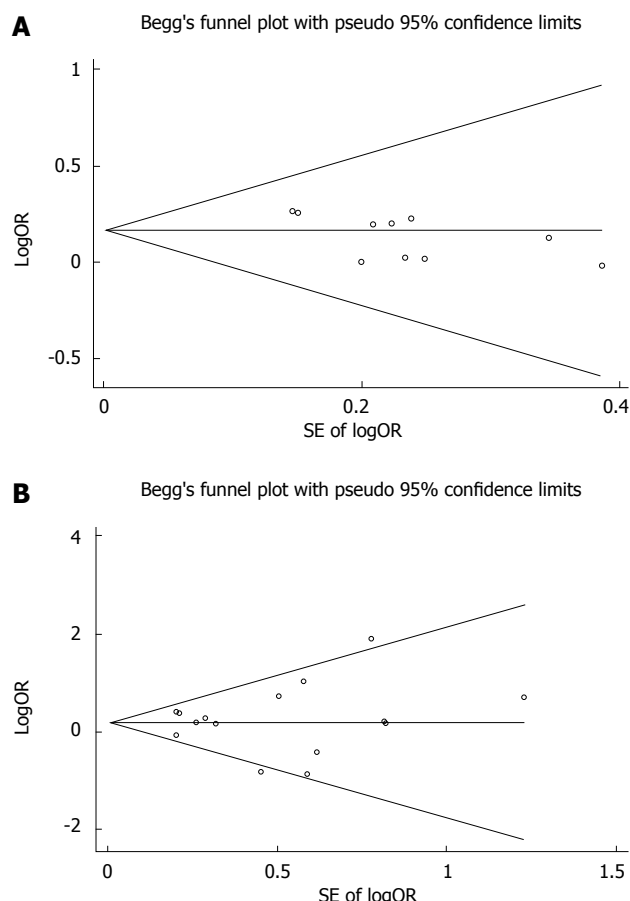


Figure 6 Funnel plots showing the associations between the xeroderma pigmentosum group D polymorphisms and esophageal cancer susceptibility. Each point represents a separate study for the indicated association. A: Funnel plot of XPD Asp312Asn for the dominant model (Asn/Asn+Asp/Asn vs Asp/Asp); B: Funnel plot of XPD Lys751Gln for the recessive model (Gln/Gln vs Lys/Gln+Lys/Lys).

large sample size, similar study designs and standardised case and control definitions. Third, the XPD gene polymorphisms may influence EC susceptibility in concert with other genes, but we did not have enough data to conduct any gene-gene interaction analyses. Finally, our results were based on single-factor evaluations without adjustment for other risk factors, including BMI, tobacco, alcohol, environmental factors, or lifestyle.

In conclusion, this meta-analysis showed that the XPD Asp312Asn polymorphism may contribute to EC susceptibility, particularly in non-Chinese populations. In addition, the analysis showed that the XPD Lys751Gln polymorphism may also contribute to EC susceptibility, particularly in Chinese individuals. Large, well-designed case-control studies are recommended in order to further enrich the present findings. Future studies should focus on gene-gene and gene-environment interactions to further shed light on the genetics of EC.

COMMENTS

Background

A small proportion of the published studies have explored the relationship be-

tween xeroderma pigmentosum group D (XPD) polymorphisms and esophageal cancer (EC) risk and have yielded inconsistent results. In order to derive a more precise estimation of this relationship, we performed a meta-analysis of all of the case-control studies that evaluated the association between the genetic polymorphisms of XPD (Asp312Asn and Lys751Gln) and EC susceptibility.

Research frontiers

The XPD enzyme plays an important role in the repair of bulky DNA adducts. Mutations at different sites in the XPD gene may render a higher risk for developing EC. However, the evidence is insufficient given the small sample size.

Innovations and breakthroughs

This meta-analysis suggested that the XPD Asp312Asn and Lys751Gln gene polymorphisms are both associated with a significantly increased risk for EC.

Applications

This study provided a potential biomarker to identify high-risk individuals for esophageal cancer.

Terminology

XPD is an evolutionarily conserved ATP-dependent helicase that plays an important role in the repair of bulky DNA adducts, such as pyrimidine dimers, photoproducts and cross-links.

Peer review

The authors clarify the effects of the XPD Asp312Asn and Lys751Gln gene polymorphisms on the risks of esophageal cancer. This is a "delicious" paper.

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Case report of acute-on-chronic liver failure secondary to diffuse large B-cell lymphoma

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HIV/HCV co-infection in the African American 1945 to 1965 birth cohort and the fact that both are risk factors for chronic liver disease and NHL we postulate that the incidence of NHL presenting as ACLF may increase.

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Key words: Diffuse large B-cell lymphoma; Acute-on-chronic liver failure; Human immunodeficiency virus; Hepatitis C virus; Hodgkin's disease; Fatal outcome

Core tip: Recognition of acute on chronic liver failure (ACLF) is vital because it may be rapidly fatal. However, many patients have underlying silent liver disease, especially, hepatitis C virus (HCV) cirrhosis. Diffuse large B-cell lymphoma is an aggressive lymphoma which is beginning to occur more frequently in the same race and birth cohort as HCV/human immunodeficiency virus related liver disease. Early recognition and potential treatment of this rapidly fatal lymphoma depends on a high index of suspicion. Due to the shared demographics the incidence of non-Hodgkin's lymphoma presenting as ACLF is likely to increase.

Abstract

Acute liver failure is a rare presentation of hematologic malignancy. Acute on chronic liver failure (ACLF) is a newly recognized clinical entity that describes acute hepatic decompensation in persons with preexisting liver disease. Diffuse large B-cell lymphoma (DLBCL) is an aggressive non-Hodgkin's lymphoma (NHL) with increasing incidence in older males, females and blacks. However, it has not yet been reported, to present with acute liver failure in patients with preexisting chronic liver disease due to human immunodeficiency virus (HIV)/hepatitis C virus (HCV) co-infection. We describe a case of ACLF as the presenting manifestation of DLBCL in an elderly black man with HIV/HCV co-infection and prior Hodgkin's disease in remission for three years. The rapidly fatal outcome of this disease is highlighted as is the distinction of ACLF from decompensated cirrhosis. Due to the increased prevalence of

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INTRODUCTION

Acute liver failure (ALF) is a well-defined clinical syndrome with associated high mortality. Acute-on-chronic liver failure (ACLF) is also a rare syndrome with diverse etiology and high mortality which is less well-defined or understood. Of the 1147 adults studied in the adult ALF

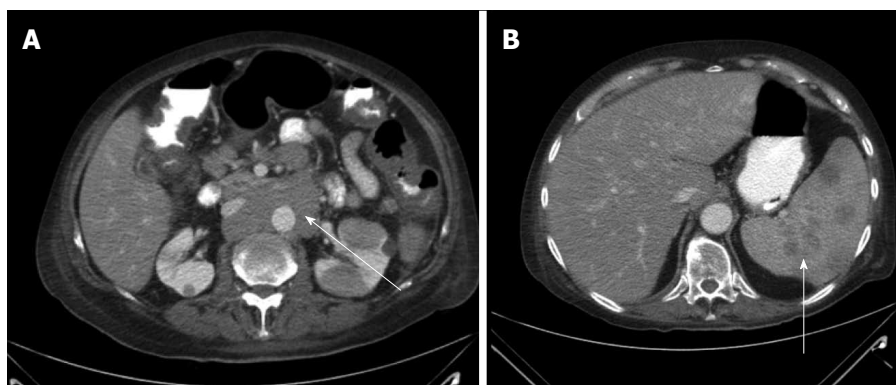


Figure 1 Abdominal computed tomography scan. A: Showing the confluent non-compressing circumaortic mass (arrow); B: Showing multiple hypodense splenic lesions (arrow).

Study Group, less than seven percent were due to malignancy^[1]. Neither the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), nor the Asian Pacific Association for the Study of the Liver (APASL) listed malignancy as a potential precipitant of ACLF. Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma (NHL) in the United States^[2,3]. Incidence rates for NHL have been increasing 3%-4% per year from 1973 to the 1990s especially among older males, females, and blacks^[3]. We present a rapidly fatal case of DLBCL presenting as ACLF in an elderly black man with HIV/HCV co-infection.

CASE REPORT

A 71-year-old African American man was brought to our emergency department (ED) by a friend who noted that he was becoming increasingly confused and lethargic. He was a former intravenous drug user now on methadone maintenance. The patient had a history of HIV/HCV co-infection (CD4 count of 116 and viral load of 214 copies/mL). Liver biopsy two years prior to admission revealed grade 2 inflammation with stage 1 fibrosis. He also reported a history of Hodgkin's disease that was now in remission after chemo-radiotherapy three years prior.

The patient was afebrile and jaundiced. Firm non-tender diffusely enlarged right cervical and axillary lymph nodes approximately 3 cm × 3 cm were noted. Heart and lung examinations were unremarkable. The abdomen was not distended. The liver and spleen were not palpable. There was no ascites. He was oriented only to self and month of the year. He was somnolent but had no asterixis. Capillary glucose was 46 mg/dL in the ED. His mental status did not improve after correction of hypoglycemia and naloxone administration.

Laboratory results showed a normal white blood cell (WBC) count, $8.7 \times 10^9/L$, hemoglobin, 9.6 g/dL, hematocrit, 27.9% and platelet count 96000 per microliter of blood. His platelet count one month prior to admission was 161000. He was mildly azotemic, blood urea nitrogen (BUN) 25 mg/dL, and serum creatinine 1.2 mg/dL.

Liver tests revealed aspartate aminotransferase (AST) of 145 IU/L (normal range: 15-37 IU/L), alanine aminotransferase (ALT) 39 IU/L (normal range: 12-78 IU/L), Alkaline phosphatase 221 IU/L (normal range: 50-136 IU/L), total bilirubin 7.3 mg/dL (normal range: 0.2-1.2 mg/dL), direct bilirubin 6.0 mg/dL (normal range: 0.0-0.2 mg/dL), total protein 5.5 g/dL (normal range: 6.4-8.2 g/dL) and albumin 2.5 g/dL (normal range: 3.4-5 g/dL). International normalized ratio (INR) was 1.6. Arterial blood gas on room air revealed pH of 7.46, pCO₂ 30 mmHg, pO₂ 65 mmHg, HCO₃⁻ 21 mmol/L, and lactate of 4.1 mmol/L (normal range: 0.5-1.6 mmol/L). Lactate dehydrogenase (LDH) was 732 IU/L (normal range: 118-273 IU/L), serum alcohol level was < 3 mg/dL and urine toxicology was positive for opiates and methadone. Acetaminophen, hepatitis A and B serologies, ceruloplasmin, and ANA were all within normal limits.

