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Editorial board member of *World Journal of Gastroenterology*, Shinji Tanaka, MD, PhD, Associate Professor, Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

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REVIEW

Treatment options for alcoholic and non-alcoholic fatty liver disease: A review

Sukhpreet Singh, Natalia A Osna, Kusum K Kharbanda

Sukhpreet Singh, Natalia A Osna, Kusum K Kharbanda, Research Service, Veterans Affairs Nebraska-Western Iowa Health Care System, Omaha, NE 68105, United States

Natalia A Osna, Kusum K Kharbanda, Department of Internal Medicine, Nebraska Medical Center, Omaha, NE 68198, United States

Kusum K Kharbanda, Department of Biochemistry and Molecular Biology, Nebraska Medical Center, Omaha, NE 68198, United States

ORCID number: Sukhpreet Singh (000000021982876X); Natalia A Osna (0000000174980556); Kusum K Kharbanda (0000000177598889).

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Correspondence to: Kusum K Kharbanda, PhD, Professor, Veterans Affairs Nebraska-Western Iowa Health Care System, Research Service (151), 4101 Woolworth Avenue, Omaha, NE 68105, United States. kkharbanda@unmc.edu

Telephone: +1-402-9953752

Fax: +1-402-9954600

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Abstract

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are serious health problems worldwide. These two diseases have similar pathological spectra, ranging from simple steatosis to hepatitis to cirrhosis and hepatocellular carcinoma. Although most people with excessive alcohol or calorie intake display abnormal fat accumulation in the liver (simple steatosis), a small percentage develops progressive liver disease. Despite extensive research on understanding the pathophysiology of both these diseases there are still no targeted therapies available. The treatment for ALD remains as it was 50 years ago: abstinence, nutritional support and corticosteroids (or pentoxifylline as an alternative if steroids are contraindicated). As for NAFLD, the treatment modality is mainly directed toward weight loss and co-morbidity management. Therefore, new pathophysiology directed therapies are urgently needed. However, the involvement of several inter-related pathways in the pathogenesis of these diseases suggests that a single therapeutic agent is unlikely to be an effective treatment strategy. Hence, a combination therapy towards multiple targets would eventually be required. In this review, we delineate the treatment options in ALD and NAFLD, including various new targeted therapies that are currently under investigation. We hope that soon we will be having an effective multi-therapeutic regimen for each disease.

Key words: Alcoholic liver disease; Non-alcoholic fatty

liver disease; Treatment options; Glucocorticoids; Liver transplantation

Core tip: Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are serious health problems worldwide. Despite extensive research on understanding the pathophysiology of both these diseases there are still no targeted therapies available. In this review, we delineate the treatment options in ALD and NAFLD, including various new targeted therapies that are currently under investigation.

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INTRODUCTION

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are serious health issues whose incidences are on the rise with each passing decade. Alcohol is responsible for approximately 4% of all deaths annually and 5% of all disabilities worldwide[1]. The Centers for Disease Control and Prevention in 2013 have estimated that in the United States acute deaths from alcohol attributable causes have outnumbered deaths from chronic diseases (44000 to 35000) (http://apps.nccd.Cdc.gov/ardi/homepage.aspx). Motor vehicle accidents have been considered the leading cause of acute death from alcohol-attributable injuries. While the incidence and prevalence of NAFLD is on the rise with each passing decade. Presently, 25%-35% and 5%-15% of the general population of Western and Asian countries, respectively, are affected by this disease[2]. This proportion is even higher in people with type 2 diabetes (60%-70%), and in those who are obese or morbidly obese (75%-92%) compared to the general population[3-5]. The prevalence of obesity in the United States has increased from 10% to 60% of the total population in the last three decades and is considered to be one of the main factors for the increasing prevalence of NAFLD[6].

The risk factors for both these diseases are well known. Patients with ALD consume an excessive amount of alcohol while NAFLD patients are usually obese; have insulin resistance and/or metabolic syndrome. Available data from various studies show that NAFLD may be the hepatic manifestation of metabolic syndrome[7]. The spectrum of both diseases ranges from benign steatosis to hepatitis to cirrhosis and hepatocellular carcinoma. Most patients with NAFLD or ALD have hepatic steatosis which is usually asymptomatic; only 20%-35% of these patients' progress to steatohepatitis or cirrhosis[8]. The role of genetic polymorphism, mainly the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene (rs738409 variant), has also recently been shown to be a risk factor for progression to advanced liver disease in both NAFLD and ALD that could explain why only a subset of patients who are chronic alcohol abusers or have high caloric intake present with progressive liver injury. Evidence from recent studies has shown this variability may be related to *PNPLA3* variant (rs738409). The single nucleotide polymorphism rs738409 variant within the *PNPLA3* causes a substitution of methionine for isoleucine at position 148. The GG phenotype of this PNPLA3 variant, rs738409, predicts a greater risk of progression to cirrhosis and HCC than the GC and CC phenotype which have shown to have a smaller risk for progression[9-12]. Despite an increased understanding of the pathophysiology and risk factors for ALD and NAFLD, we still do not have an appropriate therapeutic regimen for either disease.

The treatment options of ALD have not changed in the last four decades, and abstinence is still the cornerstone of treatment. This is supported by nutrition therapy and steroids[13,14]. Unfortunately, alcoholic hepatitis, which is the most serious manifestation of ALD, has a short term mortality of up to 50% in patients who are unresponsive to corticosteroid treatment[15]. Furthermore, limited treatment options are available for patients who are steroid non-responders or have contraindications to steroid usage (upper gastrointestinal bleed, impaired renal functions and sepsis). While the treatment for NAFLD is mainly directed toward attenuating the risk factor such as gradual weight loss by lifestyle modification with a focus on nutrition and exercise[16,17], other therapies utilizing insulin sensitizers (thiazolidinediones) and antioxidants (vitamin E) also have been found to be useful. However, their long-term safety and adverse effects have not been rigorously evaluated.

Thus, effective and safe therapeutic regimens are needed for these liver diseases. In this review, we present the current therapies as well as upcoming potential new approaches and treatment strategies for both diseases.

ALD TREATMENT

General management

For the last 50 years, abstinence has remained the primary therapy for ALD treatment. However, serious symptoms develop with the abrupt cessation of alcohol. Treating the alcohol withdrawal syndrome is thus extremely important and requires administration of fluid, calories, vitamins and minerals. Unstable patients need to be admitted to a critical care unit and airway protection is often required in patients with hepatic encephalopathy. Table 1 summarizes the treatment options and potential new options for ALD

Table 1 Treatment options for alcoholic liver disease and alcoholic steatohepatitis

General management
Abstinence
Nutritional support
Glucocorticosteroids
Pentoxifylline
Anti-TNF therapy
Antioxidants
Liver transplantation
Potential new therapies
Probiotics and antibiotics
S-adenosylmethionine
Betaine
Targeting various chemokines and interleukins
Endocannabinoids antagonists
Osteopontin inhibition
Stem cell therapy

and ASH (alcoholic steatohepatitis).

Alcohol withdrawal syndrome: This syndrome is characterized by symptoms that occur 6-24 h after abrupt cessation of alcohol in patients who drink consistently and excessively. Long acting benzodiazepines like chlordiazepoxide or diazepam are administered for prevention of seizures while intermediate acting benzodiazepines like lorazepam are recommended in withdrawal patients who are elderly or have had recent head trauma or liver or respiratory failure[18]. Antiepileptic like carbamazepine can also be used as a benzodiazepine substitute for preventing seizures. Antipsychotics like haloperidol can be used if patients have excess agitation or psychotic symptoms[18]. Alcoholics are usually malnourished and deficient in vitamins, especially vitamin B1 (thiamine), thus putting them at risk of developing Wernicke encephalopathy, so all such patients should be given thiamine[19]. Parenteral thiamine is preferred over oral thiamine because in addition to impaired gastrointestinal absorption in alcoholics, oral thiamine has poor bioavailability that does not allow for attaining a sufficient concentration in cerebrospinal fluid. However, parenteral thiamine has a short half-life, thus multiple high-dosing is required to achieve sufficient concentration for active and passive diffusion through the blood brain barrier[20-22]. An IV dose of 500 mg (three times daily for two consecutive days) is recommended, followed by 500 mg of IV or IM thiamine for five more days if a response to the therapy is seen[23-25]. Another dosing regimen is 500 mg of IV thiamine (three times daily for 2-3 d), followed by 250 mg of IV thiamine for the next 2-3 d. The intravenous regimen is then followed by oral thiamine treatment indefinitely[26,27]. Thiamine should be given before fluids containing glucose to prevent neurological damage.

Abstinence: The first step towards treatment requires the patient to accept the fact that they are dependent and addicted to alcohol. Abstinence can resolve alcoholic fatty liver disease and can improve the survival rate of cirrhotic or decompensated liver failure patients. Thus, motivating the patients to abstain from alcohol and follow the proper treatment regime are major steps. However, preventing relapse in such patients has always been a big challenge. Patients can participate in Alcoholics Anonymous group meetings for self-control and motivation. Psychological support by an addiction specialist can also help in maintaining sobriety. Recognizing and treating any associated psychiatric conditions can be helpful in such patients[28]. Pharmacotherapy also helps in maintaining sobriety; drugs like naltrexone and acamprosate assist in reducing alcohol intake in heavy drinkers[29,30]. Topiramate has found to be effective in decreasing craving and withdrawal symptoms in alcoholics[31]. Disulfiram, an acetaldehyde dehydrogenase inhibitor, is also being used. It causes accumulation of serum acetaldehyde, which produces unpleasant sensations of nausea, vomiting, abdominal pain and dizziness. Such sensations deter patients from consuming alcohol[32]. Baclofen, a γ-amino butyric acid agonist, has also been found effective in promoting abstinence[33]. Most of these drugs (disulfiram, acamprosate, naltrexone, topiramate and baclofen), are used only for treatment of alcohol dependence[34] but are not FDA approved for ALD treatment. Of these, only baclofen has been studied for its safety and efficacy in clinical trials that compared placebo and baclofen treatment for ALD patients. In this trial, baclofen (10 mg or 20 mg twice daily) was more effective than placebo in maintaining abstinence. A 53% reduction in the number of drinks per day in the 10 mg baclofen group ($P < 0.0001$) and a 68% reduction in the number of drinks per day in the 20 mg baclofen group was reported, thus demonstrating a dose response effect of baclofen[35]. Naltrexone and disulfiram should be avoided in patients with liver problems because of hepatotoxicity[36]. Moreover, naltrexone should also be avoided in patients with renal failure due to its active tubular secretion[37]. Acamprosate, topiramate and baclofen were found to be safe in such patients[33,38,39]. Another drug which can help to maintain abstinence and reduce craving is metadoxine (MTD). Although not available in the United States, MTD is approved for use in several European countries. Apart from sustaining abstinence, oral MTB is also useful in acute ethanol intoxication since it is rapidly absorbed and augments alcohol metabolism by enhancing acetaldehyde dehydrogenase activity[39]. In a clinical trial with ASH patients, MTX improved liver function tests in 1 mo compared to the placebo[40]. In another trial, improved 3-6 mo survival of severely ill ASH patients who received the combination therapy with MTX compared to those that received monotherapy with either steroids or pentoxifylline (PTX) was observed. The survival rate was higher of the MTD-treated groups at

3 mo (PTX + MTD 59.4% *vs* PTX 33.3%, $P = 0.04$; steroids + MTD 68.6% *vs* steroids 20%, $P = 0.0001$) and at 6 mo (PTX + MTD 50% *vs* PTX 18.2%, $P = 0.01$; steroids + MTD 48.6% *vs* steroids 20%, $P = 0.003$) than in the non-MTD treated groups. Furthermore, the patients receiving MTD maintained greater abstinence than those not on MTD (74.5% *vs* 59.4%, $P = 0.02$)[41]. Smoking and obesity are independent risk factor for the progression of ALD[42,43]. Hence, lifestyle modifications like weight loss and smoking cessation are also helpful. Hepatitis C virus (HCV) is another independent risk factor for ALD progression due to synergistic deleterious effects of both agents in promoting liver injury and HCC development[44,45]. The main mechanisms of this effect are that both alcohol and HCV alter cellular immunity, increase free radical oxidative damage, and in the case of alcohol exposure, promote replication of HCV. Thus, the combination often result in the presence of advanced liver disease with severe histological features at a much younger age and a decreased survival[46,47]. It has been reported that alcoholic patients with HCV infection have a 30 fold increased risk of getting cirrhosis[48] and two to eight fold increased risk of all-cause mortality compared with those without the HCV infection[49,50]. Thus, all ALD patients should be screened for HCV before starting treatment and all HCV patients should be advised to stop or reduce alcohol consumption[51].

Nutritional support: Most patients with ALD are malnourished, and disease severity often correlates with the degree of malnutrition[52]. Most of the complications of ALD are strongly associated with protein calorie malnutrition[53]. Thus, nutrition support is one of the important steps in ALD treatment. Vitamins (like folate, vitamin B6, vitamin B12[54,55], vitamin A and thiamine[56] and minerals (like selenium, zinc, copper, and magnesium) are often found to be altered in ALD and some believe that these alterations play a role in initiation and progression of liver injury[57]. Especially, zinc levels are decreased in ALD patients and in animal models, and its supplementation has been shown to improve ALD[58]. A major study has also shown that enteral nutrition reduces infectious complications and improves 1-year mortality in such patients[59,60].

The American College of Gastroenterology and the American Association for the Study of Liver Diseases guidelines recommend 1.2 to 1.5 g/kg per d of protein intake and 35 to 40 kcal/kg per d of body weight for energy intake in patients with ALD[61]. This type of malnourished patient is often predisposed to infections so empiric antibiotic treatment is also advised.

Glucocorticosteroids: There have been various clinical trials on the use of corticosteroids for treating ALD patients[62-64]. Despite mixed outcomes, corticosteroids are overall beneficial for survival of these patients. Unfortunately, 40% of patients are unresponsive to corticosteroid with virtually no other treatment options. Hence, new target oriented therapies are critically required for the management of such patients[15].

A meta-analysis which pooled data from 3 randomized control trials, found that patients with modified DF $\geqslant$ 32 or MELD score $\geqslant$ 21 treated with prednisolone at 40 mg/d for 28 d and then a tapered dose over 2-4 wk (Class I, level A), conferred a 28-d survival benefit of glucocorticoids (85%) *vs* placebo (65%), with mortality decreasing from 35% in controls to 15% in patients on steroids[64]. Early changes in bilirubin levels (at day 7 of treatment) and the Lille score were used to predict the prognosis following steroid administration[65]. A Lille's score greater than 0.45 on the 7th d after initiation of the treatment indicated that the patient was unresponsive to steroid therapy and predicted a lower survival rate of 25% at 6 mo. Recently this score has been re-classified as complete responders (score $\leqslant$ 0.16), partial responders (score 0.16-0.56), and null responders (score $\geqslant$ 0.56), and is associated with the 28-d survival rate of 91%, 79% and 53%, respectively, with $P < 0.0001$[13]. Steroids have been found to have a significant beneficial effect in complete and partial responders but not in null responders, hence discontinuation of steroid therapy is recommended for non-responders[66]. In addition to non-responders, steroids are generally avoided in patients with active infection, gastrointestinal bleeding, chronic hepatitis B virus infection or hepatorenal syndrome (HRS) because of adverse effects in these patient populations[67]. Steroids are relatively contraindicated in severe AH patients with coexistent sepsis. Thus, such patients may be treated with second line drug PTX[68]. Patients should also be screened for any infection before starting steroids and for infective complications while on steroids. Occurrence of sepsis and infective complications while the patient is on steroids is a poor prognostic sign[69]. It has been reported that patients infected after initiation of steroids had a significant lower 2-mo survival than patients with no infection (46.4% ± 6.9% *vs* 77.5% ± 3.2%, $P < 0.00001$). Thus, it is very important to differentiate infection at admission from that which occurs after starting the steroid treatment, as survival rates differ significantly. Overall, infection was more common in steroid null responders than responders[70].

PTX: Steroids are generally used as the first line of treatment in severe alcoholic hepatitis patients with DF $\geqslant$ 32, except in those with renal failure or HRS or contraindication to steroids[71]. PTX (400 mg 3 times per day for 28 d) is a substitute in such cases (Class I, level B). PTX decreases pro-inflammatory cytokines like TNF-α, has anti-fibrotic properties[72] and confers a mortality benefit by reducing the incidence of HRS[73]. A pilot study in ASH patients using PTX demonstrated reduced mortality and HRS incidence when compared to patients given a placebo[74]. These findings were later confirmed in a double-blind placebo controlled trial, where PTX decreased the 28-d mortality compared to

placebo (24.5% *vs* 46%). Also, 50% of those who died in the PTX group developed HRS, while 91.7% who died in the placebo group developed HRS, confirming that PTX reduces the incidence of HRS in such patients[75]. A study in ASH patients comparing PTX and prednisolone have shown a better survival rate of 35.29% in the PTX group *vs* 14.71% on steroids. This reduced mortality was presumably because of a decrease in incidence of HRS and gastrointestinal bleeding in the PTX group. However, this study was underpowered[76]. To date, no other study has shown any additional survival benefit of the combined PTX and corticosteroid treatment[77,78]. A recently conducted randomized, multicenter, double-blind trial (STOPAH) across 65 hospitals in the United Kingdom that recruited more than a thousand patients revealed no impact of PTX on survival or disease progression in severe non-alcoholic steatohepatitis (NASH) patients in comparison to placebo[79,80]. However, because of a lack of other treatment options, PTX is being used in some centers.

Anti-TNF therapy: Intestinal gut permeability is increased in chronic alcoholics that promotes the translocation of gut luminal antigens especially endotoxin to reach the liver and enhance TNF-α production[81]. TNF-α has been found to correlate with disease severity in severe alcoholic hepatitis patients[82], and also play a vital role in alcohol induced liver injury in various animal models of alcoholic liver injury[83]. Further, mice deficient in TNF receptor 1 do not develop liver injury when administered alcohol[83]. Based on these considerations, various human studies were undertaken using anti-TNF therapy. While initial studies were found to be promising, the results could not be duplicated in larger clinical trials. A large randomized controlled trial comparing prednisolone alone with a combination of prednisolone and infliximab had to be stopped before completion because of an increase in infection rate in the prednisolone and infliximab combination group[84]. Further, patients had to be screened for tuberculosis and nocardia infection prior to participation in the study, thus limiting its clinical utility[85].

Antioxidants: Alcohol causes oxidative stress by increasing reactive oxygen species (ROS), and decreasing endogenous antioxidant levels[86]. But to date, all trials examining antioxidants (such as lecithin, β-carotene, vitamin C, vitamin E, allopurinol, desferrioxamine, and N-acetylcysteine) either alone or in combination with steroids have been disappointing[87,88].

Liver transplantation: Liver transplantation remains the definitive therapy for end stage decompensated cirrhosis due to ALD. Severe alcoholic hepatitis patients nonresponsive to steroids have a 3 mo mortality rate of 70% and with HRS the mortality rate is ⩾ 90% unless the patients get liver transplantation[89,90]. At present, there are very few options for treating severe alcoholic hepatitis patients who are non-responsive to steroids and have a Lille score > 0.56. Thus, liver transplantation remains the only hope for such patients, but the issue of transplantation in alcoholics has always remained controversial. Concerns include the risk of recidivism, poor compliance with postoperative care, and ALD being a self-inflicted disease[91]. Recidivism following transplantation is a major challenge, which occurs at a rate of 10%-50%[92,93]. A meta-analysis reviewing factors responsible for recidivism found 3 major variables: a poor social support system, a family history of alcohol abuse/dependence and pre-transplant abstinence of 6 mo or less[94]. Thus, we need a multidisciplinary approach including the presence of an Alcohol Addiction Unit which can significantly contribute in reducing alcohol relapse after transplantation. Also, there should be psychological evaluation for any mental illness to determine patient suitability for transplantation.

Most transplant programs require the patients to undergo a 6-mo period of abstinence prior to transplantation[95]. Studies over the years have provided data both for and against the 6-mo abstinence rule. One report suggested that the 6-mo period of abstinence would allow the liver to recover with medical treatment and possibly there would be no need for transplantation[96]. Another study revealed that some recovery in liver function can take place within 3 mo of abstinence while many patients may die during the 6 mo of waiting period. This led to the suggestion of possibly reducing the period of abstinence to 3 mo[97]. Yet another study has also challenged the 6-mo abstinence rule by showing beneficial effects of early liver transplantation in steroid non-responding severe alcoholic hepatitis patients. In this study patients (with Lille score of 0.88) after 13 d of being unresponsive to steroids were put on the transplant list and it was found that the 6-mo survival rate was higher in patients who received early transplantation than those who did not (77% *vs* 23%, $P < 0.001$)[98].

However, patients who have received liver transplantation show a high incidence of de novo cancer[99,100], lymphoproliferative disorder and skin cancer. In some cases, squamous cell carcinoma of the oropharynx or esophagus has also been detected, likely due to the cumulative effects of smoking and post-transplant immunosuppressive drugs. Also, liver transplantation due to ALD is associated with a high rate of cardiovascular complications[101].

Potential new therapeutic options in ALD

Advances in basic science have helped to gain better insights into the pathophysiology of ALD that have provided new treatment options as discussed below.

Role of probiotics and antibiotics: Healthy intestinal flora is critically important for our well-being.

An alcohol-induced change in the gut microflora plays a major role in the pathogenesis of alcoholic hepatitis. Equally important in liver disease progression is the alcohol-induced increased intestinal permeability that allows for the gut luminal antigens, including endotoxin/LPS (component of the cell wall of gram negative bacteria), to reach the liver and promote the synthesis and secretion of several inflammatory cytokines[102]. Various studies have proposed the use of probiotics in restoring the normal bowel flora in patients with ALD[103]. In a study performed on patients with ALD it was shown that using probiotics (Bifidobacterium or Lactobacillus) for 4 wk enhances and normalizes neutrophil phagocytic capacity and helps in reducing endotoxin-driven elevation in cytokine levels[104]. A similar study revealed significant improvement in AST, ALT and γ-glutamyl transferase levels in ALD patients administered probiotics (Bifidobacterium or Lactobacillus) for 5 d[105]. Rifaximin, a biochemical derivative of Rifamycin, the drug for hepatic encephalopathy, given for 28 d in a clinical trial decreased systemic endotoxin levels[106]. Indeed, blood LPS levels help in predicting response to steroids and mortality of alcoholic hepatitis patients[107]. Thus, modifying the gut microbe flora by probiotics and antibiotics could be a potential therapeutic approach for treating ALD which is being actively pursued.

Role of S-adenosylmethionine and betaine: S-adenosylmethionine (SAM) is a key methyl donor that is involved in many methylation reactions critical for normal liver function. SAM also acts as an antioxidant by activating the pathway for GSH synthesis. Decreased SAM levels have been reported in ALD patients; thus, elevating SAM levels could be a potential therapy. Various animal studies have shown liver injury can be reversed by preventing a decrease in SAM levels[108]. Also, SAM administration decreases oxidative stress and hepatic stellate cell activation[109]. A randomized controlled trial using SAM or placebo for 2 years in alcohol cirrhotic patients found that the mortality and liver transplantation rates were higher in the placebo group than in the SAM group (29% *vs* 12%)[110]. Thus, there is need for long-term, high quality trials in the future to establish its effectiveness. Along the same line as SAM, betaine treatment has been very effective in improving liver injury in various animal models[111,112]. By remethylation homocysteine to generate methionine, betaine not only removes the toxic metabolites homocysteine and S-adenosylhomocysteine, but also generates SAM and normalizes the methylation potential[113]. Betaine is hepato-protective and prevents alcohol-induced steatosis, oxidative stress, apoptosis and abnormal protein accumulation[111,112], and breakdown of sulphur containing amino acid[114]. Clinical trials using betaine should be conducted.

Role of targeting various chemokines and interleukins: Chemokines play a pivotal role in the pathogenesis of alcoholic hepatitis. Studies have shown that various chemokines and their subfamily members, including CXCL5, CXCL6, CXCL10 and CCL20, are notably high in ASH livers compared to normal control livers and higher levels correlate with worse prognosis and outcomes[115,116]. Of these, CCL20 is the most elevated chemokine in ASH livers that attracts lymphocytes, monocytes, Th17 (Helper T17) cells, and dendritic cells. The consequent production of more chemokines and inflammatory mediators ultimately causes heavy neutrophilic infiltration and liver damage[117,118]. Additional studies in the future are required to determine if targeting CCL20 and other chemokines can be an effective and safe therapeutic approach for ALD patients.

IL-8 is one of the most important chemoattractant of neutrophils, which further causes hepatic infiltration as well as increased portal pressure[115]. A higher level of IL-8 in alcoholic hepatitis patients is associated with worse prognosis[115]. A therapeutic approach towards counteracting IL-8 levels should be considered as it will decrease neutrophil infiltration of the liver and prevent progressive liver damage.

IL-22 plays a critical role in bacterial infections and tissue repair. It is a part of the IL-10 family which decreases the production of various pro-inflammatory cytokines[119]. IL-22 has been found to have anti-apoptotic, antimicrobial, antioxidant and anti-steatotic effects, thus it can be used as a therapeutic option in ALD patients. It has been found that levels of T helper cells producing IL-22 correlate with improvement in alcoholic hepatitis patients[120]. Recombinant IL-22 administration showed improvement of liver injury in ethanol-fed mice[121] and in an animal model of acute hepatitis while blocking the IL-22 receptor led to worsening of the disease[122]. Thus, up regulating IL-22 levels can be a potential therapy for ALD.

IL-17 increases chemotaxis of neutrophils and various other chemokines and its levels are found to be increased in alcoholic hepatitis[123]. Secukinumab, an anti-IL-17 monoclonal antibody has shown favorable results in clinical trials of rheumatoid arthritis, psoriasis and uveitis[124]. Up until now, no study has been done in patients with liver disease using this monoclonal antibody, which can be a potential therapy.

Role of endocannabinoids: Endocannabinoids signalling through cannabinoid receptors, CB-1 and CB-2, has been implicated in the pathogenesis of ALD[125]. Studies using animal models of alcoholic liver injury revealed that CB1-deficient mice are resistant, whereas CB2-deficient mice are more susceptible to fatty liver damage[126,127]. These findings suggested that therapy targeting CB1 and CB2 receptors should be utilized as an alternative for the management of ALD.

Role of osteopontin: There is substantial evidence

Table 2 Treatment options for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

Lifestyle changes	Weight loss
	Dietary changes
	Exercise
Insulin sensitizers	Thiazolidinedione's
	Metformin
Lipid lowering agents	Statins
	Ezetimibe
Hepatoprotective agents	UDCA
Antioxidants	Vitamin E
Incretin analogues	GLP-1 agonists
	DPP-IV inhibitors
Anti-inflammatory agents	PTX
Others	Probiotics
	Angiotensin receptor blockers
	Endocannabinoid antagonists
	Bariatric surgery
	Liver transplantation
Potential new therapeutic options	Caspases inhibitors
	ASK1 inhibitors
	p38 MAPK inhibitors
	PPAR- alpha and delta agonists
	FXR agonists
	NOX-1/4 inhibitors
	Galectin-3 antagonists
	Acetyl CoA carboxylase inhibitors
	FGF-21 and FGF-19 analogues
	CCR2 and CCR5 inhibitors
	SCD-1 inhibitors
	Lysyl oxidase-like 2 inhibitors
	Sirtuins

GLP-1: Glucagon-like peptide 1; DPP-IV: Dipeptidyl-peptidase IV; PTX: Pentoxifylline; PPAR: Peroxisome proliferator activated receptor; NOX: NADPH oxidase; FGF-21: Fibroblast growth factor 21; SCD1: Stearoyl coenzyme A desaturase 1.

suggesting that osteopontin (OPN) plays a notable role in wound healing in response to injury in many organs[128]. It is an extracellular matrix protein with pro-fibrogenic properties, and is found to be highly expressed in alcoholic hepatitis patients[129]. One study demonstrated attenuation of alcohol mediated liver disease in mice lacking OPN[130]. More studies should be conducted to assess OPN as a potential therapeutic target.

Stem cell therapy: Hematopoietic stem cell transplant ion is an evolving field. Though limited research has been performed in this area, it could be a promising therapeutic approach in the future. Recent studies have suggested that stem cell transplantation may reduce liver inflammation and improve fibrosis in patients with liver cirrhosis[131]. Mesenchymal stem cells (MSC) directly inhibit the activation of hepatic stellate cells and may also induce apoptosis of hepatic stellate cells[132]. They have also been reported to stimulate proliferation of endogenous hepatocytes[133,134]. A pilot study performed on 12 patients with ALD to assess the regenerative capacity of the liver after infusion of bone marrow derived-MSC through the hepatic artery showed improvement in the histological grading with an overall decrease in TGF-β, type 1 collagen and smooth muscle actin[135]. A significant improvement in the Child-Pugh score and albumin levels was noted in another similar study on 9 cirrhotic patients given bone marrow derived stem cells *via* the portal vein[136]. Liver function was also reported to be better after stem cell therapy in cirrhotic patients[137]. Results of these studies are encouraging and stem cell therapy could serve as a potential breakthrough treatment for ALD. However, the benefits and safety of stem cells should be examined in a large sized RCT.

NAFLD TREATMENT

Like ALD there is no effective treatment to date for NAFLD. In the absence of a proven effective therapy, we must follow a multi-disciplinary approach in NAFLD treatment, where a combination of drugs and factors are taken into consideration to counter multiple pathological risk factors involved in NAFLD. These are summarized in Table 2 and are further discussed below.

Weight loss, dietary modification and changes in lifestyle

Treatment is mainly directed towards weight loss and risk factor reduction, as most patients are obese or have metabolic syndrome[138]. A weight loss of 3%-5% reduces steatosis while a ⩾ 5%-7% drop in weight has been shown to resolve NASH. Greater reductions in weight (*i.e.*, ⩾ 10%) may also improve hepatic fibrosis. Weight loss is mainly due to diet modification and exercise. However, the shortcoming of this approach is the lack of adherence and non-compliance with time[139]. Various studies have shown the benefit of weight loss in NAFLD[140]. Dietary modification also plays a key role since a carbohydrate-rich diet, especially with high fructose, is the major cause of obesity, insulin resistance and NAFLD development[141]. Thus, sugar consumption should be kept below 10% of total caloric intake in a day and a fructose-rich diet should be avoided in such patients. Food rich in omega-3 fatty acid should be included and those rich in saturated fat and omega-6 fatty acid should be excluded from the diet[142]. An omega-3 fatty acid rich diet promotes fatty acid oxidation and decreases fatty acid synthesis, thus improving the lipid profile. Fish and fish oil consumption should be promoted as they are rich in omega-3 fatty acid[143]. Thus, diet and moderate exercise are preferred methods of natural weight loss. A study also revealed that a combination of diet changes and exercise lowered ALT levels better in NAFLD patients than insulin sensitizers or other hypoglycaemic drugs[144]. Weight loss is also beneficial as it improves the cardiovascular risk profile[145]. Nevertheless, it should be noted that weight loss should be gradual, as very rapid weight loss has been associated with a worsening of steatohepatitis and an increased risk for liver failure[146] and gallstones[147].

Apart from natural weight loss, drugs like Orlistat and Sibutramine are also being used for controlling weight. Orlistat is a lipase inhibitor, that prevents fat absorption in the liver and intestine, thus causing weight loss. Sibutramine on the other hand is a serotonin reuptake antagonist which suppresses appetite. Both agents have shown to reduce serum transaminase levels and hepatic steatosis[148,149].

Insulin sensitizers

Since NAFLD is closely associated with obesity and metabolic syndrome, and both conditions cause insulin resistance, treatment strategies invariably include agents which enhance insulin sensitivity.

Thiazolidinedione: Thiazolidinediones (TZDs) are peroxisome proliferator activated receptor (PPAR)-γ agonists, which improve hepatic and peripheral insulin sensitivity[150] *via* increasing plasma adiponectin levels[151]. In addition, adiponectin is also shown to have anti-fibrotic and anti-inflammatory properties. Thus, multiple factors involved in pathogenesis of NAFLD such as high insulin resistance, low adiponectin levels and high pro-inflammatory cytokines are all targeted by these drugs. First generation TZDs (troglitazone) have shown improvement in steatohepatitis but had to be stopped due to hepatotoxicity[152]. However, it paved the way for second generation TZDs (rosiglitazone and pioglitazone) which are not hepatotoxic and showed improvement in insulin resistance, hepatic steatosis and aminotransferases levels[153,154]. A long-term therapy with second generation TZDs may be required as their benefits tend to reverse on discontinuation; however long term therapy is associated with various adverse effects like congestive heart failure, weight gain, peripheral oedema, anaemia and osteoporosis[155,156]. Also, it has been found that TZD therapy alone without nutrition and lifestyle changes is often not effective[154]. Thus, we need additional studies on a larger population with a combination of other drugs to find safe and efficacious treatment options.

Metformin: Metformin, a hypoglycaemic drug, is used for treatment of type 2 diabetes mellitus. Metformin improves hepatic and peripheral insulin resistance by decreasing hepatic gluconeogenesis, lipogenesis and glucose reabsorption from the gut and increasing fatty acid oxidation[157]. While metformin does not cause weight gain as TZDs, it can cause some minor gastrointestinal adverse effects and sometimes lactic acidosis is seen in patients with renal impairment. Various studies have documented an improved insulin sensitivity, cholesterol and aminotransferase levels in NASH patients on metformin but the results are mixed when assessing biopsy-guided improvement in steatosis and NASH activity score (NAS)[158]. Thus, while its effectiveness as a monotherapy is debatable, metformin could be a part of a multi-therapeutic regimen for the management of NAFLD patients.

Lipid lowering agents

NAFLD is often associated with obesity and metabolic syndrome which is characterized by hypercholesterolemia and hypertriglyceridemia. Therefore, the use of lipid lowering agents could be beneficial. While, clofibrate did not show any beneficial effect on the liver tests or the histological scores[159], gemfibrozil showed improvement in ALT levels in NAFLD patients compared to the placebo[160]. Statins have also been tried but have shown variable effects. Nevertheless, lipid-lowering agents should be given as most NAFLD patients are hyperlipidemic and thus have a high risk of developing cardiovascular issues.

Ezetimibe, a drug that inhibits the reabsorption of lipids from the intestine, reduces serum TNF-α levels[161], hepatic lipid content and ALT levels in a mouse model of NAFLD[162]. Human studies for this drug are awaited.

UDCA

This drug has hepatoprotective properties and has been studied in various clinical trials for NAFLD treatment. Initial small studies revealed an improvement in liver enzyme levels and hepatic steatosis[159], but a subsequent RCT showed no improvement in liver histology or aminotransferases[163]. Thus, UDCA is not approved as a monotherapy but is part of a drug combination regime in various trials on NAFLD in progress.

Vitamin E

ROS generation plays an important part in the progression of NASH[164]. Vitamin E and C decrease oxidative stress and thus have been evaluated in patients with NASH. Various clinical trials with vitamin E have revealed an improvement in liver test functions and reduction in oxidative stress markers but significantly less improvement in the histological grading of the disease has been noted[165,166]. A recent trial using a combination of vitamins E and C for 6 mo showed that these were no better than placebo for treating patients with NASH[167]. One study with a three arm trial involving placebo, UDCA and Vitamin E/UDCA combination showed improvement in histology only in the Vitamin E/UDCA combination arm[168]. Another trial comparing a combination of pioglitazone and vitamin E with vitamin E alone over a period of 6 mo showed a decrease in serum ALT in both groups, but a significant histological improvement was only seen in the combination group[169]. A meta-analysis involving high-dose vitamin E supplementation has shown an increase in all-cause mortality and cardiovascular deaths, thus decreasing the enthusiasm for vitamin E therapy[170].

Incretin analogues

Glucagon-like peptide 1 agonists: Glucagon-like peptide 1 (GLP-1) is an incretin hormone that is

produced by intestinal mucosa L cells. GLP-1 has a short half-life, as it is rapidly degraded by dipeptidyl-peptidase IV (DPP-IV). GLP-1 agonists are resistant to DPP-IV and are useful since they lower blood glucose levels by decreasing glucagon secretion, delay gastric emptying and stimulate pancreatic β cells to increase insulin secretion. Furthermore, these agonists have a central appetite suppressive effect and promote weight loss which are favorable outcomes for obese NAFLD/NASH patients[171]. In an obese mouse model, it has shown to improve insulin sensitivity and reduce hepatic steatosis[172].

In various clinical trials, liraglutide has proven to be an effective therapeutic drug for type 2 diabetics producing a good glycemic control and significant weight loss in such patients. Since diabetes is an important component of metabolic syndrome and associated NAFLD development, the effective glycemic control and weight loss makes liraglutide a suitable therapeutic option for NAFLD[173]. In a phase 2 clinical trial study (LEAN study) with 52 NASH subjects using liraglutide compared to placebo, 39% of patients using liraglutide *vs* 9% using placebo attained the primary endpoint (histological resolution of NASH without worsening of fibrosis). Two (9%) of 23 patients in the liraglutide group *vs* eight (36%) of 22 patients in the placebo group had progression of fibrosis. The trial was designed using A'Hern's single-group method, which required eight (38%) of 21 successes in the liraglutide group for the effect of liraglutide to be considered clinically significant. The liraglutide treatment group has also shown improved insulin sensitivity, reduced hepatic glucose production and lipogenesis (ClinicalTrials.gov-NCT01237119). Thus, liraglutide was safe, well-tolerated, and led to histological resolution of NASH, warranting longer term studies in such patients[174,175].

DPP-IV inhibitor: DPP-IV inactivates both incretin hormones (GIP, GLP-1), therefore DPP-IV inhibitors are used in the treatment of type 2 diabetes[176]. NASH patients exhibit higher DPP-IV expression[177]. A cross sectional study on type 2 diabetics without any evident liver disease and NAFLD patients revealed a strong positive correlation of serum DPP-IV activity and insulin resistance with liver enzymes only in NAFLD patients [178]. Serum DPP-IV activity was not increased in the type 2 diabetics with no evidence of liver disease. This led the authors to postulate that the increased serum DPP-IV reported in earlier studies in type 2 diabetics may have been due to some un-diagnosed liver disease and that the excess DPP-IV found in the serum of NAFLD patients is of hepatic origin. They further suggested that serum DPP-IV should be considered as a potential liver disease biomarker[178]. This supposition was also corroborated by another study which analyzed human liver biopsy specimens and showed a strong correlation of DPP-IV expression with stages of fatty liver and NASH[179].

Furthermore, a DPP-IV inhibitor like sitagliptin, decreases hepatic steatosis and serum transaminases levels when given to diabetic NAFLD patients[180,181]. In a recent randomized, double-blind, placebo controlled study, sitagliptin was shown to be safe but no more effective than placebo in improving hepatic steatosis and fibrosis in NAFLD patients. However, in comparison to sitagliptin, an increase in hyaluronic acid levels and increase in FIBROSpect II index (measure of liver fibrosis) was reported in the placebo arm[182]. Another RCT comparing sitagliptin to placebo also revealed no improvement in fibrosis score or NAS after 24 wk of therapy, but reported that sitagliptin increased adiponectin and decreased γ-glutamyl transferase levels[183]. There have been only a few clinical trials with sitagliptin till date. However, despite the lack of convincing evidence, the efficacy of sitagliptin in improving liver fibrosis in NAFLD cannot be ruled out. This is because not only were the trials underpowered but were possibly not long enough to evaluate its effectiveness. Hence, stagliptin effect should be assessed in clinical trials of longer duration with larger number of enrolled patients with NAFLD/NASH.

PTX

PTX can be of potential benefit in NAFLD due to its effects on reducing free radical oxidative stress, TNF-α levels, and potential anti-fibrotic properties[184]. In some trials, PTX has shown improvement in steatosis, lobular inflammation and ballooning degeneration in comparison to baseline, but improvement was not clinically significant when compared to placebo[185]. In a small RCT on NASH patients, 400 mg PTX given three times per day for a period of 1 year decreased hepatic steatosis, inflammation and NAS by ⩾ 2 points and modestly reduced fibrosis[186]. This favorable response was due to a reduction in free-radical-mediated lipid peroxidation[187]. In two recent small RCT evaluating the role of PTX has also shown beneficial effect by improving liver enzymes and histology in NAFLD patients[188,189]. In a recent meta-analysis it was found that only PTX and OCA improve fibrosis in NASH patients[190]. Therefore, further studies are warranted to determine its role in NAFLD/NASH treatment.

Others

Probiotics: Like alcoholic patients, NAFLD patients also exhibit gut bacterial overgrowth, enhanced gut permeability and increased paracellular leakage of gut luminal antigens, factors that promote NASH development. Thus, probiotics can be a therapeutic option for NASH patients[191,192]. In a RCT, improvement in liver enzymes was noted in NAFLD patients on Lactobacillus bulgaricus and Streptococcus thermophilus treatment compared to placebo[193]. In another study, patients randomized to a combination of Bifidobacterium longum with fructo-oligosaccharides plus lifestyle

modification (diet and exercise) or lifestyle modification alone for 24 wk[194], showed a significant decrease in steatosis, TNF-α, AST and NAS in the combination treatment group. Thus, probiotics could also be a part of a combination therapy for NAFLD patients.

Angiotensin receptor blockers: NAFLD is often associated with metabolic syndrome and hypertension is an important component of metabolic syndrome. Thus, angiotensin receptor blockers can be a part of combination therapy regimen of NAFLD. A small pilot study of patients with NASH showed improvements in necro-inflammation and fibrosis with losartan (an angiotensin II receptor antagonist) treatment[195]. Larger studies are required to explore their potential in the management of NAFLD.

Endocannabinoid antagonists: CB1 and CB2 are two receptors which mediate endocannabinoid (EC) activity. The CB1 receptor is mainly expressed in the brain and liver, while CB2 is mainly expressed in the immune cells. These receptors are found to be upregulated in various liver diseases[196]. Anandamide, a highly potent endogenous agonist, has been shown to promote diet-induced obesity and hepatic steatosis in mice *via* acting on the CB-1 receptors.[197] Conversely, CB-1 knockout or rimonabant (CB-1 receptor antagonist)-treated high-fat diet fed mice have less steatosis and weight gain than controls[198]. However, while rimonabant was effective in promoting weight loss of obese patients in many clinical trials, it also caused intolerable adverse effects like depression, anxiety and increased suicidal tendencies that has led to its discontinuation for routine use. Thus, novel cannabinoid type 1 receptor blockers with selectivity for peripheral receptors are required which can have favorable metabolic benefits but decreased psychiatric adverse effects[199,200].

Bariatric surgery: Steady weight loss with exercise and lifestyle modification has been found to increase insulin sensitivity and improve liver histology of NAFLD patients. But the rapid weight loss induced by bariatric surgery increases the risk of developing hepatic failure especially in the cirrhotic patients[201,202]. Bariatric surgery is mostly done in non-cirrhotic NAFLD patients who are morbidly obese. It is, however, not recommended as a primary mode of treatment in such patients as there is still a risk of developing liver failure postoperatively.

Liver transplantation: NAFLD patients with end-stage decompensated liver disease should be considered for liver transplantation. But this is not a permanent cure as NAFLD has been shown to recur in post-transplant liver[203]. This is because transplantation does not correct the multifactorial pathway alteration(s) responsible for NAFLD/NASH development. Therefore, the goals of therapy before and after transplant should be always towards weight management, proper diet consumption and adequate control of glucose and lipids.

Potential new therapeutic options in NAFLD

With advancement in the field of technology, especially bioinformatics and biogenetics, new therapies are currently being tried for managing NASH, some of which are reviewed below.

Caspase inhibition/emricasan: Caspases are enzymes which are required for completion of various apoptotic pathways and for stimulation of various cytokines and therefore, can be a potential therapeutic target. Various animal studies in the past have supported this approach[204,205]. Emricasan, a pan-caspase protease inhibitor, has been shown to inhibit apoptosis, inflammation and fibrosis in a preclinical model of NASH. A preliminary report of a phase Ⅱ clinical trial showed significantly decreased serum ALT and cCK18 levels in NAFLD patients[206]. The therapeutic effects of this drug have also been examined in various other fibrotic liver diseases where it has been shown to reduce hepatic venous pressure gradient (HVPG). A phase Ⅱ trial on NASH patients with fibrosis (ClinicalTrials.gov Identifier: NCT02686762) is ongoing to evaluate the efficacy of emricasan (10 mg and 100 mg/d for 72 wk) to improve fibrosis without worsening of NASH (primary endpoint) and to assess histological improvement or resolution of NASH (secondary endpoint).

ASK1 inhibitors/ASK1-I: Apoptosis signal regulating kinase 1/ASK1 is a MAP3 kinase (mitogen activated protein 3 kinase) which induces apoptosis and fibrosis when activated by stimuli like hyperglycaemia, TGF-β and ROS. This enzyme has been shown to be activated in patients with NASH. GS 4997, a first-in-class, oral small molecule ASK1 inhibitor, given to animals with established NASH showed a significant reduction in hepatic steatosis, fibrosis, body weight, fasting blood glucose, insulin resistance, lipogenesis, cholesterol biosynthesis, plasma AST/ALT levels, and soluble/insoluble collagen and many metabolic parameters of NASH[207-209]. GS-4997 is currently being investigated in a phase Ⅱ clinical trial of patients with NASH (ClinicalTrials.gov Identifier: NCT02466516).

p38 MAPK inhibitors: Chronic inflammation is one risk factor that contributes to progression of NAFLD. p38 mitogen activated kinases (p38 MAPK) is a stress kinase whose activation has been shown to promote inflammation[210-212]. In mammals, four p38 MAPK isoforms have been identified: p38a, b, c and d. p38 MAPK isoforms -c and -d have recently been shown to contribute to the development of steatosis and NASH in various models of NAFLD by regulating T-cell activation, neutrophil recruitment and macrophage production of TNF-α[213,214]. Studies have shown higher liver expression of p38 protein in obese individuals

with steatosis. Thus, deletion of p38 -c and -d in the myeloid cells prevents neutrophil migration to the liver, protecting these animals against diet induced steatosis and inflammation[215]. Therefore, p38 MAPK can be an effective potential target for NAFLD therapy.

PPAR-α and -δ agonists (Elafibranor): PPAR-α is mainly expressed in liver and is principally involved in lipid metabolism, while PPAR-δ is found in various tissues of the body and is involved in fatty acid oxidation and insulin sensitivity. In various animal models, PPAR has been shown to be hepato-protective *via* its effect on decreasing lipid accumulation, inflammation and fibrosis[216-218]. In a RCT (clinicaltrials.gov NCT01694849), a daily dose of 80 or 120 mg Elafibranor or placebo was given to non-cirrhotic NASH patients for 52 wk[219]. The primary endpoint of this study (i.e. resolution of NASH without worsening of fibrosis), was not met. However, it was found that patients with an initial NAS of ⩾ 4 on 120 mg/d of drug showed significant improvement in hepatic inflammation. Nevertheless, Elafibranor efficacy was described as sub-optimal. Another study is in phase 3 clinical trial to clarify Elafibranor's (GFT505) effectiveness (ClinicalTrials.gov-: NCT02704403) to improve the histological grade and reduce all-cause mortality and liver-related outcomes in patients with NASH and fibrosis.

Farnesoid X receptor/FXR agonists (Obeticholic acid): Obeticholic acid (OCA) is a Farnesoid X receptor agonist. It is a synthetic derivative of natural bile acid chenodeoxycholic acid (CDCA), with potency 100 times more than CDCA. Farnesoid X receptor is a nuclear hormone receptor which regulates bile, cholesterol, glucose and lipid metabolism[220,221]. These receptors act *via* multiple pathways; they inhibit hepatic lipogenesis, gluconeogenesis, glycogenolysis and maintain cholesterol balance and improve insulin sensitivity[222,223]. In various animal models, OCA has shown anti-inflammatory and anti-fibrotic properties and also improves insulin resistance and hepatic steatosis[224]. In an animal model, OCA was shown to reduce hepatic inflammation and fibrosis and also decreased intrahepatic vascular resistance and improved portal hypertension[225]. Also in an animal model with advanced cirrhosis, treatment with OCA was shown to reduce gut bacterial translocation from 78.3% to 33.3% ($P < 0.01$) indicating its effect in maintaining intestinal barrier integrity. Thus, it can be used as an option to prevent bacterial infection in such patients[226]. In a small pilot trial of diabetic patients with NAFLD, it was shown to decrease weight and serum γ-glutamyl transferase levels as well as an improvement in liver fibrosis[227]. The multicenter trial (FLINT trial:NCT01265498) showed a decrease in NAS, an improvement in hepatic steatosis, and a small decrease in liver fibrosis in non-cirrhotic NAFLD patients on a daily dose of 25 mg OCA compared to placebo[228]. A phase 3, double blind RCT multicenter study is ongoing to evaluate the safety and efficacy of OCA in NASH patients (ClinicalTrials.gov Identifier: NCT02548351). The effect of OCA on liver histology in non-cirrhotic NASH patients with stage 2 or 3 fibrosis will be compared to placebo. 2065 patients are randomized in 1:1:1 to receive 10 mg OCA, 25 mg OCA or placebo. An interim analysis is to be done at 18 mo and the study is expected to end in 6 years. However, an increase in total cholesterol and triglycerides with a decrease in high density lipoprotein was also seen in the OCA group when compared to placebo[228]. Two phase I studies conducted in healthy individuals given OCA for 14-20 d also reported decreased HDL and increased LDL cholesterol, regardless of the dose of OCA (5, 10 or 25 mg daily)[229,230]. These pro-atherogenic effects can be a concern for NAFLD patients that already have a high risk for cardiovascular adverse events because of dyslipidemia. Therefore, combination therapies with FXR agonist and agents that prevent atherosclerosis are warranted. Apart from OCA, various other FXR agonists such as GW4064, PX20606, GS-9674 and INT-767 are being tested. GW4064, PX20606 and GS-9674 are synthetic non-steroidal FXR agonists. INT-767 is a dual agonist for FXR and TGR5 (the transmembrane G-protein bile acid receptor) while BAR502 is a dual agonist for FXR and GPBAR1 receptors. In various animal models, these agonists have been shown to improve NASH histological features, steatosis and fibrosis [231-234]. Thus, clinical trials are anticipated for these agents as well.