Abdominal sonogram showed fatty liver infiltration, mild splenomegaly, mildly distended gallbladder without calculi, and no biliary ductal dilatation. Abdominal computed tomography with contrast showed multiple hypodense splenic lesions, a confluent non-compressing circumaortic mass, multiple para-aortic and pelvic lymph nodes, and bilateral pulmonary nodules (largest 1 cm) (Figure 1).

Right axillary lymph node excision biopsy revealed a diffuse large B-cell lymphoma. The patient's liver tests continued to worsen with ALT 37 IU/L, AST 218 IU/L, maximum total bilirubin of 10 mg/dL, (direct bilirubin 8.9 mg/dL), and INR of 2.2. Hemoglobin and hematocrit remained unchanged at 9.7 g/dL and 23.4% respectively at discharge. He was transferred to another facility for chemotherapy. There he spent an additional week, received one cycle of chemotherapy but due to continued deterioration family requested comfort care. He was transferred to a hospice facility where he died a week later.

DISCUSSION

Our patient is the first reported case of rapidly fatal DLBCL in a patient with chronic liver disease due to HCV/HIV coinfection. Because of the presence of inflammation and fibrosis on an earlier biopsy this is a presenta-

Table 1 Differences in the current definitions of acute on chronic liver failure

	APASL definition	AASLD/EASL consensus
Duration between insult and ACLF	4 wk	Not defined
Duration in which there is higher mortality	Not defined	3 mo
What qualifies as “chronic liver disease”	Chronic liver disease with/without only compensated cirrhosis	Only cirrhosis, including those with prior decompensation
What qualifies as precipitants?		
Alcohol, drugs, hepatotropic viruses, surgery, trauma	Yes	Yes
Sepsis	No	Yes
Variceal bleeding	No consensus	Yes

APASL: Asian Pacific Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver; ACLF: Acute on chronic liver failure.

tion of ACLF and not ALF. Although ALF has been well defined, a universally accepted definition for ACLF is still a subject of debate and on-going research^[4,5]. This is due to the heterogeneous proposed etiology, pathophysiology, organ dysfunction, and outcomes in different regions of the world.

ALF refers to the acute (< 26 wk) onset of severe liver injury with encephalopathy and impaired synthetic function (INR \geq 1.5) in a patient without pre-existing liver disease^[1]. APASL defined ACLF as an acute hepatic insult manifesting with jaundice (bilirubin > 5mg/dL) and coagulopathy (INR > 1.5), complicated within four weeks by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease. This definition fits our patient. An AASLD/EASL consensus statement defined ACLF as a syndrome among a subgroup of cirrhotic patients who develop organ failure following hospital admission with or without an identifiable precipitating event and have increased mortality rates. The differences in the above definitions are highlighted in Table 1^[4,5].

One particularly contentious issue is “what exactly qualifies as chronic liver disease?” For the purposes of ACLF, the APASL consensus definition for chronic liver disease included patients with compensated cirrhosis of any etiology, chronic hepatitis, non-alcoholic steatohepatitis, cholestatic liver disease, and metabolic liver disease. Simple steatosis was excluded because it is not always progressive. Decompensated cirrhosis on the other hand was also excluded because it is a distinct clinical entity that is irreversible whereas ALF and ACLF have potentially reversible acute insults. Again, our patient with mild HCV/HIV inflammation and fibrosis fits this description. However, per EASL/AASLD only cirrhotics, including subjects with prior decompensation qualify as “chronic liver disease” because these patients have distinctly different immune and cardiovascular response to injury.

The precipitating factors of ACLF may be either hepatic or extra-hepatic in origin. Hepatic causes include alcoholic hepatitis, acute viral hepatitis (HAV, HBV, and HEV), drug-induced liver injury, portal vein thrombosis, acute auto-immune hepatitis, Wilson’s disease, and ischemic hepatitis. Extra-hepatic insults comprise bacterial or fungal infections, variceal hemorrhage, surgery, trauma, and unknown hepatotoxins^[6]. A case of Hodgkin’s lymphoma induced - ACLF in an HBV patient has been reported. In that report the patient developed acute on chronic hepatitis B following treatment with entecavir. Post-mortem findings revealed co-existent early Hodgkin’s lymphoma (HL)^[7]. Our patient had ACLF secondary to DLBCL which is a NHL.

HL and NHL are two distinct hematologic malignancies. HL spreads in a contiguous manner via the lymphatic system and is characterized on hematoxylin and eosin stain by Reed-Sternberg cells which are transformed EBV positive cells in a background of granulocytes, plasma cells, and lymphocytes. DLBCL is the most common type of NHL. NHL represents 4.2% of all new cancer cases in the United States. It is the seventh most common cancer and seventh leading cause of cancer deaths. It refers to a constellation of disorders with heterogeneous clinico-pathologic features distinguished by a diffuse effacement of architecture composed mainly of large B-cells in different stages of maturation. It is an aggressive lymphoma that commonly arises from peripheral lymph nodes presenting with painless, rubbery lymphadenopathy sometimes with constitutional symptoms such as fever, night sweats, and unintentional weight loss. Several extra-nodal sites including but not limited to the bone marrow, gastrointestinal tract, brain, thyroid, testes, skin, and breast are also sites of initial presentation^[2,8,9].

About 40 cases of ALF due to any lymphoma or leukemia have been published since 1952^[10,11]. Due to its rarity it is mostly under-recognized and a high degree of clinical suspicion is required. NHL is traditionally considered to be common in those above 70 years of age, males, whites, family clusters, and is associated with chronic viral infections such as Epstein Bar Virus, HIV, HBV, and HCV. Nevertheless, the strongest predictor of risk is immune system abnormality^[3,9]. Our patient’s risk factors for NHL include male sex, advanced age, HIV/AIDS, HCV, prior Hodgkin’s and chemo-radiotherapy.

HCV is strongly associated with the occurrence of NHL in endemic areas such as Italy, Japan, and Egypt, but not in North America. Several oncogenic mechanisms have been proposed to explain the development of NHL in patients with chronic HCV such as: chronic antigen stimulation leading to monoclonal malignant proliferation; HCV genetic damage to B-cell tumor suppressor genes; HCV replication within B cells inducing oncogenic signals, and monocytes/macrophages being major target of HCV^[12-14].

In 2002, it was noted that in males 25-54 years of age the incidence rates of NHL for blacks exceeded whites. This was also true for black females aged 25-34 years.

The authors propose this increase in incidence is due to the prevalence of HIV in the African American community^[3]. The high prevalence of HCV in the African American may also have contributed to the increased of NHL that was noted.

There was no evidence of acute exacerbation of HCV in our patient as his ALT remained less than 40 IU/L throughout the decompensation of his liver function. We also doubt that the patient was truly cirrhotic as his platelet count had been normal one month before, and his ultrasound and CT scan were not suggestive of cirrhosis. In the series by Zafrani *et al*^[11], the clinical features of patients with ALF secondary to lymphoma/leukemia were characterized by average age of 49 years, hyperbilirubinemia, elevated liver transaminases, high LDH, and lactic acidosis. The majority had thrombocytopenia and prolonged prothrombin time. Our patient had all of these features. The observed disproportionate elevation in the AST compared to the ALT in our patient is similar to other reports. An AST/ALT ratio greater than 2 is characteristically seen in alcoholic liver disease and Wilson's disease. A further conundrum in deciphering the diagnosis initially was the minimal elevation of ALP which argues against malignant infiltration as a consideration. Although the reason for this is unclear, ALP elevation had the lowest frequency of liver enzyme abnormalities in other series of ALF due to lymphoreticular malignancy^[9,10]. Extreme elevations of LDH and lactic acidosis and hypoglycemia have been associated with ALF^[15].

ACLF patients manifest in various forms due to the heterogeneity of this patient population. Severe jaundice, coagulopathy, multi-organ failure with encephalopathy and renal dysfunction, and systemic inflammatory response syndrome are common findings. Proinflammatory cytokines, neutrophil dysfunction and sepsis are believed to play a major role in pathogenesis and prognosis. Elevated leukocyte counts and C-reactive protein have been found to be common as well as evidence of occult or overt infection in ACLF patients. Dysregulated inflammation is considered a critical hallmark and final common pathway of the various insults of ACLF^[16,17]. At the time of presentation our patient however, had normal white blood cell count and no evidence of infection. Further research will help define this subgroup of patients.

Several mechanisms by which NHL may cause ALF have been postulated: (1) massive hepatic sinusoidal infiltration by malignant cells could result in ischemia and hepatocyte necrosis; (2) tumor obstructing hepatic venules may also result in ischemic injury and necrosis; (3) intrahepatic bile ducts may also be infiltrated by lymphoma cells resulting in duct necrosis, cholangitis, and ALF; and (4) Rapid replacement of hepatic parenchyma by lymphomatous cells may lead to mass destruction of hepatocytes and resulting ACLF^[11,18,19].

ACLF as the presenting feature of DLBCL is uncommon and, therefore, not typically considered in the differential diagnosis of liver failure. However, given the increasing incidence of DLBCL in older black men and

patients with HCV and HIV/AIDS the diagnosis should especially be considered in this demographic group. Early diagnosis and referral requires a high index of suspicion as delayed diagnosis is usually fatal.

COMMENTS

Case characteristics

A 71-year-old African American man with human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfection presented to the emergency room with lethargy and confusion.

Clinical diagnosis

The patient was jaundiced, somnolent, disoriented to place, and had non tender cervical and axillary lymphadenopathy.

Differential diagnosis

Altered mental status secondary to hypoglycemia, drug overdose, sepsis, or hepatic encephalopathy was considered and adenopathy was biopsied to rule out infection or malignancy.