NOX-1/4 inhibitors: NADPH oxidase (NOX), is an enzyme which catalyzes the production of ROS[235]. In various animal models these enzymes are expressed on hepatic stellate cells and promote liver fibrosis and inflammation[236]. In a murine model, NOX 1/4 inhibitor (GKT137831) has been found to decrease ROS production and fibrotic gene expression, thus decreasing liver inflammation and fibrosis[235]. Therefore, these agents can have a beneficial effect in decreasing liver fibrosis in NASH patients but require further studies.

Galectin-3 antagonists: Galectins are proteins that bind to terminal galactose residues on glycoproteins[237]. They are usually expressed in immune cells and are at very low levels in the body but their levels are increased during inflammation and fibrosis[238,239]. Galectin-3 knockout mice show reduced hepatic fibrosis after liver injury. GR-MD-02, a galectin-3 inhibitor, has shown a decrease fibrosis, hepatic steatosis and collagen deposition in various animal models with NASH[240]. A Phase Ⅱ clinical trial for evaluation of the safety and efficacy of GR-MD-02 for the treatment of liver fibrosis and associated portal hypertension in patients with NASH cirrhosis is currently underway (ClinicalTrials.gov Identifier: NCT02462967). This study has enrolled

subjects with portal hypertension and biopsy proven NASH cirrhosis (excluding subjects with medium and large varices and those with decompensated cirrhosis). The expected primary completion date is October 2017 while the study is expected to complete in February 2018.

Acetyl CoA carboxylase inhibitor: Malonyl coenzyme A plays a key role in fatty acid metabolism that maintains a balance between lipogenesis and lipid oxidation[241]. It promotes fatty acid synthesis, and inhibits β-oxidation of lipids. Malonyl CoA is generated from acetyl CoA and the key enzyme regulating this process is acetyl CoA carboxylase (ACC). Therefore, inhibiting ACC prevents fatty acid synthesis and promotes its oxidation. In a murine model of NAFLD, inhibition of ACC has been shown to decrease hepatic steatosis, lipogenesis and increase insulin sensitivity and fatty acid oxidation[241]. Chronic administration of ND-630 (ACC isozyme 1 and 2 inhibitor) to diet-induced obese rats and Zucker diabetic fatty rats caused a reduction in hepatic steatosis, lowered haemoglobin A1C (0.9% reduction) and improved insulin sensitivity[242]. Also in a crossover, randomized, double-blind trial, administration of a single dose of NDI-010976 (a highly potent and selective inhibitor of both ACC1 and ACC2) to overweight/obese subjects inhibited *de novo* lipogenesis in a dose dependent manner[243]. Together, all these results suggest its usefulness in treating metabolic syndrome, type 2 diabetes mellitus, and fatty liver disease. Thus, large long term clinical trials in humans are needed.

FGF-21 and FGF-19 analogues: FGF-21 (fibroblast growth factor 21) is a hormone which is secreted mainly from the liver. It is a starvation-induced peptide hormone with pleiotropic effects whose levels are mainly increased during fasting[244,245]. While FGF-21 concentrations are elevated in human subjects with NAFLD, a lack of FGF-21 worsened the metabolic disorders in an animal model of NASH[246]. Conversely, treatment with FGF-21 analogue (BMS-986036) was found to improve insulin sensitivity, hepatic steatosis and decrease lipogenesis[247]. In another animal model of NASH, LY240531 (a FGF-21 variant) was shown to increase fatty acid oxidation by enhancing hepatic mitochondrial oxygen consumption. Also, various inflammatory markers and AST and ALT levels were reduced, suggesting an attenuation of liver injury[248]. BMS-986036 is currently being evaluated in a phase Ⅱ trial of NASH patients (ClinicalTrials.gov Identifier: NCT02413372).

FXR activation in terminal ileum by bile acid promotes FGF-19 secretion which, in turn, decreases bile acid synthesis and gluconeogenesis[245]. It also results in the activation of the FGFR4 receptor which has a proliferative impact on hepatocytes, thus raising the potential for tumorigenesis[249]. NGM-282, a variant of FGF-19 has been shown to decrease bile acid synthesis and gluconeogenesis without having a tumorigenic effect[245]. In a preliminary pre-clinical study, NGM-282 was shown to improve hepatic steatosis and histological features of NASH in an animal model[250].

CCR2 and CCR5 inhibitor (cenicriviroc): CCR2 and CCR5 are chemokine receptors which are mainly expressed in various immune cells like monocytes, macrophages, Kupffer cells, natural killer cells, T cells and stimulate hepatic stellate cells thus promoting fibrosis. These receptors can be inhibited by cenicriviroc (CVC) which is an inhibitor of the CCR2 and CCR5 receptors. CVC has been shown to decrease fibrosis and inflammation in various animal models of diet-induced NASH or substance-induced NASH[251-254]. There is an ongoing trial (ClinicalTrials.gov Identifier: NCT02217475) with CVC to examine its efficacy in NASH patients with fibrosis. It will compare shorter *vs* longer CVC treatment and assess correlations between decreased inflammation and fibrosis[255].

SCD-1 inhibitors (aramchol): Aramchol is a synthetic lipid molecule which decreases hepatic fat accumulation by decreasing lipogenesis and increasing fatty acid oxidation by inhibiting stearoyl coenzyme A desaturase 1 (SCD1) enzyme[256]. This drug was found to decrease liver fat content significantly in 60 NAFLD patients who were given 100 or 300 mg of this drug daily for 3 mo; the effect of the drug on fibrosis was not determined[256]. A phase Ⅱ clinical trial of this drug is ongoing on NASH patients with fibrosis (ClinicalTrials.gov Identifier: NCT02279524).

Lysyl oxidase-like 2 inhibitor (simtuzumab): Lysyl oxidase-like 2 inhibitor is an enzyme which causes cross linkage of collagen, thus preventing its degradation[257]. This enzyme has been found to promote fibrosis in liver diseases of various etiologies. A monoclonal antibody (simtuzumab) to this enzyme has been studied in various animal models and has shown to decrease fibrosis[258]. Two big trials are ongoing to examine the efficacy of this drug in decreasing fibrosis and preventing progression to cirrhosis in such patients (ClinicalTrials.gov Identifier: NCT01672866 and NCT01672879).

Sirtuins: Sirtuins (SIRTs) are information regulator proteins. There are various types of SIRTs found in mammals. SIRT-1, a member of this family of proteins, has anti-inflammatory effects and increases insulin secretion and sensitivity[259]. A decreased liver expression of SIRT-1 was observed in an animal model of NAFLD[260]. Since SIRT-1 activator (resveratrol) was shown to improve hepatic steatosis and insulin sensitivity[261], SIRT-1 could be a potential target for treatment of NAFLD patients' in future clinical studies.

Betaine: A phase Ⅱ clinical trial of betaine in patients with a clinical diagnosis of NAFLD is close to completion.

This trial (ClinicalTrials.gov Identifier: NCT03073343) will evaluate the effect of two doses of oral betaine in reducing ALT levels in NAFLD patients with and without diabetes.

CONCLUSION

Both ALD and NAFLD are chronic liver diseases with similar spectrums from simple steatosis to cirrhosis with basic differences only in their etiology. Despite understanding much of the pathophysiology of both diseases, there is still no effective treatment for either disease. The treatment for ALD basically relies on alcohol abstinence, nutritional support, lifestyle modifications, steroids and symptomatic treatment of complications of cirrhosis while for NAFLD, the focus of treatment is on weight loss, exercise and the use of insulin sensitizers. Removal of the cause would be the most efficient way of treating both diseases. However, the involvement of several inter-related pathways in the pathogenesis of these diseases indicates that a single therapeutic agent is unlikely to be an effective treatment strategy. Hence, a combination therapy towards multiple targets would eventually be required. Future areas of research also include the safety, efficacy, and ethical considerations of liver transplant in severe ASH for patients who are not responding to medical therapy. Various new target-oriented therapies are under investigation for both diseases and hopefully soon we will be having an effective multi-therapeutic regimen for each disease.

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REVIEW

Nonalcoholic fatty liver disease: Evolving paradigms

Amedeo Lonardo, Fabio Nascimbeni, Mauro Maurantonio, Alessandra Marrazzo, Luca Rinaldi, Luigi Elio Adinolfi

Amedeo Lonardo, Fabio Nascimbeni, Mauro Maurantonio, Alessandra Marrazzo, Azienda Ospedaliero-Universitaria di Modena, Ospedale Civile di Baggiovara, 41126 Modena, Italy

Fabio Nascimbeni, Alessandra Marrazzo, University of Modena and Reggio Emilia, 41126 Modena, Italy

Luca Rinaldi, Luigi Elio Adinolfi, Department of Medical, Surgical, Neurological, Geriatric, and Metabolic Sciences, University of Campania "Luigi Vanvitelli", 80100 Naples, Italy

ORCID number: Amedeo Lonardo (0000-0001-9886-0698); Fabio Nascimbeni (0000-0002-1051-1272); Mauro Maurantonio (0000-0003-3500-1145); Alessandra Marrazzo (0000-0001-5047-4042); Luca Rinaldi (0000-0002-6541-3821); Luigi Elio Adinolfi (0000-0001-8453-4912).

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Correspondence to: Luigi Elio Adinolfi, MD, Professor, Department of Medical, Surgical, Neurological, Metabolic, and Geriatric Sciences, University of Campania, "L. Vanvitelli", Piazza Miraglia, 80100 Naples,
Italy. luigielio.adinolfi@unicampania.it
Telephone: +39-81-5665081
Fax: +39-81-5665081

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Abstract

In the last years new evidence has accumulated on nonalcoholic fatty liver disease (NAFLD) challenging the paradigms that had been holding the scene over the previous 30 years. NAFLD has such an epidemic prevalence as to make it impossible to screen general population looking for NAFLD cases. Conversely, focusing on those cohorts of individuals exposed to the highest risk of NAFLD could be a more rational approach. NAFLD, which can be diagnosed with either non-invasive strategies or through liver biopsy, is a pathogenically complex and clinically heterogeneous disease. The existence of metabolic as opposed to genetic-associated disease, notably including "lean NAFLD" has recently been recognized. Moreover, NAFLD is a systemic condition, featuring metabolic, cardiovascular and (hepatic/extrahepatic) cancer risk. Among the clinico-laboratory features of NAFLD we discuss hyperuricemia, insulin resistance, atherosclerosis, gallstones, psoriasis and selected endocrine derangements. NAFLD is a precursor of type 2 diabetes (T2D) and metabolic syndrome and progressive liver disease develops in T2D patients in whom the course of disease is worsened by NAFLD. Finally, lifestyle changes and drug treatment options to be implemented in the individual patient are also critically discussed. In conclusion, this review emphasizes the new concepts on clinical and pathogenic heterogeneity of NAFLD, a systemic disorder with a multifactorial pathogenesis and protean clinical manifestations. It is highly prevalent in certain cohorts

of individuals who are thus potentially amenable to selective screening strategies, intensive follow-up schedules for early identification of liver-related and extrahepatic complications and in whom earlier and more aggressive treatment schedules should be carried out whenever possible.

Key words: Nonalcoholic fatty liver disease; Biomarkers; Clinical correlates; Diagnosis; Epidemiology; Genetics; Liver histology; Management; Metabolic Syndrome; Pathogenesis; Screening; Type 2 diabetes

Core tip: Nonalcoholic fatty liver disease (NAFLD) is a pandemic disease. Recent evidence highlights new concepts in clinical and pathogenic heterogeneity of NAFLD, a systemic disorder with a multifactorial pathogenesis and protean clinical manifestations. Other than the classical obese phenotype of NAFLD, a lean though metabolically abnormal variant has been recognized. Simple steatosis is no more considered a benign condition; insulin resistance is necessary but not sufficient for the disease progression, and NAFLD is not only a mere hepatic manifestation of metabolic syndrome, but may forerun the development of metabolic syndrome and cardio-renal complications. Several non-invasive diagnostic tests are now available and new drug treatment options are coming.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) features excess intrahepatic ectopic triglyceride deposition[1] in patients who are free of competing etiologies of liver disease[2]. It is the most frequent liver disease and is associated with a wide spectrum of hepatic disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC)[3]. Moreover, frequent co-morbidities of NAFLD include specific cardio-renal-metabolic conditions and increased hepatic/extrahepatic cancer risk[4].

NAFLD is a pandemic disease worldwide, which has been paralleling the ongoing epidemics of obesity, type 2 diabetes (T2D), and metabolic syndrome (MetS)[4]. The prevalence of NAFLD approaches 25%-30% in the Europe and United States general populations; but this figure surges to 80%-90% in selected cohorts of dysmetabolic individuals[5-7]. Patients with NAFLD have an increased risk of premature cardiovascular as well as of liver-related mortality[8]. Of concern, up to 50% of cases NAFLD-HCC may occur in the absence of cirrhosis, a circumstance which will often worsen the outcome[9,10].

Knowledge accumulated since 1980, when Ludwig named and described NASH[11], has challenged most of the paradigms that had been holding the scene over the previous 30 years. For example, NAFLD is now considered a multifaceted complex systemic condition[12,13] which exhibits a sexual dimorphisms[14] and follows a variable hepatic and extra-hepatic course[15,16]. In addition, recent evidence suggests that simple steatosis may progress to NASH and advanced liver fibrosis[17] challenging the previous dogma that "steatosis is a benign condition".

Insulin resistance (IR) is often associated with NAFLD but it is "necessary but not sufficient" for the disease to progress[18]. Moreover, NAFLD may occur in lean individuals[19,20] and it is not only a mere "hepatic manifestation of MetS", but may forerun the development of MetS[21,22]. NAFLD is associated with secretion of diabetogenic hepatokines and inflammatory biomarkers that increase the risk of incident T2D by adversely affecting glucose homeostasis[23]. Of concern, NAFLD and T2D are linked by a "vicious circle" which carries to accelerated worsening of liver disease[24], more difficult metabolic control and earlier appearance of T2D complications[25,26].

The historical "two-hit" pathogenic theory[27] has been replaced by a multi-factorial model, which more incisively recapitulates the complexity of the pathogenesis of the disease[28] by emphasizing the myriads of pathways leading to the same hepatic phenotype[29]. A better definition of the role played by genetics in the development of NAFLD[30] has led to the notion that, probably, two different NAFLD types exist: NAFLD associated with and NAFLD dissociated from MetS, and that each may differentially affect cardio-metabolic risk[31,32]. In addition, the declining gradient of risk of NAFLD in Hispanic patient populations compared to non-Hispanic whites and African Americans may potentially be accounted for by polymorphisms in genes that control metabolism[33]. The impressive amount of data implicating gut microbiota in the development of NAFLD may well illustrate the role of diet in increasing NAFLD risk[34-36] and significantly impact our understanding of NAFLD pathogenesis and treatment[4].

Over time, the diagnostic approach has also undergone substantial changes. Non-invasive physical and chemical biomarkers are now available[37]. Liver biopsy is reserved to selected NAFLD cases in clinical practice and is more extensively performed and repeated to evaluate novel drug agents[3,4,38,39]. Finally, new histological classifications have recently been proposed[40] and the pros and cons of each system must be carefully evaluated[41].

Within the complex frame of pathogenic and clinical heterogeneity of different NAFLD populations

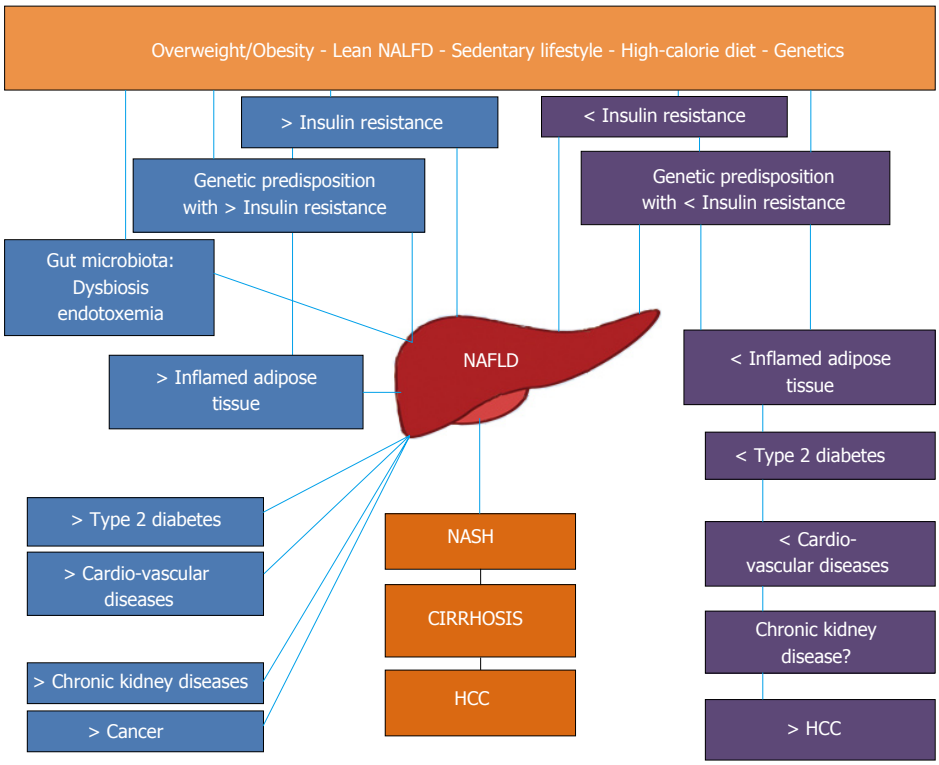

Figure 1 Nonalcoholic fatty liver disease as a pathogenically and clinically heterogeneous condition. Schematic representation of the pathogenic and clinical heterogeneity of different NAFLD populations. Left: "Metabolic" NAFLD is associated with adipose tissue dysfunction and IR and may progress towards hepatic and extrahepatic complications. Right: "Genetic" NAFLD seems to be disconnected from adipose tissue dysfunction and IR, is associated with an increased risk of liver disease progression but is probably spared from extrahepatic complications. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

synthesized in Figure 1, this review aims at disseminating the most updated paradigms on NAFLD and the possible implications for both clinical practice and research purposes.

NAFLD: TO SCREEN OR NOT TO SCREEN. PRINCIPLES OF EPIDEMIOLOGY

An ongoing debate regards whether any screening campaigns should be ever conducted to identify NAFLD individuals[38,42]. Those supporting screening would argue that NAFLD patients are prone to excess liver-related morbidity/mortality and cardiovascular risk and thus in need of intensive follow-up schedules; those against would reply that there is no point in identifying NAFLD, a condition for which no licensed drug treatment schedule is available. At any rate, given the overwhelming prevalence of NAFLD in the general population worldwide, any sensitive approach should best be conducted based on our understanding of which specific cohorts of individuals are exposed to the highest risk of disease.

In the general population, the prevalence of NAFLD has been reported to widely range from 6.3% to 51% related to the different population/ethnicity evaluated as well as to the diagnostic methods utilized to assess the amount of intrahepatic fat content[43]. In particular, the diagnostic technique used to capture cases of NAFLD has undoubtedly affected the results. For example, liver enzymes are far less sensitive than ultrasonographic scanning; in its turn, MR spectroscopy is the most sensitive although this technique is expensive and not universally available[3]. Based on meta-analytic data, Younossi *et al*[6] have reported that, in Europe and in United States, the overall prevalence of NAFLD in the general population approximates 25%. However, there are areas of the world, such as south America and middle East, wherein the average prevalence peaks > 30%; conversely, Africa has the minimum prevalence of NAFLD worldwide[6]. These impressive data clearly imply that no society worldwide can invest so many resources as to screen the general population for NAFLD. It is of interest, therefore, that modifiers of risk might assist health authorities as well as practicing physician in identifying individuals who are particularly prone to developing NAFLD. For example: male gender, middle age and Hispanic ethnicity, all reinforce the risk of NAFLD and, conversely, young women of Afro-American descent have the lowest risk of this disease[7]. Further to the above physiological modifiers, a set of metabolic modifiers have also been identified. They include arterial hypertension and the MetS (whose presence doubles the prevalence of NAFLD up to 50% of individuals); obesity, dyslipidemia and T2D[7]. In

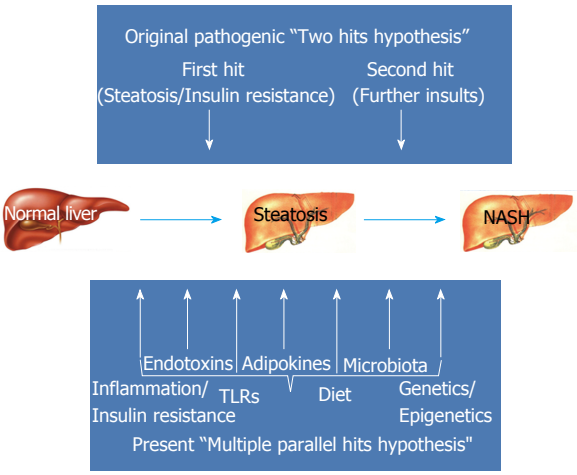

Figure 2 Nonalcoholic fatty liver disease pathogenesis: from two to multiple parallel hits. Schematic representation of the increasing grade of complexity gained in moving from earlier theories[27] to more updated pathogenic paradigms[28]. Top: Former "two hits" hypothesis: steatosis, the first 'hit', sensitizes the liver to the second "hits": oxidative stress, endotoxin, ATP depletion and adduct formation[44]. Bottom: The "multiple hits" hypothesis considers multiple insults acting together on genetically predisposed subjects to induce NAFLD and provides a more accurate explanation of NAFLD pathogenesis. Such hits include IR, hormones secreted from the adipose tissue, nutritional factors, gut microbiota and genetic and epigenetic factors[28]. NAFLD: Nonalcoholic fatty liver disease; TLRs: Toll-like receptors.

the general population, women are protected from developing NAFLD owing to their hormonal profile and/or set of chromosomes. Conversely, women with T2D are exposed to the same risk of NAFLD as men, indicating that T2D abrogates such a gender-related protection[7,14].

PATHOGENESIS OF NAFLD: FROM TWO TO MULTIPLE HITS

In recent years, it has increasingly become evident that what had initially been called the NAFLD "two hit hypothesis", in which IR acted as the "first hit" by inducing lipid accumulation in the hepatocytes and increasing the vulnerability of the liver to further insults, referred to as the "second hit", that, in their turn, promoted hepatic injury, inflammation and fibrosis, is probably over-simplistic[27,44]. Human NAFLD is rather a multi-factorial, non-communicable disease resulting from a complex interaction between multiple environmental and metabolic "hits" and a predisposing genetic background[45] (Figure 2). A "multiple parallel hits hypothesis" seems more appropriate to recapitulate the complexity of NAFLD pathogenesis[28], which results from numerous events originating within liver, adipose tissue, gastrointestinal tract and the muscle[45].

An unhealthy lifestyle, characterized by sedentariness and high-calorie diet, is crucial for NAFLD development and progression. The unbalance between caloric intake and expenditure determines the expansion of fat depots, which become inflamed and insulin resistant, and release increased amounts of free fatty acids into the bloodstream, leading to ectopic fat accumulation in the liver, skeletal muscles and pancreas[46]. The hepatic lipid content mainly derives from the increased lipolysis of triglycerides in the adipose tissue and, to a lesser extent, from dietary fats and sugars, and from de novo lipogenesis[47]. Moreover, the dysfunctional adipose tissue overproduces pro-inflammatory cytokines, such as leptin and resistin, whereas the release of anti-inflammatory adipokines, such as adiponectin, is reduced, so further enhancing NAFLD progression[48]. The role of intra-hepatocytic accumulation of specific toxic lipids, *via* lipotoxicity and inflammation, has emerged as a key player in the pathogenesis of NASH and its systemic complications[49]. Saturated fatty acids have been found elevated in NASH patients and induce inflammation and hepatocyte apoptosis through activation of JNK/endoplasmic reticulum stress and oxidative stress/mitochondrial dysfunction[50]. The accumulation of diacylglycerol and ceramide in the liver impairs hepatic insulin signaling, fuels gluconeogenesis and promotes the development of persisting hyperglycemia and, eventually, T2D[51].

Unhealthy lifestyle also results in dysbiosis, *i.e.* quantitative and qualitative changes of gut microbiota composition[52]. In NAFLD an increased Firmicutes/Bacteroidetes ratio and changes in metagenomic-based functional aspects of gut microbiota have all been described[53]. Gut microbiota may contribute to the development and progression of NAFLD by triggering (both directly or *via* end-products of bacterial metabolism) different signaling pathways, by increasing the efficiency of caloric extraction from the food, and by inducing translocation of bacterial products *via* increased gut permeability[54-57]. Circulating pathogen-associated molecular pattern (PAMPs) and damage-associated molecular patterns (DAMPs) interact with the family of pattern recognition receptors (toll-like receptors, TLRs) within the liver and induce several pro-inflammatory pathways, including over-expression of cytokines/chemokines, production of reactive oxygen species and activation of the inflammasome[58,59]. Of note, activation of Nod-like receptor protein 3 inflammasome, upregulated by TLRs in response to the presence of PAMPs and DAMPs, has been associated with a novel cell death mechanism, referred to as hepatocyte pyroptosis[60].

Several nuclear receptors with their molecular cascades are promising pharmacological targets for the treatment of NAFLD, given their pivotal role in the regulation of energy homeostasis and metabolic pathways. Owing to the development of specific targeted drugs, peroxisome proliferator-activated receptors (PPARs), farnesoid X receptor (FXR) and liver X receptors (LXRs), now deserve full attention. The PPARs superfamily includes PPAR-α, PPAR-β/δ

and PPAR-γ. PPAR-α, mainly expressed in liver and muscle, modulates the rates of fatty acids catabolism, lipogenesis and ketone body synthesis by acting as a nutrient sensor. PPAR-γ, abundantly expressed in the adipose tissue, promotes adipocyte differentiation and storage of triglycerides, and regulates glucose homeostasis. PPAR-β/δ, universally expressed in all organ tissues, regulates glucose and lipoprotein metabolism and exerts an anti-inflammatory role. FXR, mainly expressed in the liver and gut, acts as a sensor of bile acids and mediates the signaling effects exerted by bile acid on glucose and lipid metabolism. LXRs serve as lipid sensors in the liver and participate in regulating the metabolism of cholesterol and fatty acids. The role of nuclear receptors in NAFLD pathophysiology has been comprehensively reviewed elsewhere[15].

The role of skeletal muscle in the pathogenesis of NAFLD is a matter of increasing research[23]. Intramyocellular lipid storage is an early step of ectopic fat accumulation and systemic IR, leads to increased delivery of glucose to the liver, where it becomes substrate for hepatic de novo lipogenesis, and has been associated with NAFLD[61,62]. Accordingly, sarcopenia, a pathological condition featured by generalized loss of skeletal mass and strength, has recently been proposed as an additional key factor in the pathogenesis of NAFLD[63-65]. The myokines, such as irisin, which is produced in response to physical activity and exerts several beneficial metabolic effects, may partly account for these associations[66,67].

NAFLD AS A HETEROGENEOUS DISEASE ENTITY: THE ROLE OF GENETICS

NAFLD is a complex disorder with a variable natural history. Subtle inter-patient genetic variation and environmental factors combine to determine variation in disease progression[68]. Several clinical features are clues to genetics playing a major role in NAFLD. First, up to a quarter of the general population are at risk of progressive disease; however only a minority will experience associated liver-related morbidity[69]. Second, both the development and the progression of disease tend to cluster as distinct traits among "NAFLD families"[7,70]. Third, among non-diabetics, NAFLD will typically affect men more often than women[7] which, further to hormonal variations, may mirror genetic factors. Fourth, although NAFLD is strictly linked with obesity[7] not all obese subjects will develop NAFLD and, more importantly, NAFLD can also be found in non-obese individuals[20]. On these grounds, in the last few years scientific attention has focused on NAFLD genetic features.

Role of PNPLA3, TM6SF2 and MBOAT7

Among all analyzed genes patatin-like phospholipase domain-containing protein 3 (PNPLA3, also called adiponutrin) and trans-membrane 6 super family 2 (TM6SF2) are deemed to play the most significant role. More recently, membrane bound O-acyltransferase domain-containing 7 gene (MBOAT7) has also gained attention. They are all involved in the process of intrahepatic accumulation of triglycerides.

PNPLA3 gene encodes for a 481-aminoacid protein expressed in the liver and adipose tissues, with function of triglyceride hydrolase and acetytil-CoA-indipendent transacylase, suggesting a double role of catabolic lipase activity and anabolic lipogenic activity[71-73]. The nonsynonymous variant rs738409 of *PNPLA3* is a genetic polymorphism characterized by the substitution of isoleucine to methionine at position 148 (I148M). Studies consistently show a strong association between I148M variant and hepatocellular triglycerides accumulation[74-76]. I148M variant is a loss of function allele that determines an altered hepatic lipid metabolism, eventually leading to hepatic droplet lipid accumulation, lower hepatic VLDL secretion and lower adiponectin production[77]. Adiponectin is a protein with anti-inflammatory activity and onco-suppressive properties; lower circulating levels of adiponectin can account for the propensity of I148M variant to the progression from NAFLD to NASH and the increased risk of HCC[77,78].

TM6SF2, also known as KIAA 1926, is a gene at the 19p13.11 locus, with unknown biological function that encodes a protein high expressed in the small intestine, kidney and liver. It is involved in the pathophysiology of NAFLD by modifying hepatic lipid secretion; a decreased hepatic expression of this protein is associated with an increased size and number of hepatocytic lipid droplets[79]. In particular, studies have shown that a variant of *TM6SF2*, rs58542926 c.449 C>T (E167K), is associated with NAFLD evolution, also with dyslipidemia and cardiovascular risk. The minor variant allele (T) is linked with a reduction of the excretion of hepatic VLDL and a lower level of circulating triglyceride and LDL-cholesterol. Conversely, C-allele is associated with a higher hepatic excretion of VLDL, serum triglyceride and cholesterol. Thus, the minor allele exhibits a higher risk of advanced hepatic disease, though protecting from metabolic and cardiovascular disease[80]. Conversely, increased metabolic risk is associated with C-allele[81]. Morris *et al*[82]. based on their large meta-analysis have hypothesized a correlation with T-allele variant and risk developing T2D, but this theory remains to be confirmed[82]. Recently, it has been shown that TM6SF2 (*E167K*) gene variant is associated with an altered lipidomic profile featuring deficiency of polyunsaturated phosphatidylcholine and excess of polyunsaturated free fatty acids in the liver. This lipidomic profile leads to decreased concentrations of circulating VLDL and increased liver fat content[83]. Moreover, the same polymorphism has been found to modulate postprandial lipidmetabolism, nutrient oxidation and glucose homeostasis in response to dietary fat[84]. These findings

are compatible with the above reported notion that carriers of TM6SF2 polymorphisms may be specifically exposed to liver-related as opposed to vascular manifestations of NAFLD.

Finally, it has been recently shown that the rs641738 C>T variant in MBOAT7 gene, which is involved in phosphatidylinositol acyl-chain remodeling, increases the risk of developing NAFLD; inflammation and fibrosis[85,86].

Lean NAFLD

As previously reported in this review, genetic factors play a major role in development and progression of NAFLD. Such a role of genetics is further reinforced when lean NAFLD patients are taken into consideration. It is well-known that NAFLD is often found in obese and dysmetabolic patients. Accordingly, the "Lean NAFLD" phenotype has attracted interest among researchers in as much as it features selectively expanded visceral adiposity though preserving a normal body mass index and, sometimes, even a normal waist circumference. Worryingly, these individuals develop IR and atherogenic dyslipidemia similarly to obese patients and have, therefore, in the past been alluded to as "metabolically obese, normal weight"[87]. Lean NAFLD cases have been described among different ethnic population, mainly among Asians, who tend to develop the metabolic complications of obesity for BMI values inferior to those of Caucasians[88,89]. The mechanism(s) through which lean NAFLD develop(s) is/are poorly characterized and the disease goes often under-recognized. Though the role of genetic factors is likely to be more substantial in "lean NAFLD" than that found in "obese NAFLD"[90], a recent statistically sophisticated study has suggested that both lean and obese NAFLD patients share cardio-metabolic risk commonalities and that the former, despite normal body weight, nonetheless have excess of abdominal adiposity and other MetS features[19]. Accordingly, also lean NAFLD patients may benefit from increased physical activity which is effective in reducing visceral fat[91]. Of note, the findings of a very recent study confirm that NAFLD development and progression derive from a complex interplay between genetic and environmental factor, by clearly demonstrating the presence of a synergy between adiposity and the genetic risk of NAFLD conferred by multiple loci[92].

DIAGNOSTIC STRATEGIES

Diagnosis and staging of NAFLD fully rely on liver biopsy. Histological examination of liver tissue specimens is the gold-standard for quantitating steatosis, diagnosing NASH and staging fibrosis. The last of these three tasks bears major prognostic significance given that fibrosis is associated with long-term liver-related and, probably, cardiovascular outcomes[93-95]. Over the past two decades, different criteria have been suggested for scoring and staging histological lesions, and different definitions of NASH have been used[40,96-99]. Besides discrepancies and subjectivity in interpretation, liver biopsy has additional limitations such as cost, invasiveness and sampling variability which limit the feasibility of this procedure in all the patients with suspected NAFLD and have called for the development of alternative non-invasive diagnostic and staging procedures. Despite some intrinsic and specific drawbacks[100,101], these non-invasive tools, especially if used in combination or sequentially, may help in restricting the number of and assist in a better selection of potential candidates to liver biopsy[102].

Histological classification: what's new?

Steatosis, namely a minimal threshold of 5% of hepatocytes containing fat droplets in biopsy specimen, is a prerequisite for the diagnosis of NAFLD. The histological definition of adult NASH is based on a combination of three elementary lesions, *i.e.*, steatosis, hepatocellular ballooning and lobular inflammation[41,97].

Four main pathologic classifications of NAFLD have been proposed: Matteoni's, Brunt's, Kleiner's classification with the NAFLD Activity Score (NAS) and the recent Fatty Liver Inhibition of Progression (FLIP) algorithm with the Steatosis, Activity, and Fibrosis (SAF) score[40,96-99].

In 2005 the NASH Clinical Research Network designed and validated a histological feature scoring system that addressed the full spectrum of lesions of NAFLD. Currently, NAS is the most widely used histological classification for NAFLD/NASH and its use is recommended for defining and quantifying disease activity in clinical trials[98]. NAS, ranging from 0 to 8, is derived from the sum of steatosis (0-3), ballooning (0-2) and lobular inflammation (0-3). However, at least two out of its three components show a high variability among pathologists; and data increasingly suggest that NAS is poorly correlated with metabolic risk factors[103], is unable to predict fibrosis progression[104] and to prognosticate liver-related and overall mortality in NAFLD patients[93,94,105].

More recently, Bedossa and the FLIP Consortium created a simple histological algorithm (FLIP algorithm) based on a scoring system, the SAF score (Steatosis, Activity, Fibrosis) intended for pathologists to reliably diagnose NASH, assess disease severity and limit interobservervariation[40,99]. The SAF score separately assesses steatosis, activity and fibrosis. Activity, which ranges from 0 to 4, is derived from the combination of the semi-quantitative values of hepatocellular ballooning (0-2) and lobular inflammation (0-2). NASH according to the FLIP algorithm is diagnosed when steatosis, ballooning and lobular inflammation are all scored $\geqslant$ 1. The main discrepancy between NAS and SAF scores is that SAF does not include steatosis in the activity score, owing to the questionable role of steatosis *per se* as a marker of disease progression and prognosis. Moreover, fibrosis stage, the strongest

Table 1 Main features and differences between Nonalcoholic Steatohepatitis Activity score and Steatosis, Activity, and Fibrosis score

	NAS	SAF
Scoring system	Steatosis + Lobular Inflammation + Ballooning: 0-8	Steatosis, Activity (Lobular Inflammation + Ballooning), Fibrosis: S_{0-3}, A_{0-4}, F_{0-4}
Details of scoring	Steatosis	Steatosis
	0: < 5%	0: < 5%
	1: 5%-33%	1: 5%-33%
	2: 34%-66%	2: 34%-66%
	3: > 67%	3: > 67%
	Lobular inflammation	Lobular inflammation
	0:00	0:00
	1: < 2/20X	1: < 2/20X
	2: 2-4/20X	2: ⩾ 2/20X
	3: > 4/20X	
	Ballooning (number of ballooned hepatocytes)	Ballooning (size and shape of hepatocytes)
	0: None	0: normal hepatocytes
	1: Few	1: clusters, reticulated cytoplasm
	2: Many	2: enlarged hepatocytes
	Fibrosis stage	Fibrosis stage
	F0: None	F0: 0
	F1a: zone 3 perisinusoidal, delicate	F1: zone 3 perisinusoidal (all), or portal only
	F1b: perisinusoidal, dense	F2: zone 3 + portal
	F1c: portal only	F3: bridging
	F2: 1a or 1b + portal	F4: cirrhosis
	F3: bridging	
	F4: cirrhosis	

NAS: NASH activity score; SAF: Steatosis, Activity, and Fibrosis score; NASH: Nonalcoholic steatohepatitis.

predictor of outcomes in chronic liver disease, was not included in the NAS. This novel histological classification has shown excellent applicability and reproducibility among pathologists for the assessment of liver lesions in morbidly obese patients and in NAFLD patients with MetS[40,99]. However, it will have to undergo further validation in clinical practice. Table 1 shows the main features of NAS and SAF scores and highlights the main differences between these scoring systems.

Role of imaging techniques

Imaging methods are the most commonly used non-invasive tools for the diagnosis of steatosis. The main advantages of ultrasonography are its safety, low cost, wide availability and the overall scanning of abdominal organs. Traditionally, ultrasonography has been considered a technique with a low sensitivity in identifying fatty liver infiltration when less than 30% of hepatocytes are steatotic. Of note, a recent study has demonstrated that ultrasonography, especially when implemented with standardized measurements and semi-quantitative scores, is sensitive for an amount of steatosis as low as 10%[106] and may predict metabolic derangements and liver histology changes[106-108]. Further studies are necessary to confirm these novel findings. The main limitations of ultrasonography are its inability in differentiating steatosis from fibrosis, the issues with morbid obese individuals, and its operator and machinery dependency.

The controlled attenuation parameter (CAP) is a new promising screening method that measures ultrasound attenuation in the liver using signals obtained by the Vibration Controlled Transient Elastometry (TE) (Fibroscan®). Interestingly, CAP appears to be more sensitive in detecting lesser degrees of steatosis compared to other widely available imaging methods. Moreover, being coupled with liver stiffness measurements, it has the advantage of simultaneously estimating liver fibrosis[108-110]. However, the underlying disease, BMI and T2D may affect the findings and thus must be taken into consideration in the interpretation of CAP results[111].

Over the last 10 years, several ultrasound elastographic techniques have been implemented, including TE, acoustic radiation force impulse (ARFI) elastography, 2D shear-wave elastography and real-time strain elastography. TE, is the most studied elastographic method for the estimation of liver fibrosis in chronic liver disease; in NAFLD, TE can be used to confidently rule out severe fibrosis and cirrhosis (with a nearly 90% negative predictive value) and for monitoring disease progression[11,112]. Of note, the amount of steatosis has emerged as a significant factor affecting the performance of liver stiffness measurements with TE[112,113]. For this reason, it has been suggested that taking into account CAP values may improve the prediction of liver fibrosis by TE[113].

Magnetic resonance imaging techniques, and particularly magnetic resonance spectroscopy, are able to accurately quantify liver fat content; the advantages include high sensitivity for assessing small amounts of intrahepatic triglycerides and sampling a large liver volume[114]. More recently, magnetic resonance elastography and multiparametric magnetic

resonance offer the possibility of performing a thorough characterization of liver tissue by assessing steatosis, fibrosis and haemosiderosis at the same time[115,116], and have been shown to be able to predict clinical outcomes in patients with various chronic liver disease, notably including NAFLD[117]. Cost and limited availability, however, restrict their use to research purposes.

Performance and limitations of biomarkers

Several serum biomarker panels have been developed for predicting steatosis, diagnosing NASH and estimating fibrosis[118] which are, in theory, the preferred diagnostic tool for large-scale screening and epidemiological studies on NAFLD[4,38]. For example, Bedogni's Fatty Liver Index (FLI) has been largely used to capture steatosis in epidemiological studies[119]. The strength of FLI as a surrogate marker comes from the demonstration that it can predict clinical outcomes related to the MetS, such as risk of T2D, accelerated atherosclerosis and cardiovascular risk, as well as hepatic, cardiovascular and cancer-related mortality[120,121]. While clinically relevant, these associations might be conveyed independently of steatosis *per se* since FLI incorporatesvariables that are part of the MetS phenotype[101]. In fact, recent studies have found only a limited correlation between FLI and the amount of liver fat *per se*, as defined by liver biopsy or magnetic resonance spectroscopy[101,122,123]. As for the FLI, other steatosis biomarkers, such as the NAFLD liver fat score, the Hepatic Steatosis Index and the Lipid Accumulation Product, generally show good performance in identifying steatosis, though they are unable to predict liver fat content and are confounded by hepatic histological changes other than steatosis, mainly necro-inflammation and fibrosis[101,123].

With regard to the possibility of non-invasively differentiating NASH from simple steatosis, several biomarkers (acute-phase proteins, cytokines, markers of oxidative stress and apoptosis, as is the case of cytokeratin-18 fragments) and multiple complex models have been investigated, but the majority of them lack external validation, have only been tested among selected populations and have been inconsistent in their performance of detecting NASH[102,118].

Several simple non-invasive fibrosis scoring systems, notably including NAFLD Fibrosis Score and Fib-4 can, however, reliably exclude advanced fibrosis in a high proportion of NAFLD patients, allowing a more targeted use of liver biopsy[124]. Their use in clinical practice is recommended by updated hepatological guidelines[4,38], and they reliably predict liver-related and cardiovascular complications and death in NAFLD patients[125-127]. However, they were principally developed and validated in patients aged < 65 years of age, hence their cut-offs need to be adapted in the elderly[128]. It has been shown that the combination or the sequential use of different scoring systems or different non-invasive techniques (TE plus biomarker) improve the diagnostic accuracy in detecting severe liver fibrosis, thus further reducing the need for liver biopsy[129,130].

Recently, several miRNAs[131], epigenetic biomarkers[132], lipidomic[133,134], metabolomics[135] and proteomic[136] profiles have been found differently expressed in NAFLD patients and could be used as surrogate markers of progressive liver injury. Probably, a multi-domain, multi-component approach which integrates variables from a diverse set of data sources deriving from phenotypic, genomic, lipidomic, metabolomic and proteomic domains will be the avenue for future investigations in the noninvasive assessment of NAFLD[136,137].

NAFLD: CLINICO-LABORATORY FEATURES

Usually asymptomatic, NAFLD is a phenotypically polymorphic disease which, owing to its systemic nature, has a variable clinical presentation, a multitude of potentially associated disease and a rich spectrum of laboratory features[4,12,138]. Understanding this remarkable phenotypic polymorphism is key in guiding the diagnostic process in the individual patient.

Most patients will seek medical advice on the grounds of otherwise inexplicably deranged liver tests/ultrasonographic findings compatible with fatty changes. Collectively, these laboratory/ultrasonographic abnormalities tend to be often discovered either during routine medical check-up performed by asymptomatic individuals or by patients who have nonspecific and causally unrelated complaints[139,140].

Insulin resistance, hyperuricemia and atherogenic dyslipidemia

The association of NAFLD with IR and its close connection with the MetS was independently reported by three unrelated groups of investigators as early as 1999[141-143]. In particular, Marchesini *et al*[141] by comparing 46 patients with NAFLD to 92 age and sex-matched healthy controls with the homeostasis model assessment method found that NAFLD was associated with IR and hyperinsulinemia even in lean subjects with normal glucose tolerance. Consistently, Cortez-Cortez-Pinto *et al*[142] in 30 patients with NAFLD found that 80% were either obese or dyslipidemic; 50% had arterial hypertension and 33% T2D; moreover, glucose metabolism was altered in 69% and hyperinsulinemia and hyperleptinemia were common. Along the same line, Lonardo, based on his systematic search of the available literature, concluded that fatty liver most commonly affected middle-aged men with obesity, altered glucose metabolism, hyperlipidemia, and hypertension[143].

Over time, these pioneer studies suggesting an intimate connection of NAFLD with IR and the MetS have extensively been confirmed and carried further.

For example Ballestri *et al*[144] have recently shown that HOMA-IR, a widely used index of IR, along with serum uric acid concentrations, is an independent predictor of both steatosis and ballooning; waist circumference predicts both ballooning and hepatic fibrosis[144]. Moreover, the same group of investigators, also found that baseline NAFLD predicts the development of incident T2D and MetS over a median 5-year follow-up[22]. Taken collectively, these findings strongly support the notion that NAFLD should no longer be considered a mere "hepatic manifestation of the MetS" but rather, and more appropriately, both a precursor and a consequence of the MetS[21,31,145-147].

Lonardo *et al*[139] were first in reporting that both fasting insulin (a sensitive marker of IR) and uric acid were independent predictors of NAFLD. These findings have been independently confirmed by several studies[148-152], including two meta-analytic reviews[153,154]. Of interest, although serum uric acid concentrations are associated with indices of IR, both parameters are independently associated with NASH, at least in children and adolescents[155], suggesting that each probably carries an independent contribution to the development of progressive liver disease.

NAFLD is typically characterized by atherogenic dyslipidemia featuring larger and triglyceride over-enriched circulating very-low-density lipoproteins (VLDLs), small dense low-density lipoproteins (LDLs) and low and dysfunctional high-density lipoproteins (HDLs). Of note, when NAFLD progresses to severe fibrosis and cirrhosis, dyslipidemia will apparently improve, probably owing to failing hepatic synthetic capacity[144,156-159].

Liver tests, parameters of iron metabolism and non-organ specific autoantibodies

Liver tests are typically normal or mildly elevated in NAFLD. Gamma-glutamyltransferase (GGT) may be slightly elevated and is increasingly recognized as a marker of metabolic disturbances and cardiovascular risk[160]. Aminotransferases do not identify progressive disease, given that patients with normal liver enzymes are spared neither from NASH nor significant fibrosis[161].

The diagnostic process followed to investigate a suspect liver disease may include the determination of parameters of iron metabolism and non-organ specific autoantibodies (NOSA). Elevated serum ferritin, closely associated with hepatic iron deposition, is common in NAFLD and is strongly correlated with IR, more advanced disease and increased mortality[162-164]. Whether therapeutic strategies aimed at correcting iron metabolism may be beneficial in NAFLD remains controversial.

It is important to highlight that the positivity of NOSA, in the appropriate clinical and epidemiological context, is compatible with NAFLD. Loria *et al*[165], in their pilot study, were first in reporting that NOSA positivity in NAFLD was more prevalent than in the general population and that high-titer ANA was associated with IR. Bringing this line of research further, the clinical research network, in a multicenter study including a total of 864 NAFLD patients found that NOSA are frequently positive in NAFLD in the absence of autoimmune hepatitis and their occurrence is not associated with more advanced histological features[166].

NAFLD AS A MULTISYSTEM DISEASE: CARDIO-METABOLIC AND CANCER RISK

As early as 1995, was it first suggested that NAFLD was a systemic condition with a specific cardio-metabolic involvement[167], a notion which is now universally accepted[12,168].