Laboratory diagnosis

White count was normal with new anemia (hemoglobin = 9.6 g/dL), thrombocytopenia (platelet count = 96000/mL), hypoglycemia (blood glucose = 46 mg/dL), elevated lactate (4.1 mmol/L), cholestatic liver injury (direct bilirubin = 8.9 mg/dL, alkaline phosphatase = 221 IU/L, AST = 135 IU/L, ALT normal), and coagulopathy (INR = 1.6).

Imaging diagnosis

Abdominal CT scan showed a confluent circumaortic mass, multiple para-aortic and pelvic lymph nodes.

Pathological diagnosis

Lymph node biopsy revealed diffuse large B-cell lymphoma in the clinical setting of acute liver failure.

Treatment

Before transfer to another institution for chemotherapy, care was supportive with correction of hypoglycemia, lactulose for hepatic encephalopathy, oxygen, and empiric antibiotics.

Related reports

Palta reported a case of acute on chronic liver failure (ACLF) in a patient who had acute on chronic HBV and coexistent Hodgkin's lymphoma.

Term explanation

Diffuse large B-cell lymphoma (DLBCL) refers to a constellation of disorders with heterogeneous clinico-pathologic features distinguished by a diffuse effacement of architecture composed mainly of large B-cells in different stages of maturation.

Experiences and lessons

In cases of ACLF in patients with HCV and HIV, DLBCL should be considered in the differential diagnosis as the virus is lymphotropic as well as hepatotropic and early diagnosis and treatment may improve outcome.

Peer review

The authors should discuss about possibility that HCV infection of B cells might have caused lymphoma and ACLF.

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Heterotopic pancreatic tissue in the gastric cardia: A case report and literature review

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suggest that pancreatic ectopia should be a part of differential diagnosis, not only when dealing with submucosal gastric lesions, but also with those that are small, flat and/or untypically located.

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Key words: Pancreatic tissue; Ectopia; Gastric cardia; Gastroesophageal reflux disease; Epigastric pain

Core tip: The heterotopic pancreas, which is usually described as an untypical presence of pancreatic tissue without any anatomic or vascular continuity with the pancreas, is relatively rare. As an intra- or submucosal lesion, is usually found incidentally, most often in the gastric antrum. However, it may also be found anywhere in the digestive tract. We report an ectopic pancreatic lesion atypically located in the gastric cardia in a 73-year-old woman with chronic epigastric pain accompanied by heartburn.

Abstract

The heterotopic pancreas, which is usually described as an untypical presence of pancreatic tissue without any anatomic or vascular continuity with the pancreas, is relatively rare. Clinical manifestations may include bleeding, inflammation, pain and obstruction; however, in most cases it remains silent and is diagnosed during autopsy. Here, we report a case of ectopic pancreatic lesion located in the gastric cardia. The patient was a 73-year-old woman who had a history (over four months) of chronic epigastric pain accompanied by heartburn. Esophagogastroduodenoscopy revealed inflammatory changes throughout the stomach and lower esophagus, as well as a flat polypoid mass with benign features located in the gastric cardia, approx. 10 mm below the "Z" line, measuring approx. 7 mm in diameter. Endoscopic biopsy forceps were used to remove the lesion. Histological examination of the lesion revealed the presence of heterotopic pancreatic tissue in the gastric mucosa. On the basis of the presented case, we

Filip R, Walczak E, Huk J, Radzki RP, Bieńko M. Heterotopic pancreatic tissue in the gastric cardia: A case report and literature review. *World J Gastroenterol* 2014; 20(44): 16779-16781 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16779.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16779>

INTRODUCTION

The heterotopic pancreas, which is usually described as an untypical presence of pancreatic tissue without any anatomic or vascular continuity with the pancreas, was probably firstly described in the 18th century when it was found in an ileal diverticulum^[1]. It may be found at different sites in the gastrointestinal tract, with a propensity to affect the small intestine and stomach. Although a heterotopic pancreas can occur at any age, it is most common, after 50 years of age^[2,3]. Clinical manifestations may



Figure 1 Endoscopic examination showing the location of the flat polypoid mass with benign features in the gastric cardia. The lesion was approx. 10 mm below the "Z" line and measured approx. 7 mm in diameter.

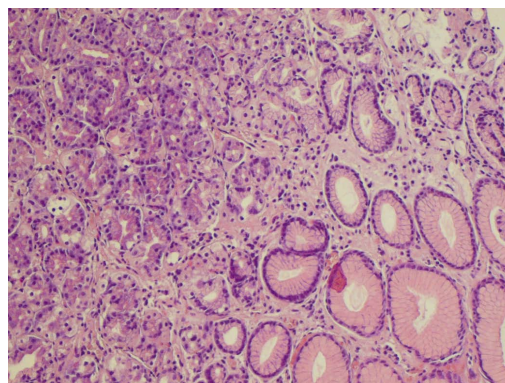


Figure 2 Pancreatic heterotopia of the cardiac mucosa. Most of the glandular structures visible below the foveolae correspond to pancreatic exocrine acini.

include bleeding, inflammation, pain and obstruction; however, in most cases it remains silent and is diagnosed during autopsy^[1,4]. On the other hand, in such tissue there is also an increased risk of malignant transformation^[5].

We report the case of a 73-year-old female with an ectopic pancreatic lesion in the gastric cardia.

CASE REPORT

In March 2011, a 73-year-old woman was admitted to hospital because of pain located in the left upper- and middle-abdomen, accompanied by heartburn, nausea and vomiting. The history of the above symptoms lasted for about four months with remarkable aggravation a few days before being admitted to hospital. Her past medical history, apart from colon diverticulosis, also contained arterial hypertension, nodular goiter and diabetes type 2. Physical examination was unremarkable, as were the results of complete blood count and routine biochemical investigations. Esophagogastroduodenoscopy revealed inflammatory changes throughout the stomach and lower esophagus, as well as a flat polypoid mass with benign features located in the gastric cardia, approx. 10 mm below the "Z" line, measuring approx. 7 mm in diameter (Figure 1). The duodenum was unremarkable. Endoscopic biopsy forceps removed the lesion, which submitted for histopathological assessment. The endoscopic diagnosis was superficial gastritis, gastroesophageal reflux disease (GERD) with the presence of a gastroesophageal junction hyperplastic polyp. Histological examination of the lesion revealed the presence of heterotopic pancreatic tissue in the cardiac mucosa with fully developed acini (Figure 2). An abdominal ultrasound was normal, apart from the presence of nephrolithiasis. After discharge from hospital, she was under the supervision of the gastroenterology outpatients clinic, where she passed colonoscopy with the polypectomy of the two tubular adenomas of approx. 6 mm and 8 mm in diameter, as well as whole gastrointestinal tract radiography with barium contrast, which was normal. Two control esophagogastroduodenoscopic examinations with random biopsies were performed, the last

in June 2012, and no abnormalities in the location of the previously described lesion were found, nor in any other location within the upper gastrointestinal tract (GIT).

DISCUSSION

The origins of pancreatic ectopia are not fully elucidated; however, the most probable theories are based on the fetal migration of pancreatic cells and on the penetration of immature gastric mucosa inside the submucosa, followed by its pancreatic metaplasia^[6]. The possible localizations within the GIT are the esophagus, stomach, small intestine, common bile duct and gallbladder, papilla of Vater, Meckel's diverticulum and mesocolon^[6,7]. However, most commonly, an ectopic pancreas is seen in the stomach - up to 38%, of which 95% are located in the greater curvature in the antrum^[8]. The occurrence in particular layers of the stomach wall is as follows: 73% in the submucosal layer, 17% in the muscularis propria layer and 10% in the subserosal layer^[7]. Notably, we could not find any information in the literature about the occurrence of ectopic pancreatic tissue on or within the epithelial layer of the gastric mucosa.

The clinical symptoms of pancreatic ectopia depend of location, size and other pathological features that may occasionally coexist, *e.g.*, secretion of pancreatic enzymes that can result in local inflammation or/and secreting the hormones that may exert a whole body effect. Lesions smaller than 15 mm in diameter remain asymptomatic until they cause local inflammation or obstruction, and are usually detected accidentally. Last, but not least, pancreatic ectopia may occasionally turn into adenocarcinoma or a neuroendocrine neoplasm^[1,2,7]. In pediatric patients, the clinical picture of pancreatic ectopia can be different. Most characteristic are GIT obstructions and intrasuspension that can also be associated with some congenital abnormalities, including granular pancreas, esophageal atresia, Meckel's diverticulum, malrotation, choledochal cyst and extrahepatic biliary atresia^[9].

Endoscopical examination usually shows a well-circumscribed submucosal tumor, sometimes with central

“umbilication”, covered with normal mucosa. Therefore, the surface biopsy results are usually inconclusive, and the final diagnosis is based on histological verification after surgery or endoscopic submucosal resection/dissection^[2]. Lymphoma, carcinoid, gastrointestinal stromal tumors, as well as some other abnormalities within the GIT, should be a part of the differential diagnosis, and available imaging techniques, including CT or MR scanning, and EUS enhanced with fine needle aspiration, allow the selection of the best resection technique^[10]. It is clear that a symptomatic heterotopic pancreas should be resected, irrespective of the method chosen; however, clear recommendations for the management of asymptomatic and histologically verified lesions have not been established to date.

In the presented case, the first diagnosis based on the endoscopic view was typical for patients with GERD-“gastroesophageal junction hyperplastic (inflammatory) polyp”; however, the histology result was very surprising, since data on the occurrence of pancreatic ectopia on the surface of the gastric mucosa in gastric cardia are lacking. In such a case, one could expect pancreatic metaplasia in the gastric mucosa, which is sometimes seen in, *e.g.*, the gastric patch^[11]; however, the histological examination revealed pancreatic ectopia. The polyp was completely and safely removed, and several further endoscopical controls did not show any relapse.

In summary, although an ectopic pancreas is relatively rare, it is always a part of differential diagnosis when dealing with submucosal or polyp-like gastric lesions. On the basis of the presented case, we suggest, that even small, flat and/or untypically located lesions (*e.g.*, gastric cardia) should also be considered to be formed from the ectopic pancreatic mass.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

A 73-year-old female with a history of recurrent epigastric pain and heartburn.

Clinical diagnosis

Ectopic pancreatic lesion located in the gastric cardia.