In particular, our understanding of the close and mutual relationship of NAFLD with the MetS has evolved and the bidirectional relationship among the two conditions is now largely acknowledged (Figure 3)[4,31,146,147]. IR, in its turn, will drive the course of liver disease to progressively fibrosing liver disease culminating with cirrhosis, but also promote the development of T2D[169]. NAFLD almost doubles the risk of incident T2D[22]. However, NAFLD patients usually die of extra-hepatic causes, frequently for cardiovascular diseases, which underpins the importance to early diagnose and aggressively treat CVD risk factors, as extensively discussed elsewhere[170]. A meta-analysis of 27 cross-sectional studies has shown a robust association between NAFLD and several markers of subclinical atherosclerosis burden, such as an increased risk, independent of classical CVD risk factors, of coronary artery calcification, carotid intima-media thickness, impaired flow-mediated vasodilatation and arterial stiffness[171]. Moreover, studies have shown that NAFLD is also associated with increased risk of atrial fibrillation and aortic valve sclerosis[172-175].

While cardiovascular mortality is the leading cause of death in NAFLD patients, malignancies, mainly affecting the gastrointestinal tract (liver, colon, esophagus, stomach, and pancreas) and extra-intestinal sites (kidney in men, and breast in women) rank second[176,177]. The association between NAFLD and colorectal cancer (CRC) has been investigated: some studies have shown a higher prevalence of CRC in NAFLD population than patients without, and this association is increased in presence of NASH[178].

NAFLD AND T2D: A DANGEROUS COMBINATION

T2D accounts for more than 90% of those 415 million people worldwide who live with diabetes; T2D may lead to micro-macrovascular complications that cause profound sufferings and put a heavy burden on health-care systems worldwide[179].

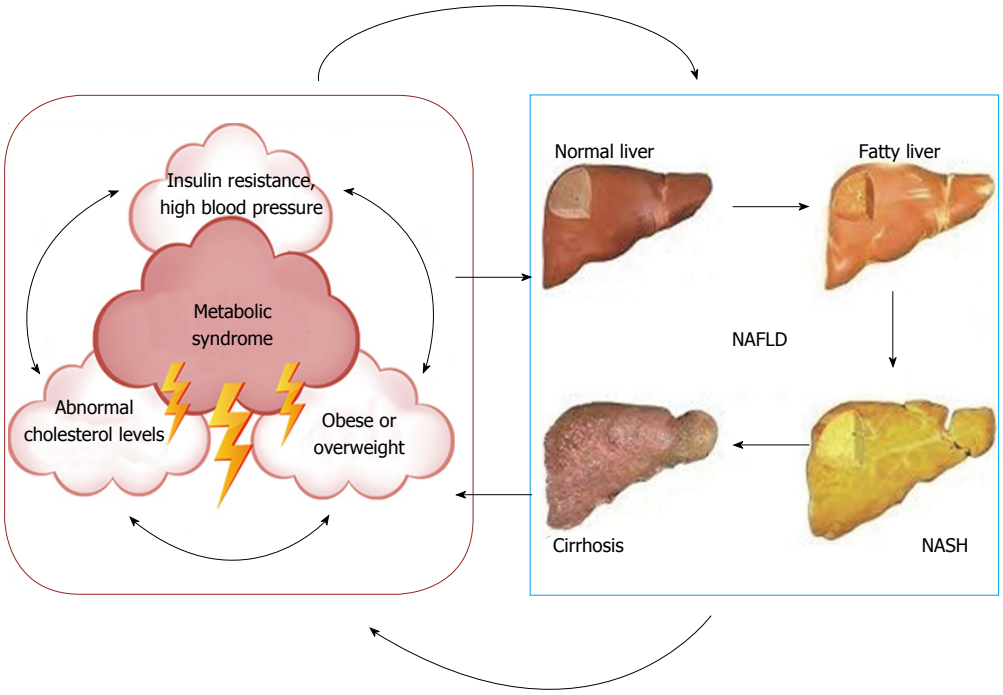

Figure 3 The closed loop nonalcoholic fatty liver disease-metabolic syndrome circuit. Schematic representation illustrating the mutual cause-and-effect relationship of NAFLD with the Metabolic syndrome[4,31,146,147]. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

The common soil and the mutual relationship

The prevalence of NAFLD is increased among those with T2D and T2D rescinds the typical gender dimorphism of NAFLD[7,180,181]. Conversely, NAFLD is associated with a 2-to-5-fold risk of developing T2D after adjustment for several metabolic confounders[22,169,182].

Common features in the epidemiology, risk factors and natural history of NAFLD and T2D can be identified: overweight and obesity[183-185]; a hypercaloric diet coupled with a sedentary behavior leading to weight gain[186,187]; IR and MetS components, including combined dyslipidemia[147,188].

As previously highlighted, NAFLD is not only a consequence, but also a cause of T2D and MetS generating a "vicious circle" and worsens the course of T2D[21,24,31,189]. It is mandatory to interrupt this circle and in this regard, it is encouraging that even transient remission/improvement of NAFLD is independently associated with reduced incidence of T2D[190,191].

The consequences of the association

T2D and NAFLD interact in enhancing the risk of promoting endothelial dysfunction, atherosclerosis, cardiovascular diseases, chronic kidney disease and advanced retinopathy[21,168,192,193]. The coexistence of T2D and NAFLD predicts the development of hepatic fibrosis[194-198]. Treatment of NAFLD, by affecting those pathogenic factors linking fatty liver with atherosclerosis (hence the so called "atherogenic liver") was predicted to be able to reduce cardiovascular risk a few years ago[199]. Consistent with such a prediction, a recent study reported that reduced CIMT progression in the entire cohort of 92 patients was independently associated with decreased liver fat content assessed with magnetic resonance spectroscopy[200].

T2D and NAFLD are also associated with an increased incidence and mortality from several cancer types and, similarly, mortality for malignancies is the second cause of mortality in patients with NAFL[178,201-203]. Similar to NAFLD, T2D enhances the risk of developing HCC and may aggravates HCC outcomes[204-206].

NAFLD in those with T2D will follow a worse prognosis *via* hastened progression to NASH, liver cirrhosis and its complications and increased risk of developing NAFLD-HCC[43,207-210]. Specularly, concurrent NAFLD will often worsen the course of T2D[211].

Diagnostic issues

An early diagnosis and treatment of NAFLD may potentially increase life expectancy of T2D patients[209]. Conversely, in subjects with NAFLD, periodical screening for T2D is encouraged, given that poor metabolic control will lead to progressive liver disease which,in its turn, is associated with enhanced cardiometabolic risk[38,193,212].

NAFLD: A LARGE SPECTRUM OF CLINICAL ASSOCIATIONS

An impressively heterogeneous spectrum of clinical conditions have been associated with NAFLD spanning from carotid atherosclerosis through polycystic ovarian syndrome[23]. Some will be shortly reviewed here; others have recently been covered in detail

elsewhere[4,213,214].

Atherosclerosis and gallstones

A pioneer study by Lonardo *et al*[215] evaluated the triangular association of NAFLD, gallstones and atherosclerosis. A decade later, we now know that the most severe NAFLD forms are strongly associated with excess cardiovascular mortality, probably as a direct/indirect result of hepatic fibrosis[193,212]. Concerning the NAFLD-gallstone duo, a systematic review and meta-analysis conducted in 12 observational studies (9 cross-sectional studies, 2 cohort studies and 1 case-control study) found that gallstones are significantly associated with NAFLD[216]. Further studies should be conducted regarding the underlying mechanism of this association. However, it is now clear that bile acids are no longer "a detergent which facilitates the absorption of fatty foodstuffs" such as it was deemed in the past; but rather, they must be regarded as signaling molecules, which coordinately regulate metabolism and inflammation acting not only in entero-hepatic tissues, but in peripheral organs as well[217].

Psoriasis

This line of research was almost anecdotally inaugurated by a report of three cases from the POLI. ST.E.N.A study[218]. Several systematic studies have subsequently confirmed this novel association and a recent systematic review and meta-analysis supports an association between psoriasis and NAFLD suggesting that screening for NAFLD may be warranted among those with psoriasis[219].

Endocrine derangements

Again, this topic was originally initiated by pioneer clinical observations. First, it was reported that endocrine derangements may eventually be conducive to NAFLD as a result of hormones being master regulators of body fat distribution and cell metabolism[220]. Next, endocrine pathways specifically leading to NASH were identified[221]. Among these, hypothyroidism has been associated to the whole NAFLD spectrum, steatosis to NASH-HCC[156, 221-224].

A large Rotterdam population-based survey, aimed at investigating the association between variations in thyroid function and NAFLD, prospectively, in 9419 adults with a median follow-up time of 10 years; the presence of fibrosis in those with NAFLD was assessed with TE[225]. Data have shown that, compared to euthyroid individuals, hypothyroid ones were exposed to a higher risk of NAFLD and of clinically relevant fibrosis; consistently, on the opposite side of the spectrum, hyperthyroidism was associated with a protection from developing NAFLD[225]. These findings may potentially disclose novel treatment strategies.

NAFLD: PRINCIPLES OF TREATMENT

It is widely accepted that lifestyle changes, namely diet and physical exercise are the mainstay of treatment of NAFLD, a condition no drugs are specifically licensed for[4,38].

Diet

A 3%-5% weight loss will improve steatosis; however, 5%-7% reductions are necessary to decrease hepatic inflammatory changes and 7%-10% to obtain remission of NAFLD/NASH and regression of fibrosis. Calorie restriction *per se* rather than specific diets are deemed to be beneficial[226]. In particular, despite emphasis is usually put in restricting dietary carbohydrates, moderate-carbohydrate diets do not induce a more profound reduction in liver enzymes (a non-validated surrogate index of NAFLD severity) than low/moderate-fat diets[227].

Physical exercise

Exercise effectively reduces visceral and perhaps liver fat. A recent meta-analysis found that aerobic exercise but not resistance training exercise was effective in reducing visceral fat in overweight/obese adults with T2D[228]. The benefits of exercise in reducing intrahepatic triglyceride content will typically occur with minimal or no body weight loss[227,229], suggesting that diet and physical exercise will improve NAFLD and should be associated whenever possible given that they act *via* different pathways. Indeed, diet combined with exercise is associated with improvement in NAFLD activity score[227].

Drugs

Drug treatment (better if within the frame of a clinical trial) should be reserved to those NASH individuals who do not respond to lifestyle changes. Drugs used to treat concurrent metabolic disorders (*e.g.*, statins and antidiabetics) should be differentiated from drugs aimed at treating NASH *per se*.

Statins, glitazones and liraglutide belong to the first group. Our appreciation of the role of statins in hepatology has undergone a dramatic evolution from under-prescription based on fears of hepatotoxicity[230] to their identification as potentially protectors from the full spectrum of liver damage in NASH, notably including liver fibrosis and HCC[231-238].

As regards the oral antidiabetic agents glitazones, a meta-analysis of five eligible randomized controlled trials showed that these drugs improved neither fibrosis nor IR; however, lobular inflammation decreased in NASH patients who received TZD treatment[239]. However, a more recent meta-analytic review was specifically aimed at investigating the role of thiazolidinediones in advanced hepatic fibrosis in those with NASH analyzed 8 RCTs (5 evaluating pioglitazone and 3 evaluating rosiglitazone) enrolling 516 patients with biopsy-proven NASH for a duration of 6 to 24 mo. Among all studies combined, thiazolidinedione therapy was associated with improved advanced fibrosis, fibrosis of any stage

Table 2 Drugs useful in treating nonalcoholic steatohepatitis - A neck-to-neck comparison of findings from two different meta-analytic studies

	Sawangjit *et al*[244]	Singh *et al*[243]
Fibrosis	OCA[1] improves fibrosis. PTX[2], TZDs plus Metformin[2], TZDs plus Losartan[2] might potentially be effective.	PTX[1] and OCA[1] improve fibrosis
Resolution of NASH	TZDs[1] and Vitamin E[1] use is associated with resolution of NASH	Vitamin E[1], TZDs[1], and OCA[1] improve ballooning degeneration
NAS	PTX[1], OCA[1], vitamin E[1], and TZDs[1] improve NAS	
Steatosis		PTX[1], Vitamin E[2], TZD[1] and OCA[2] significantly improve steatosis
Lobular inflammation		PTX[1], TZDs[1] and OCA[2] significantly improve lobular Inflammation

[1]High quality of evidence; [2]Moderate quality of evidence. NASH: Nonalcoholic steatohepatitis; NAS: NASH activity score; OCA: Obeticholic acid; PTX: Pentoxyphylline; TZDs: Thiazolidinediones.

and NASH resolution. Analyses restricted to RCTs enrolling patients without diabetes yielded similar results. All effects were accounted for by pioglitazone use[240]. Weight gain and lower limb edema occurred more frequently with thiazolidinedione therapy[240]. These novel data indicate that pioglitazone improves advanced fibrosis in NASH, even in patients without diabetes.

A phase 2 randomized study conducted on 52 patients (26 per arm) tested the efficacy of liraglutide (1.8 mg/daily *via* subcutaneous route) *vs* placebo. The use of liraglutide was associated with a 39% NASH resolution rate *vs* 9% in the placebo arm (RR = 4.3, 95%CI: 1.0-17.7, P = 0.019). This study was funded by the manufacturers of liraglutide[241].

A few drugs, such as vitamin E and obeticholic acid (OCA) have specifically been tested against NASH. Based on a meta-analysis of five RCTs vitamin E significantly reduced liver enzymes (AST, ALT, ALP); steatosis, inflammation and hepatocellular ballooning compared to the control group. Vitamin E treatment in adult NASH was also associated with reduction of fibrosis[242]. Concerns have been raised, however, on the long-term safety of Vitamin E and the lack of data in patients with T2D and cirrhosis. A network meta-analysis found that pentoxyphylline and OCA improve fibrosis, and vitamin E, TZDs, and OCA improve ballooning degeneration in patients with NASH[243]. A subsequent network meta-analysis substantially confirmed these findings by showing that OCA significantly improves fibrosis, while vitamin E and TZDs result in a significant increase in NASH resolution[244]. Table 2 compares the findings from the above two meta-analytic studies[243,244].

Thinking out of the box. Innovative treatment strategies

Further to the above discussed notions on the relationship of the entire NAFLD spectrum with endocrine and metabolic derangements, a line of innovative treatment options can be envisaged. For example, administration of thyroid receptor agonists (GC-1 and KB2115) improves NAFLD in a ob/ob mouse model and a xantine oxidase inhibitor (febuxostat) antagonizes the development of disease in a rodent NASH model[245,246].

Also our understanding of the role of nuclear receptors in the pathogenesis and natural course of NAFLD may lead to novel treatments[15]. The above-mentioned OCA exerts its effects by agonizing FXR. Elafibranor, a dual PPAR-α/δ agonist, has been shown to resolve NASH without fibrosis worsening and to improve the cardiometabolic profile in a recent phase 2 randomized trial of patients with NASH[247]. Oltipraz is a synthetic inhibitor of LXR-α whose antisteatosic properties have previously been shown in a mouse model. A recent phase 2 randomized controlled trial found that, in patients with NAFLD, a 24-wk course of oltipraz significantly reduced intrahepatic fat content assessed with magnetic resonance spectroscopy in a dose-dependent manner; however IR, liver enzymes, lipids and cytokines were unaltered[248].

CONCLUSION

NAFLD is an emerging multifaceted systemic disease with a heavy epidemiological burden. Further to hepatic risks, NAFLD also affects cardiovascular, metabolic, renal and endocrine systems as well as cancer development with a significant hepatic and extra-hepatic impact on morbidity and mortality[4]. The bi-directional relationship between NAFLD and MetS-T2D is an important emerging topic underpinning the mutual influence and the need to interrupt the *vicious circle* associating liver and dysmetabolism. However, we now know that IR is a necessary though not sufficient requisite of the development of NAFLD and that gender, genetic background and microbiota also play a major role[4,14]. It is anticipated that pathogenic complexity and multi-factoriality will have to be translated into more finely tailored diagnostic and treatment strategies. This implies that *"ad hoc"* studies are necessary to more precisely identify the individual target populations to be addressed. Presently, lifestyle changes effected *via* dietary restrictions and physical exercising remain the only recommended treatment of NAFLD; in all overweight/obese patients, weight loss is mandatory[4]. However, given that only a small minority of such individuals will succeed in maintaining their ideal body weight, and that no currently marketed

drugs are specifically licensed for NASH, data of ongoing studies are eagerly awaited to offer these numerous and heterogeneous patients effective and safe tailored drug treatment options.

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REVIEW

New therapeutic perspectives in irritable bowel syndrome: Targeting low-grade inflammation, immuno-neuroendocrine axis, motility, secretion and beyond

Emanuele Sinagra, Gaetano Cristian Morreale, Ghazaleh Mohammadian, Giorgio Fusco, Valentina Guarnotta, Giovanni Tomasello, Francesco Cappello, Francesca Rossi, Georgios Amvrosiadis, Dario Raimondo

Emanuele Sinagra, Francesca Rossi, Dario Raimondo, Gastroenterology and Endoscopy Unit, Fondazione Istituto Giuseppe Giglio, Contrada Pietra Pollastra Pisciotto, 90015 Cefalù, Italy

Emanuele Sinagra, Giovanni Tomasello, Francesco Cappello, Euro-Mediterranean Institute of Science and Technology, 90100 Palermo, Italy

Emanuele Sinagra, Giovanni Tomasello, Francesco Cappello, Department of Experimental Biomedicine and Clinical Neuroscience, Section of Human Anatomy, University of Palermo, 90100 Palermo, Italy

Gaetano Cristian Morreale, Georgios Amvrosiadis, Unit of Gastroenterology, Ospedali Riuniti Villa Sofia-Vincenzo Cervello, 90100 Palermo, Italy

Ghazaleh Mohammadian, Department of Medicine, Division of Gastroenterology and Hepatology, Karolinska Institutet, Karolinska University Hospital, Huddinge, 17176 Stockholm, Sweden

Giorgio Fusco, Unit of Internal Medicine, Ospedali Riuniti Villa Sofia-Vincenzo Cervello, 90100 Palermo, Italy

Valentina Guarnotta, Section of Cardio-Respiratory and Endocrine-Metabolic Diseases, Biomedical Department of Internal and Specialist Medicine, University of Palermo, Palermo 90127, Italy

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Correspondence to: Dr. Emanuele Sinagra, Gastroenterology and Endoscopy Unit, Fondazione Istituto Giuseppe Giglio, Contrada Pietra Pollastra Pisciotto, snc, 90015 Cefalù, Italy. emanuelesinagra83@googlemail.com
Telephone: +39-92-1920712
Fax: +39-92-1920406

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Abstract

Irritable bowel syndrome (IBS) is a chronic, recurring, and remitting functional disorder of the gastrointestinal tract characterized by abdominal pain, distention, and changes in bowel habits. Although there are several drugs for IBS, effective and approved treatments for one or more of the symptoms for various IBS subtypes are needed. Improved understanding of pathophysiological mechanisms such as the role of impaired bile acid metabolism, neurohormonal regulation, immune

dysfunction, the epithelial barrier and the secretory properties of the gut has led to advancements in the treatment of IBS. With regards to therapies for restoring intestinal permeability, multiple studies with prebiotics and probiotics are ongoing, even if to date their efficacy has been limited. In parallel, much progress has been made in targeting low-grade inflammation, especially through the introduction of drugs such as mesalazine and rifaximin, even if a better knowledge of the mechanisms underlying the low-grade inflammation in IBS may allow the design of clinical trials that test the efficacy and safety of such drugs. This literature review aims to summarize the findings related to new and investigational therapeutic agents for IBS, most recently developed in preclinical as well as Phase 1 and Phase 2 clinical studies.

Key words: Therapy; Low grade inflammation; Motility; Secretion; Irritable bowel syndrome; Immunoendocrine axis

Core tip: Irritable bowel syndrome (IBS) is a chronic, recurring, and remitting functional disorder of the gastrointestinal tract characterized by abdominal pain, distention, and changes in bowel habits. Despite there are several drugs for IBS, effective and approved treatments for one or more of the symptoms for various IBS subtypes are needed. The understanding of pathophysiological mechanisms such as the role of impaired bile acid metabolism, neurohormonal regulation, immune dysfunction, the epithelial barrier and secretory properties of the gut has led to advancements in the treatment of IBS. This literature review aims to summarize the findings relating the new and investigational therapeutic agents for IBS, most recently developed in preclinical as well as Phase 1 and Phase 2 clinical studies.

Sinagra E, Morreale GC, Mohammadian G, Fusco G, Guarnotta V, Tomasello G, Cappello F, Rossi F, Amvrosiadis G, Raimondo D. New therapeutic perspectives in irritable bowel syndrome: Targeting low-grade inflammation, immuno-neuroendocrine axis, motility, secretion and beyond. *World J Gastroenterol* 2017; 23(36): 6593-6627 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6593.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6593

INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic, recurring, and remitting functional disorder of the gastrointestinal (GI) tract characterized by abdominal pain, distention, and changes in bowel habits that do not have a known structural or anatomical explanation[1].

IBS is a global problem, with anywhere from 5% to 15% of the general population showing symptoms that would satisfy a definition of IBS[2-4]. IBS considerably affects quality of life and imposes a profound burden on patients, physicians and the health-care system[5]. For example, the IBIS-C study recently assessed the socio-economic burden of moderate-to-severe IBS with constipation in six European countries (France, Germany, Italy, Spain, Sweden and the United Kingdom), showing that IBS represents a main cause of absenteeism in the workplace[6].

Regarding the sex-related prevalence of IBS, in Western countries, the prevalence of IBS in women outnumbers that in men by 2:1[7,8], and within the patient population who have consultations with primary care physicians, women outnumber men by 3:1[7,9]. Finally, in tertiary care settings, the number of women with IBS is 4 to 5 times higher than the number of men[7-10].

According to Rome Ⅲ, IBS is defined based on the presence of: recurrent abdominal pain or discomfort at least 3 d/mo in the past 3 mo associated with two or more of the following: (1) improvement with defecation; (2) onset associated with a change in frequency of stool; and (3) onset associated with a change in form (appearance) of stool.

These criteria should be fulfilled for the past 3 mo with symptom onset at least 6 mo before diagnosis[11]. Recently, the Rome Ⅳ criteria implemented the knowledge accumulated since Rome Ⅲ was published almost ten years ago.

According to Rome Ⅳ, IBS is defined on the basis of the presence of: Recurrent abdominal pain, on average, at least 1 d per week in the last 3 mo, associated with 2 or more of the following criteria: (1) related to defecation; (2) associated with a change in frequency of stool; and (3) associated with a change in form (appearance) of stool. These criteria should be fulfilled for the last 3 mo with symptom onset at least 6 mo before diagnosis[12].

In contrast to the Rome Ⅲ criteria, the term discomfort has been deleted from the last definition and from subsequent diagnostic criteria because not all languages have the term "discomfort". This word has different meanings in different languages, which can result in ambiguity with patients[12]. Furthermore, the last definition implies a change in the frequency of abdominal pain, highlighting that patients should have symptoms of abdominal pain at least 1 d per week during the past 3 mo[12]. Finally, the sentence "improvement with defecation" was substituted in the current definition by "related to defecation", as a large subset of IBS patients do not have an improvement in abdominal pain with defecation but instead complain of worsening[12].

According to the Rome Ⅳ criteria, IBS is subtyped according to the predominant bowel habit as follows:

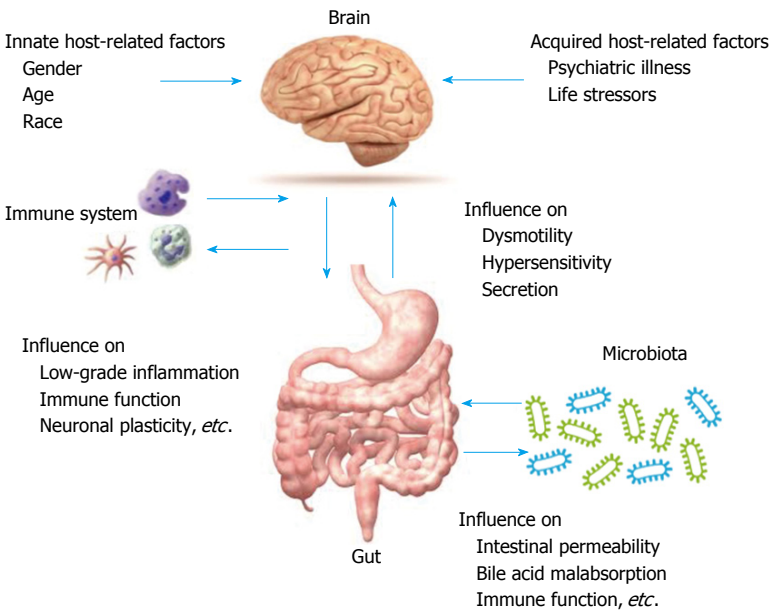

Figure 1 Complex interplay between low-grade inflammation, immuno-neuroendocrine axis, and microbiota. Brain (influenced by multiple innate and acquired factors) and gut interact bidirectionally to shape the clinical phenotype of irritable bowel syndrome (IBS). This bi-directional pathway acts not only on gastrointestinal motility, visceral sensitivity and secretion; however, the influence of both the immune system and microbiota modulates several functions that could create the definitive clinical phenotype of IBS.

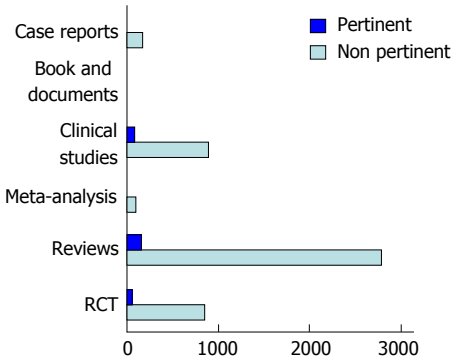

Figure 2 Literature findings on the relationship between irritable bowel syndrome and inflammation (*n* = 317). RCT: Randomized controlled trials.

IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), mixed type (IBS-M), and unclassified (IBS-U)[12]. The definition of bowel habit type is based on the patient's description of the stool form by referring to the Bristol Stool Scale[13]. Furthermore, IBS patients can be grouped into sporadic (nonspecific) and post-infectious (PI-IBS)/inflammatory bowel disease (IBD)-associated (IBD-IBS)[14,15].

Although there are several drugs for IBS in the pipeline, there is a continuous need for effective and approved treatments for one or more of the symptoms of IBS subtypes[16-18]. The understanding of pathophysiological mechanisms such as the role of altered bile acid metabolism, neurohormonal regulation, immune dysfunction, the epithelial barrier and secretory properties of the gut has led to progress in the treatment options of IBS (Figure 1)[18,19].

This literature review aims to summarize the findings relating the new and investigational therapeutic agents for IBS most recently developed in preclinical as well as Phase 1 and Phase 2 clinical studies.

MATERIALS AND METHODS

We carried out a bibliographic search in MEDLINE for the period January 1966 to December 2016 and focused on identifying publications describing the new therapeutic pharmacological approaches in IBS. Information was also obtained from abstracts and the latest results found in the Clinicaltrial.gov database. The keywords used were: irritable bowel syndrome, inflammation, immunoendocrine axis, intestinal permeability, IBS-C, IBS-D, therapy. The inclusion criteria to select articles were based on design (systematic reviews, meta-analysis, clinical trials, and experimental studies on animals) and population (adult patients > 18 years of age). We excluded articles not relevant for this topic.

According to the abovementioned criteria, 5127 studies were found and 4810 studies were excluded because they were not relevant for this topic (Figure 2).

LOW-GRADE INFLAMMATION IN IRRITABLE BOWEL SYNDROME

Recently, the scientific community has focused its attention on the pivotal role of low-grade mucosal inflammation in IBS, considering evidence showing that some patients with IBS have an increased number of inflammatory cells in the colonic and ileal mucosa, with regard to control patients[20].

In fact, the intestinal mucosa harbours a florid immune system that can be regarded as "physiologically inflamed"[20,21]. Thus, low-grade inflammation, which likely plays a multifactorial role in IBS pathophysiology, can only be evaluated using quantitative assessments[20-22].

The available data[23,24] on low-grade inflammation in IBS patients is often expressed as average numbers and are mainly focused on IBS-D. Thus, it is unclear whether this event occurs only in selected subsets of IBS patients[25].

Therefore, IBS could be considered a micro-organic disease, where there is an increased number of mucosal immunocytes (*i.e.,* mast cells, eosinophils, and T cells) in adult and paediatric patients. Several precipitating factors have been claimed, including food allergy, abnormal microbiota, bile acid malabsorption, and increased intestinal permeability[26]. The magnitude of the inflammatory response is several-fold less than that seen in acute inflammation in inflammatory bowel disease. The above-reported evidence provides a rationale to evaluate the efficacy of intestinal anti-inflammatory therapies in patients with IBS that we will touch upon in the next section.

ANTI-INFLAMMATORY THERAPIES IN IRRITABLE BOWEL SYNDROME

Corticosteroids

In the study performed by Dunlop *et al*[24], twenty-nine patients with post-infectious irritable bowel syndrome underwent a randomized, double-blind, placebo-controlled trial of 3 wk of oral prednisolone, 30 mg/d. Mucosal enterochromaffin cells, T lymphocytes and mast cells were evaluated in rectal biopsies before and after treatment, and bowel symptoms were reported in a daily diary. In this study, enterochromaffin cell counts did not change significantly after either prednisolone or placebo. Although lamina propria T-lymphocyte counts decreased significantly after prednisolone, but not after placebo, this was not linked with any significant treatment-related improvement in abdominal pain, diarrhoea, frequency or urgency[24].

Antibiotics

Rifaximin is a rifamycin derivative that acts by inhibiting bacterial ribonucleic acid (RNA) synthesis. It is virtually unabsorbed after oral administration, so it is used mainly to treat local dysfunctions within the gastrointestinal tract[27].

The Food and Drug Administration (FDA) initially approved rifaximin to treat traveller's diarrhoea caused by Escherichia coli and to prevent the recurrence of hepatic encephalopathy.

Successively, the FDA approved rifaximin in IBS-D "naive" patients at a dose of 550 mg three times a day for 14 d as well as in patients experiencing a recurrence of symptoms.

Rifaximin improves IBS symptoms through a variety of mechanisms directed at the gastrointestinal tract. In fact, much evidence from animal experiments shows that rifaximin either improves or maintains microbiota diversity and bacterial composition in IBS, reduces intestinal cytokine inflammation, provides gut-barrier protection preventing attachment and internalization of coliforms and pathogens with reduced epithelial cell inflammation and pathogen-induced inflammatory response, and reduces visceral hyperalgesia[28].

In a combined analysis of two separate Phase 3 trials (TARGET 1 and 2), a 14-d course of rifaximin 550 mg three times daily in IBS-D patients significantly increased the percentage of relief of global IBS symptoms and improved IBS-related distention and abdominal pain, discomfort, and loose or watery stools compared with placebo for up to 10 wk post-treatment[29,30].

Successively, TARGET 3 was performed to test the safety and efficacy of a repeated treatment with rifaximin in patients experiencing a recurrence of IBS symptoms. In this study, the percentage of responders during the 18-wk follow-up (in terms of pain and stool consistency improvements) to randomized repeat treatment was significantly greater with rifaximin *vs* placebo[31]. The safety profile of rifaximin in patients with IBS-D was generally similar to that observed with placebo[30].

In fact, constipation was only reported in 1 (0.3%) patient in the rifaximin group and 3 (1.0%) patients in the placebo group. Only one patient in each treatment group suspended the drug. One case of Clostridium difficile infection occurred (in a patient who had been off of rifaximin for several weeks but was receiving a concomitant systemic antibiotic)[30,31].

In conclusion, these trials show that a 2-wk course of rifaximin could improve IBS-D-related symptoms, and in the case of persistence of symptoms, retreatment may ameliorate abdominal pain and stool consistency with possible improvements in bloating and stool urgency in some patients. While patients were retreated within an 18-wk period of follow-up in the study, it is still unclear as to when and how often treatment should be given. In addition, the identification of those patients who might likely respond to rifaximin remains to be investigated.

Recently, Ghoshal *et al*[32] evaluated symptom resolution among IBS patients with or without small intestinal bacterial overgrowth (SIBO) on norfloxacin treatment and its efficacy in obtaining negative SIBO test results as compared with placebo. In this study, 80 IBS patients (Rome Ⅲ) were evaluated for SIBO by gut aspirate culture. Patients with a colony count ≥ 10 CFU/mL and those without SIBO were separately randomized to 800 mg/d norfloxacin for 10 d or placebo. The global symptom score (blind), Rome Ⅲ

criteria, aspirate culture, and glucose hydrogen breath test were assessed before and 1 mo after treatment, and patients were followed up for 6 mo. Although norfloxacin was more effective at decreasing the symptom score at 1 mo among patients with compared with those without SIBO but not placebo, the scores were comparable at 6 mo. Symptoms more often resolved to turn Rome Ⅲ negative in SIBO patients treated with norfloxacin compared with placebo at 1 mo. Patients without SIBO and a colony count of 10 CFU/mL responded more than those with a colony count less than 10 CFU/mL[32].

Mast cell stabilizers

Since mast cell activation was thought to be involved in visceral hypersensitivity, a study was undertaken by Klooker *et al*[33] to evaluate the effect of ketotifen, a mast cell stabilizer, on rectal sensitivity and symptoms in patients with IBS. In this case-control study, 60 patients with IBS underwent a barostat study to assess rectal sensitivity before and after 8 wk of treatment. After the initial barostat, patients were randomised to receive ketotifen or placebo. Ketotifen increased the threshold for discomfort in patients with IBS and visceral hypersensitivity but not placebo. This effect was not observed in normosensitive patients with IBS. Ketotifen significantly reduced abdominal pain and other IBS symptoms and improved quality of life. However, whether this effect was secondary to the mast cell stabilising properties of ketotifen or H1 receptor antagonism remains a topic of future research[33].

Successively, Lobo *et al*[34] showed a clinical Benefit of Disodium Cromoglycate (DSCG) in IBS in a double-blind, placebo-controlled clinical assay with prolonged (6 mo) oral administration of DSCG (DSCG), since it induces mast cell-mediated recovery of the healthy-like innate immunity gene expression profile in the jejunal mucosa[34].

Finally, since histamine sensitizes the nociceptor transient reporter potential channel V1 (TRPV1) and has been observed to play role in visceral hypersensitivity in animals, Wouters *et al*[35,36] investigated the role of ebastine, an antagonist of histamine receptor H1 (HRH1), in reducing symptoms of patients in a randomized placebo-controlled trial. After a 2-wk run-in period, subjects were enrolled randomly to groups given either the HRH1 antagonist ebastine or placebo for 12 wk. Rectal biopsy specimens were collected, barostat studies were performed, and symptoms were recorded (using the validated gastrointestinal symptom rating scale) before and after the 12-wk period. Patients were followed up for a further 2 wk. The primary end point of the study was the evaluation of ebastine efficacy on the symptom score evoked by rectal distension. Compared with the placebo group, patients treated with ebastine had reduced visceral hypersensitivity, increased symptom relief, and reduced abdominal pain scores[35,36].

Mesalazine

The therapeutic potential of aminosalicylates, whose benefits in chronic inflammatory bowel diseases are well known, has been focused on as a potential cure for IBS[37,38].

The largest studies on mesalazine in IBS have been conducted by Barbara and Lam. Barbara *et al*[39] conducted a phase 3, multicentre, tertiary setting, randomised, double-blind placebo-controlled trial in patients with Rome Ⅲ-confirmed IBS. Patients were randomly assigned to either 800 mg mesalazine or placebo three times daily for 12 wk and were followed for an additional 12 wk. The primary efficacy endpoint was satisfactory relief of abdominal pain/discomfort for at least half of the weeks of the treatment period. The secondary endpoint was satisfactory relief of overall IBS symptoms. The responder patients were 68.6% in the mesalazine group *vs* 67.4% in the placebo group. However, with the 75% rule or > 75% rule, there was a higher percentage of responders in the mesalazine group than placebo of 11.6% and 5.9%, respectively, although these differences were not significant. For the key secondary endpoint, in the mesalazine group, overall symptom improvement was observed and a significant difference of 15.1% *vs* placebo with the > 75% rule was reached. The authors concluded that mesalazine treatment was not superior to placebo on the study primary endpoint, but a subgroup of patients with IBS had a sustained therapy response and benefits from mesalazine therapy[39].

On the other hand, Lam *et al*[40] conducted a double-blind, randomised placebo-controlled trial of 2 g mesalazine twice daily compared with placebo for 3 mo in Rome Ⅲ criteria patients with IBS-D. The authors compared the mesalazine and placebo effects on stool frequency as the primary endpoint and secondarily assessed the effect of mesalazine on abdominal pain, stool consistency, urgency and satisfactory relief of IBS symptoms. In total, 136 IBS-D patients (82 female, 54 male) were enrolled; 10 patients withdrew from each group. The intention to treat analysis showed that the mean daily stool frequency during weeks 11 and 12 was 2.8 (SD 1.2) in the mesalazine group and 2.7 (SD 1.9) in the placebo group, with a group difference of 0.1. The authors concluded that mesalazine did not ameliorate abdominal pain, stool consistency or percentage with satisfactory relief compared with placebo during the last 2 weeks' follow-up. However a post hoc analysis in 13 post-infectious patients with IBS tended to show benefit, even though this finding needs to be confirmed in larger studies[40].

A point of weakness of these studies is that the use of endpoints for response may be easily met by patients in the placebo arm, resulting in placebo response rates of almost 70% for satisfactory relief

Table 1 Summary of the literature findings about anti-inflammatory therapies in irritable bowel syndrome

Drug	Ref.	No. of patients	Study design	Outcome
Corticosteroids (prednisolone)	Dunlop *et al*[24]	29 patients with post-infectious irritable bowel syndrome	Randomized, double-blind, placebo-controlled trial of 3 wk of oral prednisolone, 30 mg/d	Not associated with any significant treatment-related improvement in abdominal pain, diarrhoea, frequency or urgency
Antibiotics (Rifaximin)	Pimentel *et al*[29] Target 1 e 2	623 IBS patients in TARGET 1 and 637 IBS in TARGET 2	Phase 3 trials, 14 d with rifaximin 550 mg 3 times daily	Significantly increased the percentage of relief of global IBS symptoms and improved IBS-related bloating and abdominal pain, discomfort, and loose or watery stools, with regard to placebo for up to 10 wk post-treatment
Antibiotics (norfloxacin)	Ghoshal *et al*[32]	80 IBS patients evaluate for SIBO	Randomized, double-blind, placebo-controlled trial; patients were randomized to 800 mg/d norfloxacin for 10 d or placebo	Although norfloxacin was more effective at reducing the symptom score at 1 mo among patients with compared with those without SIBO but not placebo, the scores were comparable at 6 mo. Symptoms more often resolved to turn Rome Ⅲ negative in SIBO patients treated with norfloxacin compared with placebo at 1 mo
Mast cell stabilizers (Ketotifen)	Klooker *et al*[33]	60 IBS patiens	Case Control study; abarostat study to assess rectal sensitivity before and after 8 wk of treatment and, after the initial barostat, patients were randomised to receive ketotifen or placebo	Ketotifen but not placebo increased the threshold for discomfort in patients with IBS with visceral hypersensitivity, but this effect was not observed in normosensitive patients with IBS. Ketotifen significantly decreased abdominal pain and other IBS symptoms and improved quality of life
Mast cells stabilizers (DSCG)	Lobo *et al*[34]		Randomized, double-blind, placebo-controlled trial; with prolonged (6 mo) oral administration of DSCG	Induces Mast Cell-Mediated Recovery of Healthy-Like Innate Immunity Genes Expression Profile in the Jejunal Mucosa
Mast cells stabilizers (ebastin)	Wouters *et al*[35]	65 IBS patients	Double-blind placebo-controlled trial, after 2-wk run-in period, subjects were assigned randomly to groups ebastine (20 mg/d; n = 28) or placebo (n = 27) for 12 wk	Compared with subjects given placebo, those given ebastine had reduced visceral hypersensitivity, increased symptom relief, and reduced abdominal pain scores
Mesalazine	Barbara *et al*[39]	185 patients with IBS	A phase 3, multicentre, tertiary setting, randomised, double-blind, placebo-controlled trial in patients with Rome Ⅲ confirmed IBS. Patients were randomly assigned to either mesalazine, 800 mg, or placebo, three times daily for 12 wk, and were followed for additional 12 wk	Mesalazine treatment was not superior than placebo on the study primary endpoint, but a subgroup of patients with IBS showed a sustained therapy response and benefits from a mesalazine therapy
	Lam *et al*[40]	136 patients with IBS-D	A double-blind, randomised placebo-controlled trial of 2 g mesalazine twice daily compared with placebo for 3 mo	The authors concluded that mesalazine did not improve abdominal pain, stool consistency or percentage with satisfactory relief compared with placebo during the last 2 weeks' follow-up, however a post hoc analysis in 13 post-infectious patients with IBS appeared to show benefit but this needs confirmation in a larger group[40]

IBS : Irritable bowel syndrome.

of abdominal pain or discomfort and > 60% for satisfactory relief of overall IBS symptoms in the trial performed by Barbara *et al*[39] and in > 40% for satisfactory relief of IBS symptoms in the trial performed by Min *et al*[41].

It may have therefore have been preferable to use a once daily dosing schedule in both trials in order to reduce the placebo response rates, thus increasing the likelihood of detecting a statistically significant difference between mesalazine and placebo.

Based on this evidence, it is necessary that further studies prove the efficacy of mesalazine for IBS. Studies aimed at evaluating the role of aminosalicylates and other potential anti-inflammatory treatment options, including probiotics, non-absorbable antibiotics, histamine receptor antagonists and protease inhibitors on IBS symptoms or pathophysiology are now warranted[39].

Table 1 sums up the literature findings about anti-inflammatory therapies in irritable bowel syndrome.

INTESTINAL PERMEABILITY IN IRRITABLE BOWEL SYNDROME

An increase in intestinal permeability can be seen in

many conditions, such as infectious gastroenteritis and irritable bowel disease[42]. The intestinal barrier has long been a focus of gastroenterological research[43] and its role in IBS has been discussed in many studies. Most studies show an increase in intestinal permeability of patients with IBS-D and post-infectious IBS (PI-IBS)[43-46].

Among the first to describe intestinal permeability in patients with PI-IBS were Spiller *et al*[47], who detected an increased lactulose/mannitol ratio in the urine of IBS patients compared to healthy controls.

Marshall *et al*[48] also described an increase in permeability of patients with IBS after an outbreak of bacterial gastroenteritis but could not show a difference in permeability between PI-IBS and non PI-IBS.

There are genetic risk factors for developing PI-IBS and CDH1, which codes for E-cadherin, a tight junction (TJ) protein that is involved in the epithelial barrier function of the gut[49], hence suggesting the pathophysiological mechanism through which some patients experience increased permeability.

The mechanism of increased permeability in patients with IBS is suggested to involve tight junction dysfunction or involvement of the adherence proteins[44]. Among factors that could influence permeability is stress. Male soldiers were evaluated in a prospective study during and after combat training with an increase in physiological and psychological stress. Their training induced an increase in gastrointestinal symptoms and alteration in the permeability of the gut barrier[50]. Since stress has been suggested to be one of the pathophysiological factors involved in developing IBS, this mechanism could explain the reason for the gastrointestinal symptoms[51].

Another factor that has been evaluated is the intraluminal content of patients with IBS, where faecal supernatants from patients have increased the colonic permeability in mice[52,53].

Both intracellular [zonula occludens (ZO)-1, ZO-2, and ZO-3, and cingulin] and surface-membrane proteins [occludin, claudins, and junctional adhesion molecules (JAM)] are the main components of TJ[26,54]. Adherens junctions are mainly made up of e-cadherin, catenin, and actin filaments[26,55].

Inflammation has also been described to be a factor in increasing intestinal permeability, not only in inflammatory bowel disease[43] but also in IBS, where the increase in mast cells and the mediators increased the effects on the intercellular junctions[44].

Finally, other factors, such as hormonal and neurohormonal pathways, nutritional factors, ethanol consumption and several drugs (nonsteroidal anti-inflammatory drugs, methotrexate, tacrolimus, protonic pump inhibitors), could affect the intestinal barrier, a factor that needs to be further evaluated[26]. The knowledge of affection of the intestinal permeability in IBS patients will help in the development of new therapies in order to restore the gut barrier, a topic we will touch upon in the next section.

Table 2 sums up the literature findings about therapies restoring intestinal permeability in irritable bowel syndrome.

THERAPIES RESTORING INTESTINAL PERMEABILITY IN IRRITABLE BOWEL SYNDROME

Probiotics

The human intestinal microbiota represents one of the densest, biodiverse, and rapidly evolving bacterial ecosystems. The intestinal microbiome, that is, its collective genome, is an adaptive entity that varies with diet, lifestyle and environment, providing a further metabolic flexibility to the human super organism and functional traits that humans have not evolved on their own[56]. Therefore, the potential of manipulating the gut microbiota in these disorders is assessed[57].

The mechanisms through which probiotics alter the intestinal microbial flora could be direct, changing the bacterial macroenvironment of the lumen, or indirect, through the stimulation of the immune system and the improvement of mucosal function, for example, by modulating the invasion and adherence of the epithelial cells of the gut by pathogenic bacteria, thus normalizing gut permeability[58-60]. The use of probiotics in patients with IBS seems to be effective in achieving improvement in the global IBS symptoms[61,62], but how it affects the intestinal permeability is less evaluated in humans[46]. Most studies have shown that altering the intraluminal content affects the barrier functions of the gut, and studies on rodent models of IBS have shown different data[63].

Despite the growing interest of the scientific community in research in the field of probiotics, the interpretation of the scientific literature on the value of these preparations' results is difficult due to the wide variability in the species, strains and doses employed in the preparations as well as the low methodological quality of the available trials, often due to the poor design and the small sample size.

Several meta-analysis have been published on this topic[64-66], all concluding that probiotics might be efficacious in IBS, but the actual benefit and the most effective species and strains are uncertain.

In the meta-analysis performed by Ford *et al*[61], including forty-three randomized controlled trials, probiotics showed beneficial effects on global IBS, abdominal pain, bloating, and flatulence scores[61]. Probiotics appeared to be successful in chronic idiopathic constipation (CIC), but there were only two randomized controlled trials, and again, since trials for probiotics are few in number, no specific conclusions could be obtained[61].

Table 2 Summary of the literature findings about therapies restoring intestinal permeability in irritable bowel syndrome

Drug	Ref.	No. of patients	Study design	Outcome
Probiotics	Ford *et al*[61]	Forty-three RCTs were eligible for inclusion	Metanalysis	Probiotics had beneficial effects on global IBS, abdominal pain, bloating, and flatulence scores. Data for prebiotics and synbiotics in IBS were sparse. Probiotics appeared to have beneficial effects in CIC (mean increase in number of stools per week = 1.49; 95%CI: 1.02-1.96), but there were only two RCTs. Synbiotics also appeared beneficial (RR of failure to respond to therapy = 0.78; 95%CI: 0.67-0.92). Again, trials for prebiotics were few in number, and no definite conclusions could be drawn
	Mazurak *et al*[67]	Fifty-six papers	Metanalysis	The heterogeneity of the studies of probiotics in IBS questions the value of meta-analyses and the use of different bacterial strains and different mixtures of these strains, as well as different dosages, are the main contributors to this heterogeneity
Glutamine	Akobeng *et al*[73]	Two randomized trial	Cochrane analysis	Not significant difference in the permeability and no effect in the clinical remission
Larazotide acetate	Leffler *et al*[78]	342 adults with celiac disease who had been on a gluten free diet (GFD) for 12 mo or longer and maintained their current GFD during the study	Randomized, double-blind, placebo-controlled study assessed larazotide acetate 0.5, 1, or 2 mg 3 times daily	Reduce signs and symptoms in celiac disease patients on a GFD better than a GFD alone

In the last updated meta-analysis performed by Mazurak *et al*[67], including fifty-six papers (twenty-seven studies using multi-species bacterial preparations and twenty-nine using single-strain probiotics), they analysed the efficacy of probiotics regarding patients included, treatment duration, probiotic dosage, and outcome measures. According to the authors, the heterogeneity of the studies of probiotics in IBS impairs the value of meta-analyses. The use of different bacterial strains and different mixtures of these strains, as well as different dosages, may be the main factors contributing to this heterogeneity[67]. Currently, there is limited evidence for the efficacy of a small number of single-strain probiotics in IBS (mostly bifidobacteria), and this evidence leads to the performance of trials with inclusion and exclusion criteria closely following the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) guidelines for clinical trials in IBS[68,69], including the definition of minimal severity for inclusion, global primary endpoints, and adequate secondary end-points (pain, bloating, and a clinically meaningful responder definition). Such trials should include at least 8 wk of therapy, an adequate follow-up period and restriction to one of the different IBS subtypes[67].

Glutamine

Glutamine is one of the compounds that has been investigated as a treatment of conditions with leaky gut. It has been shown to regulate the protein turnover in enterocytes of pig[70], reduce intestinal permeability in intestinal cell cultures and maintain transepithelial resistance[71]. Glutamine has also been shown to maintain the integrity of the intestinal barrier in critically ill patients by reducing the incidence of infections[72]. Glutamine treatment in patients with Crohn's disease was recently reviewed in a Cochrane analysis[73]. In this review, only two randomized controls were included, and neither showed a significant difference in the permeability and neither had any effect on clinical remission.

Glutamine treatment in patients with IBS is less examined. Glutamine synthetase expression is lower in the small bowel and colonic mucosa of patients with IBS-D with increased intestinal permeability[74]. Therefore, one recent pilot study on IBS-D showed that with a higher glutamine concentration, Claudin-1 expression increases, thus improving the permeability[75]. However, further studies are needed for using glutamine as a supplement treatment for IBS.

Larazotide acetate

Larazotide acetate (LA) is a tight-junction regulator peptide preventing the opening of intestinal epithelial TJ[76]. The safety, tolerance and pharmacokinetics of LA were studied in a randomized double-blind placebo-controlled study conducted on celiac disease subjects challenged with gluten[76,77].

Recently, in a multicentre, randomized, double-blind placebo-controlled study, LA at doses of 0.5, 1, or 2 mg 3 times daily was evaluated to relieve ongoing symptoms in 342 adults with celiac disease who had been on a gluten-free diet (GFD) for 12 mo or longer and maintained their current GFD during the study. A 0.5 mg dose of Larazotide acetate appeared to reduce signs and symptoms in celiac disease patients on a

GFD better than a GFD alone. Although the results were mixed, this study resulted in the successful use of a novel therapeutic agent targeting tight junction regulation in those patients with CeD who are symptomatic despite a GFD[78].

Therefore, the modulation of the tight junction could represent a paradigm shift in the treatment of immune mediated and inflammatory diseases (Celiac Disease, IBD, IBS, *etc*).

THERAPEUTIC TARGETS IN CONSTIPATION-PREDOMINANT IRRITABLE BOWEL SYNDROME

Constipation-predominant irritable bowel syndrome (IBS-C) is a frequent disorder and represents one of the main causes of ambulatory visits. Abdominal pain and discomfort characterize IBS-C, making it different from chronic idiopathic constipation[79].

It is now well known that treatment focusing only on bowel transit does not provide complete relief to patients with IBS-C. A global evaluation of the pathophysiology of IBS-C has led to the use of sensory end points like complete spontaneous bowel movements and the FDA combined end point (abdominal pain and complete spontaneous bowel movements) in clinical trials[79].

For example, new information on the mechanisms underlying pain sensation in chronic visceral hypersensitivity as well as insights into the mechanism of action of new drugs targeting abdominal pain in IBS have recently been obtained by preclinical experiments in rodent models[80]. A number of drugs that we will touch upon in the next section are actually in development.

Linaclotide

Linaclotide (MD-1100 acetate) is a novel orally active 14-amino acid peptide of the guanylin family of cyclic guanosine monophosphate (cGMP)-regulating guanylate cyclase-C (GC-C) agonists. It has been approved by the FDA and by the EMA for the treatment of moderate to severe IBS-C in adults. Its action is focused on the increase of fluid secretion, favouring gastrointestinal transit, and has GC-C-mediated analgesic effects[81].

It is recommended at a dose of 290 μg orally once a day before meals. Linaclotide is converted to an active metabolite (MM-419447) that has the same pharmacodynamics and pharmacokinetics as the parent drug.

In 2007, Andresen *et al*[82] investigated the effect of 5 d of linaclotide on transit and bowel function in 36 women with IBS-C according to Rome Ⅱ criteria randomized in a 1:1:1 fashion for placebo, linaclotide 100 μg, and linaclotide 1000 μg.

Patients with slow colonic transit or slower transit than the mean for healthy controls were studied for 5 d at baseline and 5 d during the treatment. Patients collected all the information regarding gastric, small bowel, and colonic transit by scintigraphy and bowel function using stool diaries, which included Bristol Stool Form Scale (BSFS) scores for stool consistency, ease of stool passage scores, and completeness of evacuation.

Linaclotide did not show any effect on gastric emptying or colonic filling. It did show a significant effect on ascending colon emptying t½ times (P = 0.015) and on overall total colonic transit times at 48 h (P = 0.02) at the 1000 μg dose (P = 0.004), but not at the 100 μg dose, as well as on increased stool frequency, decreased stool consistency, improved ease of passage, and acceleration of time to first bowel movement (P < 0.001)[82].

In 2010, Johnston *et al*[83] investigated the efficacy and safety of 12 wk of linaclotide at a daily dose range of 75-600 μg in a phase Ⅱb randomized double-blind parallel-group multicentre placebo-controlled trial conducted on 420 patients with IBS-C (female patients = 92%). Patients had to meet Rome Ⅱ criteria, with fewer than three spontaneous bowel movements (SBMs) per week, and straining, lumpy/hard stools, or sensation of incomplete evacuation more than 25% of the time for at least 12 wk in the 12 mo preceding study entry. The primary endpoint was a change in the number of complete spontaneous bowel movements (CSBMs). Secondary endpoints were the effect on individual symptoms, quality of life (QOL), the number of patients who were CSBM responders (at least three CSBMs/wk and an increase of one CSBM from baseline for 75% of the study duration), and global relief responders (symptoms being somewhat, considerably, or completely relieved for 100% of the study duration or completely relieved for 50% of the study duration).

For the 75, 150, 300 and 600 μg linaclotide doses, the mean change in CSBMs per week was 2.90, 2.49, 3.61 and 2.68, respectively (P < 0.01), and the percentage of patients who were CSBM responders was 25%, 19.5%, 32% and 24%, respectively. Patients treated with linaclotide showed an adequate relief response (33%-51% *vs* 22%) and a global relief response (44%-55% *vs* 29%) compared to placebo. All doses of linaclotide significantly improved bowel habits, including frequency of short bowel movements (SBMs) ($P \leqslant 0.001$) and CSBMs ($P \leqslant 0.01$), severity of straining ($P \leqslant 0.001$), stool consistency ($P \leqslant 0.001$), and abdominal pain scores ($P \leqslant 0.05$), than placebo. Abdominal discomfort, bloating, and global IBS-C measures were also improved for all doses except for the 75 μg (abdominal discomfort) and 150 μg (bloating) doses. The linaclotide effect was observed at the first week and lasted throughout the 12 wk of treatment.

The approval of linaclotide for IBS-C was based on two randomized double-blind placebo-controlled phase Ⅲ trials similar in study design, end points, and patient demographics[84-86].

Primary end points included both the FDA-recommended combined primary end point and a more rigorous combined primary end point that required

even more CSBM responses for 9 of 12 wk. Secondary end points included patient-reported abdominal pain, discomfort, and bloating; straining severity; and weekly SBM and CSBM frequency and stool consistency. The first phase Ⅲ trial included 804 adults with IBS-C who were randomized 1:1 to receive linaclotide 290 lg or placebo daily for 26 wk, with change-from-baseline end points measured at 12 and 26 wk[84]. Attrition rates were 18.5% at 12 wk and 25.6% at 26 wk. At 26 wk, the majority of patients withdrew from the study due to adverse events in the linaclotide arm (10.2%) and perceived lack of efficacy in the placebo arm (8.2%). Over 12 wk, the FDA combined primary end point was achieved by 33.7% of patients receiving linaclotide compared with 13.9% of patients receiving placebo ($P < 0.0001$).

Linaclotide was also superior to placebo in the more rigorous investigator-defined combined primary end point that was reached by 12.7% of linaclotide-treated patients *vs* 3.0% of placebo-treated patients ($P < 0.0001$). At 26 wk, 32.4% of patients receiving linaclotide and 13.2% of patients receiving placebo ($P < 0.0001$) reached the FDA combined primary end point.

Improvements in all secondary end points occurred in the linaclotide group at weeks 12 and 26. The second phase Ⅲ trial of linaclotide was composed by a 12-wk treatment phase followed by a 4-wk randomized withdrawal phase[86]. A total of 803 adults with IBS-C were randomized to receive linaclotide 290 lg or placebo once/d for 12 wk. Approximately 78% of patients completed the entire 16-wk study, and most of the patients who suspended the study did so due to adverse events in the linaclotide arm (7.9%).

In the 12-wk active treatment phase, linaclotide demonstrated statistically significant improvements in all primary and secondary efficacy end points compared with placebo. Approximately one-third (33.6%) of patients receiving linaclotide fulfilled both components of the FDA end point compared with 21% of patients receiving placebo ($P < 0.0001$). Statistically significant improvements were observed also in abdominal pain, discomfort, and bloating in linaclotide-treated patients, with a mean reduction of about 2 points from baseline (on an 11-point scale) compared with reductions of 1.1 with placebo ($P < 0.0001$ for each measure).

In the linaclotide arm, an improvement in severity of straining, constipation, and stool consistency was observed compared with the placebo arm (all $P < 0.0001$).

Linaclotide caused diarrhoea, abdominal pain, flatulence, headache, viral gastroenteritis, and abdominal distension as adverse events. Diarrhoea, the most common, occurred in less the 20% of patients, probably due to increased fluid secretion and accelerated colonic transit[84,86].

In Johnston *et al*[83] phase Ⅱb dose-ranging trial, diarrhoea of mild to moderate severity was the primary dose-dependent adverse effect observed. It occurred in 11.4%, 12.2%, 16.5% and 18.0% of patients in the 75, 150, 300 and 600 μg linaclotide dose groups, respectively, compared with 1.2% in the placebo group. Dehydration or electrolyte disturbances were not found, although one instance of faecal impaction occurred[83]. In the studies by Rao *et al*[86] and Chey *et al*[84], 4.5%-5.7% of the linaclotide-treated patients and 0.2%-0.3% of the placebo group discontinued the study due to diarrhoea.

In a phase Ⅲ clinical trial in IBS-C, patients experienced adverse events more in the linaclotide 290-lg group (65.4%) than in the placebo (56.6%, $P < 0.05$) group[87]. In another IBS-C phase Ⅲ trial, adverse effects in the linaclotide group were reported at a similar rate to placebo (56.2% *vs* 53.0%, $P = 0.39$)[88]. Adverse events were reported by 60.5% of patients receiving linaclotide 145 lg, 55.7% of patients receiving linaclotide 290 lg, and 52.1% of patients receiving placebo[89].

In phase Ⅲ clinical trials in patients with IBSC, diarrhoea was the most frequently reported adverse event, occurring in 19.5%-19.7% of patients in the linaclotide groups compared with 2.5%-3.5% of patients receiving placebo ($P < 0.0001$).

In randomized trials, linaclotide at 145 μg/d was best tolerated with improvement in CSBM/Wand symptoms in patients with CIC. Patients with IBS-C best responded to the 290-μg daily dose[84,85]. Linaclotide appeared to be very well tolerated.

Linaclotide is approved for the treatment of IBS-C in both male and female adults at a dosage of 290 lg once/d and for the treatment of CIC at a dosage of 145 lg once/d. The medication should be taken 30 min prior to breakfast. Renal or hepatic impairment is unlikely to affect the metabolism or clearance of linaclotide or its metabolite due to its low systemic exposure.

In conclusion, linaclotide can represent a targeted approach that addresses the complexity of symptoms associated with the syndrome. Linaclotide has been reported to safely improve IBS-C abdominal pain severity, bowel movement quality, and bowel movement frequency as well as key symptoms of abdominal fullness, bloating, and discomfort, with associated improvements in QOL. Based on the United States FDA and the EMA, linaclotide fulfils the recommended endpoints with a number needed to treat (NNT) ranging from 4.39 to 7.9. It is effective and can be associated with diarrhoea as the most common adverse effect leading to suspension of the medication in approximately 5% of patients. According to recent clinical evidence, linaclotide should be considered for patients with IBS-C due to its effect on abdominal pain and bowel symptom improvement.

Plecanatide

Plecanatide is a 16-amino acid GC-C agonist currently used in phase Ⅲ clinical trials for CIC and phase Ⅱ

trials for IBS-C[90]. Plecanatide mimics the endogenous agonist of the GC-C receptor in the intestinal tract. Like that of uroguanylin, plecanatide's actions are pH-dependent, with the most favorable efficacy in the acidic environment of the duodenum. Similar to linaclotide, plecanatide luminally activates the GC-C receptor on gastrointestinal mucosal epithelial cells, leading to intracellular secretory and extracellular anti-nociceptive effects *via* a cGMP-mediated second messenger pathway[91]. A phase Ⅲ randomized double-blind trial in 951 patients with CIC treated with 0.3, 1 or 3 mg plecanatide or placebo once/d for 12 wk was conducted[92]. The primary end points were weekly (more than three CSBMs/wk and an increase of more than one CSBM/wk from baseline) or an overall study response (weekly response for 9 of 12 wk, including 3 of the last 4 wk to ensure durability of response). The percentage of overall responders was significantly higher in the plecanatide 3 mg group compared with placebo (19% *vs* 10.7%, $P = 0.009$). Weekly responder rates were also significantly higher in plecanatide 3 mg than placebo for weeks 1-12. Patients treated with 3 mg showed an improvement in stool frequency, consistency, straining, and quality of life compared with placebo. Data for other plecanatide doses were not shown.

Plecanatide potentially has low risk of adverse cardiovascular effects, as its systemic absorption is very low. According to the phase Ⅰ study for evaluation of the safety and tolerability of plecanatide in humans[93], no measurable systemic absorption was observed at any doses of oral plecanatide. Plecanatide was safe and well tolerated up to the highest dose. Diarrhoea was the most prevalent side effect, but its frequency did not statistically significantly differ between placebo and plecanatide, and appeared not to be dose-related in the plecanatide-treated subjects. Other gastrointestinal events were nausea, abdominal discomfort and pain, and vomiting. In a Phase Ⅱ dose escalation trial involving a total of 84 chronic constipation patients recruited with modified Rome Ⅲ criteria, 14 d of plecanatide therapy improved stool frequency, stool consistency, straining and overall relief of chronic constipation symptoms. To confirm the safety and efficacy of plecanatide, two Phase Ⅲ trials (NCT01982240andSP304203-00) have been planned. In the United States and Canada, the Phase Ⅲ trial NCT01982240 was initiated in November 2013 with adult chronic constipation patients and was expected to be completed in February 2015[94].

Prucalopride

Prucalopride is authorized in several countries (not in the United States) for women with CIC unresponsive to laxatives[95]. As a very highly selective 5-HT4 agonist, prucalopride has no measurable affinity for other receptors. In safety evaluation tests, prucalopride showed no h ERG (humanether-à-go-go-related gene) channel inhibitory activity. It is not arrhythmogenic, and it promotes colonic motility[96].

At dosages of 2 mg and 4 mg per day, this drug produced a low incidence of QT interval prolongation. Even up to 20 mg per day (10-fold higher than the recommended dosage), prucalopride displayed no clinically relevant effects on cardiovascular parameters in healthy volunteers. Prucalopride improved stool frequency and consistency, and it dose-dependently enhanced colonic transit in healthy controls or chronic constipation patients with no negative impact on gastric emptying or small bowel transit[97]. The patients' quality of life was significantly improved by prucalopride treatment.

In three pivotal trials, prucalopride showed a good efficacy in increasing CSBMs per week and in improving perceived disease severity and quality of life in patients with CC. A study conducted on 620 patients with CC treated with 2 or 4 mg of prucalopride for 12 wk showed that it increased one or more CSBMs per week compared to the control group[98-100]. In another trial conducted on 713 patients with CC, 2 or 4 mg of prucalopride increased the frequency to three or more CSBMs per week and improved evacuation completeness, perceived disease severity, and quality of life[101]. In another study conducted on patients 65 years or older with CIC, prucalopride at a dose of 1 mg for 4 wk did not cause any changes in an electrocardiogram or corrected QT (QTc) interval, showing its safety for the treatment of CIC in the elderly[97,102]. A study conducted on Asian subjects with CIC reported similar efficacy and safety as that observed in Western populations[103]. In a pooled analysis of the study with Asian subjects and the three pivotal trials, increased stool frequency of approximately three or more CSBMs per week was observed in Asian (34% *vs* 11%, $P < 0.001$) and non-Asian (24.6% *vs* 10.6%, $P < 0.001$) women. Prucalopride was shown to be safe and well tolerated[104], improving CIC abdominal symptoms such as abdominal discomfort, bloating, straining, and painful bowel movements[105]. Another study conducted on a small number of patients showed the efficacy of prucalopride not only in the treatment of slow transit constipation but also of obstructed defecation and IBS-C[106].

In a recent analysis, Camilleri *et al*[106] evaluated the efficacy of prucalopride using the data from six phase 3 and 4 multicentre double-blind randomized placebo-controlled parallel-group trials performed across three continents.

Over the 12-wk treatment period, prucalopride-treated patients consistently achieved a mean of 3 SCBMs/wk compared to placebo with the treatment response observed in the individual trials[98-100,102]. On the other hand, the SPD555-401 trial was the only trial that failed to demonstrate a statistically significant effect of

prucalopride on this primary endpoint after both 12 and 24 wk of treatment, without any plausible explanation of this lack of efficacy[107,108]. In the current study, no differences were found between men and women, although over time, a difference in the response rate has been reported. This could be related to differences in demographics (other than gender) and disease characteristics at baseline or to intrinsic differences in responsiveness to prucalopride between men and women. Furthermore, prucalopride was significantly more effective than placebo, as demonstrated by many secondary endpoints, including improvements in PAC-SYM (Patient Assessment of Constipation Symptoms) and PAC-QOL (Patient Assessment of Constipation Quality of Life) scores and rescue medication use. An exploratory efficacy analysis showed that prucalopride treatment was effective even in patients with very severe CIC and those with no SBMs at baseline.

In the current integrated analyses, the NNT with prucalopride used to achieve the primary efficacy endpoint in one patient was 8.8 (95%CI: 7.1-11.6). In a meta-analysis of data from three trials of linaclotide in patients with CIC, the NNT for the primary endpoint of these trials (3 SCBMs/wk and an increase of 1 SCBM/wk, for 75 % of weeks) was 7 (95%CI: 5-8)[109]. Prucalopride has a favorable safety and tolerability profile[110]. Notably, no cardiovascular safety signals were observed. Indeed, the mean QT interval corrected according to Bazett's formula (QTcB) and the mean QT interval corrected according to Fridericia's formula (QTcF) were both/470 ms. A potential limitation of this integrated analysis is moderate heterogeneity (I^2 = 56%) due to a deviation of the results of one of the six trials compared to the others.

Prucalopride was well absorbed from the gastro-intestinal tract, with an absolute oral bioavailability of more than 90%. Its main elimination route was *via* theurine (60%-70% excreted unchanged in the urine). Because prucalopride has a low level of metabolism by liver, its pharmacokinetics is unlikely to be altered by hepatic impairment, and no CYP3A4 drug interactions are anticipated. In Europe, 2 mg of prucalopride has been approved for the treatment of chronic constipation in women who have no adequate response to laxatives[111].

Headache (in 25%-30%), nausea (12%-25%), abdominal pain (16%-23%), and diarrhoea (12%-19%) were observed as adverse events.

Recently, a randomized trial compared prucalopride with Macrogol/PEG 3350 plus electrolytes in patients with CIC. Prucalopride showed a non-inferiority for the primary outcome, even though PEG showed a superiority in improving gastrointestinal transit, stool frequency, and number of spontaneous bowel movements[112]. Although no studies have yet evaluated the efficacy of prucalopride in IBS-C, it is expected that it may also be efficacious for the disease symptoms. However, the worsening of abdominal pain may limit its use in clinical practice.

YKP10811

YKP10811 is a novel substituted benzamide derivative, small molecule with high binding affinity to the 5-HT4 receptor[113]. In cellular functional assays conducted with the 5-HT4 receptor, YKP10811 showed weak agonist activity that was dose dependent and reproducible. These results indicated that YKP10811 acts as a partial agonist of the 5-HT4 receptor. YKP10811 did not show any significant off-target binding to any other receptors, enzymes, or serotonin-receptor subtypes at 1 mmol/L, except for binding to the 5-HT2A receptor and the 5-HT2B receptor. Thus, YKP10811 has 120-fold and 6-fold lower affinity, respectively, for 5-HT2A and 5-HT2B receptors than for 5-HT4. In cellular functional assays, YKP10811 showed antagonist activity at the 5-HT2B receptor with a median inhibitory concentration. In rats, YKP10811 accelerated colonic transit by 37% at a dose as low as 1 mg/kg. In dogs, 0.3 mg/kg YKP10811 accelerated colonic transit by 45.5% at 2 h after dosing. The accelerated colonic transit in dogs was associated with significantly increased colon contractions and defecation. YKP10811 significantly reduced visceral hypersensitivity in multiple pain models in rats. In a phase Ⅰ double-blind randomized 9-d placebo-controlled multiple-ascending dose study in healthy volunteers at doses of 5, 15, 30 and 45 mg once daily, YKP10811 was well tolerated with minimal side effects. In a single-center randomized parallel-group double-blind placebo-controlled study[114] in patients with functional constipation, YKP10811 enhanced gastrointestinal and colonic transit and improved bowel function during an 8-d treatment trial. The effect of YKP10811 on colonic transit was mirrored by improvements in softer stool consistency and faster time to first bowel movement, suggesting that YKP10811 has encouraging effects on these clinical end points. In addition to pharmacodynamic effects in patients with functional constipation, improvements in bowel functions are validated and measurable end points recommended for the treatment of functional constipation[115]. These findings suggest that YKP10811 may be a potential new medication for the treatment of functional constipation. YKP10811 had a robust effect on accelerating, by 30% to 40%, colonic emptying when compared with placebo. Ascending colon emptying has been reported to have the greatest contribution to overall colonic transit[116] because the ascending and transverse colon constitute the "reservoir" or storage regions of the human colon[117]. Among the other 5-HT4-receptor agonists previously studied with the same method, 4 mg prucalopride and 30 and 50 mg velusetrag[118] also accelerated AC emptying. Emptying of the proximal colon correlates linearly with faecal weight[119], which largely reflects

stool water content, and as expected based on prior studies, the overall colonic transit was correlated linearly with stool consistency, with less significant association with the number of bowel movements per day. The results also showed the dual action (agonist/antagonist) of YKP10811 seen in *in vitro* studies. YKP10811 facilitated the electrical field stimulation-induced neurogenic twitch of guinea pig ileum at lower concentrations. This type of dual action (agonist/antagonist) of YKP10811 under the same assay conditions was also shown in the peristaltic reflex test, with an EC50 of 0.5 mmol/L and an IC50 of 21 mmol/L. There is a significant gap in concentration ranges (> 40-fold difference) for stimulatory *vs* inhibitory effects of YKP10811 *in vitro* (unpublished data; SK Life Science, Inc). Two participants, 1 receiving placebo and 1 receiving 20 mg YKP10811, had prolonged QTc (> 470 ms). Both participants discontinued the study on the advice of the investigators, even though the QTc prolongation was minimal (functions in patients with functional constipation). Thus, YKP10811 is likely to be of benefit to patients with functional constipation without rectal evacuation disorders. The safety and efficacy of this novel agent should be studied in larger multicentre clinical trials. With further studies, the current data suggest that YKP10811 would expand the therapeutic options beyond the recently approved secretagogue medications for the treatment of functional constipation, lubiprostone and linaclotide[120,121].

YKP10811 was reported to be safe and tolerable in healthy volunteers. Except for a Phase Ⅱ clinical trial in C-IBS patients (NCT02082457)[122], there were only two registered Phase Ⅱ trials that evaluated the efficacy and safety of YKP10811 in comparison with placebo in subjects with CIC (NCT015 23184, NCT01989234)[123,124]. Collectively, 420 eligible subjects were enrolled to be treated with different doses of YKP10811 or placebo once daily for 8 d and 12 wk in two trials. The results have not been completed for reporting yet. This drug is pending to pass Phases Ⅱ and Ⅲ of clinical trials, expected in 2016.

Renzapride

Renzapride (a novel benzamide substitute) is a full agonist for the 5HT4 receptor and an antagonist to 5HT2b and 5HT3 receptors. It can accelerate the gastrointestinal tract transit and motility stimulating the 5HT4 and 5HT2b receptors[125], and it appears to be a promising therapeutic agent for constipation, which is predominant in IBS patients. It is safe and has only a few adverse effects[126,127]. Several clinical trials have been performed to evaluate its potential efficacy in IBS patients, confirming that renzapride does not cause cardiac arrhythmias in clinical doses, unlike cisapride[126,127]. It is excreted renally and is not metabolized by cytochrome P450 enzymes. Thus, no drug interactions *via* affecting cytochrome P450 enzymes have been reported[125,127]. Renzapride stimulates colonic transit and reduces transit time and pain in IBS patients due to its prokinetic property, providing a benefit in those patients with constipation[128]. In addition, a dose-dependent efficacy of this drug has been demonstrated[126]. In a phase Ⅱ study of 46 women with IBS-C, renzapride at a dose of 4 mg q.d. favoured colonic transit and increased ascending colon emptying compared to placebo[129]. A large multicentre European trial confirmed the effects of 4 mg renzapride q.d. in the improvement of frequency of bowel movements and stool consistency in IBS-C[130]. Much pharmacodynamic data support renzapride's prokinetic effects. As for the prior European study, statistically significant differences in the frequency of bowel movements and stool consistency in favour of renzapride 4 mg q.d. were relatively small. In addition, renzapride did not improve the feelings of completeness of bowel movements or the amount of straining. Several systematic reviews have shown the efficacy of 5HT receptor modulators in IBS patients. In 2009, Ford *et al*[131] conducted a meta-analysis by reviewing placebo-controlled clinical trials up to 2008 on the efficacy of known 5-HT3 antagonists and 5-HT4 agonists in IBS. They observed that renzapride and cisapride were not more effective than placebo in IBS patients.

Other investigators also evaluated the efficacy of combined 5HT3 antagonists/5HT4 agonists (cisapride and renzapride) in IBS patients[132] and observed that 1 and 2 mg of renzapride was ineffective in relieving IBS symptoms, supporting the results obtained by Ford. However, these authors showed that 4 mg of renzapride was significantly more effective than placebo.

Recently, a meta-analysis[133] from randomized placebo-controlled clinical trials, including 2528 C-IBS, non-C-IBS, and non-D-IBS patients according to the Rome criteria, was performed. The study confirmed that renzapride had no significant effects in relieving symptoms in IBS patients compared to placebo. To reach a convincing conclusion on the effectiveness of renzapride, a clinical trial compared with placebo was performed. Renzapride at a dose of 4 mg was compared to placebo for 5 wk or less and more than 5 wk. Although the differences were not statistically significant, the results were clinically important and significant for both treatment durations. Therefore, these results could be considered for renzapride 4 mg, while more trials are necessary to determine the effectiveness of this novel drug more precisely. As regards adverse effects, no statistically significant differences between renzapride and placebo were found, except for diarrhoea occurrence, which was higher in patients treated with renzapride. In addition, renzapride caused more withdrawals due to adverse effects and/

or low efficacy in patients. One of the limitations of this meta-analysis was the evaluation of trials with different patient inclusion criteria (age, sex, lifestyle and compliance). In addition, the trials evaluated had different durations of treatment and endpoints. The treatment durations ranged from 2 wk[134] to 12 wk[135]. To avoid heterogeneity, patients were divided into two groups according to treatment duration and time of reporting the results (5 wk or less and more than 5 wk), although there were few data in each group. The safety data from these phase Ⅲ studies indicated that renzapride was generally well tolerated, even though ischaemic colitis was reported in the long-term study in 3 patients. However, evaluating the total of patients treated with renzapride during the study, the overall rate of ischaemic colitis appeared comparable with that reported for other 5-HT3 receptor antagonists[136]. In conclusion, renzapride is not only superior to placebo in relieving IBS symptoms (abdominal pain and discomfort), but it also causes increased diarrhoea occurrence compared with placebo and appears to be associated with many drop-outs. Therefore, this drug might be a cost burden to patients, without any advantages in efficacy. Indeed, during the trial, no improvements in frequency of bowel movements, straining, or completeness of evacuation were observed in patients treated with renzapride. Taken together, these data suggest that renzapride is unlikely to provide clinically meaningful improvement in IBS symptoms.

Velusetrag

Velusetrag is an orally administrated available 5-HT4 agonist developed by Theravance. The binding affinity of this drug for the 5-HT4 receptor is more than 500-fold that of other 5-HT receptor subtypes[137]. The major metabolite detected in plasma after oral velusetrag is THRX-830449, which is a full agonist and is approximately equipotent to velusetrag. Metabolism occurs through the CYP3A4 system. In healthy subjects[138], at steady a state, the THRX-830449 to velusetrag AUC ratio is approximately 0.5 following once-daily dosing of velusetrag (15 mg).

Increased smooth muscle contractility of the antrum, fundus, duodenum and jejunum was observed in velusetrag-treated dogs[139]. Velusetrag increased guinea-pig colonic transit and produced dose-dependent relaxation of the rat esophagus[140]. Relief of constipation using velusetrag was also confirmed in chronic constipation patients[141].

Velusetrag was approximately 6- or 86-fold more potent than cisapride or mosapride after intravenous dosing and 9- or 18-fold more potent than tegaserod or cisapride, respectively, after intraduodenal administration[141].

Its low risk for cardiovascular events has been confirmed in an *in vitro* investigation demonstrating no effect on hERG channel conductance[140]. In a preclinical study that compared *in vivo* activity of velusetrag *vs* tegaserod in guinea pig, the subcutaneous administration of velusetrag increased colonic transit more than tegaserod did. Velusetrag was more potent than tegaserod when orally administered in a dog GI smooth muscle contractility model[142]. Velusetrag exhibited an acceptable oral bioavailability in rats and dogs[140], while the systemic effect of the drug was increased by an increase in the administered dose in healthy volunteers[143-145]. Both single (up to 70 mg) and multiple (up to 50 mg, for 2 wk) dosing of velusetrag in healthy subjects showed a dose-dependent effect on GI motility[145].

There have been two Phase Ⅱ clinical trials to evaluate the clinical efficacy of this drug. In one of these two studies[138], 60 healthy volunteers were randomly assigned, in double-blind fashion, to placebo or 5, 15, 30 or 50 mg velusetrag, with transit measurements after single and 6-d dosing.

The GI transit was evaluated in a randomized double-blind placebo-controlled study conducted on 60 healthy subjects randomly assigned to receive velusetrag at a dose of 5, 15, 30 or 50 mg or placebo either as a single dose or for 6 d[137]. Velusetrag at single dose (30 and 50 mg) favoured colonic transit, evaluated by colonic filling at 6 h and geometric center at 24 h, while this effect was not observed in patients treated with placebo. Similarly, velusetrag at multiple doses (15-50 mg doses) favoured gastric emptying compared with placebo (P = 0.002). In this study by Manini *et al*[115], an improvement of stool frequency and consistency by velusetrag in a subset of 11 patients with chronic constipation was also reported. Pharmacokinetic evaluations demonstrated a similar profile in healthy and CIC subjects[143]. Velusetrag was well tolerated in the Phase Ⅰ study when administered in single and repeated doses in healthy subjects. In the Phase Ⅰ clinical trial, the most commonly reported adverse event was diarrhoea, which is expected because of velusetrag's mechanism of action[141,143]. In a Phase Ⅱ randomized, double-blind, placebo-controlled trial, the efficacy and safety of velusetrag were compared with placebo in 401 subjects with CC. SBM frequency, CSBM and other associated symptoms with CIC were significantly improved compared with placebo in patients who received velusetrag for 4 wk. The most effective dose was 15 mg once daily. Most of the adverse events, such as diarrhoea, headache, nausea and vomiting, were mild to moderate. These adverse events were common in the first days of treatment with the dose of 50 mg once daily. The number of withdrawals due to adverse events was 18 *vs* 1 for the velusetrag- and placebo-treated subjects, respectively. The number of withdrawals were 4, 3 and 11 in the 15-, 30- and 50-mg treated groups, respectively. However, the medicine was well tolerated with no cardiac complications[146]. Another Phase Ⅱ study of

velusetrag in 401 patients with chronic constipation treated for 4 wk showed that there were significant treatment effects on the average daily number of bowel movements compared with placebo[147].

The most common adverse effects of velusetrag were those frequently associated with 5-HT4 agonists, including diarrhoea, headache and nausea. These dose-dependent adverse effects were mild to moderate and usually occurred within the initial days of dosing. Clinically relevant doses of velusetrag in animals or humans did not generate severe side effects on blood pressure, heart rate or electrocardiogram. In isolated porcine or canine coronary arteries, velusetrag showed no contractile activity[148]. In the randomized, double-blind, placebo-controlled study in 60 healthy subjects, there was no significant treatment effect on heart rate recorded by ECG after treatment for the prior 5-6 d. In this study, there were also no serious adverse events, and predictable GI effects such as diarrhoea and altered bowel movement were the main adverse events recorded[140]. These results suggest that velusetrag appears to be well tolerated. Further careful clinical studies will be required to further evaluate the safety and tolerability of this drug.

Naronapride

Naronapride (ATI-7505)[149] is a benzamide 5-HT4 receptor agonist that activates 5-HT4 receptors but has almost no actions on the other 5-HT subtypes. The design of ATI-7505 was based on the prototypical agent, cisapride. However, unlike cisapride, which is a mixture of (3R, 4S) and (3S, 4R) isomers of substituted piperidine-based scaffolds, ATI-7505 is the pure (3S, 4R) isomer. ATI-7505, with its (R)-quinuclidinyl moiety, is metabolized by ubiquitous carboxyl esterases to a single metabolite, ATI-7500.

This potent and selective 5-HT4 receptor agonist showed different pharmacodynamic and pharmacokinetic properties from previous nonselective 5-HT4 agonists. Hydrolytic esterase metabolism, unlike oxidative CYP450 metabolism, is a large-capacity metabolic system that can easily handle therapeutic amounts of xenobiotics. This large-capacity system implies that other drugs metabolized by esterases are not expected to induce drug-drug interactions of ATI-7505 with other drugs. There is also no interaction with drugs metabolized by a different enzymatic system, such as CYP450[150]. Naronapride is not metabolized by CYP450 enzymes, and thus, less drug-drug interaction occurs.

A thorough QT study showed that naronapride had no obvious effect on cardiac repolarization at either therapeutic or supratherapeutic doses. The structure of naronapride is similar to that of cisapride, but it is more selective than cisapride and thus interacts minimally with hERG channels and 5-HT3 receptors[150]. ATI-7500, the main metabolite of naronapride, is 100-fold less active than the parent drug. Unlike prucalopride and velusetrag, neither naronapride nor ATI-7500 can pass the blood-brain barrier, therefore reducing the incidence of side effects. This new benzamide exhibited GI prokinetic effects, stimulated colonic transit and reduced stool consistency in healthy male and female subjects[151]. One Phase Ⅱ randomized double-blind placebo-controlled dose definition study evaluated several doses of orally administered naronapride (20, 40, 80 and 120 mg twice a day) in 210 patients with CC. This study evaluated the clinical effects of 9 days' treatment with three doses of ATI-7505 at 3, 10 and 20 mg on GI and colonic transit using a validated scintigraphic method. There were borderline effects on gastric emptying at half-time. However, ATI-7505 stimulated colonic transit at 24 h and ascending colonic emptying. There was looser stool consistency as measured by the Bristol stool form scale with the 10- and 20-mg t.i.d. doses. This finding suggests that ATI-7505 appears to have prokinetic properties in both stomach and colon in healthy subjects and, particularly, in the colon. Further clinical trials of larger numbers of patients with functional gastrointestinal disorders, such as patients with CIC, are required to evaluate clinical efficacy.

The inhibition of the delayed rectifier K+ current in response to ATI-7505 in patch-clamped HEK293 (human embryo kidney) cells transfected with the human IKr channel is very weak, suggesting that there would be an adequate safety window between activity in the GI tract and potential cardiac toxicity. In addition, the primary metabolite ATI-7500 is 100-fold less active than the parent drug at the 5-HT4 receptor and, as with ATI-7505, has no detectable HERG channel inhibitory activity at concentrations up to 100 μmol/L. Preliminary data on intensive cardiac safety monitoring suggest that ATI-7505 is safe as regards the cardiac profile[149].

The most common drug-related adverse events were headache, diarrhoea, nausea and vomiting. Headache and abdominal pain were reported more frequently by the maximum dose of naronapride[152].

Chenodeoxycholic acid

Chenodeoxycholic acid (CDCA) is a bile acid that can induce colonic electrolyte secretion by acting on the membrane-bound bile acid GPBA receptor (TGR5) on enterocytes, subsequently leading to the stimulation of cAMP generation and electrogenic chloride secretion. Supplementation with specific bile acid analogues or by using drugs that inhibit ileal bile acid reabsorption may benefit constipation patients.

Oral chenodeoxycholic acid at doses of 750-1000 mg/d can increase bowel movements, decrease stool consistency, and reduce the time to defecation in IBS-C[153].

They were previously used for the dissolution of

gallstones, and they are known to favour diarrhoea at high doses in healthy controls and constipation patients[154]. The effects of CDCA on gastrointestinal and colonic function have been evaluated in healthy volunteers and patients with irritable bowel syndrome with constipation. In a randomized controlled trial, 500 mg and 1000 mg CDCA given to 60 healthy volunteers for 4 d led to dose-dependent acceleration of colonic transit. In addition, significant increases in stool frequency, decreases in stool consistency, and improvements in ease of stool passage were reported with CDCA[155]. In a double-blind placebo-controlled study, Rao *et al*[153] demonstrated that sodium chenodeoxycholate[156] stimulated colonic transit and improved bowel function in 36 women with irritable bowel syndrome with constipation. Increased stool frequency, greater ease of stool passage and looser stool consistency were observed in patients treated with sodium chenodeoxycholate 500 mg or 1000 mg for 4 d as compared with controls. Unfortunately, over 40% of sodium chenodeoxycholate-treated patients had light abdominal cramping or pain. Whether these side effects could be mitigated at a lower dose remains to be determined.

Elobixibat

Elobixibat is an orally administrated available potent inhibitor of ileal bile acid transporter with minimal systemic exposure[157]. Elobixibat (A3309) reduces bile acid enterohepatic recirculation and upregulates bile acid synthesis as measured by serum C4 levels. It also depletes liver cholesterol and reduces serum LDL[158], thus increasing the delivery of bile acids to the proximal colon, which in turn increased fluid secretion, colonic motility and stool frequency, and it improved stool consistency and relieved constipation-related symptoms in chronic idiopathic constipation patients[159,160].

In a phase Ⅰ trial, elobixibat stimulated colonic transit in a dose-dependent way. In a randomized phase Ⅱ trial, elobixibat at doses of 15 and 20 mg/d showed an improvement of stool consistency and of stool passage, increased the number of SBMs and reduced straining in female patients with CIC[161]. In a dose-finding randomized trial, elobixibat increased C4, reduced LDL cholesterol, increased colonic transit from 3 to 1.9 d and increased the number of SBM and CSBM/wk in patients with CIC compared to placebo. The treatment with elobixibat also resulted in an improvement of bloating severity, but no effects on abdominal pain or discomfort were reported[157]. The well-tolerated doses were 5-10 mg, with a discontinuation rate during the phase IIb trial of 13%, rising to 23% for the 15 mg group. Fifty-four percent of patients developed adverse events, such as abdominal cramps, relieved by defecation, and diarrhoea. However, the side effects were not different from those of the placebo group[162]. In a large randomized trial conducted on patients with CIC, the 10- and 15-mg doses increased SBMs and reduced the time to SBM (12 h with the 10-mg dose, 7 h with the 15-mg dose and 24 h with the placebo). In patients treated with elobixibat, an increased spontaneous laxation within 24 h was observed compared with placebo (75 % on 15 mg/d and 45 % on placebo).

The side effects of elobixibat are mainly gastrointestinal tract-related. Although higher dosages of elobixibat caused abdominal pain and diarrhoea more frequently, no severe adverse effects occurred in the Phase Ⅰ and Phase Ⅱ clinical trials. The Phase Ⅲ clinical trials are ongoing to determine the best tolerated dose and to examine the effects of long-term administration.

Complete spontaneous bowel movements per week increased in a dose-dependent way. An improvement of stool consistency and bloating was observed at the 10- and 15-mg doses. Side effects such as abdominal pain and diarrhoea were also dose-dependent, notably for the 15-mg dose[157].

Elobixibat is a promising anti-constipation drug. However, there are no studies in cancer or in OIC (opioid induced constipation) patients. Due to its prokinetic activity, elobixibat is not recommended in patients with mechanical bowel obstruction.

According to the results of Phase Ⅱ trials in chronic idiopathic constipation patients, elobixibat was safe and generally well to lerated, even at a dose up to 20 mg per day.

As illustrated by elobixibat, the advantages of IBAT inhibitors may be especially attractive, which may boost research on other IBAT inhibitors, such as SC-435, S-8921 and S-0960[163-165].

Lubiprostone

Lubiprostone, a first-in-class drug for the treatment of chronic idiopathic constipation and irritable bowel syndrome in adult women with constipation is believed to be a highly selective locally acting activator of ClC-2 channels[166]. Lubiprostone can tautomerize between the inactive form Ⅰ and the active form Ⅱ[167]. Lubiprostone acts mainly by activating specific type-2 chloride channels (ClC-2) on the apical membrane of the enterocytes[166] that are involved in ion and fluid transport across the epithelial membrane. Once channels are opened, chloride enters the enterocyte in the basal membrane through the action of Na-K-2Cl active cotransporters. This mechanism results in an electrochemical gradient favouring chloride secretion. It leads to an overall concentration-dependent raise in intestinal fluid secretion without any impairment on serum sodium and potassium levels. These mechanisms explain how lubiprostone increases the number of colonic spontaneous bowel movements per week. However, lubiprostone efficacy on the abdominal pain score is only partially known

and needs further investigation. Lubiprostone also activates a prostaglandin receptor (EP4), which in turn activates cystic fibrosis conductance regulators (CFTR)[168]. The activation of EP4 receptors favours colonic smooth muscle and gastric longitudinal muscle *via* vagal nerve endings[169]. Lubiprostone changes mucin, which improves the gut microbiome, creating an anti-inflammatory environment[170]. Unlike linaclotide, lubiprostone does not increase pain thresholds[171].

Lubiprostone pharmacokinetics is not impaired by renal failure. However, great adverse events with a standard dose of lubiprostone can result in cases of mild-to-moderate hepatic impairment (Child-Pugh class A and B), which increases the lubiprostone metabolite M3. Thus, in cases of liver impairment, a reduction of lubiprostone starting doses is required. Lubiprostone metabolism does not involve cytochromes. The catabolism is mediated by carbonyl reductase in the stomach and jejunum[172]. Lubiprostone is unlikely to have major drug interactions.

In healthy subjects, a reduction in gastric emptying, an increase in gastric fasting volume, a reduction in maximum tolerated gastric volumes and a stimulation of small bowel and colon transit was observed with lubiprostone at a dose of 24 μg twice daily[173]. However, the effects on gastric motility may mask the nausea side effect.

Two 12-wk double-blind randomized multicentre placebo-controlled phase Ⅲ clinical trials[174] and one 36-wk open-label extension study[175] contributed to the FDA's approval of lubiprostone for the treatment of IBSC in women. A total of 1171 patients were randomized 2:1 to receive either 8 μg lubiprostone or placebo twice/d. The primary end point of each study was the evaluation of response rate, measured by patient-reported improvements from baseline in IBS-C symptoms. As secondary end points, monthly responder rates, changes from baseline in SBM frequency, stool consistency, straining, distention, abdominal pain/discomfort (each measured on a 5-point Likert scale) and change in health-related quality of life were evaluated.

The discontinuation rate in both studies was 24%, firstly due to withdrawal of consent and secondly to adverse events and perceived lack of efficacy. Lubiprostone was superior in the primary end point compared to placebo (17.9% *vs* 10.1%, P = 0.001). Patients treated with lubiprostone reported more improvements in all secondary end points than placebo. Lubiprostone was associated with a more significant improvement in abdominal pain/discomfort than placebo from baseline to month 2 (0.43 *vs* 0.35, P = 0.039) and month 3 (0.45 *vs* 0.36, P = 0.028).

Lubiprostone significantly changed the mean SBM frequency from baseline to month 1 compared with placebo, even though the numerical data were not included.

Two 4-wk phase Ⅲ randomized double-blind placebo-controlled multicentre clinical trials were conducted on a total of 479 patients to evaluate the short-term efficacy and safety of lubiprostone in patients with CIC with identical study designs and primary end points. After a 2-wk baseline period, eligible subjects received 24 lg lubiprostone or placebo twice/d.

The number of patient-reported SBMs, defined as any BM occurring 24 h or longer after the use of an alternative drug used to relieve constipation (rescue medication), during the first week of treatment was the primary end point of each study[176,177]. Lubiprostone was associated with a statistically higher frequency of SBMs during the first week of treatment than placebo.

Improvements in other secondary end points, such as stool consistency, straining, and constipation severity were also observed in patients treated with lubiprostone compared with placebo in all 4 wk in both studies. However, significant improvement in abdominal distention and discomfort compared with placebo was not observed in either study.

As regards side effects, a similar percentage of patients reporting at least one treatment-related adverse event for IBS-C was observed in the lubiprostone (50%) and placebo (51%) groups. The most common side effects were gastrointestinal (19% with lubiprostone *vs* 14% with placebo). Serious adverse events were similar between the two groups (1%). Nausea was the most frequent treatment-related event[11,12], although it may be reduced by administering lubiprostone with meals.

A unique adverse effect occurred with the initial dose. In rare cases, acute transit dyspnea and ischemic colitis were observed[176,178].

Caution in the use of lubiprostone should be used for infants of breastfeeding mothers due to the risk of diarrhoea[179]. Limited data are available on the lubiprostone effects in paediatric patients, and further, larger studies are required. In an open-label 4-wk clinical trial conducted on paediatric patients with CIC (mean age 10.2 years)[179], lubiprostone was efficacious and well tolerated at daily doses of 12-48 lg. The recommended dose of lubiprostone for the treatment of CIC in both adult men and women is 24 lg twice/d, while for the treatment of IBSC in adult women, it is 8 lg twice/d. Due to its minimal systemic absorption and its metabolism through a cytochrome P450-independent pathway, lubiprostone-drug interactions are unlikely, even though *in vitro* studies have suggested that methadone may decrease the efficacy of lubiprostone by reducing chloride channel type 2 activation[179].

In patients with moderate or severe hepatic impairment (Child-Pugh class B or C), a dose reduction might be suggested, while in patients with renal failure, no dosage adjustment is recommended.

A small percentage (8%-13%) of patients over 65 years were included in clinical trials with lubiprostone. The safety profile was similar in elderly and younger patients, even though, due to the limited the number of patients over 65 years, no differences in clinical response were observed[179], and further studies are needed.

Like linaclotide, lubiprostone is contraindicated in mechanical bowel obstruction. To confirm the indications in the treatment of IBS-C in adults, more and larger trials are required. Due to the chronic nature of IBS-C and CIC, post-marketing studies are necessary to confirm the long-term efficacy and safety of lubiprostone. All randomized clinical trials were of limited duration (12-26 wk). However, in open-label extension studies, a safety over 52 wk was demonstrated.

Despite the efficacy, the side effects (*e.g.,* nausea, abdominal pain) and the high cost may limit the use of lubiprostone.

Tenapanor

Tenapanor, also known as AZD1722 or RDX5791, is a first-in-class orally available inhibitor of NHE3 that is minimally absorbed in the gastrointestinal tract-this constitutes a significant therapeutic benefit, as it may act on the drug target[180,181]. Consequently, tenapanor increases intestinal Na^+ contents, which leads to an increase in intestinal fluid volume and accelerates the whole GI transit, as shown in rats. Moreover, tenapanor inhibits the absorption of phosphorus, which is independent of typical phosphorus transporters in the intestines, namely, sodium-dependent phosphate transport protein 2B (NaPi2b) and Na (+)-dependent phosphate transporter (PiT1). Tenapanor is stable at room temperature and is formulated into tablets ranging from 1 to 50 mg. Absorption, distribution, metabolism and excretion (ADME) studies have revealed that tenapanor is minimally absorbed and metabolized. For example, experiments in rats showed 92.2% ± 1.6% recovery of tenapanor in faeces upon oral administration[182]. In humans, the inactive metabolites of tenapanor were found in plasma, but they were only approximately 9% of the parent compound. In pharmacokinetic studies, tenapanor was observed at relatively low concentrations in plasma (average < 3 ng/mL) of rats and dogs, but only sporadically (29/76 and 0/92, respectively).

Oral administration of tenapanor (at doses of 0.1 and 3 mg/kg) produced a dose-dependent increase in faecal water content and stool consistency in rats. The effect of tenapanor at a dose of 50 mg/kg twice daily on stool form was assessed in cynomolgus monkeys. The animals were observed for 4 d before treatment. Soft or watery stools were observed in monkeys on tenapanor treatment, and stool consistency was normalized on day 6 of the experiment. Under physiological conditions, tenapanor given orally at doses of 3, 10, 30 and 50 mg/kg did not affect visceral sensitivity or the changes in intestinal volumes induced by colorectal distension in comparison with the control and tegaserod-treated (5 mg/kg administered per os) groups[183]. However, tenapanor (30 and 50 mg/kg) had a dose-dependent antinociceptive effect in the acute restraint stress-induced intestinal hypersensitivity to colorectal distension. The antinociceptive potential of tenapanor was comparable with that of the tegaserod-treated group.