Differential diagnosis

Hyperplastic polyp, gastric adenoma, fundic gland polyp, gastrointestinal stromal tumor (GIST), lymphoma.

Laboratory diagnosis

Blood morphology, metabolic panel and liver function tests were within normal limits.

Imaging diagnosis

Esophagogastroduodenoscopy revealed inflammatory changes throughout the

stomach and lower esophagus, as well as a flat polypoid mass with benign features located in the gastric cardia.

Pathological diagnosis

Histology revealed heterotopic pancreatic tissue in the cardiac mucosa with fully developed acini.

Treatment

Endoscopic biopsy forceps removed the lesion.

Related reports

The diagnosis may be complex because of the gross similarity of pancreatic heterotopia with GIST, carcinoid, lymphoma, adenoma or even gastric carcinoma.

Experiences and lessons

The presented case report suggests that even small, flat and/or untypically located lesions (*e.g.*, cardia) should also be considered to be formed from the pancreatic ectopic mass.

Peer review

This article describes a pancreatic ectopia mimicking a flat polyp, atypically located in the gastric cardia.

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IgG4-related disease manifesting as an acute gastric-pericardial fistula

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Abstract

IgG4-related disease is a recently recognized entity linked initially to autoimmune pancreatitis and has been subsequently described in nearly every organ system. Men over the age of 50 represent the most affected demographic group and a comprehensive set of diagnostic criteria has been developed to aid treating clinicians. Though elevated levels of IgG4 in the serum are suggestive of the disease, definitive diagnosis is made on histopathology. Treatment is tailored to the clinical presentation with corticosteroid therapy known to have proven efficacy. Gastric manifestations of the IgG4-related disease primarily come in two varieties, notably chronic ulceration or pseudotumor formation. Autoimmune pancreatitis conveys increased risk for IgG4-related disease of the stomach, which is independent of *Helicobacter pylori* status. In this case report, we present an acute gastric-pericardial fistula secondary to IgG4-related disease that required urgent operative management. To our knowledge, this is the first report in the medical literature describing this complication of IgG4-related disease.

Key words: IgG4-related disease; Autoimmune pancreatitis; Gastric ulcer

Core tip: IgG4-related disease has been an increasingly recognized entity affecting multiple organ systems. Lesions may mimic neoplasms, yet corticosteroid therapy is highly efficacious. In the stomach, manifestations include ulceration and pseudotumor formation. This case report describing a complication of the disease, notably an acute gastric-pericardial fistula has yet to have been described in the medical literature.

Frydman J, Grunner S, Kluger Y. IgG4-related disease manifesting as an acute gastric-pericardial fistula. *World J Gastroenterol* 2014; 20(44): 16782-16785 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16782.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16782>

INTRODUCTION

IgG4-related disease was first recognized as a new clinicopathological entity by Kamisawa *et al*^[1] in 2003 when extrapancreatic manifestations were diagnosed with higher frequency in patients with autoimmune pancreatitis (AIP). Multiple organ systems are now known to be affected by the disease, including gastrointestinal, hepatobiliary, pulmonary, genitourinary, cardiovascular, lymphatic, skin, salivary, endocrine and central nervous system^[1-9]. Furthermore, with respect to gastric disease, autoimmune pancreatitis is a risk factor for high prevalence of chronic gastric ulceration, which is independent of *Helicobacter pylori* (*H. pylori*) infection status^[10,11]. In this case report, we describe a patient who underwent urgent repair of a gastric-pericardial fistula secondary to chronic gastric ulceration by a dense lymphoplasmacytic infiltrate of IgG4-positive plasma cells. To our knowledge, this complication of IgG4-related disease has not yet been described in the medical literature.

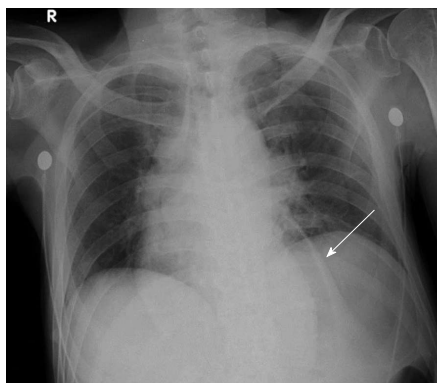


Figure 1 Chest radiograph. Arrow demonstrates air outlining cardiac silhouette. R: Right.

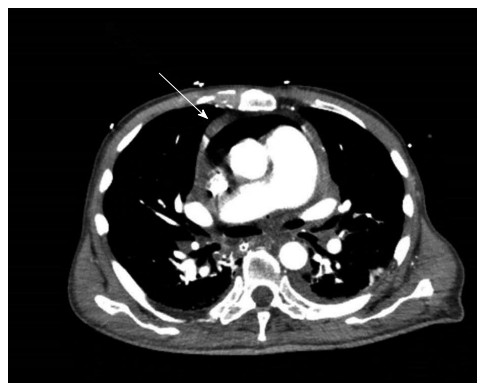


Figure 2 Computed tomography axial section at thoracic level revealing pneumopericardium, as denoted by arrow.

CASE REPORT

The patient is a 65 years old male with a history of tobacco abuse and poor hygiene, who three years prior to the current presentation underwent an emergent antrectomy with Roux-en-Y reconstruction secondary to massive bleeding from erosion of a posterior duodenal ulcer into the gastroduodenal artery (GDA) and penetration into the pancreatic head. The bleeding was arrested by undersuturing of the GDA in four quadrants, and a tube duodenostomy was utilized to manage the difficult stump. Post-operative course was unremarkable and he was discharged home after 10 d. He was followed by the gastroenterology service with findings of a benign marginal ulcer at the gastro-jejunal anastomosis on endoscopy which was treated conservatively, though the patient was poorly compliant and lost to follow-up.

At this time he presented to the emergency department with a three day history of intense upper abdominal and inter-scapular back pain, associated with fever up to 38.4 °C. The patient appeared anxious and malnourished. Heart rate was regular at 86 bpm with blood pressure of 90/50 mmHg. Abdomen was soft, non-distended without peritoneal signs and there was no melena on rectal exam. Laboratory analysis was significant for leukocytosis ($WBC = 14.4 \times 10^3/\mu L$ with bandemia of 7%), severe anemia ($Hgb = 6.7$ g/dL) and renal insufficiency ($Cr = 1.5$ mg/dL). He was found to have a metabolic acidosis ($pH = 7.33$, $Lactate = 9.0$ mmol/L), elevated troponin-I (0.3 ng/mL) and nutritional depletion (albumin = 2.2 g/dL). Diagnostic work-up included an abdominal ultrasound which was negative for an abdominal aortic aneurysm or free peritoneal fluid. Chest X-ray was remarkable for pneumopericardium (Figure 1). Computed tomography (CT) of the chest demonstrated a large pneumopericardium (Figure 2) as well as pneumomediastinum, whose source was a possible fistula emanating from the upper GI tract below the diaphragm (Figure 3). Upper gastrointestinal endoscopy (performed without air insufflation secondary to risk of cardiac tamponade) confirmed a large, necrotic gastric ulcer emanating from the cardia with fistulization toward the pericardium.

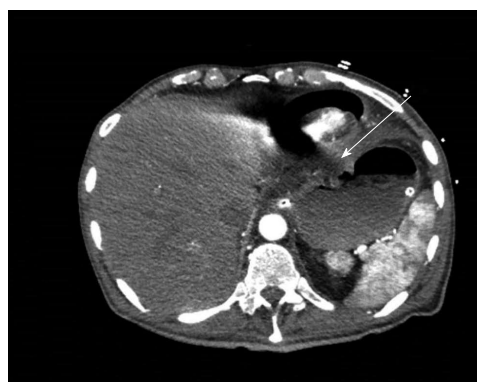


Figure 3 Computed tomography axial section at upper abdominal level revealing the presence of a gastric-pericardial fistula, as denoted by arrow.

After a short period of fluid resuscitation, the patient was brought to the operating room for an exploratory laparotomy. An inflammatory mass was found in the stomach remnant with evidence of fistulization via the diaphragm into the pericardium. A completion gastrectomy was performed with Roux-en-Y esophagojejunostomy and the pericardium was drained. The post-operative course was complicated by respiratory failure requiring percutaneous tracheostomy, as well as treatment for pneumonia and pulmonary embolus. Final pathology revealed a chronic gastric ulcer with extensive fibrosis and chronic inflammatory changes. On immunohistochemistry, multiple IgG4 positive plasma cells were scattered in the fibrous stroma with an IgG4:IgG ratio > 40%. Furthermore, serum IgG4 was elevated at 620 mg/dL [normal range (NR): 8-140 mg/dL] with total IgG 1350 mg/dL (NR: 680-1560 mg/dL). The patient was initiated on corticosteroid therapy and ultimately discharged to a rehabilitation facility. On clinical follow-up, the patient's overall health improved significantly, with weight gain and return to his normal daily activities.

DISCUSSION

Stemming from a histopathological study of organs in patients with AIP, the pancreatic lesion was found to be a

manifestation of a systemic process, notably a new clinicopathological entity known as IgG4-related disease^[1]. It has since been described in multiple succeeding reports in nearly every organ system^[1-9]. From epidemiologic studies, the majority of patients affected by IgG4-related disease are men over the age of 50, although the true prevalence of the disease is likely underestimated secondary to the lack of familiarity with this disease entity^[12]. Indeed, the diagnosis of IgG4-related disease must be considered in the differential diagnosis where appropriate, as the clinical and radiographic characteristics may mimic neoplasm, resulting in unnecessary surgical resection^[13]. In order to facilitate the diagnosis, a comprehensive set of clinical diagnostic criteria has been devised by the Ministry of Health, Labor, and Welfare of Japan: a characteristic diffuse or localized swelling or mass in single or multiple organs with elevated IgG4 serum levels, or distinctive pathological findings, notably tumefactive lesions with a dense lymphoplasmacytic infiltrate rich in IgG4-positive plasma cells and storiform fibrosis^[12,13].

Gastric manifestations from this pathological basis are two-fold, either chronic ulceration or pseudotumor formation. Shinji *et al*^[11] evaluated EGD findings in patients undergoing ERCP for obstructive jaundice. Compared to the control group, patients with AIP were more likely to have gastric ulcer (34.8% *vs* 13.5%, *P* = 0.007). In addition, the gastric ulcers in the AIP group were more likely to be located on the lesser curvature with linear appearance perpendicular to the incisura angularis.