The safety and tolerance of tenapanor were assessed in a randomized, double-blind, placebo-controlled study[184,185]. Eighty healthy volunteers were included in the study (male and female). Tenapanor was given orally at the doses ranging from 10 to 900 mg (as a single administration) and for 7 consecutive days at doses ranging from 3 to 100 mg to assess the safety of tenapanor administration. Tenapanor was also beneficial for the percent of days with a spontaneous bowel movement. Finally, no serious side effects were observed, and there were very few adverse events[186]. Phase Ⅱa In a Ⅱa double-blind randomized placebo-controlled study on 181 patients with IBS-C[187], tenapanor was given orally at doses of 10, 30 and 100 mg once daily for 4 consecutive weeks with 2 wk follow-up. The primary end point (change in complete spontaneous bowel movements from baseline to week 4) was not met in this study, and the incidence of diarrhoea was comparable with that of the placebo group. However, an improvement in bloating and abdominal pain was noted in IBS-C patients. In Phase Ⅱb In a Ⅱb randomized double-blind placebo-controlled multicentre study, 371 IBS-C patients were divided into four groups: placebo and tenapanor (5, 20 and 50 mg) treated twice daily for 12 wk with 4 wk follow-up. The primary efficacy end point was met in 60.7% of the tenapanor-treated group (at a dose of 50 mg) *vs* 33.7% of the placebo-treated group. The overall responder was met in 50.0% of the tenapanor-treated group (50 mg) *vs* 23.6% for placebo (after 12 wk). After 12 wk, adequate relief in IBS-C symptoms was observed in 63.1% of the tenapanor-treated group (50 mg twice daily) *vs* 39.3% in placebo. The effectiveness of tenapanor therapy was maintained during entire time of the clinical study. The treatment satisfaction patient scale questionnaire showed that tenapanor-treated (50 mg) IBS-C patients were quite or very satisfied (65% *vs* 38% for the placebo-treated group). The drug was well tolerated in all groups, and no serious adverse effects were noted. The most common adverse effect was diarrhoea in the tenapanor-treated group (50 mg twice daily), reported in 11.2% of IBS-C patients *vs* 0% in placebo. Safety and tolerability In the preclinical studies in rats, tenapanor did not influence gastric emptying[187].

Piromelatine

Melatonin is engaged in the regulation of gastrointestinal motility and sensation. When administered

orally in pharmacological doses, it has shown beneficial effects on abdominal pain in IBS patients without any effects on sleep disturbances[188]. It was also shown that oral melatonin significantly stimulated colonic transit time in healthy subjects, and it may be a promising option for future research on the agents modulating bowel motility[189]. Melatonin synthesized in the enteroendocrine cells of the intestinal mucosa reaches the liver *via* the portal vein[190]. Melatonin is a potent accelerator of duodenal mucosal bicarbonate secretion, which neutralizes the acid content of the stomach in the duodenum, and it seems to be engaged in the acid-induced stimulation of the secretion[191]. Melatonin protects the gastrointestinal mucosa due to an antioxidant action, a decrease in secretion of hydrochloric acid, stimulation of the immune system, promotion of epithelial regeneration, and increased microcirculation[192,193].

Recently, it was shown that patients with IBS had significantly lower 6-SMLT (6-sulphatoxymelatonin)/creatinine level compared with healthy controls[194]. The lack of statistical difference in 6-SMLT/creatinine levels between the constipation and diarrhoea groups is difficult to explain. In some patients, the symptoms could be recurrent, or there could be some subjects with mixed (IBS-M) or unsubtyped (IBS-U) IBS. This study's results agree with those obtained by Bultman[195] and Lu *et al*[196] who performed the study on female patients with IBS and found decreased salivary melatonin and urine 6-SMLT level compared to non-IBS volunteers. Low melatonin levels were observed in women with eating disorders. Low melatonin concentrations have been associated with increased depressive symptoms, such as sadness, bodily discomfort, inner tension, difficulties in attention concentration and pain.

Serotonin, an endogenous amine and the precursor of melatonin, synthesized and released from enteroendocrine cells of the gastrointestinal mucosa is thought to play an important role in the pathogenesis of IBS[197]. Antagonists of the serotonin 5-HT3 receptor are beneficial in patients with IBS-D, whereas the partial agonist of the serotonin 5-HT4 receptor (tegaserod) alleviates symptoms of IBS with constipation, especially in females. The role of melatonin as the regulator of circadian and seasonal rhythmicity has been established[198,199]. Patients with functional disorders of the gastrointestinal tract also had sleep disorders, and some of them suffered from increased neural excitability and anxiety[199,200]. There were speculations concerning a possible role of melatonin in functional dyspepsia (FD), particularly ulcer-like dyspepsia. In two types of FD, one with epigastric pain and another with postprandial distress syndrome, the melatonin level is varied, and different dyspeptic symptoms may be related to differences in melatonin secretion. Sleep disturbances are common in patients with IBS and are among the most important extraintestinal symptoms, markedly affecting quality of life and psychosocial well-being[201]. In a double-blind placebo-controlled study, Camilleri *et al*[202] showed that melatonin improves abdominal pain in IBS patients with sleep disturbances. Currently, conventional treatment for irritable bowel syndrome is quite unsatisfactory. Despite multiple therapeutic interventions, no long-term effect has been achieved. On the other hand, up to 80% of patients with IBS treated with hypnotherapy showed an improvement of their symptoms[203]. These observations emphasize the possible role of melatonin in the pathogenesis of irritable bowel syndrome and in its therapy[204,205].

Daikenchuto

Daikenchuto (TU-100), a traditional Japanese drug (Kampo medicine), is indicated in the treatment of adhesive bowel obstruction[206,207]. TU-100 is a mixture of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and maltose powder. In many trials, the TU-100 prokinetic effect has been demonstrated to be useful in treating GI hypomotility[208]. Studies conducted on postoperative patients after gastrointestinal surgery showed that TU-100 prevented postoperative ileus, but little is known about the TU-100 effects in patients who did not undergo major gastrointestinal surgery[209]. Iturrino *et al*[210] performed a randomized controlled trial to evaluate the effects of oral TU-100, 2.5 g t.d.s. or 5 g t.d.s. compared to placebo t.d.s. on gastrointestinal and colonic transit, rectal compliance and sensation thresholds, anal sphincter pressures and bowel function in women with functional constipation. In this study, there were no significant effects on gastrointestinal and colonic transit, rectal compliance, anal sphincter pressures, recto-anal pressure difference, or rectal sensation thresholds. The highest dose was associated with lower rectal sensation thresholds for first sensation and gas. There were no treatment effects on psychosensory symptoms, stool frequency, stool consistency or quality of life[211].

On the other hand, Manabe *et al*[211] reported that TU-100 provided a clinically significant promotility effect in small bowel and ascending colon transit in healthy subjects. TU-100 is quite safe and well tolerated and is a potential treatment for IBS-C and functional constipation[210].

Recently, however, Acosta *et al*[212] did not report any significant effects of TU-100 on rectal sensation ratings, sensation thresholds, rectal fasting or postprandial tone, rectal compliance, bowel function, abdominal pain or bloating scores, or IBS quality of life. Further randomized controlled trials in patients with IBS-C or functional constipation using both clinical and validated biomarkers are required.

DA-6886

DA-6886, a gastrointestinal prokinetic benzamide

derivative, is a novel 5-HT4 receptor agonist. Experimental studies showed that it may represent a highly potent and selective 5-HT4 receptor agonist to stimulate colonic transit in mice, having a favorable safety profile in patients with IBS-C and chronic constipation[213]. Currently, a phase Ⅰ dose block-randomized double-blind placebo-controlled single/multiple dosing dose escalation clinical trial with an open-labelled food effect is being conducted to evaluate the safety and pharmacokinetics of single-dose DA-6866 in healthy male subjects[214].

Table 3 sums up the literature findings about irritable bowel syndrome-C therapies.

THERAPEUTIC TARGET IN DIARRHOEA-PREDOMINANT IRRITABLE BOWEL SYNDROME

To date, the treatment options for IBS-D are limited and frequently unsuccessful. However, the incidence of IBS-D is currently increasing, thus causing a heavy economic burden both for patients and health care systems worldwide. As for IBS-C, a complete understanding of IBS-C pathophysiology has favoured the use of sensory end points such as complete spontaneous bowel movements and the FDA combined end point (abdominal pain and complete spontaneous bowel movements) in clinical trials[79].

Furthermore, also in the setting of IBS-D, preclinical studies in rodents have recently improved the understanding of the mechanisms underlying the alterations in gastrointestinal motility, sensitivity and secretion. A number of drugs that we will touch upon in the next section are actually in development.

Ramosetron

Ramosetron is a potent and selective 5-HT3 receptor antagonist. 5-HT3 receptors can be widely found in the central and peripheral nervous system[215]. Intraluminal stimuli favour the release of 5-HT from enterochromaffin cells located in the mucosa[215]. When secreted, 5-HT can activate 5-HT3 receptors located on intrinsic primary afferent neurons with submucosal terminals. Thus, the peristaltic reflex and intestinal secretion can occur[215,216]. 5-HT also activates 5-HT3 and 5-HT4 receptors located on primary afferent neurons of both splanchnic and vagal fibres, which are involved in sensory and motor responses[217].

In experimental studies, corticotrophin-releasing hormone (CRH) exogenously administered or released from the central nervous system by stress peripherally activates the release of 5-HT, which in turn promotes defecation through the 5-HT3 receptor. Ramosetron decreased defecation by CRH in a dose-dependent way[218-219].

The first 5-HT3 receptor antagonist to be introduced was Alosetron, which has been demonstrated to be effective in the treatment of female patients with IBS-D[220]. However, due to serious gastrointestinal events (ischemic colitis and severe constipation), it is still only available in the United States and is indicated for women with severe D-IBS refractory to conventional therapy.

Ramosetron was first tested by Lee *et al*[221] in a multicentre randomized open-label trial on 343 men with IBS-D. Patients were randomized to a 4-wk treatment of ramosetron 5 mg once daily or mebeverine 135 mg three times daily. An improvement in abdominal pain/discomfort and bowel habits in the ramosetron and mebeverine groups was observed during the treatment period. A significant reduction in abdominal pain/discomfort and urgency, stool form score, and stool frequency severity scores in both treatment arms was reported compared with the baseline.

Adverse events were observed in 7% and 4% of patients treated with ramosetron and mebeverine, respectively, even though no statistical significant differences were reported. Additionally, all the side effects were mild or moderate[221].

Successively, Fukudo *et al*[222] performed a randomized double-blind placebo-controlled trial to determine whether ramosetron reduces diarrhoea in 296 male outpatients with IBS-D. Patients were treated with 5 mg of oral ramosetron (n = 147) or placebo (n = 149) once daily for 12 wk after a 1-wk baseline period. The primary end point was increased stool consistency in the first month. Secondary end points were the relief of overall IBS symptoms and the improvement of IBS-related quality of life. In the first month, patients on ramosetron treatment (74, 50.3%) showed an improvement of stool consistency compared to placebo (29, 19.6%) (P < 0.001). In patients treated with ramosetron, the monthly relief of overall IBS symptoms and IBS-related quality of life was demonstrated compared with placebo. Safety was evaluated in all 296 patients, with side effects occurring in 46.9% and 51.7% of ramosetron and placebo patients, respectively. All constipation and hard stools experienced in the ramosetron group that were related to the pharmacologic actions of ramosetron were classified as mild and resolved early without using rescue drugs[222].

In another randomized double-blind placebo-controlled trial performed by Fukudo *et al*[223] on 576 female outpatients with IBS-D, patients were given either 2.5 μg ramosetron or placebo once daily for 12 wk. Patients treated with ramosetron reported global improvement, increased stool consistency, a significant decrease in abdominal pain and discomfort and significant improvement in QOL compared with placebo. Of the patients tested with ramosetron, 11.0% complained of constipation[223]. Successively, in a phase Ⅲ open-label uncontrolled long-term safety

Table 3 Summary of the literature findings about irritable bowel syndrome-C therapies

Drug	Ref.	No. of patients	Study design	Outcome
Linaclotide	Andresen *et al*[82]	36 women with IBS-C	Phase Ⅱa randomized, double-blind, placebo-controlled trial. Patients were randomized in a 1:1:1 fashion to placebo, linaclotide 100 μg, and linaclotide 1000 μg and was evaluated the effect of 5 d	No treatment effects were seen for gastric emptying or colonic filling with linaclotide. Significant treatment effects were found for ascending colon emptying t ½ times (P = 0.015) and overall total colonic transit times at 48 h (P = 0.02), for the 1000 μg dose (P = 0.004) but not the 100 μg dose, as well as overall treatment effects on increased stool frequency, decreased stool consistency, improved ease of passage, and acceleration of time to first bowel movement (P < 0.001)[82]
	Johnston *et al*[83]	420 patients with IBS-C	Phase Ⅱb randomized, double-blind, parallel-group, multicenter, placebo-controlled trial evaluate 12 wk of linaclotide at a daily dose range of 75-600 μg	Compared with placebo, all doses of linaclotide significantly improved bowel habits, including frequency of SBMs and CSBMs, severity of straining, stool consistency, as well as abdominal pain scores. Abdominal discomfort, bloating, and global IBS-C measures were also improved, for all doses except for the 75 μg (abdominal discomfort) and 150 μg dose (bloating). Effects were present for the first week, and sustained throughout the 12 wk of treatment
	Chey *et al*[84]	804 adults with IBS-C	Phase Ⅲ trials randomized, double-blind, placebo-controlled to receive linaclotide 290 lg or placebo daily for 26 wk, with change-from-baseline end points measuredat 12 and 26 wk	Over 12 wk, the FDA combined primary end point was achieved by 33.7% of patients receiving linaclotide compared with 13.9% of patients receiving placebo (P < 0.0001)
	Videlock *et al*[109]	7 trials of linaclotide in patients with IBS-C or CC	A meta-analysis from MEDLINE, EMBASE, and the Cochrane central register of controlled trials were searched for randomized, placebo-controlled trials	The NNT for the primary endpoint of these trials (3 SCBMs/wk and an increase of C1 SCBM/wk, for 75% of weeks) was 7 (95%CI: 5-8)
	Rao *et al*[86]	803 adults with IBS-C	Phase Ⅲ trials randomized, double-blind, placebo-controlled to receive linaclotide 290 lg or placebo once/d for 12 wk	Linaclotide demonstrated statistically significant improvements in all primary and secondary efficacy end points compared with placebo Severity of straining, constipation, and stool consistency also improved in the linaclotide group compared with the placebo group
Plecanatide	Miner *et al*[92]	951 patients with CIC	Phase Ⅲ, randomized, double-blind trial, received plecanatide 0.3, 1 or 3 mg, or placebo once/d for 12 wk	The proportion of overall responders was significantly greater with plecanatide 3 mg compared with placebo (19% *vs* 10.7%, P = 0.009); weekly responder rates were also significantly greater for plecanatide 3 mg than placebo for weeks 1-12. Improvements in stool frequency, consistency, straining, and quality of life were also noted with the 3-mg dose *vs* placebo. Data for other plecanatide doses were not reported
Prucalopride	Quigley *et al*[88]	620 patients with CC	A double-blind, placebo-controlled study. Patients receiving 2 or 4 mg of prucalopride for 12 wk	Increased one or more CSBMs per week compared to patients in the control group
	Camilleri *et al*[98]	713 patients with CC	A double-blind, placebo-controlled study. Patients receiving 2 or 4 mg of prucalopride for 12 wk	Increased frequency of three or more CSBMs per week, and improved evacuation completeness, perceived disease severity, and quality of life
	Müller-Lissner *et al*[97]	Elderly patients aged 65 years and older with CC	A double-blind, placebo-controlled study Patients receiving 2 or 4 mg of prucalopride for 12 wk	No changes in electrocardiogram or corrected QT (QTc) interval were reported, indicating its safety for the treatment of CC in the elderly
	Ke *et al*[102]	4 Randomized, Placebo-controlled Studies	A Pooled Analysis	Safe and well-tolerated It was also effective in improving the abdominal symptoms of CC such as abdominal discomfort, bloating, straining, and painful bowel movements
YKP10811	Shin *et al*[114]	55 patients	A single-center, randomized, parallel-group, double-blinded, placebo-controlled study were assigned randomly to groups given YKP10811 10 mg (n = 15), 20 mg (n = 16), 30 mg (n = 15), or placebo (n = 11) daily for 8 d	Enhanced gastrointestinal and colonic transit and improved bowel function during an 8-d treatment trial. In general, the 10-mg and 20-mg doses were the most effective in accelerating colonic transit. No serious adverse events were observed
Renzapride	Camilleri *et al*[101]	46 women with IBS-C	In a phase Ⅱ study	Renzapride 4 mg q.d. accelerated colonic transit and increased ascending colon emptying *vs* placebo
	George *et al*[130]	510 patients	Multicentre, randomized, placebo-controlled, double-blind study men and women were randomized to placebo or renzapride (1, 2 or 4 mg/d) for 12 wk	4 mg renzapride q.d. in terms of improving frequency of bowel movements and stool consistency

	Ford *et al*[131]	29 RCTs were eligible for inclusion	Meta-analysis of placebo-controlled clinical	Renzapride and cisapride were not more effective than placebo in IBS patients
	Mozaffari *et al*[133]	2528 C-IBS and non C-, non D-IBS patients	Meta-analysis from randomized placebo-controlled clinical trials	Renzapride has no significant advantage over placebo in relieving symptoms in IBS patients
Velusetrag	Manini *et al*[115]	60 healthy volunteers	Phase Ⅱ clinical trials, pt were randomly assigned, in double-blind fashion, to placebo, 5, 15, 30 or 50 mg velusetrag, with transit measurements after single and 6-d dosing	Single doses of velusetrag (30 and 50 mg), but not placebo, accelerated colonic transit, as measured by colonic filling at 6 h and geometric center at 24 h
	Goldberg *et al*[140]	401 subjects with CC	In a Phase Ⅱ randomized, double-blind, placebo-controlled trial	Short bowel movement (SBM) frequency, complete SBM and other associated symptoms with CC were significantly improved in comparison with placebo in patients who received velusetrag for 4 wk
Naronapride	Dennis *et al*[149]	210 patients with CC.	Phase Ⅱ, randomized, double-blind, placebo-controlled, dose definition study (orally 20, 40, 80 and 120 mg twice a day) to evaluate the clinical effects of 9 days'	There were borderline effects on gastric emptying at half-time; however, ATI-7505 accelerated colonic transit at 24 h and ascending colonic emptying
Chenodeoxycolic acid	Odunsi-Shiyanbade *et al*[155]	60 healthy volunteers	Randomized controlled trial, CDCA 500 mg and 1000 mg given for 4 d	Significant increases in stool frequency, decreases in stool consistency, and improvements in ease of stool passage were reported with CDCA
	Rao *et al*[153]	36 female patients	Double-blind placebo-controlled study	Accelerated colonic transit and improved bowel function
Elobixibat	Simrén *et al*[162]	30 patients	Dose-finding randomized trial five dose-levels (range: 0.1-10 mg/d) or to placebo	Increased C4, reduced LDL cholesterol and increased colonic transit from 3 to 1.9 d, and increased the number of SBM and CSBM/W in patients with CIC
	Chey *et al*[157]	190 patients	Were randomized to 5, 10, or 15 mg A3309 or placebo once daily. 8-wk, multicenter, randomized, double-blind, placebo-controlled, parallel group, phase Ⅱb study,	A3309 increased stool frequency and improved constipation-related symptoms in CIC; effects were maintained over 8 wk of treatment
Lubiprostone	Drossman *et al*[173] Chey *et al*[174]	1171 patients in total	Two double-blind, randomized, multicenter, placebo-controlled phase Ⅲ clinical trials', a 12-wk randomized 2:1 to receive either lubiprostone 8 lg or matching placebo twice/d with food; a 36-wk open-label extension study	Lubiprostone was superior to placebo in the primary end point of overall responders, greater improvements in all secondary outcome measures compared with placebo
	Johanson *et al*[175] Barish *et al*[82]	479 patients in total	Two 4-wk phase Ⅲ, randomized, double-blind, placebo-controlled, multicenter clinical trials	Patients treated with lubiprostone had a statistical higher frequency of SBMs during the first week of treatment compared with placebo
	Hyman *et al*[176]	127 pediatric patients with CIC (mean age 10.2 yr)	An open-label 4-wk clinical trial	Demonstrated that lubiprostone was efficacious and well tolerated at total daily doses of 12-48 lg
AZD1722	Rosenbaum *et al*[185]	181 patients with IBS-C	Phase Ⅱa In a Ⅱa double-blind, randomized placebo-controlled study, Tenapanor was given orally at the doses of 10, 30 and 100 mg once daily for 4 consecutive weeks with 2 wk follow-up	The primary end point [change in complete spontaneous bowel movements (CSBM) from baseline to week 4] was not met in this study and the incidence of diarrhea was comparable with placebo group. However, improvement in bloating and abdominal pain was noted in IBS-C patients
	Rosenbaum *et al*[186]	371 IBS-C patients	Phase Ⅱb In a Ⅱb randomized, double-blind, placebo-controlled, multicenter study	The overall responder was met in 50.0% of tenapanor-treated group (50 mg) *vs* 23.6% for placebo (after 12 wk). After 12 wk, adequate relief in IBS-C symptoms was observed in 63.1% of tenapanor-treated group (50 mg twice daily) *vs* 39.3% in placebo
	Rosenbaum *et al*[187]	356 patients	A double-blind, placebocontrolled, randomized phase 2b trial 12-wk dose-ranging study evaluating tenapanor 5 mg, 20 mg or 50 mg b.i.d. *vs* placebo (1/2)	Tenapanor 50 mg b.i.d. significantly improved CSBM responder rate (primary endpoint) compared with placebo in patients with IBS-C. Tenapanor 50 mg b.i.d. also improved key secondary endpoints compared with placebo, including overall responder rate, abdominal pain responder rate and stool frequency. In addition, improvements were observed in several exploratory endpoints addressing a range of symptoms in patients with IBS-C. Tenapanor was generally well tolerated and had minimal systemic availability. Tenapanor shows promise as a future treatment option for patients with IBS-C

Neu p11 (piromalatine)	Camilleri *et al*[202]	40 IBS patients	In double blind placebo controlled study were randomly assigned to receive either melatonin 3 mg (n = 20) or matching placebo (n = 20) at bedtime for two weeks	Melatonin 3 mg at bedtime for two weeks significantly attenuated abdominal pain and reduced rectal pain sensitivity without improvements in sleep disturbance or psychological distress. The findings suggest that the beneficial effects of melatonin On abdominal pain in IBS patients with sleep disturbances are independent of its action on sleep disturbances or psychological profiles

IBS : Irritable bowel syndrome.

trial, the long-term safety and efficacy of 2.5 and 5 μg of ramosetron treatment in women with IBS-D was reported. However, the authors of the study concluded that "clinicians should be aware that one-fifth of women with IBS-D receiving ramosetron may suffer from constipation during treatment"[224].

Finally, a recent systematic review and meta-analysis performed to explore of the safety and efficacy of ramosetron both in male and female patients with IBS-D concluded that ramosetron is efficacious in both male and female patients with diarrhoea-predominant IBS, even if large-scale studies are needed to assess its effects on different ethnicities[225].

LX-1031

LX-1031 is a tryptophan hydroxylase (TPH) inhibitor that reduces peripheral serotonin production. It is indicated for conditions characterized by excess 5-HT expression such as IBS-D and, possibly, carcinoid diarrhoea. The blocking of excess 5-HT effects is well established[226]. Previously, pharmacological attempts aimed at inhibiting 5-HT synthesis, such as para-chlorophenylalanine, but the central adverse effects due to the inhibition of brain 5-HT synthesis with consequent affective disorders blocked their use[227].

LX-1031 does not cross the blood-brain barrier and, thus, does not pose risk of depression[228].

In a phase Ⅱ multicentre randomized double-blind placebo-controlled proof-of-concept study[229] performed on 155 patients, the subjects were assigned randomly in a double-blind fashion to 1 of 2 doses of LX1031 (250 mg 4 times/d or 1000 mg 4 times/d) or placebo taken daily during the 28-d treatment period[229]. Patients treated with LX1031 at the dose of 1000 mg significantly improved the primary efficacy end point, namely, the relief of IBS pain and discomfort, compared with placebo at week 1. No significant improvements were observed at weeks 2, 3 or 4. Adverse effects reported were generally mild, self-limited, and evenly distributed across the placebo and both LX1031 treatment arms[229]. The relationship between clinical improvement and reduction in serotonin synthesis shown in this study supports LX1031's proposed mechanism of action in IBS and thus supports serotonin synthesis inhibition in the GI tract as a novel therapeutic strategy for the treatment of IBS-D[229].

ASP-7147

Activation of the Bombesin-2 receptor may be involved in the regulation of gastrointestinal motility and intestinal secretion. ASP7147 is a novel small molecule Bombesin-2 receptor antagonist that reduces motility and intestinal secretions. Indeed, ASP 7147-mediated inhibition of this receptor may improve symptoms in patients with IBS-D. In the RCT performed by Lembo *et al*[230] on 64 patients during a 4-wk study, ASP7147 showed promise as a safe and effective new therapy for both men and women with IBS-D, demonstrating improvement in multiple symptoms of IBS-D. The persistence of treatment effect suggests the possibility of retained efficacy with less-frequent dosing in follow-on trials[230].

JNJ-27018966

JNJ-27018966 is a dual μ-opioid agonist and δ-opioid receptor antagonist that has been shown to have benefits in patients with IBS-D[231]. A randomized controlled double-blind study compared JNJ-27018966, at doses of 25, 100 and 200 mg twice daily, to placebo in 807 patients with IBS-D. Diarrhoea and pain were significantly improved in patients treated with JNJ-27018966 at the doses of 25 and 200 mg twice-daily compared to placebo (12, 13.8 and 5.7%, respectively, $P < 0.05$ for both comparisons to placebo)[232].

ROSE-010

ROSE-010 is a glucagon-like peptide 1 analogue that decreases gastric emptying and motility[1]. Hellström *et al*[233] conducted a randomized crossover placebo-controlled trial on 160 patients with IBS and associated abdominal pain treated with ROSE-010 100 μg once daily, 300 μg once daily or placebo. ROSE-010 was associated with a twofold greater response to abdominal pain compared to placebo ($P < 0.05$ for all comparisons) and significantly higher patient-reported satisfaction ($P < 0.05$). The most frequent treatment-related side effect was nausea, which was experienced by 19, 37 and 0% of ROSE-010 100 μg, ROSE-010 300 μg and placebo, respectively[233-234].

AST-120

AST-120, also known as kremezin, is an orally administered intestinal sorbent that has been reported to slow

chronic kidney disease (CKD) progression and to delay the initiation of dialysis by reducing the levels of renal toxins or their precursors in the gastrointestinal (GI) tract[235,236].

It has been shown that AST 120 exerts its properties in IBS by acting on intestinal permeability, reduction of visceral sensitivity and alteration of gut motility[236]. In a randomized double-blind controlled study conducted on 115 non-constipation-related IBS patients, AST-120 at a dose of 2 g three times daily significantly improved the percentage of patients with at least a 50% decrease in the number of days with abdominal pain compared to placebo (26.8% *vs* 10.2%, respectively). Additionally, AST-120 significantly improved bloating and stool consistency compared to placebo. The safety profile of AST-120 was similar to that of placebo[237].

Ibodutant

Antagonists of NK2 receptors have been suggested to modulate gastrointestinal chemical-induced impaired motility and stress-induced impaired bowel habits in humans, as recent phase 2 clinical trials have reported[238]. In a randomized double-blind controlled trial conducted on 559 IBS-D patients, ibodutant, a neurokinin-2 receptor antagonist, significantly improved abdominal pain, overall symptoms and quality of life compared to placebo. Ibodutant at doses of 1, 3 or 10 mg once daily showed superiority over placebo, with the 10 mg once daily dose being the most effective and women showing a better response than men[239,240]. Considering the limited number of effective available therapeutic options for IBS-D, ibodutant may become an important and safe treatment option, depending on whether ongoing phase 3 studies will confirm the efficacy observed in phase 2 studies[238].

Asimadoline

Asimadoline, a kappa-opioid receptor agonist, acts peripherally, inducing analgesic and antidiarrheal effects[241]. Action in the central nervous system is not required for asimadoline efficacy in the treatment of IBS. Asimadoline reduces sensation in response to colonic distension at subnoxious pressures in healthy subjects and in IBS patients without impairment of colonic compliance. Asimadoline decreased the appetite and enhanced the postprandial gastric volume (in healthy women). However, there were no significant effects on gastrointestinal transit, colonic compliance, fasting or postprandial colonic tone. In a clinical trial conducted on 40 patients with functional dyspepsia (according to Rome Ⅱ criteria), asimadoline did not significantly impair appetite or symptoms over 8 wk. However, asimadoline at a dose of 0.5 mg significantly reduced the appetite in patients, with higher postprandial fullness scores and daily postprandial fullness severity (over 8 wk). Patients treated with asimadoline at a dose of 1.0 mg showed borderline significant effects. In a clinical trial conducted on patients with IBS, the average pain 2 h post-on-demand treatment with asimadoline was not significantly decreased. At the post hoc analyses, asimadoline was demonstrated to be effective in mixed IBS[242]. Successively, in a randomized controlled double-blind trial conducted on 596 patients with IBS-D, asimadoline at doses of 0.15, 0.5 and 1 mg twice daily was compared to placebo. Patients treated with asimadoline at a dose of 0.5 mg twice daily had a twofold significant improvement in the total number of months with adequate relief of IBS pain, pain scores, urgency and frequency[243,244].

Colesevelam

Bile acids have several physiologic functions and are actively reabsorbed (up to 95%) in the terminal ileum[245,246]. Disruption of the enterohepatic circulation of bile acids due to ileal disease (inflammatory bowel diseases) or idiopathic bile acid malabsorption is responsible for chronic diarrhoea[246]. Faecal concentrations of bile acids in IBS-D or functional diarrhoea are unknown. While earlier studies suggested up-regulation of the ileal active transporter[247] as a result of chronic loss of bile acids (which may reduce the bile acids reaching the colon), other data suggest increased delivery to the colon may occur if the ileal reabsorptive capacity for bile acids is exceeded[246].

Odunsi-Shiyanbade *et al*[155] showed in 12 IBS-D patients that colesevelam modestly affected overall colonic transit (in patients treated with colesevelam, the emptying of the ascending colon was approximately 4 h longer compared to placebo). Furthermore, colesevelam favoured stool passage and somewhat firmer stool consistency. No effects on mucosal permeability or safety were found[246]. Successively, Camilleri *et al*[248] performed a 10-d single-center unblinded single-dose trial on the effects of colesevelam in 12 IBS-D patients. They demonstrated that colesevelam accelerates the delivery of BAs to stool, while improving stool consistency. It also stimulates hepatic BA synthesis, avoiding steatorrhea in patients with IBS-D. The overall effects are due to luminal BA sequestration by colesevelam[248]. All the abovementioned studies suggest that there is an opportunity to diagnose and specifically treat the cause of symptoms in IBS-D.

Solifenacin

Solifenacin is a muscarinic type 3 receptor antagonist recommended in the treatment of overactive bladder (OAB) in adults[249]. Since 1967, M3 receptor antagonists such as mepenzolate bromide have been used in Japan as modulating agents of gastrointestinal motility. However, no clinical trials had been designed to evaluate the efficacy for IBS defined under the modern Rome criteria. Given the high rate of comorbidity between IBS

and OAB[250], and considering that solifenacin acts on bowel dysfunction similarly to darifenacin, a selective M3 receptor antagonist with equivalent potencies, solifenacin was evaluated on symptomatic relief in 20 IBS-D patients in an open-label trial[249]. After a 2-wk observation period, solifenacin was administered for 6 wk. Later, solifenacin was suspended, and ramosetron, a serotonin 3 receptor antagonist, was given for 4 wk. Solifenacin was not inferior to ramosetron in the treatment of IBS with diarrhoea[249].

The results of this study suggested the potential therapeutic application of solifenacin in the treatment of IBS-D. However, the possible placebo effect could not be excluded. Therefore, further placebo-controlled parallel group studies are required to confirm the efficacy of solifenacin[249].

Tiropramide

Tiropramide, a derivative of tyrosine, has a spasmolytic effect on the intestine, decreasing Ca^{2+} release into intestinal smooth muscle[251-253]. In a double-blind placebo-controlled randomized trial performed by Lee *et al*[251], tiropamide was associated with an improvement of total symptom scores for 4 wk compared with 3 wk in the placebo group. In addition, only patients treated with tiropramide improved abdominal pain at week 4[254].

Lee *et al*[251] successively performed a multicentre, randomized, non-inferiority trial involving 287 patients with IBS randomly assigned to either tiropramide 100 mg or octylonium 20 mg t.i.d. (means 3 times a day) for 4 wk[1]. The visual analogue scale (VAS) scores of abdominal pain at week 4 were significantly reduced in both tiropramide and octylonium groups, even though the change from baseline was similar in the 2 groups[251]. In both groups, abdominal pain and discomfort assessed using VAS scores, diaries and IBS-QoL were improved, and no differences in the changes from baseline were observed. Side effects were similar in both groups. No severe side effects involving either drug were observed[251].

Despite the useful results of the abovementioned study, further studies are required to elucidate tiropramide's pharmacodynamic and pharmacokinetic properties and its mechanism of action on the intestine[251].

Eluxadoline

Eluxadoline is a μ- and κ-opioid receptor agonist and δ-opioid receptor antagonist. Its action is directed to the enteric nervous system, with slight side effects in the central nervous system. Its use was approved by the United States Food and Drug Administration on May 2015[255-257].

Patients with IBS-D receiving eluxadoline (100 mg twice daily) in a phase Ⅱ dose-ranging study had greater efficacy compared with patients receiving placebo after 12 wk[258]. Eluxadoline improved the number of daily bowel movements and decreased the episodes of urgency and incontinence experienced by patients during the 3-mo treatment period[258]. Eluxadoline had an overall favorable safety profile, with nausea, abdominal pain, vomiting, and constipation the most commonly reported AEs[258].

Subsequently, in two large Phase 3 trials (IBS-3001 and IBS-3002), the efficacy of eluxadoline in patients with IBS-D was shown[259].

Finally, Cash *et al*[260] reported pooled safety and tolerability data from Phase 2 and 3 clinical studies for the approved doses of eluxadoline: 75 and 100 mg. The authors demonstrated that constipation and nausea were the most common adverse events[260]. Consistent with the known adverse effects of opioid agonists, clinically apparent sphincter of Oddi spasm events were observed in eluxadoline-treated patients without a gallbladder. The majority of these cases were observed in patients on the higher dose of eluxadoline, thus suggesting a possible association[260].

Table 4 sums up the literature findings about irritable bowel syndrome-D therapies.

CONCLUSION

IBS currently remains a field of intense therapeutic research, in which most of the aforementioned studies focus on stool-pattern-specific subcategories of patients with this condition. Multiple further drugs are also under evaluation. Among these, alpha galactosidase (AG) was shown to reduce meteorism associated with black bean ingestion, even though it is unknown whether it may have a benefit on IBS[261]. However, in a subsequent study performed by Hillilä *et al*[262], no evidence to support the use of AG routinely in IBS patients was found.

With regards to therapies restoring intestinal permeability, multiple studies with prebiotics and probiotics[263] are ongoing, even if to date their efficacy has been limited. In parallel, much progress has been made in targeting low-grade inflammation, especially through the introduction of drugs such as mesalazine and rifaximin, even if a better knowledge of the mechanisms underlying the low-grade inflammation in IBS may support the design of clinical trials aimed at evaluating the efficacy and safety of such drugs.

On the other hand, the non-pharmacological treatment of IBS is often viewed as attractive. Faecal microbiota transfer, dietary interventions, holistic and integrative medicine approaches currently represent possible future therapeutic alternatives in this setting.

In conclusion, long-term studies and comparative studies with pharmacotherapy, as well as elucidation of the underlying mechanisms of action, are still needed to find the correct algorithm to manage IBS patients.

Table 4 Summary of the literature findings about irritable bowel syndrome-D therapies

Drug	Ref.	No. of pt	Study design	Outcomes
Ramosetron	Lee *et al*[213]	343 male pt	A multicenter, randomized, open-label trial male patients with IBS-D; pt were randomized to either a 4-wk treatment of ramosetron 5 mg once daily ,or a 4-wk treatment of mebeverine 135 mg three times daily	Global IBS symptoms, abdominal pain/discomfort and abnormal bowel habits in the ramosetron and mebeverine groups significantly increased during the treatment period. The severity scores of abdominal pain/discomfort and urgency, the stool form score, and the stool frequency in both treatment arms were significantly reduced, compared with the baselines
	Fukudo *et al*[222]	296 male pt	A randomized, double-blind, placebo-controlled trial in male patients with IBS-D Patients were given 5 mg oral ramosetron (n = 147) or placebo (n = 149) once daily for 12 wk after a 1-wk baseline period	Improving stool consistency in the first month. The ramosetron group had significantly higher monthly rates of relief of overall IBS symptoms and IBS-related quality of life than the placebo group. Adverse events occurring in 46.9% and 51.7% of ramosetron and placebo patients, respectively
	Fukudo *et al*[223]	576 female pt	A randomized, double-blind, placebo-controlled trial. The subjects received either 2.5 μg ramosetron or placebo once daily for 12 wk.	Global improvement, an increased stool consistency a significant reductions in abdominal pain and discomfort and greater improvement in QOL compared with placebo
Lx1031	Brown *et al*[229]	155 patients	A phase-Ⅱ multicenter, randomized, double-blind, placebo-controlled, the subjects were assigned randomly in a double-blind fashion to 1 of 2 doses of LX1031 (250 mg 4 times/d or 1000 mg 4 times/d) or placebo, taken daily during the 28-d treatment period	Improved significantly in patients given 1000 mg LX1031 compared with those given placebo, at week 1, together with nonsignificant improvements at weeks 2, 3 and 4. Adverse Effects reported were generally mild, self-limited, and evenly distributed across the placebo and both LX1031 treatment arms
ASP-7147	Lembo *et al*[230]	64 patients	RCT performed on during a 4-wk	Demonstrating improvement in multiple symptoms of IBS-D. The persistence of treatment effect suggests the possibility of retained efficacy with less frequent dosing in follow-on trials
JNJ-27018966	[232]	807 patients	A randomized, controlled, double-blind study, 25, 100, and 200 mg twice daily to placebo	The composite of diarrhea and pain was significantly improved in the JNJ-27018966 25 and 200 mg twice-daily groups compared to placebo
ROSE-010	Hellström *et al*[233]		A randomized placebo-controlled trial. Patients were randomized to ROSE-010 100 μg once daily, 300 μg once daily or placebo	Treatment with ROSE-010 resulted in a two fold greater response to abdominal pain compared to placebo and significantly greater patient-reported satisfaction with ROSE-010. The most common treatment-related adverse effect was nausea
AST-120	Tack *et al*[95]	160 patients 115 non-constipation-related IBS patients	A randomized, double-blind, controlled study	AST-120 2 g three times daily significantly improved the proportion of patients with at least a 50% reduction in the number of days with abdominal pain compared to placebo. AST-120 resulted in significantly improved bloating and numerically improved stool consistency compared to placebo. The safety profile AST-120 was similar to placebo
Ibodutant	Trinkley *et al*[231]	559 IBS-D patients	A randomized, double-blind, controlled trial	Improved abdominal pain, satisfactory relief of overall symptoms, and quality of life compared to placebo. All three doses of ibodutant (1, 3, 10 mg once daily) were superior to placebo, but 10 mg once daily was most effective and females responded better than males
Asimadoline	Trinkley *et al*[231]	596 IBS-D patients	A randomized, controlled, double-blind trial compared asimadoline 0.15, 0.5 and 1 mg twice daily to placebo	Asimadoline 0.5 mg twice daily significantly improved by two fold the total number of months with adequate relief of IBS pain, pain scores, urgency and frequency
Colesevelam	Odunsi-Shiyanbade *et al*[155]	12 IBS-D patients	Single center trial	Colesevelam modestly affected overall colonic transit (emptying of the ascending colon took an average 4 h longer in patients given colesevelam compared to placebo). Furthermore, colesevelam was associated with greater ease of stool passage and somewhat firmer stool consistency. No effects on mucosal permeability or safety were identified
	Camilleri *et al*[240]	12 IBS-D patients	A 10-d single-center, unblinded, single-dose trial	Colesevelam increases delivery of BAs to stool while improving stool consistency, and increases hepatic BA synthesis, avoiding steatorrhea in patients with IBS-D

Solinefacin	Fukushima *et al*[249]	20 IBS-D patients	An open-label trial. After a 2-wk observation period, all participants received solifenacin for 6 wk. Subsequently, the administration of solifenacin was discontinued and ramosetron, a serotonin 3 receptor antagonist, was administered for 4 wk	The efficacy of solifenacin in the treatment of IBS with diarrhea was not inferior to that of ramosetron
Tiropamide	Lee *et al*[251]	287 patients	A multicenter, randomized, non-inferiority Patients randomly allocated to either tiropramide 100 mg or octylonium 20 mg t.i.d (means 3 times a day) for 4 wk	Tiropamide led to symptom improvement in terms of total symptom scores for 4 wk, compared with 3 wk in the placebo group; in addition, at week 4 abdominal pain was only improved in the tiropramide group. The incidence of adverse events was similar in the 2 groups, and no severe adverse events involving either drug were observed

IBS : Irritable bowel syndrome.

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MINIREVIEWS

Glucocorticosteroid therapy in inflammatory bowel diseases: From clinical practice to molecular biology

Karen Dubois-Camacho, Payton A Ottum, Daniel Franco-Muñoz, Marjorie De la Fuente, Alejandro Torres-Riquelme, David Díaz-Jiménez, Mauricio Olivares-Morales, Gonzalo Astudillo, Rodrigo Quera, Marcela A Hermoso

Karen Dubois-Camacho, Daniel Franco-Muñoz, Marjorie De la Fuente, Alejandro Torres-Riquelme, David Díaz-Jiménez, Mauricio Olivares-Morales, Gonzalo Astudillo, Marcela A Hermoso, Innate Immunity Laboratory, Immunology Program, Institute of Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Santiago 8380453, Chile

Payton A Ottum, Neuroimmunology Laboratory, Immunology Program, Institute of Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Santiago 8380453, Chile

Marjorie De la Fuente, Division of Research, Clínica Las Condes, Santiago 7591046, Chile

Rodrigo Quera, Gastroenterology Department, Inflammatory Bowel Disease Program, Clínica Las Condes, Santiago 7591046, Chile

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Correspondence to: Marcela A Hermoso, PhD, Full Professor, Innate Immunity Laboratory, Immunology Program, Institute of Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Independencia 1027, Santiago 8380453, Chile. mhermoso@med.uchile.cl
Telephone: +56-2-29785672
Fax: +56-2-29786979

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Abstract

Inflammatory bowel diseases (IBDs), such as ulcerative colitis and Crohn's disease, are chronic pathologies associated with a deregulated immune response in the intestinal mucosa, and they are triggered by environmental factors in genetically susceptible individuals. Exogenous glucocorticoids (GCs) are widely used as anti-inflammatory therapy in IBDs. In the past, patients with moderate or severe states of inflammation received GCs as a first line therapy with an important effectiveness in terms of reduction of the disease activity and the induction of remission. However, this treatment often results in detrimental side effects. This downside drove the development of second generation GCs and more precise (non-systemic) drug-delivery methods. Recent clinical trials show that most of these new treatments have similar effectiveness to first generation GCs with fewer adverse effects. The remaining challenge in successful treatment of IBDs

concerns the refractoriness and dependency that some patients encounter during GCs treatment. A deeper understanding of the molecular mechanisms underlying GC response is key to personalizing drug choice for IBDs patients to optimize their response to treatment. In this review, we examine the clinical characteristics of treatment with GCs, followed by an in depth analysis of the proposed molecular mechanisms involved in its resistance and dependence associated with IBDs. This thorough analysis of current clinical and biomedical literature may help guide physicians in determining a course of treatment for IBDs patients and identifies important areas needing further study.

Key words: Inflammatory bowel diseases; Ulcerative colitis; Crohn's disease; Glucocorticoid dependence; Glucocorticoid resistance

Core tip: Glucocorticoids (GCs) are widely used in patients with Inflammatory Bowel Diseases who have moderate or severe disease activity; however, some of them do not respond to treatment or become dependent. Knowledge of both the clinical approach of GCs treatment as well as the molecular basis underlying their effects will help physicians prescribe drugs that will lead to better outcomes for patients.

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INTRODUCTION

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are multifactorial disorders comprised of both environmental and genetic factors. Even though they are different disease entities, UC and CD are grouped under the generic term of IBDs given their similar activity/remission stages, chronicity, immunological pathophysiology and uncertain etiology[1-3]. The prevalence of UC is 505 per 100000 people in Europe and 249 per 100000 people in North America, and CD is 322 per 100000 people in Europe and 319 per 100000 people in North America[4]. An increased incidence has been reported in some South American and mid-eastern Asian countries such as Chile[5], Brazil[6], Israel[7], and Malaysia[8]. This tendency might be related to the industrialization phenomenon and the influence of environmental risk factors[4].

IBDS: A MULTIFACTORIAL COMPLEX DISORDER

Various genetic and environmental factors have been associated with development of IBDs. Polymorphisms in a vast number of genes which impair functions such as lymphocyte activation, autophagy, pathogen sensing, stress response, antigen presentation, and chemotaxis, among others have been described[9]. The presence of such genetic variants may contribute to an imbalance in the immune response and increased predisposition to IBDs[10,11]. Moreover, environmental factors, such as diet, smoking, appendectomy, breast-feeding behavior in childhood, vitamin D deficiency and infections, play key roles in inflammatory manifestations, and have been described as risk factors for IBDs[12,13]. Furthermore, intestinal microbiome composition differs in IBDs patients compared to healthy subjects: IBD patients have a more predominant *Proteobacteria* population and decreased *Firmicutes* and *Bacteroidetes* populations[14,15]. Environmental factors might be involved in creating such imbalance in microbiome composition, and this may give rise to a constant inflammatory antigenic stimulus in the gut that causes chronic inflammation in genetically susceptible individuals[14].

These multifactorial components involved in the origin and progression of IBDs, has prevented the development of a specific treatment applicable to all patients. Current treatment options seek to diminish inflammation in order to control symptoms and keep the patient in a state of remission or symptom improvement. For example, aminosalicylates (5-asa derivatives) are used to treat mild disease, while immunosuppressants such as azathioprine/6-mercaptopurine and methotrexate, and biological therapy such as anti-TNF antibodies or anti-integrin are prescribed for moderate to severe disease[16]. Finally, glucocorticoids (GCs) are used to achieve, but not to maintain, remission in patients with moderate to severe activity[17].

GC THERAPY IN IBDs: ADMINISTRATION AND EFFECTIVENESS

The first study that demonstrated the usefulness of GCs (cortisone) in controlling severe UC attacks was published in 1955[18]. Since the 70s, the first generation of GCs (prednisone, methylprednisolone, hydrocortisone) has been used to induce clinical remission in IBDs patients. However, significant adverse effects have led to the development of second generation GCs (budesonide, budesonide MMX, Beclomethasone dipropionate)[19-21]. A characteristic of GC treatments and their use is summarized in Table 1.

Table 1 Glucocorticoids of first and second generation and his medical uses

Glucocorticoids	Component	Indications	Ref.
1st generation	Prednisone	Moderate to Severe cases	[3,25,27]
	Methyl-Prednisolone	of IBDs	[26]
	Hydrocortisone	Short duration of treatment	[29]
2nd generation	Budesonide	Moderate CD cases	[32-34]
	Budesonide MMX	Mild to moderate UC cases	[36,37]
	Beclomethasone dipropionate	Topical administration	[40,41]
	Erythrocyte - Mediated Delivery of Dexamethasone	In research for long term treatments	[42]

IBDs: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

First generation GCs: more effective than 5-asa and immunomodulatory treatment with significant secondary effects

Several studies have compared the efficacy and safety of traditional GCs to other treatments by measuring the rates of remission and adverse side effects. Two independent placebo-controlled double-blind randomized trials showed that 47% of active CD patients treated with prednisone (1 mg/kg, ranging from 40-60 mg/d) achieved clinical remission, defined as CDAI score < 150 (for a review on clinical and endoscopy scoring see references[22-24]), compared to 26% of patients receiving placebo after 4 mo of treatment. Neither sulfasalazine (0.5-1 gr/15 kg) nor azathioprine (2-2.5 mg/kg per day) achieved significant effectiveness[25]. Later, a placebo-controlled study showed that high doses of methylprednisolone (12-48 mg/d), sulfasalazine (3 gr/d), or both in active CD patients, were significantly effective compared to the placebo, and patients treated with methylprednisolone (alone or in combination with sulfasalazine) showed faster improvement compared to sulfasalazine[26].

Despite the effectiveness of GCs demonstrated in patients with severe CD, a prospective multicenter study comprised of patients with active CD on prednisone treatment (1 mg/kg per day) concluded that while 92% achieved clinical remission, only 29% of them reached endoscopic remission[27]. The presence of stenosis or fistula secondary to CD which occurs in patients who do not reach endoscopic remission, indicates a poor response to GC therapy and requires surgical intervention[27]. This suggests that GCs are efficient in improving the inflammatory response but other strategies are needed to increase mucosal healing.

Regarding UC, systemic GCs are the first choice therapy in cases of severe colitis[3]. In acute and critical UC, 76% of the patients treated with prednisolone (20 mg/d), plus a nightly rectal drip of hydrocortisone succinate sodium (100 mg in solution), presented clinical remission *vs* 52% of patients who were treated with sulfasalazine (8 gr/d the first week, 4 gr/d the second week)[28] demonstrating the clinical efficacy of this treatment. Likewise, two consecutive trials in UC patients with left side colitis determined that oral prednisone (1 mg/kg per day) was effective in severe stages of the disease compared with calcium lactate (1-3 gr; placebo), and prednisone treatment led to clinical remission more quickly and more often than those treated with salazopyrin (4 gr/d; a sulfasalazine derivate) and topic hydrocortisone treatments (100 mg/150 mL of sterile water administered as an enema)[29].

Despite the effectiveness of first generation GCs, they are characterized by several serious side effects that limit their long-term use. Among these, some of the most important are: metabolic (altered distribution of fat, Cushing's face or steroid-induced diabetes), eye-related (cataracts and glaucoma), dermatological (bruising and urticarial), gastrointestinal (gastric ulcer and gastrointestinal bleeding), and musculoskeletal side effects (osteopenia to osteoporosis). Additionally, adverse effects on the central nervous system have been described (insomnia, anxiety, hyperactivity, psychotic processes), along with hypertension, hypothalamic-pituitary-adrenal axis suppression and increased susceptibility to infections due to immunosuppression[19]. However, its use in IBDs patients has not be associated with atherosclerosis progression[30]. Finally, there is evidence that GC use increases the likelihood of sepsis of gastrointestinal origin; therefore it should be prescribed with caution in these patients[31].

Second generation GCs: high anti-inflammatory activity and fewer side effects.

The previously described serious side-effects related to first-generation GCs drove the development of second-generation steroids, which maximize the amount of corticosteroid locally available for distal ileum and proximal colon and minimize systemic bioavailability[3,26]. These new formulations of GCs with limited absorption include budesonide, budesonide MMX (multimatrix delivery system MMX) and beclomethasone dipropionate[3,19].

Budesonide, similar effectiveness with fewer adverse effects: Budesonide undergoes hepatic inactivation before reaching the systemic circulation (first-pass metabolism) which reduces its bioavailability[3]. Various placebo-controlled clinical studies have provided evidence about the efficacy of budesonide in IBDs patients. A multicenter randomized, placebo-controlled, double-blind clinical

trial was conducted in CD patients who received three different budesonide doses (3, 9 and 15 mg/d)[32]. After 8 wk of treatment, 51% of those receiving 9 mg budesonide progressed to remission in CD patients with ileum and proximal colon disease activity. Thus, this dose was effective and safe but depended on disease location. A randomized, double-blind, double-dummy controlled trial conducted in patients with mild to moderately active CD demonstrated that budesonide (9 mg/d for 2 mo) was as effective as prednisone (40 mg/d for the first 2 wk, 30 mg/d the third week, and gradually tapered to 5 mg/wk at the end of the study lasting 2 mo) in patients with terminal ileum, cecum, and/or ascending colon inflammation[33].

Subsequently, a multicenter double-blind, randomized trial was conducted in CD patients with moderate activity in the distal ileum and ascending colon. More patients that received budesonide (4.5 mg twice daily, or 9 mg once daily) achieved clinical remission compared to placebo 2 wk after beginning treatment, and this difference was statistically significant[34]. In addition, a pooled analysis of trials in CD patients found significantly fewer GC-related adverse events compared to patients treated with conventional corticosteroids (RR = 0.64, 95%CI: 0.54-0.76)[35].

An improved oral formulation of budesonide uses the colonic delivery technology Multi-Matrix System (MMX) to extend drug release in the colon (Budesonide MMX). Due to this characteristic, it is indicated for the treatment of UC patients that do not respond to standard doses of salicylates. A placebo-controlled study demonstrated that a higher proportion of UC patients with mild to moderate activity treated with budesonide MMX (9 mg), reached clinical remission and symptom resolution compared to placebo. In contrast, a lower dose of budesonide MMX (6 mg) and mesalamine, another salicylate treatment (2.4 gr/d), did not significantly improve either parameter[36]. Furthermore, the higher dose of Budesonide MMX was efficient in inducing remission in patients with mild to moderate UC disease who did not achieve remission with 5-asa treatments such as mesalamine or sulfasalazine[36,37]. Currently, physicians prescribe Budesonide MMX in UC patients that do not respond to traditional maintenance therapy before treating with azathioprine/6-mercaptopurin.

In 2015, data from 5 clinical studies, including double-blind, randomized, and open label studies, showed that rates of adverse effects were similar between budesonide MMX 9 mg and 6 mg (54.5% and 60.6% respectively) and placebo (50.5%) in patients with mild to moderate UC[38]. Furthermore, the open label studies showed less frequency of adverse effects with budesonide MMX (3 mg or 9 mg) compared to placebo. The most common adverse effects in these studies were headache, nausea and urinary tract infection demonstrating that second generation GCs not only have fewer side effects, but those effects are less severe compared to first generation GCs side effects.

Beclomethasone dipropionate, a second generation option with low systemic activity: Beclomethasone dipropionate (BDP) is a second-generation corticosteroid with topical effects and minimal systemic activity. It is administered as a pro-drug and is partially metabolized in the lower gastrointestinal tract[39]. The effectiveness and tolerability of BDP *vs* budesonide have been evaluated in CD patients whose clinical characteristics did not include complications such as stenosis or fistulas. A study showed that BDP (10 mg/d) was less effective than budesonide (9 mg/d) in CD patients treated for 2 mo. They concluded that this was due to the pharmacokinetic properties of budesonide[40].

A study was conducted to evaluate the efficacy and safety of BDP enemas *vs* prednisone enemas in active distal UC after 1 mo of treatment. They determined that clinical and endoscopic remission occurred in 29% of patients who received BDP and in 25% of patients who had standard topical GC treatment, and both groups experienced few adverse effects[41]. This evidence leads to the conclusion that BDP has similar efficacy to traditional GCs.

GC delivery technology, the next step to avoid side effects

Novel drug delivery systems capable of slowly releasing drugs into the bloodstream at a low concentration such as the erythrocyte-mediated drug delivery have been proposed. The membrane characteristics of autologous erythrocytes render them ideal drug carriers because their permeability allows for the diffusion of small molecules. This has been demonstrated using dexamethasone 21-phosphate (Dex 21-P), a biologically inactive compound[42]. Once encapsulated, an enzyme resident in the red blood cell dephosphorylates Dex 21-P to form the corresponding active metabolite, dexamethasone (Dex), which is then released into the bloodstream by passive diffusion through the cytoplasmic membrane of erythrocytes[42].

A study recruited 40 patients with mild to moderate UC, who had not responded to mesalamine and randomly assigned them to one of the following three treatment groups: Dex 21-P encapsulated into erythrocytes (DEE) delivered *via* two infusions 14 d apart, oral prednisolone infusions (0.5 mg/kg for 2 wk by 6 mg/wk tapering), and placebo. The group of DEE and oral prednisolone achieved a higher rate of clinical and endoscopic remission (75%) after 2 mo compared to placebo[42]. DEE-treated patients showed no adverse effects associated with GCs, in contrast to 80% of prednisone group, in whom acne, hirsutism and weight gain were reported. This delivery tool is an attractive choice for patients with IBDs to avoid most of GCs' adverse effects.

Resistance and dependence, an unresolved issue for GC treatment

More than half of IBDs patients on GC therapy respond to treatment, approximately 28% have a partial response, and 19% are non-responders (GC-resistant). However, about 20% of IBDs patients on long-term GC therapy become dependent[43,44]. Therefore, clarifying this GC resistance/dependency is crucial to making appropriate decisions regarding patient treatment. GC resistance or refractoriness is the inability of GCs to exert their effects on target tissues, thus limiting the efficacy of the therapy. In IBDs, GC resistance is defined as the persistence of an active manifestation of the pathology, despite having received standard treatment of 0.75 mg/kg per day for 4 or more weeks[44].

On the other hand, GC dependency is the need for GCs to maintain remission. Patients are considered GC-dependent if they fail to taper to steroid doses below 10 mg within 4 mo (starting dose 0.75-1 mg/kg oral prednisone-equivalent) or if they relapse within 3 mo after the discontinuation of GC treatment[44]. Usually, long-term treatment with GCs is associated with its resistance or dependency and a more aggressive clinical phenotype in IBD patients[45].

MOLECULAR BASIS OF GLUCOCORTICOID TREATMENT

Exogenous GCs are highly lipophilic compounds, making them widely bioavailable. Similar to endogenous cortisol, GCs are primarily transported in the bloodstream bound to corticosteroid-binding globulin and, to a lesser extent, to albumin[46]. GCs have the ability to passively diffuse through cell membranes and interact with the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors[47].

Molecular characteristics of GR and its impact on Immune system

GR is a cytosolic protein with a molecular weight of 94 kDa encoded by the *NR3C1* gene (5q31.3). It contains nine exons that mainly code for two transcripts formed by alternative splicing: the α and β isoforms[48]. In addition to GRβ, three less-well-characterized isoforms have been reported: GRγ, GR-A and GR-P[49]. Moreover, GRα and GRβ can also undergo alternative translation initiation in exon 2, which generates eight additional GR isoforms with truncated N-terminals giving them distinct properties[49].

The amino terminal region of GRα contains a ligand-independent transactivation domain (AF-1), a highly conserved central DNA binding domain (DBD), and a hinge segment. The carboxyl-terminal region contains the ligand-binding domain, which includes an AF-2 region that interacts with co-regulators in a ligand-dependent manner[50]. The GRβ is a shorter protein which differs from GRα in its C-terminal domain and antagonizes the activity of GRα[51]. After GC binding, the receptors undergo conformational changes and expose the DBD, interact with chromatin, and regulate gene expression[50].

The GR-GC complex can inhibit proinflammatory proteins such as nuclear factor κB and AP-1 through protein-protein interactions. These molecular mechanisms down-regulate the expression of proinflammatory cytokines and chemokines, such as IL-1α, IL-1β and IL-8. In addition, GCs up-regulate the expression of other cytokines that suppress the production of inflammatory mediators: such as transforming growth factor-β3 (TGF-β) and IL-10, increasing its anti-inflammatory function. They also inhibit T and B lymphocyte proliferation, and promote a tolerant macrophage profile (M2) (Figure 1)[46,52,53].

Molecular basis of GC resistance, a clue for personalized treatment

The molecular mechanisms of GC resistance in IBDs have been associated with changes in the GR isoform levels, polymorphisms in *NR3C1* or genes involved in GC bioavailability, and impaired cytokine production[46,48,54,55]. The most important genes associated to GC resistance are summarized in Table 2. Changes in levels of GRα and β isoforms have been associated with GC resistance. The α-isoform is the most well-studied protein, but only increased levels of the β-isoform have been associated to GC resistance in clinical trials in UC, asthma, nasal polyposis, cancer and chronic lymphocytic leukemia[53]. The GRβ protein is located primarily in the nucleus and is found mostly in T lymphocytes, macrophages, neutrophils, eosinophils, and peripheral blood mononuclear cells; however, it has also been reported in brain, lung, and heart tissue[56,57]. It has been proposed that GRβ may act as a dominant negative to GRα, since GRβ does not bind to GCs, but rather it interacts with glucocorticoid element response (GRE) causing GC resistance. This mechanism alters GRα signaling independent of GRα-GRβ heterodimer formation[58]. An *in vitro* study showed that overexpression of GRβ in a colonocyte cell model induces a vast deregulation of gene expression without Dex treatment, and a proportion of these genes have been shown to be altered in IBDs patients[59]. Thus, GRβ may directly change transcriptional gene activity of proinflammatory molecules. This effect is independent of GRα function and may promote the GC-resistance.

Furthermore, GRβ can bind to RU-486, a GRα antagonist, in such a way as to change the cellular location of this GRα, and alter its ability to regulate gene expression[60]. In addition, it has previously been shown that the expression of GRβ can be transcriptionally activated in regulatory T lymphocytes and neutrophils by proinflammatory cytokines[61]. Hence, GRβ expression may affect the immune response of these cells promoting inflammation and non-response to GC[55,57,62].

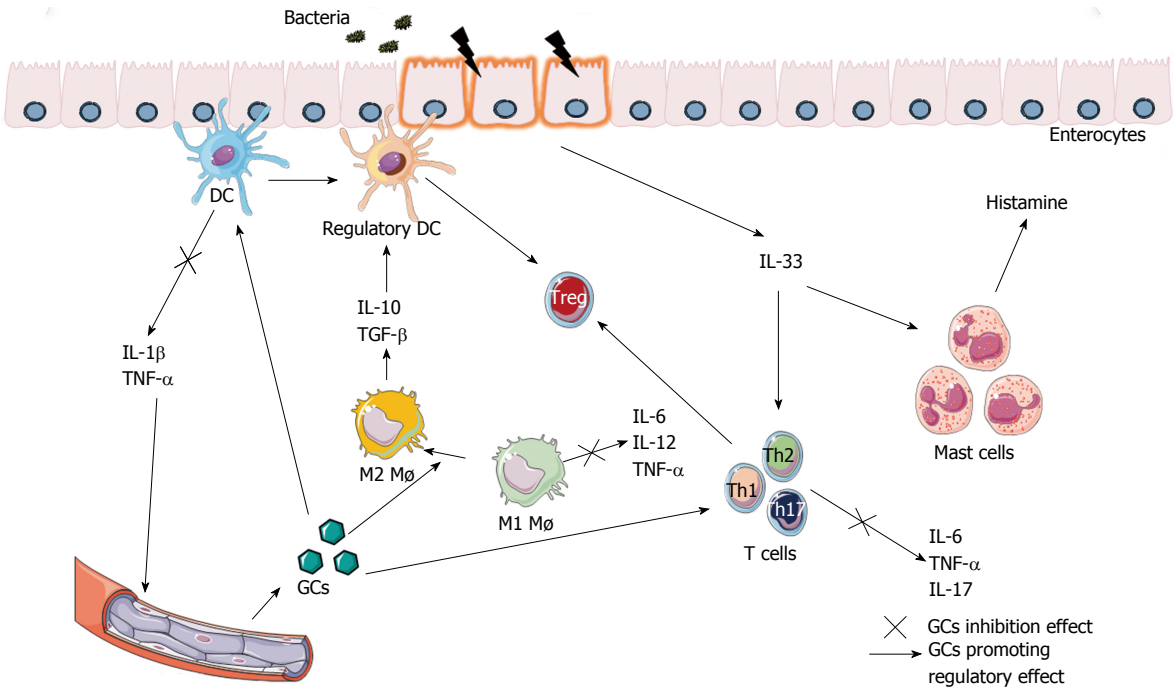

Figure 1 Immunologic dynamics and the responses triggered by glucocorticoids in inflammatory bowel diseases. During activation of the immune response in inflammatory bowel diseases (IBDs), the invasion of bacteria and the cytokine cascade triggered by the disruption of the epithelial barrier prompt an inflammatory response in the intestinal mucosa. Among the different cells that participate in the pathology of IBDs, the most important are represented in this diagram. When bacterial organisms interact with dendritic cells (DC), or the intestinal epithelium is disrupted due to an inflammatory response, the production of proinflammatory cytokines and the release of DAMPs (Damaged- Associated Molecular Patterns) such as TNF-α and IL-33, respectively, is elicited. This leads to the infiltration of immune cells such as monocytes which differentiate into proinflammatory M1 macrophages (Mø) and lymphocytes T (T cells), along with mast cell activation. In the presence of glucocorticoids (GCs), the infiltrating monocytes differentiate into a regulatory M2 profile Mø and CD103$^+$ DCs. These tolerogenic cells produce anti-inflammatory cytokines, skew the infiltrating naïve T cells toward a T regulatory phenotype, and control the inflammatory response. Also, the action of GCs blocks the production of proinflammatory cytokines by the M1 Mø, T cells and DCs, leading to the healing of the intestinal mucosa. Figure 1 was produced using Servier Medical Art from http://smart.servier.com. TGF-β: Transforming growth factor-β; IL: Interleukin.

Table 2 Molecular highlights on responses to steroidal treatment

Gene/protein	Function	Molecular alteration or genetic variant	Effect on GC response	Ref.
NR3C1	GC receptor	Increased expression of the isoform	Block signaling of the isoform/GC resistance	[58]
		rs6189 rs6190	Altered transactivation of GC receptor/GC resistance	[54,63]
		rs6195	Enhanced sensitivity to GC	[65]
		rs41423247	Alternative splicing of receptor/GC resistance	[54,63]
MDR1	Export of drugs from the cell	rs1045642	Decreased MDR1 levels/GC resistance in Brazilian CD patients	[68]
		rs1128503 rs1045642	Possible misfolding/GC dependence in Chinese CD patients	[75-77]
IL-10	Anti-inflammatory cytokine	rs1800896	Lowered production of IL-10/GC dependence in UC and CD patients	[45]
EBF3	Transcription factor	Decreased levels of EBF3	More severely inflamed phenotype/GC dependence	[72]
PAR2	G protein coupled receptor	Methylation of gene promoter	GC resistance/dependence in UC patients.	[82]

UC: Ulcerative colitis; CD: Crohn's disease; GCs: Glucocorticoids.

In 2009, a study was conducted to measure the expression of GRβ in intestinal mucosa of IBD patients that were refractory to treatment with GCs[55]. High levels of the GRα isoform were found in both GC-resistant and responsive patients; however, GC-resistant patients expressed higher levels of GRβ, in comparison with the GC-responder group. In the intestinal mucosa, the cells that primarily expressed the GRβ receptor were CD4$^+$ and CD8$^+$ T lymphocytes, macrophages and B cells. Fibroblasts and vascular endothelial cells expressed GRβ in a minor proportion. These results suggest that a higher ratio of GRβ/GRα in inflammatory mononuclear cells disturbs the effects of GCs resulting in refractory outcomes.

Single nucleotide polymorphisms (SNPs) in *NR3C1* gene, have been associated with GC resistance[54],

and these SNPs may result in different effects on the receptor structure and its signal transduction[54]. The most-studied SNPs that could induce a refractory phenotype in IBDs patients are: ER22/23EK (rs6189 and rs6190), N363S (rs6195), and BcII (rs41423247)[54]. The ER22/23EK is produced by a change in the amino acid residues 22 and 23 in the GR, where two glutamic acids (E) are replaced by an arginine (R) in position 22, and a lysine (K) in position 23[63]. These SNPs affect the transactivation process in *in vitro* assays, where the receptor is less likely to change its conformation and induce gene transcription in the presence of GCs[64]. The SNP N363S is in codon 363 of exon 2 and results in an amino acid change from asparagine (N) to serine (S). This SNP was reported to be associated with enhanced sensitivity to GCs, as was demonstrated by a Dex suppression test[65]. The BclI SNP was identified as a cytosine-guanine substitution 646 nucleotides downstream from exon 2, yielding 2.2 and 3.9 kb long fragments[63], which could suggest alternative splicing. A meta-analysis that included a mostly Caucasian population, analyzed the association of each of these 3 SNPs with GC resistance in IBDs patients[54]. Although no association was found, the analysis of the presence of all three SNPs, (or combinations of two SNPs) as a polygenic additive effect, could show risk associations of these variants with GC resistance.

Like the GR, the P-glycoprotein (P-gp) has also been associated with GC resistance in IBDs. P-gp is a trans-membrane glycoprotein with a molecular weight of 170 kDa encoded by the *MDR1* gene, which is located on the long arm of chromosome 7. P-gp is responsible for the absorption, distribution, metabolism and excretion of various drugs including GC[66]. A relationship between increased P-gp levels in circulating lymphocytes and intestinal epithelial cells with GC-resistant IBDs patients has been reported[67]. Polymorphisms in the *MDR1* gene have been shown to alter P-gp function, which in turn modifies the response to GCs[67]. A case-control study in a Brazilian population demonstrated that SNP C3435T (rs1045642), located in exon 26 of *MDR1* was associated with GC resistance in CD but not in UC patients[68].

Additionally, higher expression of proinflammatory cytokines has been associated with GC resistance. Overexpression of IL-6 and IL-8 has been associated with non-responsiveness to GC treatment[69]. In pediatric patients with UC, a similar correlation was found between the expression levels of IL-6 and unresponsiveness to GC[70]. In this study, the patients who did not respond to GC had higher levels of IL-6, compared to responders. Given the pleiotropic nature of IL-6, this correlation could reflect a cause-effect relationship between the signaling of the GC receptor and the expression of proinflammatory cytokines, but the real reason for the failure to respond remains unclear[70].

Similarly, in CD a correlation between cytokine production and resistance to GC was found. A clinical study showed that in intestinal mucosa from GC-resistant patients, the rate of apoptosis of T and B cells, presence of caspase-3 and IL-10 production were diminished compared to healthy and GC-responsive individuals[71]. These findings suggest a strong relationship between cytokine signaling and the inhibition of the GR effect in inflammatory diseases.

Analyses of gene expression using microarrays have been conducted to associate gene deregulation with GC resistance from colonoscopic biopsies of IBDs patients. *ATG16L1*, a gene related to the autophagy process was down-regulated in GC-resistant patients[72]. A low expression of the ATG16L1 protein has been associated with altered secretion of mucus by Paneth cells[73]. Furthermore, inhibition of autophagy led to Dex-resistance in a lymphoma cell line and in a mouse model[74]. This evidence indicates the importance of autophagy in the GC-resistant phenotype.

GC dependence: another genetic issue

Contrary to GC refractoriness, the molecular mechanisms behind GC dependence are poorly studied. There is evidence that associates *MDR1* SNPs (rs1128503 and rs1045642) with GC-dependent CD patients in a Chinese population[75]. Both SNPs correspond to a change from cytosine (C) to thymine (T) and are in coding regions of the gene (exon 12 and 26, respectively). Based on computational protein analysis, these SNPs, as haplotypes, seem to affect protein folding which could alter P-gp levels[76], but this mechanism has not been experimentally demonstrated. Nevertheless, the rs1045642 - CC was frequently found in individuals with significantly higher P-gp levels in plasma along with increased protein activity (measured by rhodamine efflux in $CD56^+$ natural killer cells), compared to carriers of the TT genotype[77]. This study suggests that increased GC transportation out of cells might drive a decreased GC response leading to the dependent phenotype[75].

SNPs in the *IL-10* gene have been associated with GC dependency as well. The SNP -1082 A/G (rs1800896) is located in the promoter sequence of the IL-10 gene, and a case control study of this variant showed an association with GC dependence in UC and CD patients[45]. This SNP was also related to a lower production of IL-10 in IBDs patients. Together, this evidence suggests that intrinsically inferior levels of IL-10 may generate GC dependency because exogenous GCs would be necessary to reach the anti-inflammatory IL-10 content necessary to achieve sustained remission. Other mechanisms, similar to those of refractoriness, such as altered expression of GR that impair intracellular signaling, have been proposed[78].

Downregulation of EBF3 was related to GC dependence in IBD patients[72]. The transcription factor EBF3 is a downstream target of SMAD2/3, which are components of the TGF-β signaling pathway[79]. This anti-inflammatory mechanism might be impaired in the context of low EBF3 expression[80]. Moreover,

EFB3 down-regulation has been associated with a low rate of apoptosis and increased cell proliferation[81]. This evidence suggests that decreased EBF3 levels might allow for a more severely inflamed phenotype making necessary a continual GC administration to maintain remission in IBD patients and driving their dependence.

Epigenetics knowledge in GCs dependence/resistance in IBDs

Epigenetic changes may also cause GC dependence/resistance. In the context of IBDs, higher methylation of the *Protease-Activated Receptor 2* (*PAR2*) gene, has been associated with steroid-dependent and resistance phenotypes in UC patients[82]. PAR2 is a G protein coupled receptor that is involved in pro-inflammatory responses and play a role in IBDs pathogenesis[82]. However, its specific role in GCs dependence/resistance has not been described.

Various studies relate the epigenetics changes such as hypermethylation of promoter genes with severe disease and risk of neoplasia[83-85]. There is evidence that epigenetic shifts can occur in the early stages of embryonic development in genes known as metastable epialleles, and these events can appear, as shown in rat and murine models, by environmental factors such as stress[86-88]. However, more analyses are needed to clarify the role of epigenetics in controlling the molecular mechanisms that underlie dependence or resistant to GCs in IBDs patients.

CONCLUSION

It has been over half a century since the first use of GC in IBD, yet amazingly, it currently remains a widely used therapeutic tool, specifically in disease exacerbations. The latest pharmacological applications, known as second-generation steroids, have contributed to the reduction of adverse reactions, however, increased effectiveness would need to be demonstrated in order for them to replace traditional GC therapy.

Despite findings showing positive GC responses, some patients remain resistant or become dependent to this drug. Deepening our understanding of the molecular basis of these undesired effects will help us generate novel, personalized GC therapies, depending on a patient's genetic background and cytokine profiles.

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Basic Study

Attenuation of MET-mediated migration and invasion in hepatocellular carcinoma cells by SOCS1

Yirui Gui, Md Gulam Musawwir Khan, Diwakar Bobbala, Claire Dubois, Sheela Ramanathan, Caroline Saucier, Subburaj Ilangumaran

Yirui Gui, Md Gulam Musawwir Khan, Diwakar Bobbala, Claire Dubois, Sheela Ramanathan, Subburaj Ilangumaran, Department of Pediatrics, Immunology Division, Faculty of Medicine and Health Sciences, University of Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

Caroline Saucier, Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

Author contributions: Gui Y, Bobbala D and Khan MGM performed experiments; Dubois C helped with the 3D-invasion assay and data analysis; Ramanathan S generated cell lines and provided mice; Saucier C helped with experiments, participated in data analysis and manuscript preparation; Gui Y, Saucier C and Ilangumaran S designed the study and wrote the manuscript; Saucier C and Ilangumaran S share senior authorship.

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Correspondence to: Subburaj Ilangumaran, PhD, Department of Pediatrics, Immunology Division, Faculty of Medicine and Health Sciences, University of Sherbrooke, 3001 North 12th avenue, Sherbrooke, Québec J1H 5N4, Canada. subburaj.ilangumaran@Usherbrooke.ca

Telephone: +1-819-3461110-14834

Fax: +1-819-5645215

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Abstract

AIM

To investigate the role of suppressor of cytokine signaling 1 (SOCS1) in regulating MET-mediated invasive potential of hepatocellular carcinoma (HCC) cells.

METHODS

Stable derivatives of mouse (Hepa1-6) and human (hep3B, HepG2) HCC cell lines expressing SOCS1 or control vector were evaluated for their ability to migrate towards hepatocyte growth factor (HGF) in the transwell migration assay, invade extracellular matrix in response to HGF stimulation in a 3-D invasion assay by confocal microscopy, and to undergo anchorage-independent proliferation in semisolid agar. Following intravenous and intrasplenic inoculation into NOD.scid. gamma mice, the ability of Hepa cells to form othotopic tumors was evaluated. Following HGF stimulation of Hepa and Hep3B cells, expression of proteins implicated in epithelial-to-mesenchymal transition was evaluated by western blot and qRT-PCR.

RESULTS

SOCS1 expression in mouse and human HCC cells inhibited HGF-induced migration through matrigel. In the

3-D invasion assay, HGF stimulation induced invasion of HCC cells across type- Ⅰ collagen matrix, and SOCS1 expression significantly reduced the depth of invasion. SOCS1 expression also reduced the number and size of colonies formed by anchorage-independent growth in semisolid agar. Following intravenous inoculation, control Hepa cell formed large tumor nodules that obliterated the liver whereas the SOCS1-expressing Hepa cells formed significantly smaller nodules. Tumors formed by SOCS1-expressing cells showed reduced phosphorylation of STAT3 and ERK that was accompanied by reduced levels of MET protein expression. HGF stimulated Hepa cells expressing SOCS1 showed increased expression of E-cadherin and decreased expression of EGR1, SNAI1 and ZEB1. Comparable results were obtained with Hep3B cells. SOCS1 expressing HCC cells also showed reduced levels of EGR1 and SNAI1 transcripts.

CONCLUSION

Our findings indicate that loss of SOCS1-dependent control over epithelial-to-mesenchymal transition may contribute to MET-mediated migration, invasion and metastatic growth of HCC.

Key words: Migration; Invasion; MET; Hepatocellular carcinoma; Suppressor of cytokine signaling 1

Core tip: The suppressor of cytokine signaling 1 (*SOCS1*) gene is frequently repressed in primary hepatocellular carcinoma (HCC) specimens, and mice lacking SOCS1 are highly susceptible to experimental HCC. The tumor suppressor functions of SOCS1 in HCC are not yet fully understood. We have shown that SOCS1 regulates hepatocyte growth factor signaling *via* the MET receptor in HCC cells and inhibits their growth. In this study, we characterize SOCS1 as a regulator of MET-mediated migration and invasion of HCC cells. We propose that *SOCS1* gene expression status could be exploited as a selection marker for precision therapies targeting MET in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers and a leading cause of worldwide cancer mortality[1]. HCC develops slowly over two to three decades and most cases present advanced disease at the time of diagnosis. Treatment options for HCC are constrained by the extent of disease, and are very limited and less effective in patients with advanced disease. Therefore, reducing HCC-associated mortality is critically dependent on the development of new treatment methods targeting molecular signaling pathways that promote HCC pathogenesis and diagnostic tools to facilitate targeted therapies[2-4].

Invasive intrahepatic dissemination is a key factor in malignant growth of HCC and its poor prognosis[5,6]. Up to 65% of HCC patients also present extrahepatic metastasis at autopsy[7-9]. HCC can also metastasize to stomach *via* direct invasion[10]. Detachment from the tumor mass and invasion of the extracellular matrix and the basement membrane are important steps in tumor cell invasion and metastasis. Cancer cells gain these abilities through epithelial-mesenchymal transition (EMT), a developmental genetic program that is crucial for embryogenesis and wound healing response[11,12]. A wide spectrum of paracrine (from the tumor stroma) and aurocrine cytokines and growth factors elicit and modulate the EMT program[12]. Even though TGFβ is the most important inducer of EMT, growth factor receptor tyrosine kinase (RTK) signaling induced by hepatocyte growth factor (HGF), epidermal growth factor and platelet-derived growth factor can also activate the EMT program in carcinomas[12,13].

The HGF receptor c-MET is overexpressed in many human cancers including HCC[14]. MET not only promotes neoplastic growth of HCC cells but also facilitates tumor metastasis by promoting EMT[14-16]. Recent studies have implicated the microRNAs miR-148a and miR-449a in regulating EMT in HCC cells by targeting the MET receptor[17,18]. Previously, we have shown that suppressor of cytokine signaling 1 (SOCS1) is an important regulator of HGF signaling hepatocytes. SOCS1 deficiency in primary mouse hepatocytes increases MET signaling and cell proliferation, whereas stable expression of SOCS1 in human HCC cells attenuates HGF-induced cell growth[19,20]. We have also shown that SOCS1 binds to the MET receptor and promotes its ubiquitination and proteasomal degradation[20]. The *SOCS1* gene is frequently repressed in HCC, and *Socs1*-deficient mice show high susceptibility to experimental HCC with larger and more numerous tumor nodules[21-23], suggesting a role for SOCS1 in controlling MET-mediated tumor cell invasion.

In this study, we examined the role of SOCS1 in MET-mediated migration and invasion of HCC cells and their growth following delivery to the liver. Our findings show that SOCS1 inhibits the invasive potential of HCC cells and reduces orthotopic tumor growth that could result at least partly from modulating the expression of proteins implicated in EMT.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

HCC cell lines of mouse (Hepa1-6; Hepa) and human origin (Hep3B, HepG2) were obtained from ATCC

and grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS, from Sigma). Cells stably expressing full-length HA-SOCS1 through lentiviral transduction have been previously described[20]. Recombinant mouse and human HGF were from Peprotech (Rocky Hill, NJ). Phospho-ERK (T202/Y204; #9106), phosphor-STAT3 (Y705; #9131), E-cadherin (24E10), ZO-1 (D7D12), N-cadherin (D4R1H), vimentin (D21H3), EGR1 (44D5), SNAI1 (C15D3) and ZEB1 (D80D3) antibodies were from Cell Signaling Technology (Beverly, MA). Antibodies (Ab) against total ERK (sc-93, sc-153), STAT3 (sc-483) and MET (sc-161) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb against β-actin (A4700) was from Sigma Aldrich (Oakville, ON, Canada). Secondary antibodies were from Jackson Immunoresearch Laboratories Inc. (Cedarlane, Burlington, ON, United States). Calcein was from Calbiochem (San Diego, CA, United States).

Orthotopic tumor growth

Male NOD.*scid.gamma* (NSG) mice (8-12 wk old) purchased from the Jackson Labs (Bar Harbor, ME, United States) were used to evaluate tumor growth *in vivo* under protocols approved by the Université de Sherbrooke ethical committee on animal care and use. To evaluate the growth of hepatoma cells in the liver, cells were injected *via* intravenous or intrasplenic/portal route. For intravenous inoculation, 10^6 Hepa-vector or Hepa-SOCS1 cells in 100 μL volume were injected *via* the caudal vein. For intrasplenic inoculation, mice were anesthetized with ketamine (10 mg/kg) and the spleen was exposed through a small abdominal incision[24]. Tumor cells (10^6 cells in 100 μL) were injected into the spleen and mice were splenectomized 2 min later. Tumor nodules in the liver were examined 20 d later when the animals began to show distress. The images of hematoxylin and eosin (H and E) -stained sections of the liver were acquired using Nanozoomer Slide Scanner and analyzed by the Nanozoomer Digital Pathology (NDP) software (Hamamatsu Photonics, Japan).

Soft agar colony formation

After adding the bottom layer of 0.6% agar in 2 × DMEM containing 20% FBS to 100 mm Petri dishes, 5000 HCC cells suspended in 0.3% agar in 2 × DMEM containing 10% were added FBS as the top layer. Cells were fed every 3-5 d by overlying fresh 500 μL of DMEM containing 10% FBS. After 3 wk culture, colonies were stained with 1 mg/mL methylthiazol tetrazolium for 3 h, photographed and counted using the NIH ImageJ software.

Transwell migration assay

Migration of cells was assessed using transwells (Corning) with 8 μmol/L pore polycarbonated inserts, coated with growth factor-reduced matrigel (BD Pharmingen Biosciences, Palo Alto, CA, United States). The upper chamber contained 2 × 10^4 cells and the lower chamber contained 0.7 mL of complete medium with or without HGF. Migration through the membrane was determined after 24 h of incubation at 37 ℃. Cells remaining on the top side of the transwell membrane were removed using a cotton swab, and cells migrated to bottom were stained with 0.5% crystal violet and examined by microscopy. Cells in five random fields of the lower side of the membrane were counted. Data represent means ± SEM of three independent experiments.

3-dimensional invasion assay

The wells of a 96-well microtiter plate was covered with 50 μL of 1% agarose containing 10% FBS as the bottom layer and overlaid with 50 μL of fibrillar type-Ⅰ collagen (3 mg/mL) (R and D Systems, Minneapolis, MN, United States). Control and SOCS1-expressing HCC cells were serum starved overnight and layered on the top (20000 cells/well in 100 μL volume) in the presence or absence of HGF. After 48 h incubation, the cells were labeled with CellTrace™ Calcein Green, AM (Invitrogen, CA, United States) for 1 h, washed with PBS, fixed with 3% glutaraldehyde and confocal images were acquired using the FV1000 Olympus confocal microscope. The collagen matrix was scanned along the Z-axis at incremental 5 μm depths in order to reconstruct 3-D images. The images were analyzed to assess the depth of cell migration, which is expressed as ratio of the fluorescence intensity at each 5 μm layer over the fluorescence intensity of the non-invaded cells at the top 5 μm layer.

Western blot

Lysates of liver tissues harboring the tumor nodules were prepared in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0 and protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN) for 2 min using the bead mill MM 400 (Retsch, Hann, Germany). After adding equal volume of the same buffer containing 0.2% SDS, 1% Triton X-100 and 1% sodium deoxycholate, the lysates were kept on a shaker at 4 ℃ for 30 min. Following centrifugation at 15000 *g* for 20 min, the supernatant was collected and protein concentration determined using the RC-DC Protein Assay Kit (Bio-Rad, Mississauga, ON, United States). Cultured cells were lysed directly in 1 × SDS-PAGE sample buffer. Aliquots of 30-50 μg proteins were analyzed by western blot. Secondary antibodies and enhanced chemiluminescence reagents were from GE Healthcare Life Sciences (Pittsburg, PA, United States). Western blot images were captured by the VersaDOC 5000 imaging system (Bio-Rad). Densitometric quantification of the western blot bands was carried out using the NIH ImageJ 1.62 software.

Real-time RT-PCR

For RT-PCR analysis, total RNA was isolated from 1 × 10^6 cells, either unstimulated or stimulated with HGF for the indicated periods of time, using RiboZol™ (AMRESCO,

Solon, OH, United States). After verifying the RNA quality by UV absorption, the first complementary strand was made from 1 μg total RNA using QuantiTect® reverse transcription kit (Qiagen). The primers for gene expression analysis are as follows: Mouse *Egr1* (NM_007913): AGCGCCTTCAATCCTCAAG and TTTGGC TGGGATAACTCGTC; *Snai1* (NM_011427): GTGAAGAGA TACCAGTGCCAG and AAGATGCCAGCGAGGATG; Human *EGR1* (NM_001964): TGTCACCAACTCCTTCAGC and TCCTGTCCTTTAAGTCTCTTGTG; SNAI1: TCTAGGCCCTG GCTGCTACAA and ACATCTGAGTGGGTCTGGAGGTG; housekeeping genes: mouse *Gapdh* (NM_008084.3): ATGACATCAAGAAGGTGGTGAA and GTCTTACTCCTTGG AGGCCATGT; Human *GAPDH* (NM_002046.5): GATGA CATCAAGAAGGTGGTGAA and GTCTTACTCCTTGGAGG CCATGT. All primers showed more than 90% efficiency with a single melting curve in the MyQi5® cycler (Bio-Rad, Mississauga, ON, United States). Expression levels of the housekeeping gene were used to calculate fold induction of the specific genes modulated by the presence or absence of SOCS1.

Statistical analysis

Statistical significance of the differences in cell proliferation, tumor growth and protein expression was evaluated by student's *t*-test.

RESULTS

Previously we have shown that SOCS1 inhibited HGF-induced MET signaling, cell proliferation and migration in mouse (Hepa) and human (Hep3B, HepG2, SNU-423) HCC cell lines[19,20]. Here, we investigated whether SOCS1 also inhibits HGF-induced invasion of the extracellular matrix in some of these cell lines. At the concentration previously found to be effective in inducing cell proliferation and migration[19], HGF induced cell scattering in murine Hepa cells and human Hep3B cells, which was attenuated by SOCS1 (Figure 1A). In a trans-well migration assay, Hepa cells expressing the control vector showed marked ability to invade the matrigel in response to HGF, whereas the SOCS1-expressing cells showed significantly reduced ability (Figure 1B). Similar reduction of HGF-induced matrix invasion by SOCS1 was also observed with the human HCC cell line Hep3B (Figure 1C).

Next, we used a 3-D matrix invasion assay using collagen as matrix, where the depth of invasion was evaluated by confocal microscopy and quantified (Figure 2A and B). HGF stimulation increased the ability of Hepa, Hep3B and HepG2 cells to invade the collagen matrix, and this invasive potential was markedly pronounced in Hep3B and HepG2 cells, as indicated by the maximum depth of invasion (Figure 2A and C). In all the three cell lines, SOCS1 reduced the HGF-induced invasive potential (Figure 2A and C). Even though the SOCS1-mediated inhibition of HGF-induced invasion was only marginal in Hepa cells, Hep3B-SOCS1 and HepG2-SOCS1 cells showed significant reduction in HGF-induced invasive potential (Figure 2D). These results indicated that SOCS1 is an important regulator of MET signaling that promotes cell migration and invasion.

Tumor dissemination requires not only the ability to invade but also the ability to survive and undergo anchorage-independent proliferation. Therefore, we compared the ability of control and SOCS1-expressing HCC cells to form colonies in semi-solid agar. As shown in Figure 3A, Hepa-SOCS1 cells form fewer and smaller colonies than Hepa-V cells. Similar reduction of colony forming ability was also observed in Hep3B-SOCS1 and HepG2-SOCS1 cells compared to controls (Figure 3B).

To evaluate whether SOCS1 attenuates invasive tumor growth within the liver, we delivered control and SOCS1-expressing HCC cells *via* parenteral inoculation to the liver. For this purpose, we used murine Hepa1-6 cells, which can utilize endogenous HGF and other mouse growth factors better than human hepatoma cells[25]. Following intravenous injection, the control cells formed numerous orthotopic tumor nodules that obliterated the liver, whereas Hepa-SOCS1 cells induced fewer nodules (Figure 4A). The lungs of these mice did not show macroscopically visible tumor, suggesting that Hepa cells preferentially colonized the liver. We also delivered the HCC cells to the liver *via* intrasplenic route through the portal circulation. In the latter setting, Hepa-vector cells obliterated the liver with numerous macroscopically visible nodules, whereas Hepa-SOCS1 cells formed markedly smaller nodules (Figure 4B and C). Orthotopic tumors formed by Hepa-SOCS1 showed markedly diminished phosphorylation of STAT3 and ERK (Figure 4D), presumably through SOCS1-mediated inhibition of HGF and other cytokine and growth factor signaling[19,26,27]. Moreover, the SOCS1 expressing tumors showed significantly reduced levels of MET expression (Figure 4E), in agreement with our previous report that SOCS1 targets the activated MET receptor to the proteasomal degradation machinery[20]. These results indicated that SOCS1 inhibits the invasive growth potential of HCC cells and that at least part of this invasive growth could result from attenuation of HGF-induced MET signaling.

To understand the mechanisms underlying SOCS1-mediated attenuation of the invasive growth of HCC cells, we evaluated the expression of proteins implicated in EMT that promote tumor cell spreading following HGF stimulation[28-30]. Even though HGF stimulation did not appreciably affect the expression of E-Cadherin in control or SOCS1-expressing Hepa and Hep3B cells, the SOCS1-expressing cells showed increased basal expression of E-Cadherin (Figure 5A). SOCS1-expresing Hepa cells but not Hep3B cells showed discernible increase in ZO-1 expression, whereas the expression of N-Cadherin was not affected by SOCS1 in both cell

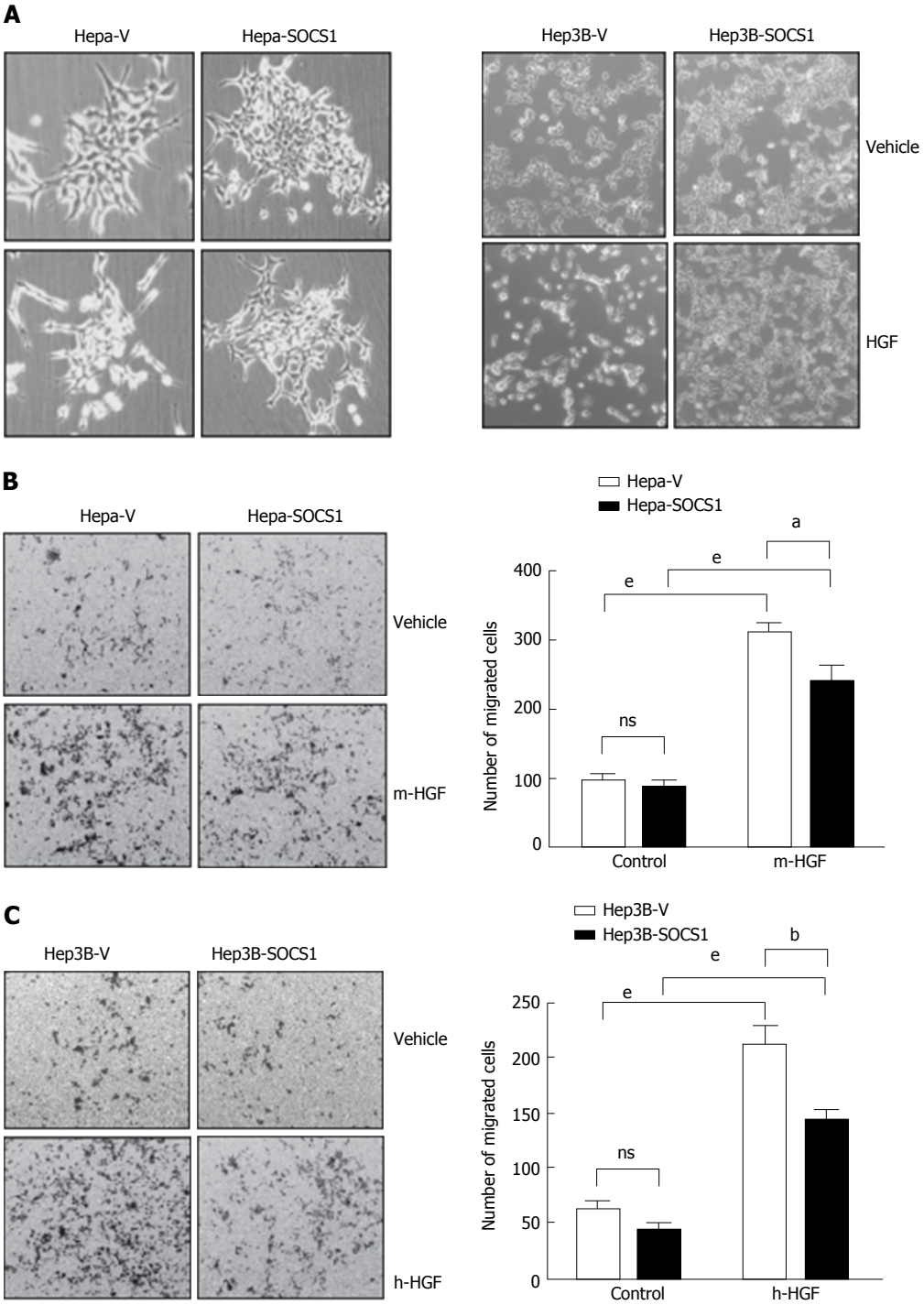

Figure 1 Suppressor of cytokine signaling 1 attenuates hepatocyte growth factor-induced scattering and migration of hepatocellular carcinoma cells. A: Hepa and Hep3B cells expressing SOCS1 (Hepa-SOCS1, Hep3B-SOCS1) or control vector (Hepa-V, Hep3B-V) were grown to less than 50% confluent state, starved in serum-free medium for 16 h and exposed to murine or human HGF (HGF, 25 ng/mL), respectively. After 48 h, the culture plates were examined by phase contrast microscopy to visualize HGF-induced cell scattering; B: Hepa-SOCS1 and Hepa-V cells were seeded onto Matrigel-coated trans-well migration chambers, and migration towards m-HGF was evaluated after 48 h. Representative images of cells that had migrated across the membrane and the number of migrated cells in five random fields from three separate experiments (mean ± SE) are shown; C: Migration of Hep3B cells expressing SOCS1 (Hep3B-SOCS1) or control vector (Hepa-V) towards human HGF (h-HGF, 10 ng/mL). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$. SOCS1: Suppressor of cytokine signaling 1.

lines. Analysis of gene expression showed marked reduction in the induction *Egr1* and *Snai1* genes in Hepa-SOCS1 cells, and *EGR1* and *SNAI2* genes in Hep3B-SOCS1 cells (Figure 5B). Western blot analysis showed reduced expression of EGR1, SNAI1 and ZEB1 in Hepa-SOCS1 cells compared to Hepa-V cells, whereas only SNAI1 showed appreciable reduction in Hep3B cells (Figure 5A). These findings suggest that SOCS1 may modulate diverse components of the EMT signaling machinery during MET-mediated invasion of

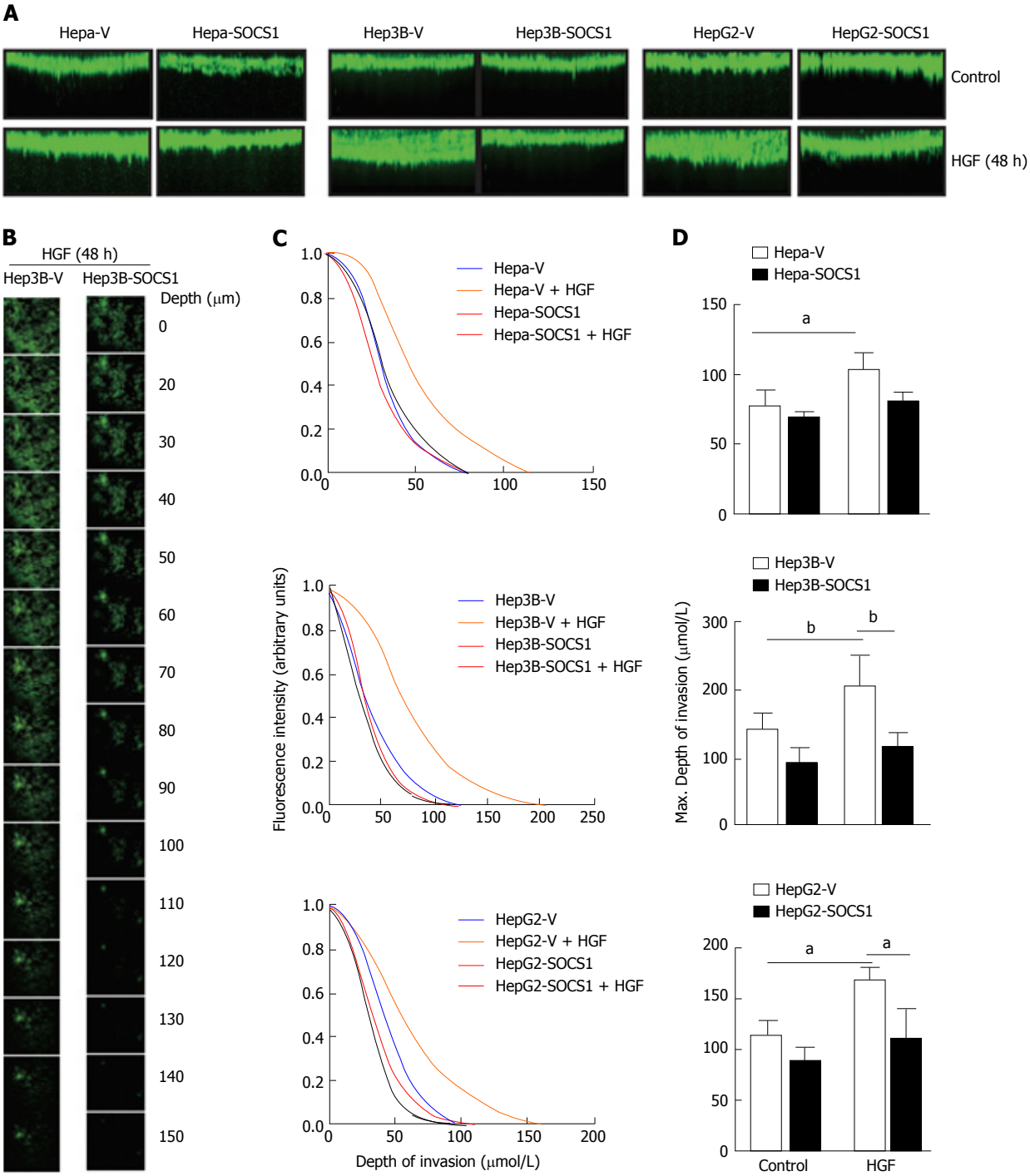

Figure 2 Inhibition of hepatocyte growth factor-induced invasion of collagen matrix by suppressor of cytokine signaling 1 in hepatocellular carcinoma cells. Hepa-V and Hepa-SOCS1, Hep3B-V and Hep3B-SOCS1, and HepG2-V and HepG2-SOCS1 cell lines were evaluated for their ability to invade collagen matrix in a 3-D invasion assay in the presence or absence of mouse HGF (25 ng/mL for Hepa cells) or human HGF (30 ng/mL for Hep3B, HepG2 cells). After 48 h, cells were fluorescently labeled with calcein Green, fixed and examined by confocal microscopy. A: The reconstructed cross-sectional images of cell migration, representative of two independent experiments, are shown; B: Representative images of Hep3B-V and Hep3B-SOCS1 cells migrated to different planes of the collagen-agarose matrix along the z-axis; C: Efficiency of migration (ratio of the fluorescence intensity at each 5 μm layer over the fluorescence intensity of the non-invaded cells at the top 5 μm); D: Maximum depth of invasion by control and SOCS1-expressing cells in the absence or presence of HGF. $^{a}P < 0.05$, $^{b}P < 0.01$. SOCS1: Suppressor of cytokine signaling 1.