Two recent reports have illustrated the potential for chronic gastric ulceration^[14,15]. Fujita *et al*^[14], describe a patient with a chronic gastric ulcer which failed to heal after standard treatment regimens including proton pump inhibitor therapy and *H. pylori* eradication. Biopsy revealed the presence of an intense infiltration of plasma cells containing IgG4, though the patient subsequently refused corticosteroid treatment. Similarly, Bateman *et al*^[15], report a patient who underwent a partial gastrectomy for a chronic gastric ulcer due to concern for occult malignancy in which final pathology yielded the causative factor as IgG4-related disease.

With respect to pseudotumor formation, multiple reports have documented the etiology of IgG4-related disease with findings of either multiple gastric polyps^[16], or more commonly solitary sclerosing nodular lesions of the stomach^[17-19]. The diagnosis of IgG4-related disease in these cases was secured on final pathology after laparoscopic wedge resections^[17,18] and endoscopic submucosal resection^[19]. It is speculative whether these lesions would have responded completely to corticosteroid therapy had the causative factor been elucidated preoperatively.

In the current report, we describe a novel presentation of IgG4-related disease involving the stomach, notably a chronic gastric ulcer which fistulized to the pericardium requiring urgent operative management. The final pathology met diagnostic criteria for IgG4-related disease and elevated serum level of IgG4 was confirmatory. Given the increased recognition of this disease entity in

the stomach, early diagnosis based on the typical clinical, serological and histological findings, followed by treatment with corticosteroid therapy may ameliorate gastric complications of IgG4 related-disease.

COMMENTS

Case characteristics

Authors report a case of a 65-year-old man diagnosed with an acute pneumopericardium secondary to a gastric-pericardial fistula, ultimately attributed to IgG4-related disease of the stomach.

Clinical diagnosis

The patient presented with 3 d history of intense interscapular and upper abdominal pain associated with fever.

Differential diagnosis

Differential diagnosis based on clinical presentation is broad, including, but not limited to, acute myocardial infarction, dissecting thoracic aneurysm, pneumothorax, esophageal perforation, strangulated paraesophageal hernia, mesenteric ischemia and perforated peptic ulcer.

Laboratory diagnosis

Initial laboratory findings included leukocytosis with bandemia, anemia, elevated troponin, prerenal azotemia and metabolic acidosis, with later significantly elevated serum level of IgG4.

Imaging diagnosis

Chest X-ray demonstrated pneumopericardium, computed tomography suggested a gastric-pericardial fistula and upper endoscopy revealed gastric ulceration with fistulization from the cardia toward the pericardium.

Pathological diagnosis

Immunohistochemistry revealed a dense lymphoplasmacytic infiltrate rich in IgG4 plasma cells with an IgG4: IgG ratio greater than 40%.

Treatment

Given the acute presentation in this case, an emergent gastrectomy was required, though the vast majority of IgG4-related disease is treated effectively by corticosteroid therapy.

Experiences and lessons

IgG4-related disease is an increasingly recognized entity, requiring a high index of suspicion by the treating clinician to make the diagnosis based on typical clinical, serological and histological findings.

Peer review

The authors present a novel case of IgG4-related disease of the stomach causing an acute gastric-pericardial fistula requiring surgical treatment, although the vast majority of chronic IgG4-related gastric ulcers will heal with corticosteroid therapy.

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E- Editor: Wang CH



Duodenum-preserving resection and Roux-en-Y pancreatic jejunosomy in benign pancreatic head tumors

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Author contributions: Xiu DR and Zhang TL designed the report; Yuan CH and Tao M collected the patient's clinical data; Jia YM and Xiong JW carried out the immunoassays; Yuan CH and Zhang TL wrote the paper.

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no deaths or complications observed during the perioperative period. All patients had no signs of recurrence of the BTPH within a follow-up period of 48-76 mo and had good quality of life without diabetes. Partial pancreatic head resection with Roux-en-Y pancreatic jejunosomy is feasible in selected patients with BTPH.

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Key words: Pancreatic benign tumor; Pancreatic head; Partial resection; Roux-en-Y pancreatic jejunosomy; Postoperative complications

Core tip: This study elucidated an innovative technique for local pancreatic head resection and Roux-en-Y pancreatic jejunosomy in four patients with benign tumors of the pancreatic head and showed that local resection of the pancreatic head in combination with Roux-en-Y pancreatic jejunosomy not only completely resected the pancreatic tumor but also retained optimal pancreatic function and reduced the incidence of pancreatic leakage.

Yuan CH, Tao M, Jia YM, Xiong JW, Zhang TL, Xiu DR. Duodenum-preserving resection and Roux-en-Y pancreatic jejunosomy in benign pancreatic head tumors. *World J Gastroenterol* 2014; 20(44): 16786-16792 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i44/16786.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i44.16786>

Abstract

This study was conducted to explore the feasibility of partial pancreatic head resection and Roux-en-Y pancreatic jejunosomy for the treatment of benign tumors of the pancreatic head (BTPH). From November 2006 to February 2009, four patients (three female and one male) with a mean age of 34.3 years (range: 21-48 years) underwent partial pancreatic head resection and Roux-en-Y pancreatic jejunosomy for the treatment of BTPH (diameters of 3.2-4.5 cm) using small incisions (5.1-7.2 cm). Preoperative symptoms include one case of repeated upper abdominal pain, one case of drowsiness and two cases with no obvious preoperative symptoms. All four surgeries were successfully performed. The mean operative time was 196.8 min (range 165-226 min), and average blood loss was 138.0 mL (range: 82-210 mL). The mean postoperative hospital stay was 7.5 d (range: 7-8 d). In one case, the main pancreatic duct was injured. Pathological examination confirmed that one patient suffered from mucinous cystadenoma, one exhibited insulinoma, and two patients had solid-pseudopapillary neoplasms. There were

INTRODUCTION

Pancreaticoduodenectomy, duodenum-preserving pancreatic head resection, and pancreatic head tumor removal are the main surgical procedures in most cases of benign tumors of the pancreatic head (BTPH) based on specific circumstances of lesions and the experience level of each surgeon. In recent years, the surgical removal

of pancreatic head tumors and duodenum-preserving pancreatic head resection has been gradually replacing the traditional approach of pancreaticoduodenectomy^[1,2]. The aim of the present study was to elucidate an innovative technique for partial pancreatic head resection and Roux-en-Y pancreatic jejunostomy used in four patients with BTPH. Furthermore, the recurrence of disease and post-operative quality of life were observed in a follow-up exam.

CASE REPORT

Case 1

A 31-year-old female was routinely examined after she was admitted to the hospital and diagnosed with a pancreatic tumor by B-ultrasonic examination. The B-ultrasonic wave images revealed a hypoechoic mass at 3.3 cm × 2.8 cm in the pancreatic head. In association with a computed tomography (CT) scan, a low density mass at 3.5 cm × 3.2 cm was detected in the pancreatic head. The tumor displayed an ill-defined borderline and striped calcified lesions (Figure 1A). Preoperative examination showed that the serum tumor markers alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and carbohydrate antigen (CA)199 were within normal ranges. However, both intraoperative frozen section analysis and pathological examination confirmed the presence of a solid pseudopapillary tumor of the pancreas (Figure 1B). The patient underwent partial pancreatic head resection and Roux-en-Y pancreaticojejunostomy (Figure 1C and D). The wound surface area of the pancreas was 6.3 cm × 6.5 cm. Because the tumor was very close to the main pancreatic duct, approximately 1.5 cm of the duct was removed. The proximal pancreatic end of the main pancreatic duct was ligated, and a stent was placed in the distal duct. The operation was performed in 210 min, and the amount of intraoperative blood loss was 155 mL. The patient had a smooth, uncomplicated postoperative recovery, started taking oral food on day 3 and was discharged on day 7 after the surgery. There was no recurrence of the tumor observed during the follow-up period of 48-76 mo. Moreover, quality of life for this case was satisfactory, and fasting blood glucose levels were normal.

Case 2

A 21-year-old female was diagnosed with a pancreatic tumor in a B-ultrasonic examination after her hospital stay. Contrast-enhanced abdominal CT scan of the upper abdomen showed oval-shaped lesions in the uncinate process of the pancreas with a diameter of approximately 4.2 cm, and an uneven density was significantly strengthened around the enhanced loci (Figure 2A). Preoperative examination showed that the serum tumor markers AFP, CEA, and CA199 were within the normal range. In the surgical exploration, the size of the uncinate process of the pancreas was 3.6 cm × 3.5 cm (Figure 2B). The patient underwent partial pancreatic head resection and Roux-en-Y pancreatic jejunostomy (Figure 2C and D).

The wound surface area of the pancreas was 7.0 cm × 6.5 cm. The operation was performed in 226 min, and the amount of intraoperative blood loss was 210 mL. A solid pseudopapillary tumor of the pancreas was confirmed in postoperative pathology outcomes. The patient had a smooth postoperative recovery with no complications, started taking oral food on day 3, and was discharged on day 8 after the surgery. Recurrence of the tumor was not observed during the follow-up period of 54 mo. Moreover, a good quality of life was associated with fasting blood glucose in the normal range.