HCC cells.

DISCUSSION

The *SOCS1* gene is frequently repressed in HCC by promoter CpG methylation[21,31,32]. Recent meta-analyses of published works on DNA methylation status of several HCC-associated genes have shown that *SOCS1* gene promoter hypermethylation is an important risk factor and predictive biomarker for HCC[33,34]. Consistent with the role of SOCS1 as a putative tumor suppressor in HCC, others and we

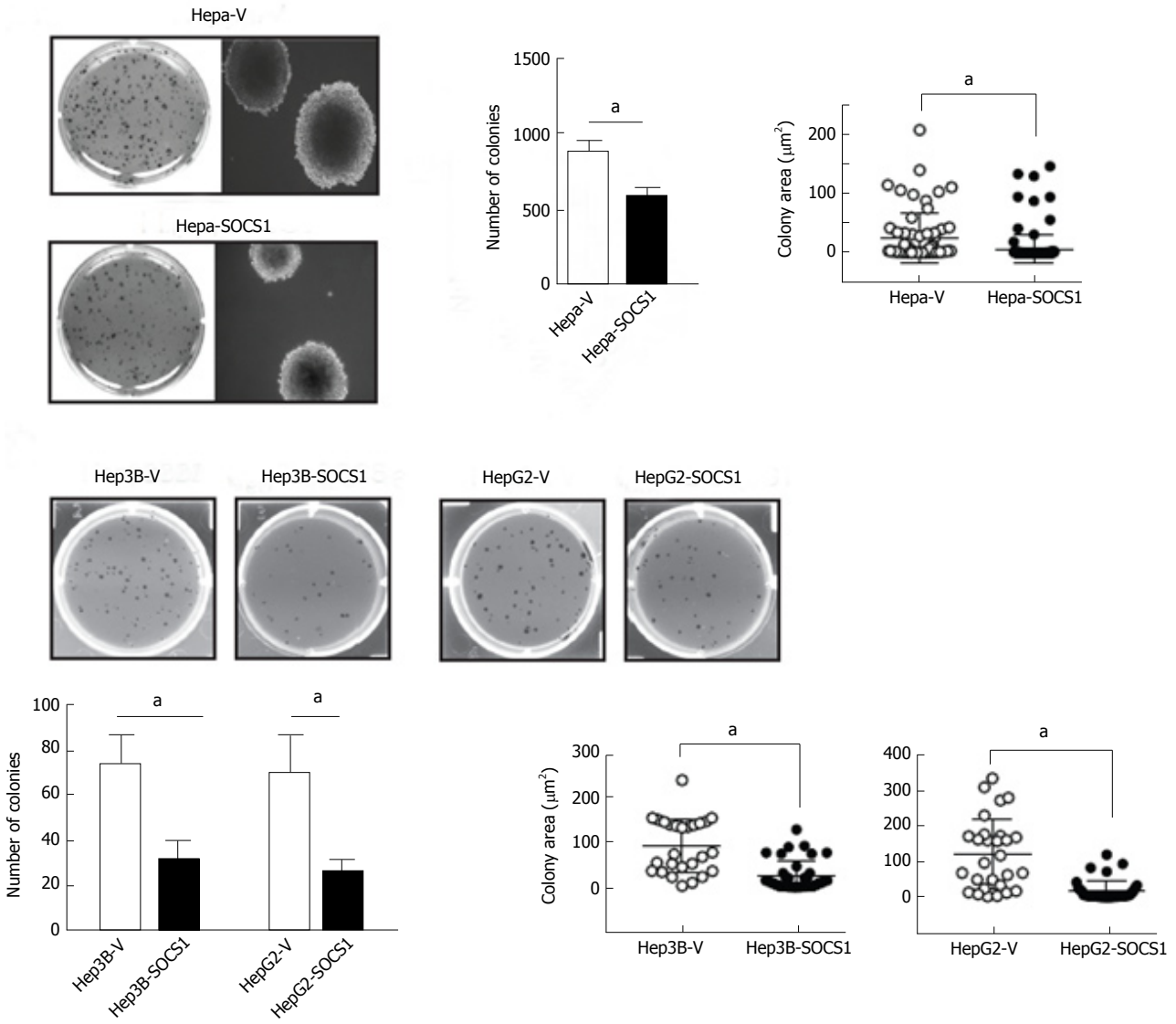

Figure 3 Reduced anchorage-independent proliferation of hepatocellular carcinoma cells expressing suppressor of cytokine signaling 1. SOCS1-expressing and control mouse Hepa (A) and human Hep3B and HepG2 (B) HCC cell lines were evaluated for their ability to form colonies in soft agar. After 3 wk culture, the colonies were photographed, and the number and size of colonies (area; mean ± SE) were evaluated from triplicates of two separate experiments. $^{a}P < 0.05$. SOCS1: Suppressor of cytokine signaling 1.

have shown that loss of *Socs1* gene expression in mice increases susceptibility to HCC induction[22,23]. The loss of SOCS1 expression might contribute to HCC progression *via* multiple mechanisms. We have shown that SOCS1 is an important regulator of oncogenic MET RTK signaling and suppresses the oncogenic potential of P21CIP in hepatocytes[19,20,23]. These reports suggested that the loss of SOCS1 expression in HCC by epigenetic mechanisms could promote cancer cell survival, migration and proliferation. The findings of the present study indicate that the loss of SOCS1-dependent regulation of HGF/MET signaling may also contribute to invasive growth of HCC cells through, at least partly, *via* promoting EMT.

Aberrant MET signaling that facilitate tumor progression and metastasis occurs in many cancers mainly *via* receptor overexpression, gene amplification or through the loss of MET-targeting microRNAs, although activating mutations and increased autocrine HGF stimulation can also amplify MET signaling[14]. While gain-of-function mutations and gene amplification of MET are rare in HCC, MET overexpression has been frequently reported[14]. The elevated MET expression would not only lead to ligand-independent activation but also confer increased responsiveness to des-gamma-carboxy prothrombin, which is highly expressed in HCC[35]. The molecular basis of MET overexpression in HCC has not been elucidated yet[14]. The high frequency of SOCS1 promoter methylation in HCC[33,34] and the ability of SOCS1 to promote MET degradation by proteasomes[20] suggest that MET overexpression in HCC could result at least partly from the loss of SOCS1 mediated control of MET expression.

The MET signaling pathway is an important mediator of EMT in HCC progression *via* intra- and extra- hepatic metastases[36,37]. EMT is mediated *via* induction of transcription factors (*SNAI1, SNAI2, ZEB1, ZEB2, TWIST1*), which repress genes coding for proteins

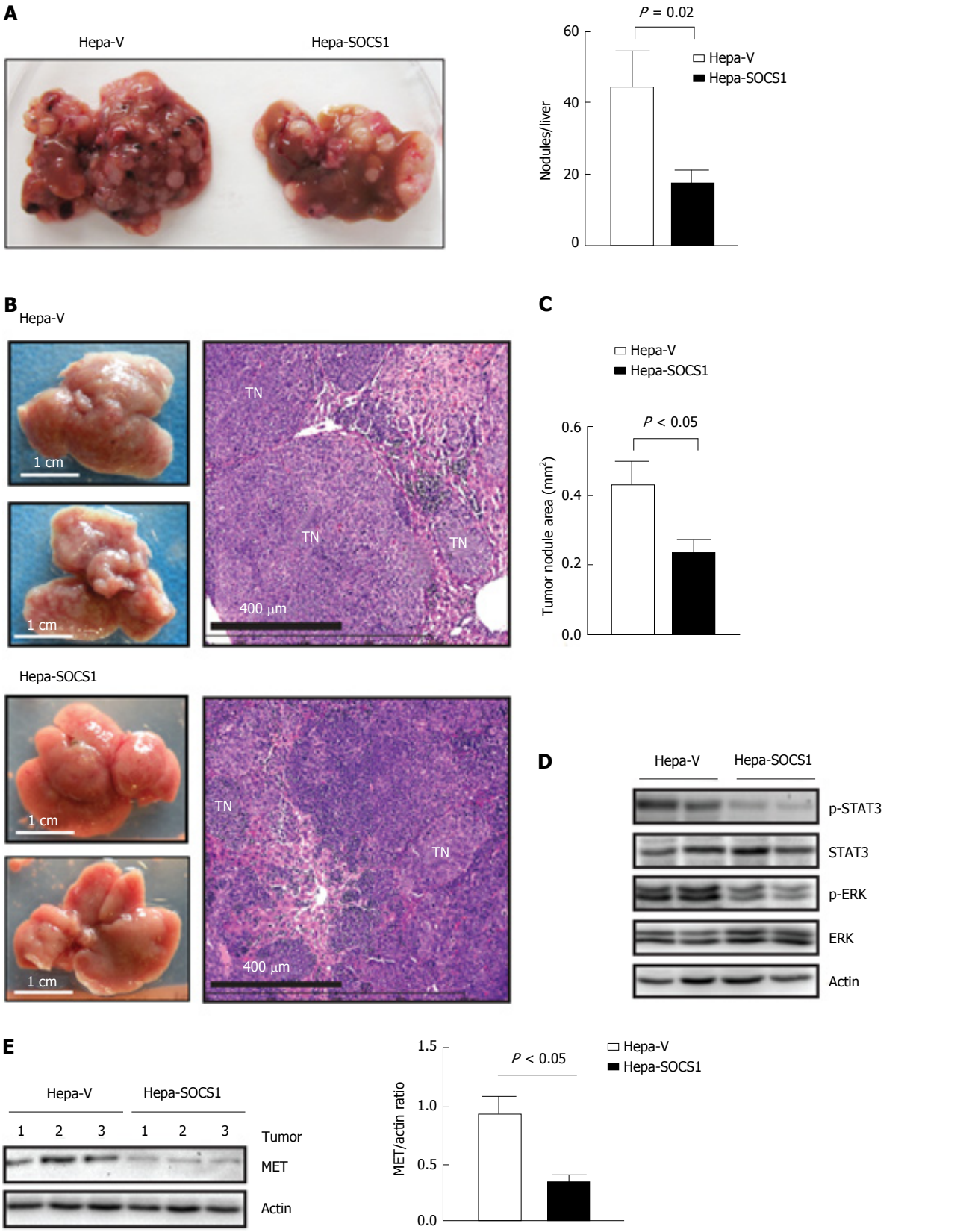

Figure 4 Suppressor of cytokine signaling 1 attenuates invasive growth of orthotopic Hepa cell tumors. A: Hepa-V and Hepa-SOCS1 cells were injected *via* intravenous route into NOD.*scid.gamma* (NSG) mice and the livers were examined macroscopically after 20 d. Representative images (left) and quantification of the liver tumor nodules (right) from four mice per group are shown; B-D: Hepa-vector and Hepa-SOCS1 were injected *via* intrasplenic route into NSG mice (1×10^6 cells per mouse; $n = 6$ per group from two separate experiments) and the mice were splenectomized. After 20 d, the liver tissues were examined macroscopically for tumor nodules. The dorsal and ventral views of representative livers (B, left) and H and E-stained sections of a representative liver for each group (B, right) are shown; C: For each group, the areas of thirty random tumor nodules from three different mice were measured digitally using the NDP software and compared by Student's paired *t* test; D: Western blot evaluation of STAT3 and ERK phosphorylation in the tumors from 2 mice per group; E: Western blot evaluation of MET expression and its quantification in the tumors from 3 mice per group. SOCS1: Suppressor of cytokine signaling 1.

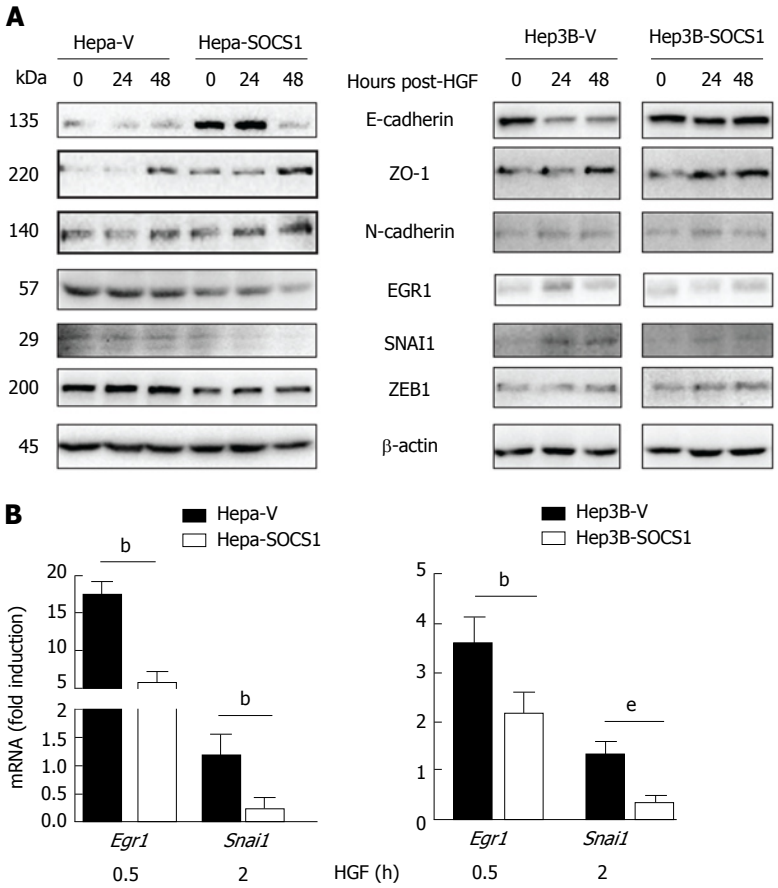

Figure 5 Effect of suppressor of cytokine signaling 1 on the expression of proteins modulated during epithelial-mesenchymal transition in hepatocyte growth factor-stimulated hepatocellular carcinoma cells. A: Hepa-V and Hepa-SOCS1, and Hep3B-V and Hep3B-SOCS1 were cultured in low-serum medium (0.25%) overnight and stimulated or not with m-HGF or h-HGF for 24 or 48 h. Unstimulated and HGF-stimulated cells were lysed in SDS sample buffer and analyzed by western blot for the indicated proteins. Representative data from two experiments are shown; B: Cells were stimulated with HGF for 30 min or 2 h and the expression of *Egr1* and *Snai1* genes was evaluated by qRT-PCR. Fold-induction relative to the housekeeping gene *Gapdh* was evaluated from triplicates of two separate experiments (mean ± SE). $^{b}P < 0.01$, $^{e}P < 0.001$. SOCS1: Suppressor of cytokine signaling 1.

that maintain epithelial cell integrity[12,13]. During EMT, progressive loss of epithelial markers (E-cadherin, occludin, ZO-1) and acquisition of mesenchymal markers (N-cadherin, vimentin) cause tumor cells to disrupt intercellular junctions, become motile, remodel cell-matrix interactions and become invasive. However, these changes may not be absolute and intermediate phenotypes may represent partial EMT[37]. Reduced expression of E-cadherin occurs in 50%-60% of primary HCC that correlates with increased *SNAI1* expression and poor prognosis[38]. Similar changes were reported in human and murine HCC cell lines with high invasive potential[36,38]. Indeed, elevated MET expression has been associated with mesenchymal phenotype characterized by reduced expression of E-Cadherin and increased expression of the *CDH1* gene repressor *ZEB2* in human HCC cell lines MHCC97-L and MHCC97-H that display increased metastatic potential[16]. Similar findings have been reported in murine HCC cell lines[15]. A murine HCC line-derived circulating tumor cells isolated following intrahepatic implantation displayed elevated MET expression with reduced levels of E-Cadherin, implicating the EMT process in HCC dissemination[39]. Our findings that SOCS1 expression reduces HGF-induced invasive growth in HCC cells that is accompanied by increased expression of E-cadherin and discernibly reduced expression of EGR1, SNAI1 and ZEB1, as well as the markedly diminished orthotopic growth of SOCS1-expressing HCC cells associated with reduced MET expression support the idea that SOCS1 is an important regulator of MET-mediated cancer progression *via* EMT. Thus, our findings add SOCS1 to the growing list of endogenous regulators of MET-mediated EMT, namely miR-148a, miR449a, SENP1[17,30,40].

Molecular mechanisms underlying MET-mediated EMT are not yet fully elucidated. In HepG2 human HCC cells, HGF-induced cell scattering requires ERK activation, leading to induction of *EGR1* (Early growth response factor), a transcriptional activator of SNAI1, which represses *CDH1* encoding E-cadherin[28]. The HGF-induced morphological and molecular changes of EMT in HCC cells could be reversed by blocking ERK activation using sorafenib that targets RTKs, or by U0126 that inhibits the ERK upstream kinase MEK1[29]. Given that the MET signaling pathways are implicated in multiple aspects of cancer progression, attenuating MET signaling will also disrupt MET-mediated EMT[37].

Several therapeutics targeting the MET signaling pathway including multikinase inhibitors, selective MET inhibitors, antibodies targeting HGF or MET, agents that neutralize HGF and molecules that disrupt MET signaling[14]. Given the possible emergence of drug resistance to agents that target signaling pathways, and that MET can be activated by alternate ligands such as des-gamma-carboxy prothrombin and heparin[35,41], alternate strategies are needed to effectively target MET-mediated HCC invasion and metastasis. In this context, anti-MET Ab such as DN-30 that promotes MET degradation[42] and strategies to disrupt MET receptor expression[14] could represent promising approaches. However, this would require identification of HCC patients who would benefit from aggressive MET-targeting therapy[14]. Although MET overexpression could be a direct marker, clinical grade MET Ab are still under development[43]. Given that SOCS1 is an important regulator of MET protein expression, and that loss of SOCS1 expression predominantly occur by promoter methylation in HCC, assessing SOCS1 methylation status could be a useful approach to identify HCC patients who would benefit from MET-targeting therapies.

In conclusion, our findings show that SOCS1 attenuates migration and invasion properties of HCC cells at least partly *via* modulation of MET-mediated EMT, and controls invasive tumor growth. Based on these findings, we propose that the *SOCS1* gene methylation and expression in primary HCC may serve as useful markers to identify patients for targeted MET therapy.

COMMENTS

Background

Invasive intrahepatic dissemination is a key factor in malignant growth of hepatocellular carcinoma (HCC) and its poor prognosis. HCC patients also present extrahepatic metastasis that may occur *via* direct invasion. Cancer cells gain invasive potential through epithelial-mesenchymal transition (EMT) induced by cytokines and growth factors that stimulate receptor tyrosine kinases (RTK). An important RTK implicated in EMT is the HGF receptor MET. Suppressor of cytokine signaling 1 (SOCS1) is an important regulator of MET signaling in hepatocytes.

Research frontiers

Emerging data implicate EMT in cancer growth and metastasis in HCC and many other cancers. Among the strategies to target EMT, the MET signaling pathway holds promise. The present study investigates the role of SOCS1 in regulating MET signaling involved in the invasive tumor growth of HCC.

Innovations and breakthroughs

This study shows that SOCS1 inhibits the invasive growth of HCC cells *in vitro* and *in vivo*, and adds SOCS1 to the growing list of endogenous regulators of MET-mediated EMT.

Applications

SOCS1 expression in HCC biopsies could be a useful biomarker of invasive and metastatic potential to identify patients suitable for treatment with MET-targeting therapies. Development of therapeutics to restore SOCS1 expression in cancer cells could be another approach to attenuate the invasive potential and metastatic growth of HCC.

Terminology

SOCS1 is a negative feedback regulator of cytokine and growth factor signaling. SOCS1 inhibits HGF-induced MET signaling pathways that promote invasive growth, which can be assessed *in vitro* using a 3-dimensional invasion assay and *in vivo* by orthotopic tumor growth.

Peer-review

In this study, the authors investigated the role of SOCS1 in the invasion ability of HCC. Extending the findings of their previous work, the authors show that SOCS1 inhibits the invasive growth of HCC cells by targeting MET signaling.

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ORIGINAL ARTICLE

Basic Study

Protective effects of oral glutathione on fasting-induced intestinal atrophy through oxidative stress

Hiroyuki Uchida, Yukari Nakajima, Kazuo Ohtake, Junta Ito, Masahiko Morita, Ayako Kamimura, Jun Kobayashi

Hiroyuki Uchida, Yukari Nakajima, Kazuo Ohtake, Junta Ito, Jun Kobayashi, Division of Pathophysiology, Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Science, Josai University, Sakado, Saitama 350-0295, Japan

Masahiko Morita, Ayako Kamimura, Kyowa Hakko Bio Co., Ltd. Healthcare Products Development Center, Tsukuba-shi, Ibaraki 305-0841, Japan

Author contributions: Uchida H and Nakajima Y contributed equally to this work; Uchida H, Nakajima Y, Morita M, Kamimura A and Kobayashi J designed the study; Uchida H, Nakajima Y, Ohtake K and Ito J conducted the experiments and analyzed the data; Uchida H, Ito J, Morita M and Kamimura A wrote the paper; Uchida H and Kobayashi J critically revised and reviewed the manuscript for important intellectual content.

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Correspondence to: Dr. Hiroyuki Uchida, Division of Pathophysiology, Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Science, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan. mrhiro@josai.ac.jp
Telephone: +81-49-2717204
Fax: +81-49-2717204

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Abstract

AIM

To determine whether oral glutathione (GSH) administration can alleviate the effects of fasting-induced intestinal atrophy in the small intestinal mucosa.

METHODS

Rats were divided into eight groups. One group was fed *ad libitum*, another was fed *ad libitum* and received oral GSH, and six groups were administrated saline (SA) or GSH orally during fasting. Mucosal height, apoptosis, and cell proliferation in the jejunum were histologically evaluated. iNOS protein expression (by immunohistochemistry), nitrite levels (by high performance liquid chromatography, as a measure of NO production), 8-hydroxydeoxyguanosine formation (by ELISA, indicating ROS levels), glutathione/oxidized glutathione (GSH/GSSG) ratio (by enzymatic colorimetric detection), and γ-glutamyl transpeptidase (*Ggt1*) mRNA levels in the jejunum (by semi-quantitative RT-PCR) were also estimated.

RESULTS
Oral GSH administration was demonstrated to drastically reduce fasting-induced intestinal atrophy in the jejunum. In particular, jejunal mucosal height was enhanced in GSH-treated animals compared to SA-treated animals [527.2 ± 6.9 for 50 mg/kg GSH, 567.6 ± 5.4 for 500 mg/kg GSH *vs* 483.1 ± 4.9 (μm), $P < 0.01$ at 72 h]. This effect was consistent with decreasing changes in GSH-treated animals compared to SA-treated animals for iNOS protein staining [0.337 ± 0.016 for 50 mg/kg GSH, 0.317 ± 0.017 for 500 mg/kg GSH *vs* 0.430 ± 0.023 (area of staining part/area of tissue), $P < 0.01$ at 72 h] and NO [2.99 ± 0.29 for 50 mg/kg GSH, 2.88 ± 0.19 for 500 mg/kg GSH *vs* 5.34 ± 0.35 (nmol/g tissue), $P < 0.01$ at 72 h] and ROS [3.92 ± 0.46 for 50 mg/kg GSH, 4.58 ± 0.29 for 500 mg/kg GSH *vs* 6.42 ± 0.52 (8-OHdG pg/μg DNA), $P < 0.01$, $P < 0.05$ at 72 h, respectively] levels as apoptosis mediators in the jejunum. Furthermore, oral GSH administration attenuated cell proliferation decreases in the fasting jejunum [182.5 ± 1.9 for 500 mg/kg GSH *vs* 155.8 ± 3.4 (5-BrdU positive cells/10 crypts), $P < 0.01$ at 72 h]. Notably, both GSH concentration and Ggt1 mRNA expression in the jejunum were also attenuated in rats following oral administration of GSH during fasting as compared with fasting alone [0.45 ± 0.12 *vs* 0.97 ± 0.06 (nmol/mg tissue), $P < 0.01$; 1.01 ± 0.11 *vs* 2.79 ± 0.39 (Ggt1 mRNA/Gapdh mRNA), $P < 0.01$ for 500 mg/kg GSH at 48 h, respectively].

CONCLUSION
Oral GSH administration during fasting enhances jejunal regenerative potential to minimize intestinal mucosal atrophy by diminishing fasting-mediated ROS generation and enterocyte apoptosis and enhancing cell proliferation.

Key words: Intestinal atrophy; Glutathione; Apoptosis; Cell proliferation; Inducible nitric oxide synthase

Core tip: We have previously demonstrated that the intestinal mucosal atrophy consequent to fasting is due in large part to increased apoptosis in jejunal villi and decreased cell proliferation in jejunal crypts, with concomitant increased reactive oxygen species (ROS) and NO production and decreased glutathione (GSH). Here we demonstrate protection against fasting-induced intestinal mucosal atrophy by minimizing ROS induction in the intestinal mucosa through supplemental oral administration of an antioxidant such as GSH during fasting in rats. In particular, oral GSH administration during fasting enhances jejunal regenerative potential by diminishing enterocyte apoptosis and enhancing cell proliferation.

Uchida H, Nakajima Y, Ohtake K, Ito J, Morita M, Kamimura A, Kobayashi J. Protective effects of oral glutathione on fasting-induced intestinal atrophy through oxidative stress. *World J Gastroenterol* 2017; 23(36): 6650-6664 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6650.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6650

INTRODUCTION

Fasting induces small intestinal mucosal atrophy including increased epithelial permeability and compromised tight junctions, which can lead to bacterial translocation, particularly in patients receiving a prolonged course of total parenteral nutrition (TPN)[1,2]. Many surgeons involved in nutritional support therapy desire the identification of bioactive substances that may be efficacious in protecting against intestinal injury during long-term TPN administration, particularly with respect to intestinal barrier function and subsequent septic complications. Fasting and other states of malnutrition are associated with increased reactive oxygen species (ROS) formation, which has been implicated in the loss of intestinal mucosal structure and function under conditions of inflammation, injury, and shock[3,4]. In addition, these fasting states are also accompanied by depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa[5-7]. Therefore, GSH is the most prevalent and important low-molecular-weight thiol in mammalian tissues[8]. As GSH deficiency in tissues is associated with increased oxidative stress, aging, and increased risk of numerous chronic diseases as well as fasting[9], the maintenance of tissue levels of GSH is critical for maintaining health and preventing disease and age-related biological insults.

GSH sources in the intestinal mucosa include intracellular synthesis, biliary supply, and dietary intake. The intestinal lumen receives a large quantity of hepatic GSH from biliary secretion[10] and dietary GSH from fresh fruits, vegetables, and many types of meat[11]. As the only enzyme of the γ-glutamyl cycle located on the outer surface of the plasma membrane, γ-glutamyl transpeptidase (GGT) plays a key role in GSH homeostasis by breaking down extracellular GSH and providing cysteine, the rate-limiting substrate for intracellular *de novo* GSH synthesis[12]. The two intracellular enzymes, γ-glutamylcysteine synthetase and GSH synthetase, catalyze intracellular GSH synthesis from glutamate, cysteine, and glycine, which are translocated into cells by amino acid transporters on the surface of the plasma membrane following the resolution of extracellular GSH by GGT. Intracellular GSH plays an important role in antioxidant defense and the regulation of pathways essential for *in vivo* homeostasis through its catalysis by glutathione S-transferase (GST) and glutathione peroxidase[9]. Studies of oral GSH supplementation in humans and laboratory animals have shown that the enhancement of intestinal mucosal GSH levels by oral GSH

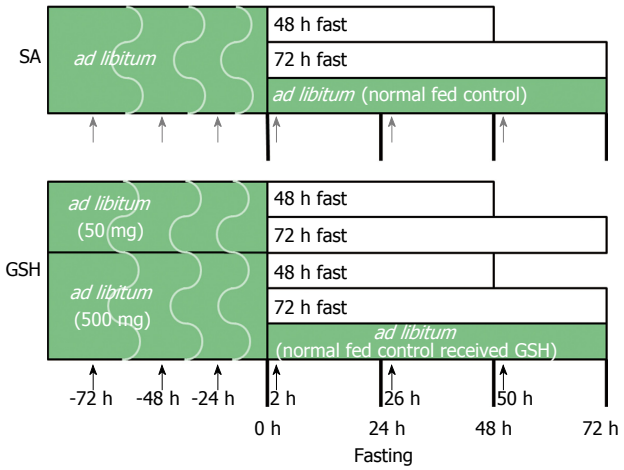

Figure 1 Experimental design. Rats were divided into eight groups, one of which was fed ad libitum, another of which was fed ad libitum and received oral glutathione (GSH), and six of which were administered saline (SA) or GSH orally prior to and after fasting. The large block arrows represent treatment with SA (closed gray arrow) and GSH (closed block arrow).

supplementation under conditions in which intracellular GSH status is compromised (*i.e.*, GSH deficiency-related biological insults) can restore tissue GSH and promote ROS metabolism[10,13,14]. Thus, it has been described that orally administered GSH acts as backup for GSH-deficient tissue.

As an alternative, the ability to control extracellular redox provides another possible role for intestinal luminal GSH. Recent studies have shown that the thiol/disulfide redox status in the extracellular compartment regulates the ROS that are generated primarily in the intracellular compartment and serve an important function in the activation of proteins and up-regulation of antioxidant and detoxification systems[15-17]. Furthermore, many proteins including transporters, receptors, and enzymes present on the surface of the plasma membrane and located in extracellular fluids contain cysteine and methionine residues that are subject to oxidation. Accordingly, these proteins respond to variations in the extracellular thiol/disulfide redox environment, which can alter their activity[18]. Notably, recent demonstrations that treatment with GSH leads to a significant increase in the number of free sulfhydryls on the cell surface have led to the conclusion that extracellular GSH modulates the interaction between bacteria and epithelial respiratory cells and inhibits bacterial invasion into these cells[19].

We previously suggested that fasting for 2-3 d causes intestinal mucosal atrophy resulting from increased apoptosis in jejunal villi, nitric oxide (NO) and ROS production following elevated inducible nitric oxide synthase (iNOS) expression, and decreased cell proliferation in jejunal crypts[20]. Conversely, these apoptosis mediators are all suppressed by treatment with aminoguanidine, a selective iNOS inhibitor, with consequent mucosal recovery, suggesting that iNOS, which induces the production of ROS, is likely to constitute a significant upstream factor promoting fasting-induced apoptosis in enterocytes. Furthermore, refeeding repaired fasting-induced jejunal atrophy by inhibiting these apoptosis mediators, including ROS, and also showed an association between ROS inhibition and increased cell proliferation for decreasing intestinal mucosal atrophy[21]. Therefore, we hypothesized that protection against intestinal mucosal atrophy might be provided through the elimination of induced ROS in the intestinal mucosa by introducing an antioxidant such as GSH during a fasting period in rats.

Therefore, the objective of the present study was to investigate the effects of oral GSH administration on the manifestation of fasting-induced intestinal atrophy through the reduction of oxidative stress, with a particular focus on the possible participation of intracellular GSH levels and GGT expression on the cell surface as parameters for *de novo* intracellular GSH synthesis.

MATERIALS AND METHODS

Animals and experimental design

The experimental protocol and design were approved by the Institutional Animal Care and Use Committee at the Life Science Center of Josai University. Male Wistar rats at 9 wk of age were purchased from SLC (Shizuoka, Japan) and housed in wire-bottomed cages. The rats were placed in a room illuminated from 7:00 am to 7:00 pm (12:12-h light:dark cycle). The animals were allowed free access to deionized water and standard rat chow (CE-2, CLEA Japan) until the study began. At 10 wk of age, 59 rats were randomly divided into eight groups (Figure 1). Each group received saline (SA) or GSH (50 or 500 mg/kg b.w./d) using oral gavage needles at 24, 48, and 72 h before fasting, including a normally fed control group and a normally fed control that received GSH, with the following fasting durations in each group: (1) SA + *ad libitum* (normal fed control); (2) SA + 48-h fast; (3) SA + 72-h fast; (4) 500 mg/kg GSH + *ad libitum* (normal fed control received GSH); (5) 50 mg/kg GSH + 48-h fast; (6) 50 mg/kg GSH + 72-h fast; (7) 500 mg/kg GSH + 48-h fast; and (8) 500 mg/kg GSH + 72-h fast. All rats received SA or GSH by oral gavage for 2, 26, and 50 h after fasting. The GSH (Setria® reduced glutathione) used in this work was provided by Kyowa Hakko Bio Co., Ltd. The rats were weighed daily.

Collection of intestinal mucosa

After fasting, the rats were anesthetized and then euthanized by exsanguination. The entire small intestine was carefully removed and placed on ice. Then, 10 cm of tissue from the oral (duodenum) side was removed and the remainder of the intestine was divided into two segments: proximal (jejunum) and distal (ileum). The segments used in analyses comprised the jejunum from 3 to 5 cm distal to the duodenum[22]. Samples with a

length of approximately 3 cm were fixed in 10% neutral buffered formalin for measurement of mucosal height and immunohistochemistry. The remaining segments were snap-frozen in liquid nitrogen and stored at -80 ℃.

Histopathological analysis of mucosal height and apoptotic index

Fixed tissue samples were embedded in paraffin and sectioned (2-3 μm thickness) prior to being stained with hematoxylin and eosin (H&E). Mucosal height (villous height plus crypt depth) was measured using a microscope (BX41; Olympus, Tokyo, Japan) and a digital camera system (Penguin 150CL; Pixera, San Jose, CA, United States). Mucosal height was measured for at least 30 villi per animal.

To detect enterocyte apoptosis in the jejunum, apoptotic index (AI) using conventional light microscopy of H&E-stained specimens was used. We followed previously published methods[20,23]. In brief, jejunal sections as used above for histopathological analysis were examined in a blinded manner for the typical attributes of apoptotic cells. We assessed 50 villus-crypt columns per rat. For each column, the number and position of apoptotic cells and the total number of cells were recorded. To account for the effects of fasting and GSH treatment on apoptosis, the average number of apoptotic cells in villi and crypts was determined along with AI. To identify the locations of apoptosis along the villi and crypts, AI distribution curves were constructed on the basis of group means, in which cell position *vs* AI was plotted at each position. Here, AI was defined as the total number of apoptotic cells at each cell position and is expressed as the percentage of the total number of cells counted at that cell position. Cell position 1 was set as the cell at the crypt-villus junction and the cell at the base of the crypt column for villus and crypt data, respectively.

Immunohistochemical assessment of iNOS expression and 5-bromo-2'-deoxyuridine (5-BrdU)-positive cells

Immunohistochemical staining was performed using a rabbit anti-iNOS polyclonal antibody[24]. The specimens were dewaxed and treated for antigen retrieval by boiling in 10 mmol/L citrate buffer (pH 6.0)[25]. After being washed with PBS, the specimens were incubated in 6% hydrogen peroxide and nonspecific binding was blocked with a 20% goat serum solution in PBS. Specimens were subsequently incubated with an anti-iNOS primary antibody (1:100; BD Transduction Laboratories, Lexington, KY, United States), except for the control sections, for which no primary antibody was used. A biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, United States) was used as a secondary antibody. The sections were then treated using the VECTASTAIN Elite ABC Kit (Vector Laboratories), and reaction products were detected using color development with diaminobenzidine. Finally, the sections were counterstained with hematoxylin and examined under a light microscope and a digital camera system. For each rat, 5 clearly dyed sections were chosen randomly and 5 random fields for each section were assessed (at 40 × magnification). The content of iNOS was quantitatively measured based on the average optical density using a digital camera and ImageJ software (National Institutes of Health, Bethesda, Maryland, United States)[26].

To assess cell proliferation in the crypt, the cell proliferation index was determined using conventional light microscopy of specimens immunohistochemically stained for 5-BrdU[27]. Rats were intraperitoneally injected with 100 mg/kg 5-BrdU prior to euthanasia. After paraffin embedding and sectioning, tissue sections were dewaxed and immersed in 3% hydrogen peroxide-methanol solution. The specimens were washed with PBS and denatured in 2 N hydrochloric acid. Following further PBS washing, the specimens were immersed in 0.1 mol/L boric acid buffer (pH 8.5), incubated with 20 μg/mL proteinase K at 37 ℃, and then the reaction was terminated with PBS containing bovine serum albumin. The sections were incubated with a mouse anti-5-BrdU monoclonal antibody (1:50; Chemicon International, Billerica, MA, United States), with the exception of control samples, for which the primary antibody was omitted. A biotinylated goat anti-mouse IgG (1:200; Vector Laboratories) was used as a secondary antibody. The sections were then treated using the VECTASTAIN Elite ABC Kit (Vector Laboratories) and antibody binding was detected by color development after addition of diaminobenzidine. Finally, the sections were counterstained with hematoxylin and examined under a light microscope with a digital camera system. The number of labeled cells in at least 10 well-oriented longitudinal crypts was determined for each rat. Results are expressed as the number of 5-BrdU-labeled cells per crypt.

Jejunal nitrite concentrations

Nitrite concentrations in the jejunum were measured using a dedicated high-performance liquid chromatography (HPLC) system (ENO-20; EiCom, Kyoto, Japan)[28]. Jejunal segments were homogenized with an equal volume of methanol and centrifuged for deproteinization at 12000 *g* at 4 ℃ for 5 min. The samples were then applied to the HPLC system. The nitrites and nitrates were separated using a reverse-phase column (NO-PAK; EiCom), after which nitrate was reduced to nitrite in a reduction column packed with copperized cadmium (NO-RED; EiCom). These nitrites were then mixed with the Griess reagent in a reaction coil and the change in absorbance was monitored at 540 nm.

DNA oxidation analysis

Oxidative stress in the jejunum was evaluated by quantifying 8-hydroxydeoxyguanosine (8-OHdG) present in DNA[29]. 8-OHdG is a product of oxidative

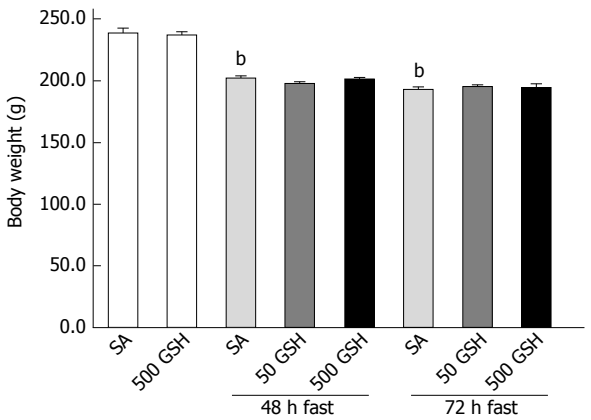

Figure 2 Effects of fasting and glutathione treatment on body weight. Fasting caused gradual decreases in body weight in both SA- and GSH-treated groups. There was no difference in weight loss between the GSH treatments and the respective SA-treated groups in each fasting period. Values represent the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine base and is thought to constitute a marker of oxidative DNA damage, reflecting the DNA repair rate[30]. Jejunal DNA was purified using the DNA Extractor TIS Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan)[31]. DNA samples were hydrolyzed to nucleosides by sequential incubation with 6 U of nuclease P1 (Wako) followed by 2 U of alkaline phosphatase (Wako). Hydrolysates were filtered through a VIVASPIN 500 MWCO 10000 filter (Sartorius Stedim Biotech, Gottingen, Germany). The levels of 8-OHdG in the filtered samples were determined using an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan).

Jejunal GSH and GSSG concentrations

GSH and GSSG concentrations were measured by using a GSSG/GSH Quantification Kit (Dojindo Molecular Technologies, Inc., Rockville, MD, United States). Jejunal segments were homogenized with 5% sodium sulfosalicylate (Wako) and centrifuged to remove proteins. For each sample, the supernatant was added to a well to which coenzyme working solution and enzyme working solution had been previously added, and incubated at room temperature. Then, the substrate working solution was added to the well and it was incubated at room temperature. The absorbance of samples and the GSH standard was measured at 405 nm using a microplate reader (TECAN GENios, Grodig, Austria). For measurement of GSSG, the supernatant was treated with masking solution and then added to a well as described above. The absorbance of samples and standard GSSG was measured at 405 nm.

Analysis of Ggt1 mRNA expression by semi-quantitative reverse transcription-PCR

Total RNA was purified using the TaKaRa RNAiso Reagent (TaKaRa Bio, Kusatsu, Japan). Reverse transcription (RT)-PCR was performed with total RNA using the RNA PCR kit (AMV) Version 3.0 (TaKaRa Bio): 1 cycle at 42 ℃ for 30 min, 99 ℃ for 5 min, and 5 ℃ for 5 min for reverse transcription, and 30 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min for PCR. The following primer pairs (synthesized by TaKaRa Bio) were used: *Ggt1* forward primer, 5′-ACCACTCACCCAACCGCCTAC-3′; *Ggt1* reverse, 5′-ATCCGAACCTTGCCGTCCTT-3′ (product size: 317 bp)[12]. Expression of target mRNAs was measured relative to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), which was determined using the following primers: *Gapdh* forward, 5′-GGCACAGTCAAGGCTGAGAATG-3′; *Gapdh* reverse, 5′-ATGGTGGTGAAGACGCCAGTA-3′ (143 bp, TaKaRa Bio ID: RA015380). A portion of each PCR mixture was electrophoresed on a 2% agarose gel in Tris-borate-EDTA buffer and DNA bands were visualized using ethidium bromide staining. PCR product intensity was measured using the Gene Genius Bioimaging System (Syngene, Cambridge, United Kingdom).

Statistical analysis

Statistical analyses were performed using SPSS ver. 22 for Windows. All values are expressed as the mean ± SE. One-way analysis of variance (ANOVA) followed by a Bonferroni multiple-comparison test was used for analyzing the statistical difference between the fasting periods in SA- or GSH-treated groups. Two-way ANOVA followed by a Bonferroni multiple-comparison test was used for analyzing the statistical difference between the SA- and GSH-treated groups for the fasting periods. Statistical significance was accepted at $P < 0.05$. The statistical analysis of this study was performed by Uchida H, who acquired biostatistics expertise during his training in public health.

RESULTS

Body weight changes

Figure 2 shows body weight changes in SA-treated and GSH-treated rats. Decreases in body weight in both groups were observed along with fasting. Rats fasted for 48 and 72 h with SA treatment showed approximately 15% ($P < 0.01$) and 19% ($P < 0.01$) body weight loss, respectively, compared with normally fed controls. There was no difference in weight loss between the GSH treatments and the respective SA-treated groups at each fasting period.

Histological characterization of jejunal mucosal atrophy

Jejunal mucosal atrophy was assessed by jejunal mucosal height. Decreased jejunal mucosal height in both SA- and GSH-treated rats was observed along with fasting (Figure 3). Rats fasted for 48 and 72 h with SA treatment showed a significant decrease ($P < 0.01$) in mucosal height compared with normally fed controls. However, significant differences ($P < 0.01$) in jejunal mucosal height were found between the SA-

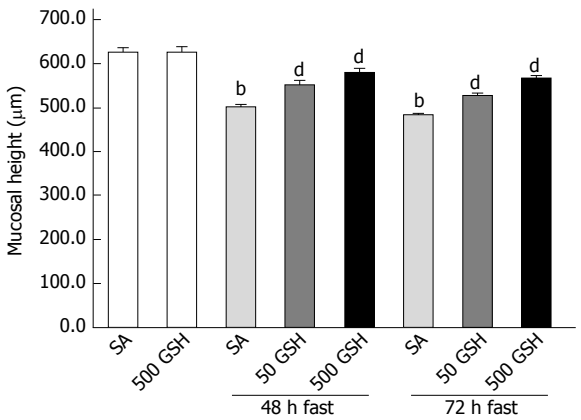

Figure 3 Effects of fasting and glutathione treatment on jejunal mucosal atrophy. Jejunal mucosal atrophy was assessed by jejunal mucosal height. Values represent the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. $^{d}P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

and GSH-treated rats with 48 and 72 h of fasting.

iNOS protein expression in the jejunum

As shown in Figure 4A, iNOS protein staining was localized almost exclusively in the mucosal epithelial monolayer in the 48- and 72-h fasting periods compared with the normally fed controls, and a decreased level of staining occurred with GSH treatment. Quantitative measurement using the average optical density revealed that this expression significantly increased following fasting for 48 and 72 h with SA treatment ($P < 0.01$), whereas GSH treatments significantly decreased the fasting-induced enhancement of jejunal iNOS protein expression ($P < 0.05$ *vs* SA at 48 h fasting, $P < 0.01$ *vs* SA at 72 h fasting; Figure 4B).

Nitrite level in the jejunum

We next measured the levels of nitrite, which is a stable oxidation product of endogenous NO that is induced by iNOS. In SA-treated groups, fasting significantly increased the jejunal nitrite concentration with 48 and 72 h of fasting compared with the normally fed controls ($P < 0.01$; Figure 5). Conversely, GSH treatments significantly decreased the fasting-induced enhancement of the jejunal nitrite concentration compared with the respective SA-treated group for each fasting period ($P < 0.01$).