Case 3

A 37 year-old male had symptoms that included intermittent drowsiness, dizziness, malaise, irritability, sweating, slurred speech, and cognitive impairment without any obvious reason for 15 mo prior to a hospital stay. He was diagnosed with carbon monoxide (CO) poisoning in a local hospital, and his blood glucose level reached 0.8 mmol/L. The patient's symptoms were well controlled after treatment. However, the symptoms recurred frequently, and he was admitted to our hospital for further treatment. Levels of fasting glucose, serum insulin and C-peptide were shown as 1.9 mmol/L, 83.5 mIU/L, and 1140.4 pmol/L, respectively. Enhanced abdominal CT scan of the upper abdomen showed oval-shaped lesions in the uncinate process of the pancreas with a diameter of approximately 3.3 cm, whereas the density around the enhanced loci was significantly strengthened (Figure 3A). During the surgical procedure, ultrasound was used to identify the uncinate process of the pancreas (Figure 3B). The patient underwent partial pancreatic head resection of the tumor (3.2 cm × 3.5 cm) (Figure 3C) and Roux-en-Y pancreaticojejunostomy (Figure 3D). The wound surface area of the pancreas was 5.2 cm × 5.0 cm. Insulinoma was diagnosed intraoperatively by a postsurgical pathology examination. The duration of the surgery was 186 min, and the amount of intraoperative blood loss was 105 mL. The patient had a smooth postoperative recovery with no pancreatic leakage. The serum levels of his fasting glucose, insulin, and C-peptide were 5.5 mmol/L, 12.5 mIU/L, and 320.8 pmol/L, respectively, on day 7, and he was discharged on day 8 after surgery. No recurrence of the tumor was observed during the postoperative follow-up period of 50 mo. The patient maintained good quality of life, and fasting blood glucose, insulin, and C-peptide were within the normal range.

Case 4

A 48-year-old female had a one month history of intermittent abdominal pain before her visit. Ultrasonic evaluation showed "low or no echo placeholder beneath the right bottom of the pancreas." Enhanced abdominal CT scan (Figure 4A) and enhanced magnetic resonance imaging (MRI) indicated the uncinate process of the multilocular cystic placeholder (diameter of 4.3 cm). Preoperative examination showed that the serum tumor markers AFP, CEA, and CA199 were within the normal range. In the

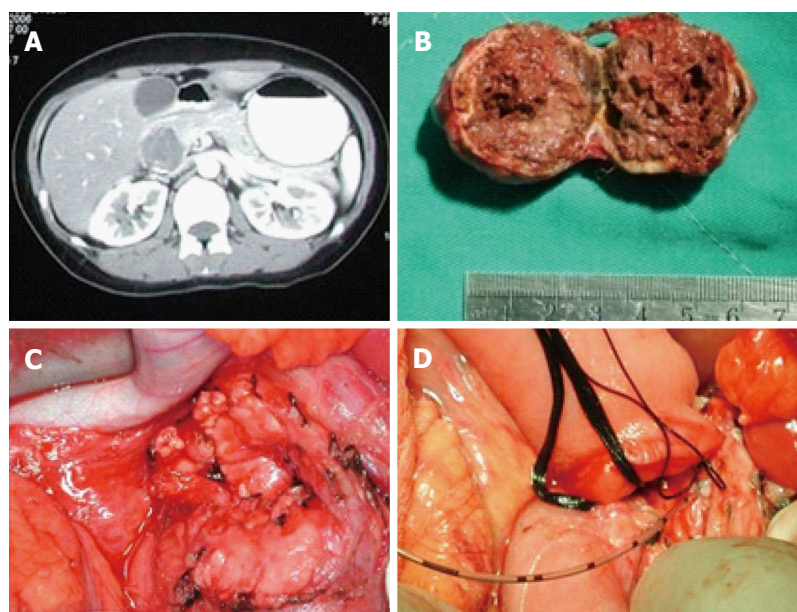


Figure 1 B-ultrasonic examination and computed tomography scan of case 1. A: Enhanced computed tomography image of BTPH shows a low density in the pancreatic head mass at 3.5 cm × 3.2 cm. The tumor displayed ill-defined borderline in calcified lesions; B: Shape and structure of BTPH. The tumor is an oval shape and some necrotic tissue existed inside the tumor; C: Wound surface area of the pancreas is 6.3 cm × 6.5 cm after the tumor removed; D: Illustration for operative repair with Roux-en-Y pancreaticojejunostomy is shown with proximal pancreatic end ligated and a stent is put into distal pancreatic resection. BTPH: Benign tumors of the pancreatic head.

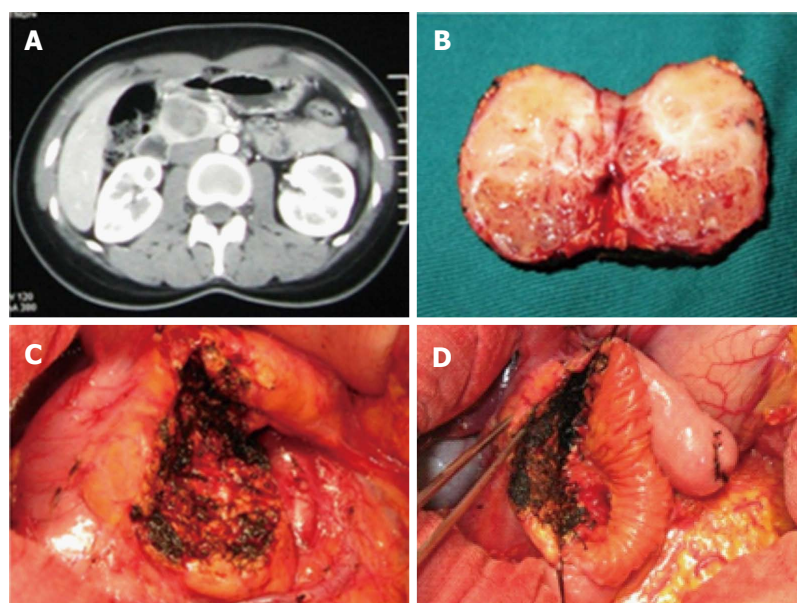


Figure 2 B-ultrasonic examination and computed tomography scan of case 2. A: Enhanced computed tomography image image of BTPH shows oval-shaped lesions at approximately 4.2 cm in diameter with significantly uneven density around the enhanced loci; B: The uncinate process carcinoma of the pancreas is a solid mass with an uneven texture in area of 3.6 cm × 3.5 cm; C: The wound surface area of the pancreas is measured at 7.0 cm × 6.5 cm after the surgery; D: Illustration for wound repair with Roux-en-Y pancreaticojejunostomy. BTPH: Benign tumors of the pancreatic head.

surgical exploration, the uncinate process of the cystic placeholder was measured at 4.5 cm × 4.8 cm (Figure 4B). The patient underwent partial pancreatic head resection and Roux-en-Y pancreaticojejunostomy (Figure 4C and D). The wound surface area of the pancreas was 7.2 cm × 6.1 cm. Pancreatic mucinous cystic adenoma was confirmed in intraoperative and postsurgical pathological examination. The operation was performed in 165 min, and the amount of intraoperative blood loss was 82 mL. The patient had a smooth postoperative recovery with no complications, and was discharged on day 7 after the surgery. No return of the tumor was observed during the postoperative follow-up period of 48 mo, and her upper abdominal pain symptoms disappeared. The patient maintained good quality of life, and fasting plasma glucose was within the normal range.

This project focuses on four patients who underwent pancreatic head resection and Roux-en-Y pancreaticojeju-

nostomy (Table 1). This is a retrospective study approved by the Peking University Third Hospital Institutional Review Board. The surgical technical details and outcomes were analyzed. All operations were performed using a supraumbilical transverse incision under general anesthesia. First, a paramedian incision was made on the upper right side of the abdomen. Isolation and exposure: The gastrosplenic and duodenum ligaments were slit to expose the pancreas. The duodenum and the pancreatic head region were found. The side peritoneum from the duodenal bulb to the descending part was isolated from the inferior vena cava. The tumor was detected by preoperative enhanced CT/MRI, intraoperative exploration, and ultrasound when necessary. The tumor was carefully isolated from normal pancreatic tissue to avoid injury or ligation of the common bile duct and the pancreatic duct. Specifically, with preserving the right gastroepiploic artery, the anterosuperior pancreaticoduodenal artery was identified

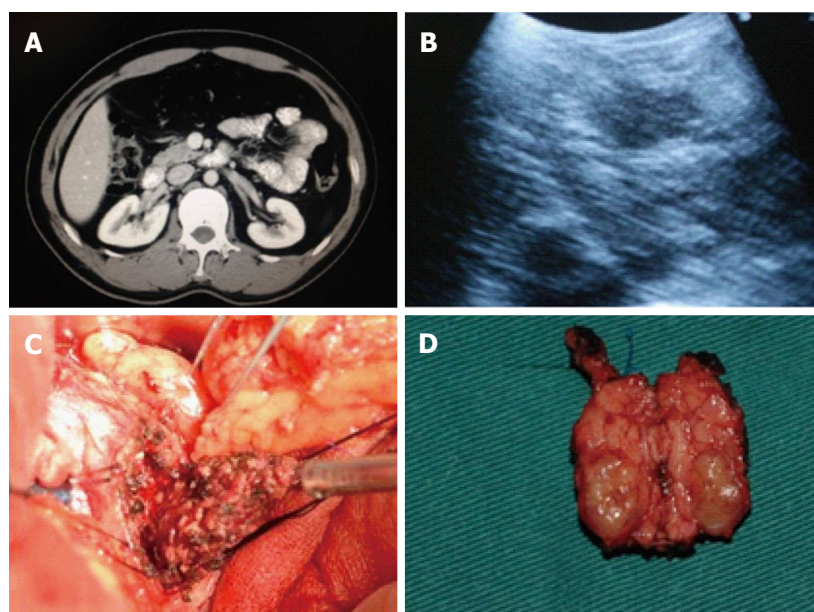


Figure 3 B-ultrasonic examination and computed tomography scan of case 3. A: Enhanced computed tomography image of BTPH shows oval solid lesions measuring 3.3 cm in diameter in the uncinate process. BTPH had significantly strengthened density around the enhanced loci; B: The uncinate process carcinoma of the pancreas is solid texture with low density in area of 3.6 cm × 3.5 cm; C: The uncinate process carcinoma of pancreas is a solid mass with uneven texture in area of 3.2 cm × 3.5 cm; D: Pancreaticojejunostomy Roux-en-Y anastomosis shows wound surface area measuring 5.2 cm × 5.0 cm. BTPH: Benign tumors of the pancreatic head.

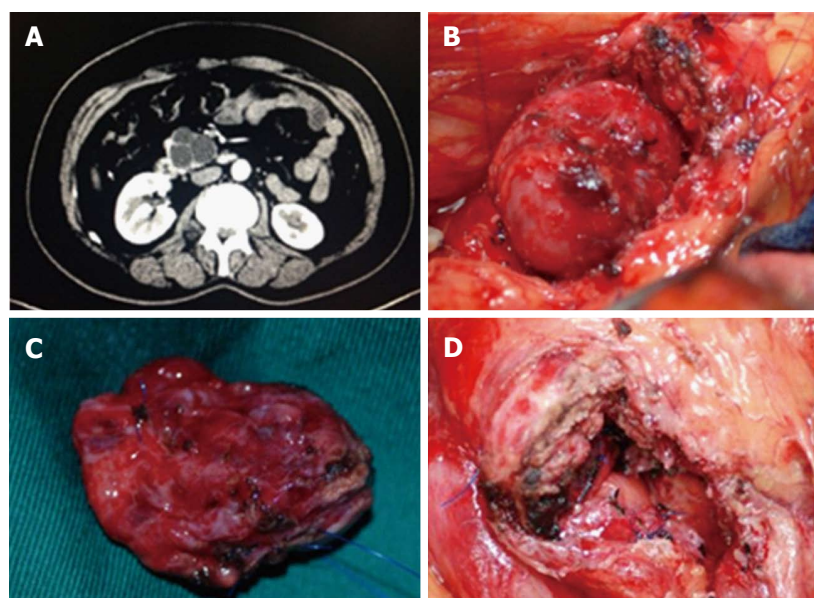


Figure 4 B-ultrasonic examination and computed tomography scan of case 4. A: Enhanced computed tomography image of BTPH Uncinate process of multilocular cystic placeholder measured at 4.3 cm in a diameter; B: The tumor mass is carefully isolated along its edge; C: The uncinate process of multilocular cystic placeholder detected at 4.5 cm × 4.8 cm; D: After the resection of pancreatic head tumor, the wound surface area of the pancreas measured at 7.2 cm × 6.1 cm. BTPH: Benign tumors of the pancreatic head.

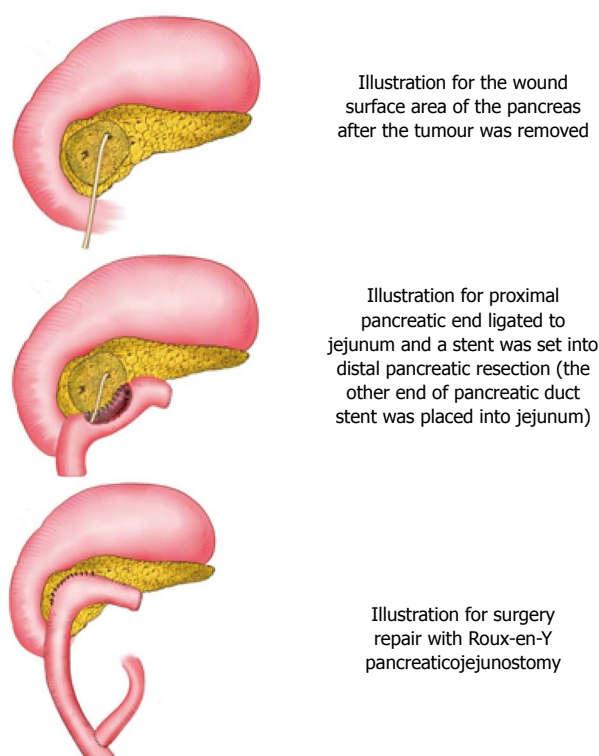
and divided. The origin of the posterior superior pancreaticoduodenal artery was identified and the attached pancreatic tissues were separated downward, preserving the vessels. Leaving both the posterior superior pancreaticoduodenal artery and common bile duct intact, the pancreatic tissues surrounding the common bile duct and intervening between the posterior superior pancreaticoduodenal artery and the common bile duct were carefully dissected. The tumor within the head of the pancreas, as well as some pancreatic tissue in the pancreatic head, was completely removed from the tightly attached papillary area of the second portion of the duodenum, and the tumor tissue was prepared for frozen section biopsy to confirm the benign tumor. Pancreaticoduodenectomy would have been carried out if a malignant tumor was proven. Further management of surgical-site bleeding: If the tumor was close to the main pancreatic duct and

the pancreatic duct was clearly injured, we had to carry out ligation of the proximal pancreatic duct and set up an internal stent at the distal position (subject to Roux-en-Y pancreatic jejunostomy). If there was no injury found in the main pancreatic duct, Roux-en-Y pancreatic jejunostomy was directly performed. Roux-en-Y pancreatic jejunostomy: We cut off the jejunum at the location 15 cm away from Treitz ligament, pulled the distal end to the pancreas through the rear of the colon, sutured the transected end, and constructed side-to-side anastomosis of the pancreas wound with the jejunum (pancreatic duct stent placed into jejunum). Then, end-to-side anastomosis was performed between the transected ends of jejunum and the location of approximately 40 cm from the anastomotic stoma, and a drainage tube was placed under the pancreatic anastomotic stoma. Figure 5 is the schematic sketch of some key steps.

Table 1 Demographic data of the cases

	Sex	Age (yr)	Symptoms	Tests	Preoperative diagnosis	Operation time (min)	Blood loss (mL)	Postoperative hospital stay (d)	Pathology	Size (cm)
Case 1	F	31	Incidental	US CT	SPT	210	155	7	SPT	3.5
Case 2	F	21	Incidental	US CT	SPT or MCN	226	210	8	SPT	3.6
Case 3	M	37	Hypoglycemia	US CT	Insulinoma	186	105	8	Insulinoma	3.5
Case 4	F	48	Abdominal pain	US CT MRI	MCN	165	82	7	MCN	4.8
Mean		34.3				196.8	138.0	7.5		3.85
(range)		(21-48)				(165-226)	(82-210)	(7-8)		(3.5-4.8)

SPT: Solid pseudopapillary tumor; MCN: Mucinous cystic neoplasam; US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging.

**Figure 5** Schematic sketch of some key steps.

DISCUSSION

BTPH includes pancreatic exocrine tumors (serous cystic adenoma, mucinous cystadenoma, intraductal papillary mucinous tumor, and solid pseudopapillary tumor) and endocrine tumors (insulinoma, pancreatic gastrinoma, and non-function neuroendocrine tumors)^[5]. With the development of diagnostic imaging techniques, many pancreatic lesions now can be confirmed as benign lesions before surgery^[4,5]. It is the continual pursuit of the surgeon to maximize the integration of the pancreas, gastrointestinal tract, and biliary tract organs to facilitate the functional recovery and preservation of the organ while regulating a balance between tumor removal and injury control^[6]. In patient selection for specific surgical procedures, the surgeon should conduct a comprehensive assessment based on local changes around the lesion with its systemic response and based on the patients' understanding of their family history of the disease^[7]. Because

pathological types of pancreatic lesions are diverse and complex, it may be effective to make a decision on organ preservation when a surgery is applied to a patient. Currently, the main procedures for treating BTPH include pancreaticoduodenectomy, duodenum-preserving pancreatic head resection, and surgical excision of pancreatic cancer^[8].

The main surgical approach for pancreatic head tumors has been pancreaticoduodenectomy. Because pancreaticoduodenectomy has a high incidence of complications, organ damage, and loss of exocrine and endocrine pancreatic function, it is controversial as to whether pancreaticoduodenectomy should be conducted for low-grade malignant lesions and benign lesions^[9]. The surgical excision of pancreatic cancer is suitable for benign tumors growing outside of the pancreas with a small tumor bed and the ability to avoid injury of the main pancreatic duct during the removal process. Kiely *et al*^[10] and Crippa *et al*^[2] conducted enucleation of benign tumors of the pancreas in 11 and 61 patients, and the incidence of postoperative pancreatic fistula was 27% and 38%, respectively^[10]. The postoperative incidence of pancreatic leakage in the surgical excision of pancreatic cancer was 10% higher than in pancreaticoduodenectomy^[6]. The following presents the main reasons for a higher incidence of pancreatic leakage remaining in the surgical excision: (1) tumor excision from the deep pancreatic parenchyma, particularly for a tumor of the dorsal pancreas, which can easily injure the main pancreatic duct; (2) an unnoticed injury of the duct after pancreatic ductal ligation; (3) the vice pancreatic duct course varied greatly, which was susceptible to traumatic injury; (4) partial resection of the tumor resulted in a relatively recessed pancreatic section base, which hinders a clear vision of the small pancreatic duct section with possible ligation completed, easily leading to postoperative pancreatic leakage; and (5) the tumor subjected to resection is normally located in the periphery of the pancreatic tissue and does not block the main pancreatic duct. The pancreas is not susceptible to chronic inflammation, but the pancreas and pancreatic duct are soft and can rupture or tear. This was not conducive to achieving healing of the transected ends with wound closure.

How to control the extent of surgical injury and reduce the incidence of pancreatic defects and leakage has been a pressing issue for this surgical treatment^[11].

To maintain the advantages of surgical excision of the pancreatic cancer as well as to reduce the incidence of pancreatic leakage, we conducted partial pancreatic head resection and pancreatic Roux-en-Y anastomosis reconstruction for the selected cases. We summarized the indications for the surgery with the following causes: (1) tumor size of 3-5 cm; (2) large surface area wound highly susceptible to pancreatic leakage after partial resection; and (3) clear damage during surgery, the proximal pancreatic duct was ligated, and a stent needed to be placed in the distal pancreatic duct by pancreaticojejunostomy. In some cases, although there was not an explicit statement on pancreatic injury, the tumor was close to the pancreatic duct; therefore, we could not eliminate the possibility that the pancreatic duct was damaged. The modified operation completely removed the lesion in the tissue and reconstructed the digestive tract with Roux-en-Y pancreatic jejunostomy, which may simplify the surgical procedures and reduce postoperative complications. Prior to surgery, a field lesion assessment should be performed using detailed images, especially with an enhanced CT scan, and an appropriate approach of surgical treatment should be chosen after judging the relationship of the lesions between the main pancreatic duct and the surrounding vessels. The diagnosis must be clearly made. The analysis on quick frozen section was performed when it was difficult to determine the nature of the lesions. If a malignant tumor was demonstrated, radical dissection would have been chosen^[12]. For instance, special attention should be paid to prevent the ligation of the main pancreatic duct during the local excision of tumors; otherwise, postoperative complications will be increased. In the process of tumor removal, we did not over-free the duodenum and tissues at the rear of the pancreas to prevent pancreatic penetration, which may increase surgical risk and even failure.

In this retrospective study, four cases received local pancreatic resection and surgical wound repair with Roux-en-Y pancreatic jejunostomy. We found that the procedure had the following benefits: preservation of organ function; no injury of the biliary system; intact stomach and duodenum; precise dissection of the pancreas; and maximization of the retention of a healthy pancreas, thereby avoiding or reducing the risk of postoperative diabetes. The four patients did not suffer from postoperative diabetes or pancreatic leakage. The injury was under control, thus reducing or avoiding pancreatic leakage caused by pancreatic duct injury in the process of local tumor resection. The drainage channel from the pancreas into the digestive tract was rebuilt by precise and exact pancreaticojejunostomy; therefore, the impaired pancreatic exocrine function was restored. It was not necessary to reconstruct the biliary system to avoid bile leakage. All of these factors may improve surgical procedure safety and good preservation and injury control of the pancreas.

The authors suggested that the local resection of the pancreatic head and the surgical wound repair with Roux-

en-Y pancreatic jejunostomy may be beneficial in reducing complications; these benefits should be recognized, and the main pancreatic duct should be protected during surgery. The intraoperative detection of tumors using B-ultrasonic scans can be performed when necessary. It was necessary to keep the tumor border 2-3 mm away from the main pancreatic duct to ensure a clean cutting edge without main pancreatic duct injury^[13]. Tumor size was not an absolute indication for local excision procedure. Instead, tumor location, the size of its base section, and the relationship between the tumor and the main pancreatic duct are important factors required for the consideration of local excision^[14]. During the surgery, the injured blood vessels or small pancreatic duct should be ligated or sutured with appropriate management of the pancreatic trauma. If there is a clear pancreatic duct injury, ligation should be performed at the proximal end and a stent should be placed at the distal end of the pancreatic duct. If B-ultrasonic images examination for preoperative assessment of tumor location show that the tumor is close to the pancreatic duct, surgery should be managed by stent implantation with nasal endoscopy. This technique was helpful not only to search for the pancreatic duct in the surgery but also to reduce pancreatic injury and to examine whether there was any pancreatic duct injury. However, this operation was invasive, with an increased risk of pancreatitis; therefore, risk and benefits must be fully considered before choosing this method.

In summary, our study indicates that a 3-5 cm BTPH with a large pancreatic wound after local resection existed at a high risk of pancreatic leakage. Local resection of the pancreatic head in combination with Roux-en-Y pancreatic jejunostomy not only completely resected the pancreatic tumor but also retained optimal pancreatic function and reduced the incidence of pancreatic leakage.

COMMENTS

Case characteristics

Four patients (three female and one male) with a mean age of 34.3 years presented with benign tumors of the pancreatic head with diameters of 3.2-4.5 cm.

Clinical diagnosis

Benign tumor of the pancreatic head.

Differential diagnosis

CO poisoning.

Laboratory diagnosis

While the preoperative serum tumor markers alpha-fetoprotein, carcinoembryonic antigen, and carbohydrate antigen 199 were within the normal ranges in case 1, case 2 and case 4, the levels of fasting glucose, serum insulin and C-peptide of case 3 were shown as 1.9 mmol/L, 83.5 mIU/L and 1140.4 pmol/L, respectively.

Imaging diagnosis

B-ultrasonic examination, computed tomography scan, and enhanced magnetic resonance imaging were used to assist the diagnosis of benign tumors of the pancreatic head in the 4 cases.

Pathological diagnosis

Intraoperative frozen section analysis and pathological examination confirmed the presence of solid pseudopapillary tumors of the pancreas in case 2.

Treatment

The 4 patients were cured by partial pancreatic head resection in combination with Roux-en-Y pancreaticojejunostomy.

Related reports

How to maintain the advantages of surgical excision of the pancreatic cancer as well as to effectively reduce the incidence of pancreatic leakage is a pressing issue that is unclear for this surgical treatment.

Term explanation

Benign tumors of the pancreatic head include exocrine tumors (serous cystic adenoma, mucinous cystadenoma, intraductal papillary mucinous tumor, and solid pseudopapillary tumor) and endocrine tumors (insulinoma, pancreatic gastrinoma, and non-function neuroendocrine tumors).

Experiences and lessons

This case report indicates that the local resection of the pancreatic head in combination with Roux-en-Y pancreatic jejunostomy not only completely resected the pancreatic tumor but also retained optimal pancreatic function and reduced the incidence of pancreatic leakage.

Peer review

In this manuscript, the authors report 4 cases of duodenum-preserving pancreatic head resection for benign pancreatic lesions. Previous reports on duodenum-preserving pancreatic resections claimed clinical benefits such as less complication rates and shorter hospital stay, low hospital mortality rate of < 1% and superior long-term outcomes.

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Individualized proximal margin for early gastric cancer patients

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Abstract

There is no robust evidence to define a safe proximal margin by distance for early gastric cancer (EGC). The discussion on resection margin should not only focus on the oncologic safety, but also the postgastrectomy quality of life. The distance 1-10 mm is only acceptable for those endoscopic treatment fit EGC patients. For endoscopic unfit EGC cases, if the borderline of tumor is able to be clearly determined intraoperatively, the distance 1-3 cm is recommended for proximal resection margin. If there is any uncertainty on the tumor borderline, the distance 3-5 cm should be considered for proximal margin.

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Key words: Early gastric cancer; Gastrectomy; Margin; Oncologic safety; Quality of life

Core tip: There is no robust evidence to define a safe proximal margin by distance for early gastric cancer (EGC). The distance 1-10 mm is only acceptable for those endoscopic treatment fit EGC patients. For endoscopic unfit EGC cases, if the borderline of tumor is able

to be clearly determined intraoperatively, the distance 1-3 cm is recommended. If there is any uncertainty on the tumor borderline, the distance 3-5 cm should be considered.

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TO THE EDITOR

We read with great interest the article by Kim *et al*^[1], in which they investigated the oncologic safety of distances from early tumor borderline to resection margin. It is concluded that the distance from proximal gastric margin more than 1 mm is adequate for the oncologic safety in early gastric cancer (EGC). It is really a finding that challenges our current understanding of tumor resection margin. Actually, there is no robust evidence to define a safe margin by distance for EGC by now. Japanese Gastric Cancer Association (JGCA) guideline suggests that a gross resection margin of 2 cm is necessary for T1 diseases^[2]. National Comprehensive Cancer Network guideline still recommends typically no less than 4 cm from gross tumor as adequate resection for T1b diseases^[3].

In general practice, surgeons might think that the recommendation of > 1 mm margin is merely theoretical but not practical. Particularly in China, the proportion of EGC is merely 10%-20%, and most of EGC patients undergo surgical treatment^[4]. Since there is no serosal invasion in all EGC cases, it is impossible to determine the edges of tumor visually. Usually, surgeons require the guide by preoperative endoscopic clipping or straining, and even direct palpation. Therefore, basically, it is hard to mark a resection margin only 1 mm to the tumor based on gross findings, particularly for irregular shaped

tumors. Hence, with regard to oncologic safety, a practical proximal margin should not be just recommended as 1 mm at least.

The discussion on resection margin should not only focus on the oncologic safety, but also the post-gastrectomy quality of life. Commonly, digestive tract reconstruction pattern is considered a principal factor for postgastrectomy quality of life. Besides, we think that the volume of the remnant stomach may be also an influencing factor among patients undergoing subtotal gastrectomy^[5]. A greater margin allows to obtain higher oncologic safety, but correspondingly, a smaller stump volume would impair the postoperative quality of life due to less intake per meal and more severe reflux symptom. In fact, the recurrence rate of EGC is very low, so too great distance of margin seems not “cost-effective”. Therefore, the decision-making on optimal margin must balance concerns of both oncologic safety and quality of life (Table 1).

It is easy to understand that neither < 1 mm nor > 5 cm is suitable for EGC surgery. The distance ranging from 1 to 10 mm is only acceptable for endoscopic treatment [*i.e.*, endoscopic submucosal dissection (ESD)] fit EGC patients. For endoscopic unfit EGC cases, if the borderline of tumor is able to be clearly determined intraoperatively, the distance from 1 to 3 cm is recommended for proximal resection margin. It is helpful to use preoperative endoscopic clipping or straining for guiding the tumor borderline. However, for some particular EGC cases, the tumor borderline is really hard to determine. Therefore, if there is any uncertainty on the tumor borderline, the distance from 3 to 5 cm should be considerable for proximal margin.

Additionally, for the ESD candidates, the oncologic safety not only concerns the distance of resection margin, but also encounters another issue: the risk of lymph node metastasis. Since ESD cannot control lymph node metastasis, ESD-fit cases should be strictly and highly selected. According to the JGCA treatment guideline, the standard indications are (1) cT1a tumor; (2) cN0 status; (3) no more than 20 mm in diameter; (4) without ulceration; and (5) histologically differentiated adenocarcinoma^[2]. Beyond the above criteria, any other ESD candidates should be considered to be selected according to expanded criteria for endoscopic treatment of EGC,

Table 1 Association of proximal margins with oncologic safety and postgastrectomy quality of life in early gastric cancer patients

Distance	Oncologic safety	Quality of life	Recommendation
< 1 mm	Dangerous	No impact (ref.)	Denied
1-10 mm	Marginal	No impact	Endoscopic fit cases only ¹
1-3 cm	Probably safe	No impact	Surgical cases with clear tumor borderline
3-5 cm	Safe	Probable impact	Surgical cases with uncertain tumor borderline
> 5 cm	Safe (ref.)	Clear impact	Denied

¹Indications: (1) cT1a tumor; (2) cN0 status; (3) no more than 20 mm in diameter; (4) without ulceration; and (5) histologically differentiated adenocarcinoma^[2].

and therefore ESD is only performed for investigation purposes in this condition. Although they are relatively strict criteria for ESD candidates, there is still a pitfall for endoscopic treatment, *i.e.*, the false negative prediction of node metastasis, which would lead to a fatal consequence.

In short, the optimal proximal margin for EGC patients is still controversial, and it is better to be decided in an individualized manner.

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access (OA) journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1362 experts in gastroenterology and hepatology from 67 countries.

Aims and scope

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In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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