8-OHdG level in the jejunum

As we previously determined that fasting causes jejunal apoptosis *via* ROS production and induction of NO following increased iNOS expression, in the present study we measured levels of iNOS expression, nitrite (indicating NO production), and 8-OHdG (as a marker of ROS presence). Consistent with the abovementioned changes in iNOS expression, fasting increased intestinal nitrite levels, whereas the degree of increase was significantly reduced by GSH treatment. Furthermore, the elevated jejunal 8-OHdG levels observed after fasting were significantly diminished by both 48 h and 72 h of GSH treatment ($P < 0.01$ for 50 mg/kg GSH *vs* SA with 72 h of fasting, $P < 0.05$ for 500 mg/kg GSH *vs* SA with 72 h of fasting; Figure 6).

Evaluation of enterocyte apoptosis

Apoptosis was determined by histomorphometry, which is preferable to terminal deoxynucleotidyl transferase dUTP nick-end labeling for quantitative assessment. Using histomorphometric assessment of jejunal cells, we evaluated the contribution of reduced apoptosis to the recovery from fasting-induced mucosal atrophy mediated by oral GSH treatment. The representative apoptosis changes as visualized by conventional light microscopy of H&E-stained specimens are shown in Figure 7A. AI distribution profiles in the villi and the crypt are also shown in Figure 7B. From the AI distribution profiles, increased apoptosis in the lower half of the villus (cell positions 1 to 40) was evident with 48 and 72 h of fasting in the SA-treated groups, and this increase was diminished by GSH treatment. In the SA-treated groups, fasting significantly increased jejunal villus AI with 48 and 72 h of fasting compared with that in the normally fed controls ($P < 0.01$; Table 1). GSH treatment significantly decreased the fasting-induced enhancement of villus AI compared with that in the respective SA-treated group for each fasting period ($P < 0.05$ for 50 mg/kg GSH *vs* SA with 48 or 72 h of fasting, $P < 0.01$ for 500 mg/kg GSH *vs* SA with 48 or 72 h of fasting). From the AI distribution profiles, increased apoptosis in the lower two-thirds of crypts (cell positions 1 to 20) was evident with 48 and 72 h of fasting in the SA-treated groups (Figure 7B), and this effect was diminished by GSH treatment. In the SA-treated groups, fasting significantly increased jejunal crypt AIs with 48 and 72 h of fasting compared with the normally fed controls ($P < 0.01$; Table 1). GSH treatments significantly ameliorated the fasting-induced increase in crypt AI compared with the respective SA-treated group for each fasting period ($P < 0.01$).

Evaluation of cell proliferation

GSH is known to exhibit an important function related to the regulation of cell proliferation by protecting against the damaging effects of ROS[32]. To evaluate the effect of oral GSH administration on the reduced cell proliferation in fasting-induced jejunal mucosal atrophy, we assessed 5-BrdU incorporation, a proliferation indicator, in the jejunum. Although cell proliferation decreased with 48 and 72 h of fasting compared with that in the normally fed controls ($P < 0.01$; Figure 8), animals treated with GSH exhibited significantly higher levels of jejunal cell proliferation compared with those in the respective SA-treated group for each fasting period ($P < 0.01$ for 50 mg/kg GSH *vs* SA at 48 h fasting, and for 500 mg/kg GSH *vs*

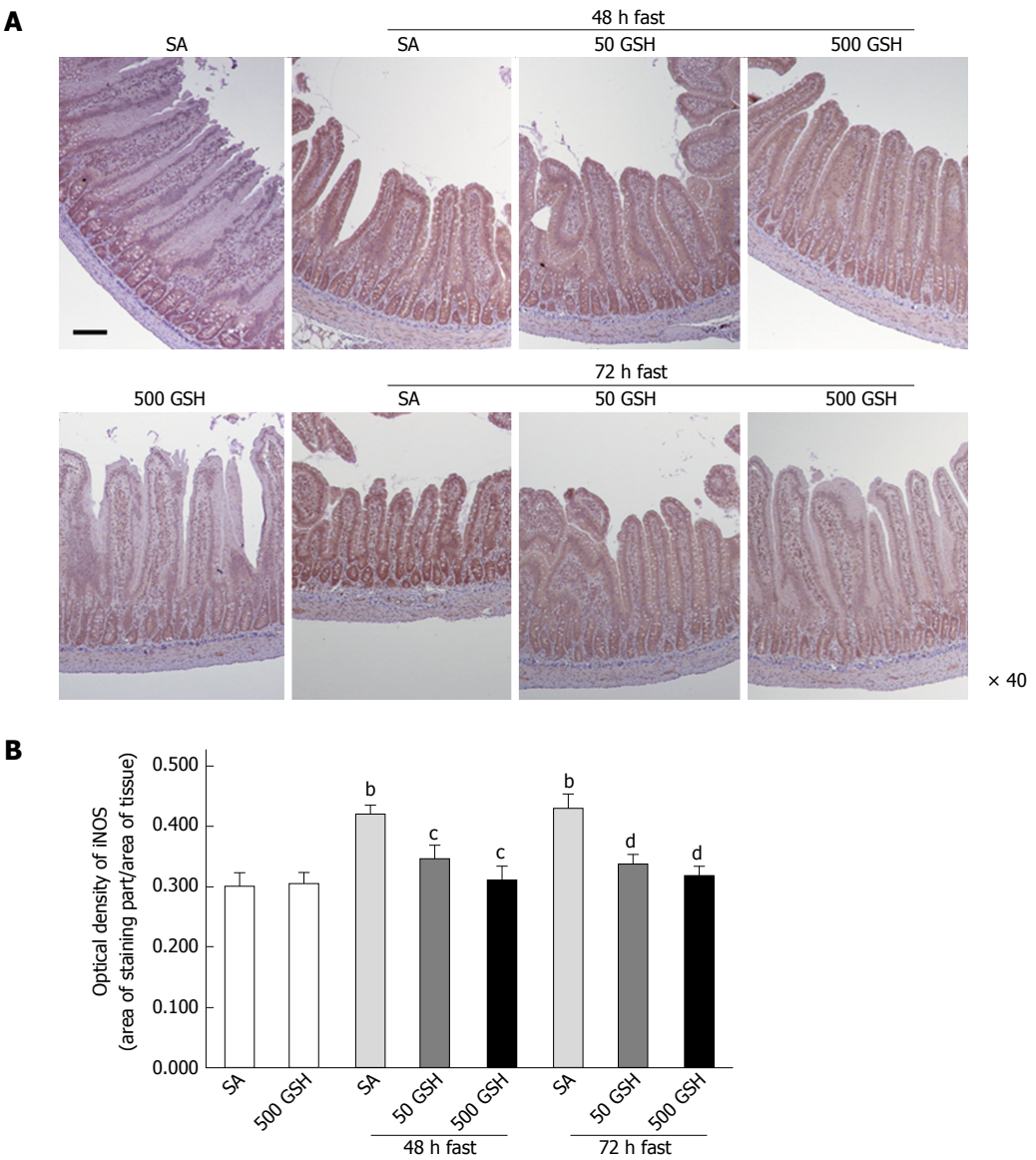

Figure 4 Effects of fasting and glutathione treatment on inducible nitric oxide synthase protein expression in the jejunum. A: Light micrographs of immunohistochemical staining for iNOS. In SA-treated groups, iNOS protein staining was localized almost exclusively in the mucosal epithelial monolayer with 48 and 72 h of fasting compared with the normally fed control. Decreased staining occurred with GSH treatment. Bar = 100 μm. B: Optical density of iNOS protein. The content of iNOS protein was quantitatively measured by averaging the optical density. Values represent the mean ± SE. $^{b}P < 0.01$ *vs* normally fed controls. $^{d}P < 0.01$, $^{c}P < 0.05$ *vs* the respective SA group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline; iNOS: Inducible nitric oxide synthase.

SA at 48 and 72 h fasting).

GSH and GSSG levels in the jejunum

GSH is one of the most important scavengers of ROS; additionally, GSH and oxidized GSH (*i.e.*, GSSG) are used as markers of oxidative stress[33]. To evaluate GSH redox balance and GSH levels in the jejunum consequent to oral GSH administration during fasting, we assessed GSH and GSSG levels in the jejunum. Fasting significantly decreased the GSH concentration in the jejunum ($P < 0.01$; Figure 9A). GSH treatment significantly further decreased the fasting-induced decreases in the jejunal GSH concentration for each fasting period ($P < 0.01$). Conversely, GSSG concentration in the jejunum was similar to that of normally fed controls except for the 500 mg/kg GSH treatment group with *ad libitum* feeding (Figure 9B).

Ggt1 mRNA expression in the jejunum

Fasting significantly increased *Ggt1* expression in the jejunum at 48 h of fasting ($P < 0.05$) (Figure 10) whereas GSH treatment significantly reduced *Ggt1* elevation at 48 h of fasting ($P < 0.05$, $P < 0.01$ for 50 mg/kg GSH, 500 mg/kg GSH *vs* SA at 48 h of fasting, respectively). A similar tendency was obtained for the 72-h fasting group, although this difference did not reach statistical significance.

DISCUSSION

Previous reports have suggested the involvement of GSH in fasting-induced intestinal mucosal atrophy; in

Table 1 Enterocyte apoptosis of the jejunal villus and crypt evaluated by conventional light microscopy of hematoxylin and eosin-stained specimens

	SA	500 GSH	48 h fast			72 h fast		
			SA	50 GSH	500 GSH	SA	50 GSH	500 GSH
Villus								
Cells per villus column, *n*	70 ± 1	69 ± 1	56 ± 2[b]	64 ± 1[d]	67 ± 2[d]	56 ± 2[b]	63 ± 1[d]	65 ± 2[d]
Apoptotic cells per villus column, *n*	0.06 ± 0.01	0.09 ± 0.01	0.20 ± 0.03[b]	0.12 ± 0.01[d]	0.11 ± 0.01[d]	0.23 ± 0.03[b]	0.18 ± 0.01	0.14 ± 0.02[c]
Apoptotic index, %	0.09 ± 0.01	0.12 ± 0.01	0.38 ± 0.06[b]	0.19 ± 0.02[c]	0.18 ± 0.02[d]	0.42 ± 0.06[b]	0.29 ± 0.02[c]	0.21 ± 0.02[d]
Crypt								
Cells per villus column, *n*	28 ± 0.2	27 ± 0.4	22 ± 0.4[b]	25 ± 0.3[d]	27 ± 0.4[d]	22 ± 0.3[b]	23 ± 0.4[c]	26 ± 0.3[d]
Apoptotic cells per villus column, *n*	0.06 ± 0.01	0.06 ± 0.01	0.18 ± 0.01[b]	0.08 ± 0.01[d]	0.09 ± 0.01[d]	0.17 ± 0.02[b]	0.10 ± 0.01[d]	0.11 ± 0.01[d]
Apoptotic index, %	0.21 ± 0.04	0.22 ± 0.02	0.81 ± 0.05[b]	0.32 ± 0.03[d]	0.33 ± 0.03[d]	0.78 ± 0.10[b]	0.41 ± 0.06[d]	0.40 ± 0.05[d]

Apoptosis was measured as in Figure 7A. 7-8 rats were tested in each group. Values represent the mean ± SE. [b]$P < 0.01$ *vs* the normally fed control; [d]$P < 0.01$ or [c]$P < 0.05$ *vs* the SA-treated group in each fasting period. GSH: Glutathione; SA: Saline.

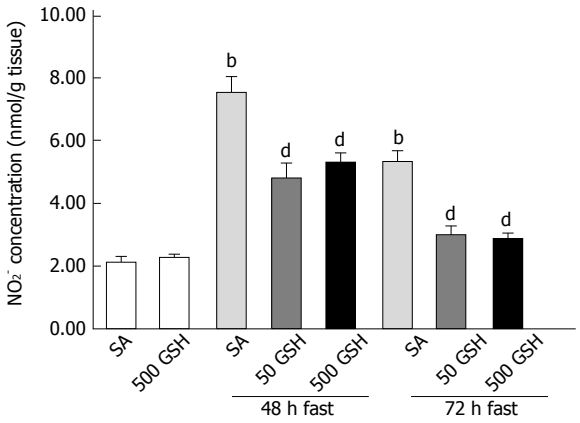

Figure 5 Effects of fasting and glutathione treatment on nitrite concentration in the jejunum. Nitrite concentrations were measured by HPLC using postcolumn derivatization with Griess reagent. Values represent the mean ± SE. [b]$P < 0.01$ *vs* the SA-treated group. [d]$P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

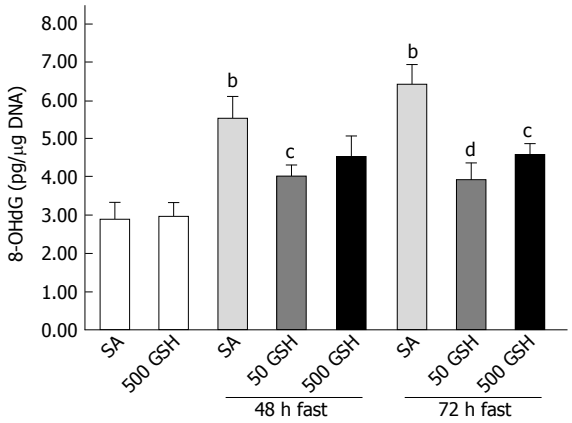

Figure 6 Effects of fasting and glutathione treatment on DNA oxidative damage in the jejunum. DNA oxidative damage was assessed by the level of 8-hydroxydeoxyguanosine (8-OHdG) in the jejunum. Values represent the mean ± SE. [b]$P < 0.01$ *vs* the normally fed control. [d]$P < 0.01$, [c]$P < 0.05$ *vs* the SA-treated group with 48 and 72 h of fasting. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

addition, fasting has been associated with increased ROS formation and depletion of the critical antioxidant GSH in the intestine[4,6,34-36]. However, the roles of GSH in intestinal mucosal recovery from this condition as mediated by oral GSH administration have not yet been described. The objective of the present study was to investigate the effects of oral GSH administration on the development of fasting-induced intestinal atrophy, with a particular focus on the possible participation of intracellular GSH level and GGT expression on the cell surface as potential parameters of intracellular *de novo* GSH synthesis.

During fasting, the intestinal lumen receives very little or no GSH from two of the three primary sources, *i.e.*, biliary secretion[10,37] and dietary intake[11]. Therefore, the overall GSH levels of the intestinal mucosa decrease during fasting because the remaining GSH source, intracellular GSH, is primarily synthesized from GSH in the intestinal lumen.

There is growing evidence that dysfunctional GSH homeostasis is involved in the etiology of several diseases. The previously reported conditions associated with GSH depletion include liver disease[38], immune disorders[39], neurodegenerative disease[40], cardiovascular disease[41], pulmonary disease[42], arthritis, diabetes[43], and the aging process itself[44]. Thus, oral supplementation with GSH to increase the depressed GSH level has been studied extensively as a potential means to prevent these diseases by countering the negative effects of oxidative stress, which is one of their underlying causes. Several studies have shown that GSH supplementation in laboratory animals is effective, with benefits including enhancement of immune function[45] and protection against carcinogenesis[46]. In addition, daily consumption of GSH supplements in humans was effective at increasing body compartment stores of GSH, which may be of importance as the maintenance of tissue levels of GSH might be critical for maintaining health and preventing disease as well as age-related biological insults[47]. Thus, it has been

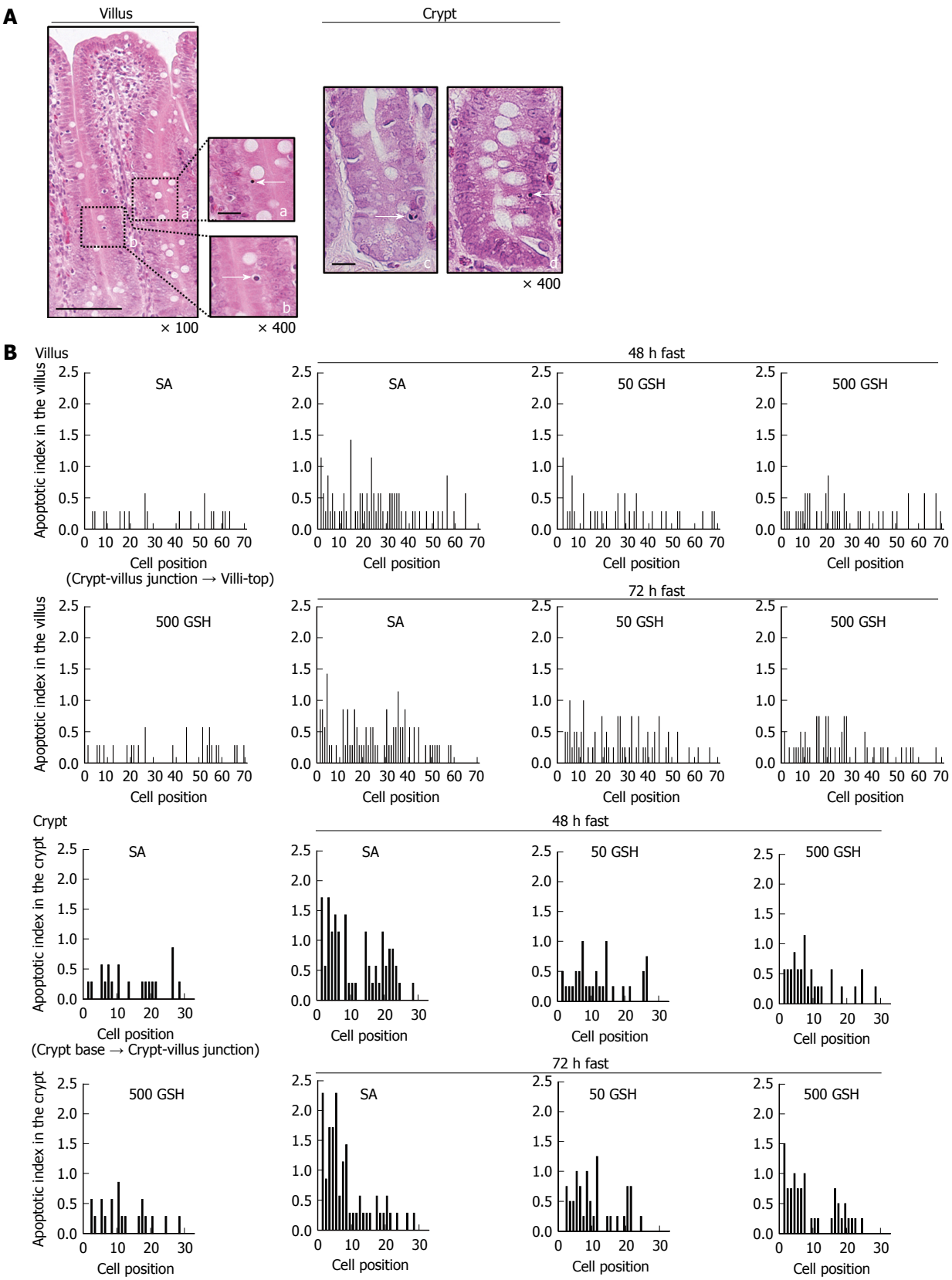

Figure 7 Effects of fasting and glutathione treatment on apoptotic index in the jejunal villus and crypt. Representative apoptotic changes determined by conventional light microscopy of hematoxylin and eosin (HE)-stained sections of the jejunal mucosa are shown in A. Left side: A jejunal villus from a 72-h fasted rat with 500 mg/kg GSH treatment. Apoptotic cells in the villus are indicated by an arrow showing an apoptotic corpuscle (a) and condensed chromatin (b). Bar = 100 μm (low magnification). Bar = 20 μm (high magnification). Right side: Jejunal crypts from 48- and 72-h fasted rats. Apoptotic cells in the crypt are indicated by an arrow showing an intensely eosinophilic cytoplasm and nuclear fragmentation (c) and condensed chromatin (d). Bar = 20 μm. AI distribution curves in the villus and the crypt are shown in B. AI is defined as the total number of apoptotic cells at each cell position and is expressed as a percentage of the total number of cells counted at that cell position. Cell position 1 is defined as the cell at the crypt-villus junction and the cell at the base of the crypt column for the villus and crypt data, respectively. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

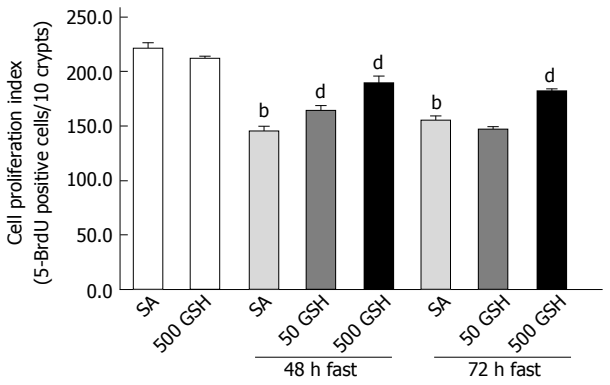

Figure 8 Effects of fasting and glutathione treatment on cell proliferation in the jejunum. Cell proliferation in the jejunal crypt was histologically assessed by 5-bromo-2'-deoxyuridine (5-BrdU) incorporation. The fraction of 5-BrdU-positive cells was expressed as the cell proliferation index (5-BrdU-positive cells/10 crypts). Values represent the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. $^{d}P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

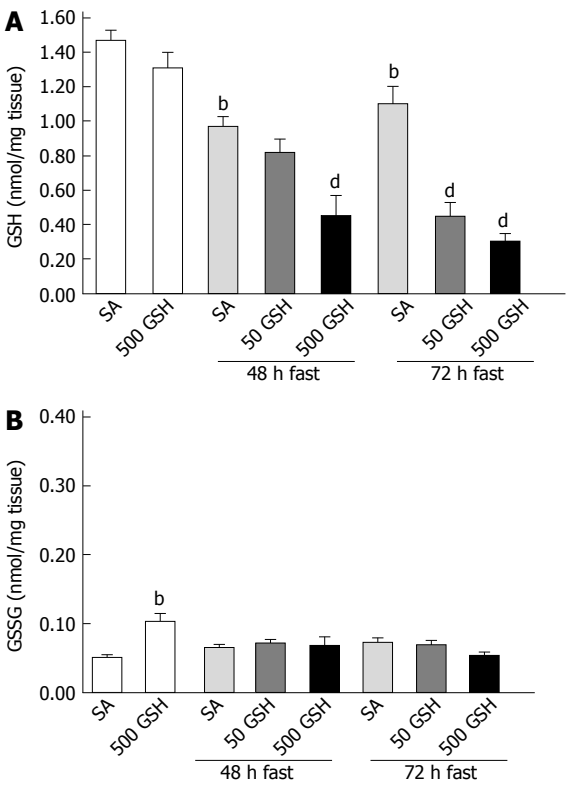

Figure 9 Effects of fasting and glutathione treatment on glutathione and oxidized glutathione concentration in the jejunum. GSH and GSSG concentration in the jejunum was determined by measuring the absorption derived from a colorimetric reaction with DTNB [5, 5'-dithiobis (2-nitrobenzoic acid)] coupled with the enzymatic recycling system. A: GSH concentration in the jejunum; B: GSSG concentration in the jejunum. Values are the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. $^{d}P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline; GSSG: Oxidized glutathione.

suggested that orally administered GSH may act as a backup for intracellular GSH for GSH-deficient tissue. Therefore, the enhancement of intestinal mucosal GSH levels by oral GSH supplementation under conditions of intracellular GSH deficiency-related biological insults such as fasting may restore tissue GSH and promote ROS metabolism, thus facilitating the recovery from intestinal mucosal atrophy.

Our previous studies suggested that fasting-induced intestinal mucosal atrophy could be alleviated by directly inhibiting the fasting-mediated NO production by iNOS and subsequent ROS enhancement associated with increased jejunal apoptosis *via*, *e.g.*, iNOS inhibitor treatment[20] or refeeding[21]. Notably, an association was also observed between inhibition of ROS and increasing cell proliferation[21]. The intestinal epithelial layer is uniquely organized for rapid self-renewal, which is achieved by the well-regulated processes of crypt-to-villus apoptosis and crypt stem cell proliferation. The intestinal epithelium sits at the interface between the intestinal mucosa and the intestinal lumen, and as such is prone to oxidative damage induced by luminal and intracellular oxidants. A previous study[16] reviewed the GSH/GSSG redox mechanism, which modulates intestinal cell transition through cell proliferation, differentiation, or apoptosis and can govern the regenerative potential of the intestinal mucosa. Therefore, we hypothesized that protection against intestinal mucosal atrophy could be provided by controlling apoptosis and cell proliferation through the elimination of induced ROS in the intestinal mucosa by orally administering an antioxidant such as GSH during fasting in rats.

Here, we found that there was no difference in weight loss between fasting alone and oral GSH treatment during fasting (Figure 2) with the GSH levels used for this study. Conversely, jejunal mucosal atrophy was significantly increased by fasting but was remedied by oral GSH treatment during fasting (Figure 3), with significantly greater improvement resulting from the high *vs* low GSH dose. Furthermore, because the etiology of fasting-induced intestinal atrophy was shown to comprise ROS genesis from the NO produced by iNOS[20,21], we measured iNOS protein expression in the jejunum. We identified an inverse relationship between intestinal mucosal atrophy and iNOS protein expression (primarily in the mucosal epithelial monolayer) associated with oral GSH treatment during fasting, most particularly with high levels of GSH administration (Figure 4). This is supported by our previous observations that iNOS inhibitor treatment during fasting and refeeding after fasting decreases fasting-induced atrophy and suppresses iNOS expression in the jejunum.

Oral GSH treatment during fasting inhibited the fasting-mediated generation of the apoptosis mediators NO and ROS concomitant with decreased iNOS expression (Figures 5 and 6). To determine the importance of apoptotic changes in the jejunum for intestinal recovery by oral GSH treatment (Figure 7A), we performed a histomorphometric assessment including AI score (Table 1) and distribution profiles

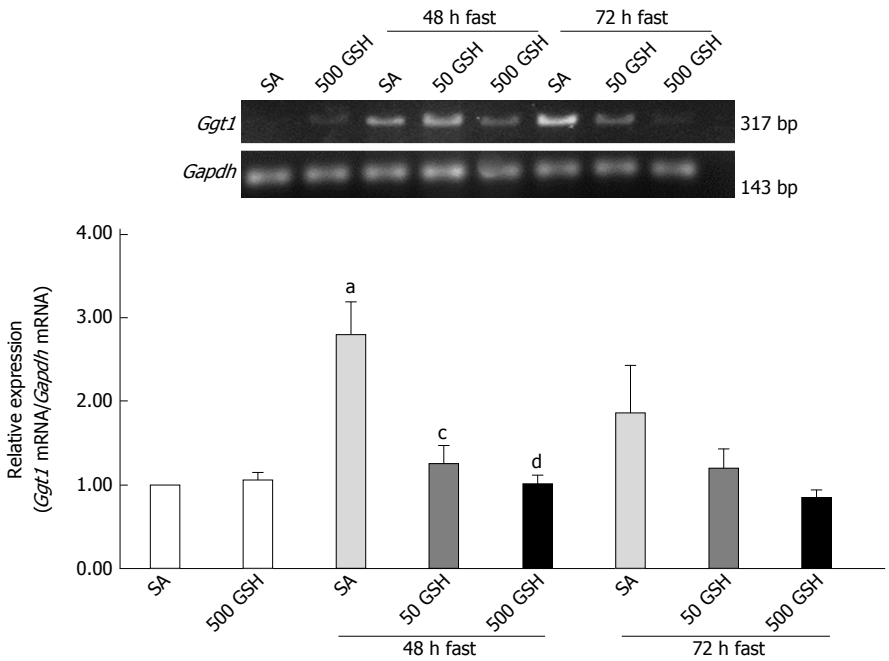

Figure 10 Effects of fasting and glutathione treatment on *Ggt1* mRNA expression in the jejunum. GGT is the only enzyme of the gamma-glutamyl cycle located on the outer surface of plasma membrane and plays key roles in GSH homeostasis by breaking down extracellular GSH. Values are the mean ± SE. $^{a}P < 0.05$ *vs* the normally fed control. $^{d}P < 0.01$, $^{c}P < 0.05$ *vs* the SA-treated group with 48 h of fasting. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline; GSSG: Oxidized glutathione.

(Figure 7B). The increased AI recorded in the villi and crypts after fasting decreased with oral GSH treatment (Table 1). Specifically, the lower half of the apoptotic enterocytes distributed across the entire jejunal villi and the apoptotic cells primarily located in the lower two-thirds of the crypt (Figure 7B) were particularly affected by oral GSH treatment during fasting. These results were broadly consistent with changes in iNOS, NO, and ROS as apoptosis mediators, suggesting that oral GSH administration during fasting may inhibit enterocyte apoptosis by diminishing the ROS in the jejunum generated by fasting.

GSH treatment also was found to alleviate the decreased jejunal cell proliferation mediated by fasting (Figure 8), with significantly greater improvement in fasting-induced jejunal mucosal atrophy resulting from high *vs* low dose GSH. These observations are consistent with the role of GSH in cell proliferation through protection against damage from ROS[16,32]. Together, these findings indicate that oral GSH administration leads to less fasting-mediated enhancement and therefore decreased overall ROS levels in the jejunal mucosa, thereby functioning to increase cell proliferation. This suggests that oral GSH administration may provide regenerative potential to the mucosa during fasting.

Notably, although our findings replicated prior results[6,34-36] that fasting significantly decreased jejunal GSH concentrations (Figure 9A), we found that the concentrations were further significantly decreased by oral GSH supplementation during fasting, with a greater effect from high *vs* low GSH dose. Conversely, the jejunal concentrations of the GSH oxidation product GSSG were not changed by any feeding regime (Figure 9B). Furthermore, oral GSH treatment during fasting significantly decreased the fasting-induced ROS levels in the jejunum (Figure 6), but we consider it unlikely that this effect depends on antioxidant activity given the depressed GSH concentration in the jejunum. These results demonstrate that oral GSH administration during fasting did not contribute to the supply of GSH in the jejunum, and an increase in GSH oxidation was not observed, suggesting that oral GSH administration during fasting does not provide a backup for the jejunum-depleted GSH.

The synthesis of intracellular *de novo* GSH, which is critical for ROS removal and maintenance of GSH homeostasis, relies upon the enzyme activity of the plasma membrane protein GGT to break down extracellular GSH[12]. In the present study, we demonstrated that oral GSH treatment significantly ameliorated the increased jejunal *Ggt1* mRNA expression observed following 48 h of fasting (Figure 10). In comparison, Jonas *et al*[4] indicated that fasting for 72 h did not change *Ggt1* mRNA levels in the ileum, although this was determined *via* ribonuclease protection assay rather than by semi-quantitative RT-PCR as used in the present study. The different findings may also be a consequence of the disparity in fasting duration. Notably, Ogasawara *et al*[34] showed that starvation causes a striking decrease of GGT activity in the jejunum, which is consistent with our finding of fasting-induced increases in *Ggt1* mRNA levels, as the mRNA expression of GGT might be upregulated by the

decline of GGT activity[12]. Furthermore, Zhang *et al*[12] described that *Ggt1* mRNA expression is increased during oxidative stress, which was considered to constitute an adaptation to such adverse conditions. Similarly, we found that fasting increased jejunal ROS levels and up-regulated *Ggt1* mRNA expression in the jejunum in this study. However, considering that oral GSH treatment during fasting significantly decreased both jejunal GSH concentration and *Ggt1* mRNA expression, the supplemented GSH in the intestinal lumen may not be involved in intracellular *de novo* GSH synthesis.

The ability of GSH to control extracellular redox provides another possible role for intestinal luminal GSH during fasting, as the present study demonstrated that intracellular GSH levels of the jejunum mucosa were not increased by intestinal luminal GSH administration during fasting, although the GSH administration attenuated the fasting-induced ROS in the intestinal mucosa. Reversible redox reactions of intracellular thiol/disulfide pairs such as GSH/GSSG regulate diverse biological processes *in vivo*, including signaling for apoptosis and cell proliferation, enzyme catalysis, gene expression, and molecular folding and trafficking[16-18,48,49]. Most studies have focused on the major intracellular thiols such as GSH[50,51]; however, relatively little is known regarding the effects of changes in extracellular thiol/disulfide pairs. Biliary GSH is an important intestinal luminal source of GSH and attenuates ROS of the intestinal mucosa by increasing the GSH levels of the intestinal mucosa. However, it has been suggested that biliary GSH controls the status of the intestinal lumen and contributes to protection of the intestinal mucosa[10]. The GSH of the intestinal lumen may act on the intestinal mucosa *via* both of these two pathways, and it is important to consider the control of the redox status of the intestinal lumen. In addition, the roles of extracellular (*i.e.*, intestinal luminal) GSH in the recovery from fasting-induced intestinal mucosal atrophy enabled by oral GSH treatment have not yet been described.

A few studies have examined the ability of the extracellular thiol/disulfide redox state to regulate apoptosis and cell proliferation. Circu *et al*[16] demonstrated that the luminal/extracellular redox environment is determined by the cysteine/cystine (Cys/CySS) redox pair with contributions from the GSH system, with the majority of Cys in the intestinal lumen originating from GGT enzymatic hydrolysis of GSH obtained from dietary intake and biliary supply. Intestinal cell proliferation and apoptosis are associated with quantitative changes in the redox potential (Eh) of the extracellular GSH/GSSG or Cys/CySS redox pair. In the present study, the changes in apoptosis and cell proliferation in the intestinal mucosa resulting from oral GSH administration during fasting may derive from Eh changes mediated by GSH and Cys in the intestinal lumen, which in turn may ultimately control signaling proteins, enzyme catalysis, and gene expression. For example, Devadas *et al*[52] linked ROS formation with the progression of Fas-induced apoptosis *via* the protective effects of extracellular GSH, which are ascribed to its known capacity as an antioxidant. Specifically, extracellular GSH was deduced to inhibit Fas-mediated apoptosis following ROS reduction in cells because Fas crosslinking induced rapid generation of ROS well before the appearance of characteristic apoptotic changes. Furthermore, Fujise *et al*[22] suggested that fasting-induced apoptosis in the rat small intestine occurred *via* a receptor-mediated type I apoptotic pathway including induced expression of FasL, Fas, and TNFR1. Selleri *et al*[53] and Viard-Leveugle *et al*[54] provided evidence that iNOS expression and NO production are involved in the mechanism of FasL upregulation and Fas-mediated apoptosis. The iNOS-NO-ROS-FasL pathway represents a potential link between the apoptosis and intestinal atrophy observed in the fasting.

In the present study, fasting-induced intestinal mucosal atrophy resulting from increased apoptosis was caused by increased production of NO and ROS as apoptosis mediators following elevation of iNOS expression. Because the iNOS-NO-ROS-FasL pathway is part of the apoptotic mechanism in the intestinal mucosa atrophy occurring after fasting[22,52-54], oral GSH treatment during fasting might inhibit Fas-mediated apoptosis following reduction of ROS levels in the jejunal mucosa resulting from Eh changes mediated by GSH and Cys in the intestinal lumen. Additionally, because iNOS protein expression was also induced by ROS produced from NO in fasting-induced intestinal mucosal atrophy in a previous study[20], the decrease in ROS levels in the intestinal mucosa reduces iNOS protein expression and NO production. Moreover, Jonas *et al*[55] showed that the extracellular thiol/disulfide redox state modulates cell proliferation and that this system interacted with growth factor signaling in a human colon carcinoma cell line. Thus, the change in extracellular thiol/disulfide redox state in response to peptide growth factors indicated an interaction of growth factor-activated pathways and thiol/disulfide metabolism during intestinal cell proliferation.

Therefore, oral GSH administration during fasting may regulate the redox state of the intestinal lumen and consequently may both relieve Fas-mediated apoptosis and increase growth factor-mediated cell proliferation by ROS removal in jejunal epithelial cells (Figure 11). However, because our present study focused on intracellular GSH, further studies focusing on extracellular GSH are required to confirm this model. In particular, a study utilizing measurements of GSH/GSSG and Cys/CySS levels in the intestinal lumen along with Fas and growth factor signaling in the jejunum may be necessary to identify the mechanism underlying the recovery of the intestinal mucosal atrophy mediated by oral GSH administration during fasting.

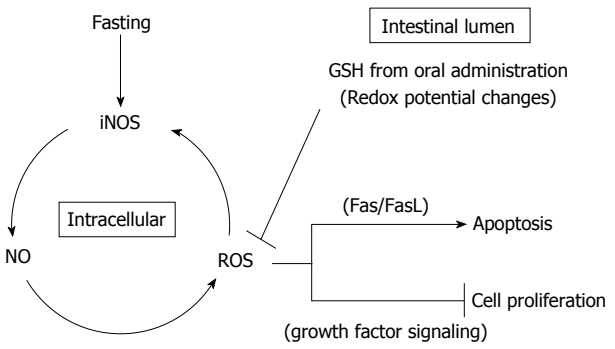

Figure 11 Schematic diagram of protective effects of glutathione in the intestinal lumen against fasting-induced intestinal atrophy, mediated through oxidative stress. The schematic diagram depicts a possible role of intestinal lumen redox status in the regulation of jejunal mucosa apoptosis and cell proliferation in fasting-induced intestinal atrophy, mediated through oxidative stress. Fasting causes increased production of NO and ROS as apoptosis mediators following elevation of iNOS expression. The changes in apoptosis and cell proliferation in the intestinal mucosa resulting from oral GSH administration during fasting may derive from intracellular ROS removal by redox potential changes mediated by GSH and Cys (originating from enzymatic hydrolysis of GSH) in the intestinal lumen. Intracellular ROS removal is considered to inhibit Fas-mediated apoptosis and increase growth factor-mediated cell proliferation. GSH: Glutathione; ROS: Reactive oxygen species.

In conclusion, oral GSH administration during fasting may provide regenerative potential to the jejunal mucosa through control of enterocyte apoptosis and cell proliferation. The GSH in the intestinal lumen provided through oral administration during fasting may not be involved in intracellular *de novo* GSH but may be useful to prevent intestinal mucosal atrophy by diminishing the levels of ROS in the jejunum generated by fasting.

COMMENTS

Background

Fasting induces small intestinal mucosal atrophy, which can lead to bacterial translocation, particularly in patients receiving a prolonged course of total parenteral nutrition (TPN), and is associated with increased reactive oxygen species (ROS) formation. This fasting state is also accompanied by depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa. As GSH deficiency in tissues is associated with increased oxidative stress and fasting, the maintenance of tissue levels of GSH is critical for protection of small intestinal mucosa.

Research frontiers

The roles of GSH in intestinal mucosal recovery from fasting as mediated by oral GSH administration have not been investigated.

Innovations and breakthroughs

GSH treatment in the intestinal lumen could be a promising strategy to improve clinical outcome in patients receiving a prolonged course of TPN.

Applications

Oral GSH administration during fasting enhances jejunal regenerative potential to minimize intestinal mucosal atrophy by diminishing fasting-mediated ROS generation and enterocyte apoptosis and enhancing cell proliferation.

Peer-review

The manuscript by Uchida *et al* describes effects of oral GSH administration on fasting-induced intestinal atrophy in the small intestinal mucosa. The authors found that oral GSH administration of rats during fasting significantly enhanced jejunal regenerative potential to minimize mucosal atrophy. This reduction of intestinal atrophy correlated with correction of histopathological outcomes and many biochemical parameters. The authors' conclusion that oral GSH improves intestinal atrophy is supported by convincing results.

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ORIGINAL ARTICLE

Basic Study

Faecal and mucosal microbiota in patients with functional gastrointestinal disorders: Correlation with toll-like receptor 2/toll-like receptor 4 expression

Li-Na Dong, Jun-Ping Wang, Ping Liu, Yun-Feng Yang, Jing Feng, Yi Han

Li-Na Dong, Central Laboratory, Shanxi Provincial People's Hospital, Shanxi Medical University, Taiyuan 030012, Shanxi Province, China

Jun-Ping Wang, Yun-Feng Yang, Jing Feng, Yi Han, Department of Gastroenterology, Shanxi Provincial People's Hospital, Shanxi Medical University, Taiyuan 030012, Shanxi Province, China

Ping Liu, Department of Gynaecology, Shanxi Provincial People's Hospital, Shanxi Medical University, Taiyuan 030012, Shanxi Province, China

ORCID number: Li-Na Dong (0000-0003-3922-5912); Jun-Ping Wang (0000-0002-7517-9936); Ping Liu (0000-0002-1983-4238); Yun-Feng Yang (0000-0001-1609-0644); Jing Feng (0000-0001-8415-6296); Yi Han (0000-0002-4688-1582).

Author contributions: Dong LN and Wang JP contributed equally to this study; they performed the majority of experiments, analysed the data, and drafted the manuscript; Wang JP and Liu P participated in the design of this study and reviewed the manuscript; Yang YF, Feng J and Han Y collected the specimens; all authors have read and approved the final manuscript.

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Correspondence to: Jun-Ping Wang, Professor, Department of Gastroenterology, Shanxi Provincial People's Hospital, Shanxi Medical University, 29 Shuangta Road, Taiyuan 030012, Shanxi Province, China. wangjp8396@sohu.com
Telephone: +86-351-4960141

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Abstract

AIM

To investigate the intestinal luminal microbiota (LM) and mucosa-associated microbiota (MAM) in Chinese patients with functional gastrointestinal disorders (FGIDs) and examine the association between these communities and the expression of toll-like receptor (TLR) 2 and TLR4.

METHODS

Thirty-two Chinese subjects who suffered from symptoms of FGIDs, as confirmed by gastroenterologists, were enrolled in this study. Fresh faecal samples and descending colonic mucosal biopsies were collected from the subjects before (faecal) and during (mucosal)

flexible colonoscopy. For analysis of the samples, we performed high-throughput sequencing of the V3-V4 region of the *16S rRNA* gene and reverse transcription (RT)-PCR to detect the expression of colonic TLR2 and TLR4. Differences in the stool and mucosal microbiota were examined and a correlation network analysis was performed.

RESULTS

The microbiota of faecal samples was significantly more diverse and richer than that of the mucosal samples, and the LM and MAM populations differed significantly. TLR2 expression showed a significant positive correlation with TLR4 expression. In the MAM samples, the genera *Faecalibacterium* and *Ruminococcus*, which belong to the family Ruminococcaceae, were inversely correlated with TLR4 expression (r = -0.45817, P = 0.0083 and r = -0.5306, P = 0.0018, respectively). *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression (r = -0.5573, P = 0.0010 and r = -0.5435, P = 0.0013, respectively). In the LM samples, examination at phylum, class, or order level revealed no correlation with TLR4 expression. *Faecalibacterium*, which belongs to Ruminococcaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression (r = -0.5743, P = 0.0058 and r = -0.3905, P = 0.0271, respectively).

CONCLUSION

Microbial compositions of LM and MAM in Chinese patients with FGIDs are different. Expression of TLRs may be affected by the type of bacteria that are present in the gut.

Key words: Gastrointestinal microbiota; *16S rRNA* gene; Toll-like receptors; High-throughput sequencing

Core tip: To explore which bacteria regulate the expression of toll-like receptors (TLRs) and thereby affect intestinal functions, we performed high-throughput pyrosequencing of the bacterial *16S rRNA* gene, compared the microbial communities in the faeces and mucosa of Chinese patients with functional gastrointestinal disorders, and studied their association with the expression of colonic mucosal TLR2 and TLR4. Samples of luminal microbiota were different from those of mucosa-associated microbiota (MAM), and MAM samples were closely associated with TLR2/4 expression. The abundance of *Faecallibacterium* and *Ruminococcus* was lower in patients with gut disease, while the expression of TLRs is higher than in healthy controls. The presence of *Faecallibacterium* and *Ruminococcus* was significantly and negatively correlated with TLR4 expression, suggesting that these two bacteria, which colonize on the colonic mucosa, play a key role in gut diseases by regulating mucosal TLR4 expression. *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression. Because the two genera contained not only pathogenic species but also probiotic species, it will be important to establish a better understanding of the relationship between TLRs and bacterial strains in future studies.

Dong LN, Wang JP, Liu P, Yang YF, Feng J, Han Y. Faecal and mucosal microbiota in patients with functional gastrointestinal disorders: Correlation with toll-like receptor 2/toll-like receptor 4 expression. *World J Gastroenterol* 2017; 23(36): 6665-6673 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6665.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6665

INTRODUCTION

The intestinal microbiota is a complex community of bacteria, archaea, viruses, and eukarya. A wide variety of bacterial species in the gastrointestinal tract exerts numerous effects on the host and influences a variety of gastrointestinal functions[1]. Faecal samples (representing the luminal niche) are examined in most studies of the intestinal microbiota because their collection is simple. However, recent studies have shown that the microbial compositions of the luminal microbiota (LM) and the mucosa-associated microbiota (MAM) differ, suggesting that these two distinct microbial populations play different roles within the intestinal microbiota ecosystem[2]. LM is present in the whole intestine, whereas MAM represents a special niche. Because MAM is in close contact with the host, it may play a more prominent role in the intestine, whereas LM may play a key role in metabolic activities and nutrient harvest[3]. Toll-like receptors (TLRs) are pattern recognition receptors expressed by various cells in the gastrointestinal tract. TLRs detect conserved microbial products and play a central role in the activation of innate and adaptive immune pathways. TLR2 and TLR4 are two of the best characterized TLRs that respond to microbial membrane components. A number of studies have examined the role of TLR signalling in microbiota-induced chronic inflammation and immunopathology[4]. The microbiota may directly interact with the TLRs and regulate gut immune responses[5].

In some intestinal diseases, such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS), the expression of TLR2 and TLR4 is increased and the dysbiosis is observed. However, limited data are available on the correlation between the intestinal microbiota and TLRs in humans. To explore which bacteria regulate the expression of TLRs and thereby affect intestinal functions, we performed high-throughput pyrosequencing of the bacterial *16S rRNA*

gene, compared the microbial communities in the faeces and mucosa of Chinese patients with functional gastrointestinal disorders (FGIDs), and studied their association with the expression of colonic mucosal TLR2 and TLR4.

MATERIALS AND METHODS

Study subjects

Thirty-two Chinese subjects aged between 18 and 65 years, who were diagnosed with FGIDs, were recruited at the Department of Gastroenterology, Shanxi Provincial People's Hospital from 2013 to 2014. None of the subjects enrolled in the study had taken corticosteroids, opioids, probiotic and prebiotic supplements or antibiotics in the 6 mo preceding the study, no one had systemic comorbidity, and no one had a history of excessive alcohol intake (> 20 alcoholic drinks per week). Patients with a prior history of gastrointestinal surgery or intestinal organic disease were excluded. All subjects provided signed informed consent before participation. The study was performed in accordance with the principles of the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of Shanxi Provincial People's Hospital, China.

Stool sample processing and DNA extraction

Faecal samples were collected at home before bowel preparation, frozen immediately at -20 ℃, and transported within 6 h to the study centre, where they were stored at -80 ℃ until analysis. Bacterial DNA was extracted from the faecal samples with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA concentrations were quantified with an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany).

Mucosal sample collection and genomic DNA extraction

Colonic mucosal biopsies were collected from each subject during an unsedated flexible colonoscopy. Colonic mucosal biopsies were taken from the descending colon. After removal from the colon, the biopsies were immediately frozen at -20 ℃ and transported within 6 h to the study centre, where they were stored at -80 ℃ until analysis. Total genomic DNA was extracted from the colon samples with the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The concentration and purity of the genomic DNA were measured with an Eppendorf BioSpectrometer.

PCR amplification of the V3-V4 region of bacterial 16S rRNA gene and Illumina sequencing

The bacterial genomic DNA was used as the template for amplification of the V3-V4 hypervariable region of the *16S rRNA* gene with the forward primer 5′-GACTACHVGGGTATCTAATCC-3′ and the reverse primer 5′-CCTACGGGNGGCWGCAG-3′.

Bioinformatics analysis

Pairs of reads from the original DNA fragments were merged using FLASH, which was designed in case the original DNA fragments were shorter than twice the read length. The sequencing reads of each sample were given a unique barcode and analysed with the Quantitative Insights Into Microbial Ecology (QIIME) software and the UPARSE pipeline. In brief, the reads were filtered with the QIIME quality filters using the default settings for Illumina processing and the operational taxonomic units (OTUs) were selected using the UPARSE pipeline. The samples were sequenced on an Illumina MiSeq Benchtop Sequencer and the bioinformatic analysis was performed by Genesky Biotechnologies Inc. (Shanghai, China).

The size of the bacterial groups is expressed as percentage of the total bacteria.

Quantitative real-time polymerase chain reaction

Total mucosal RNA was extracted from the colonic biopsies using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan), according to the manufacturer's instructions. After reverse transcription with PrimeScript Reverse Transcriptase Mix (TaKaRa), the expression of *TLR2* and *TLR4* genes was determined by quantitative real-time polymerase chain reaction (qPCR) and SYBR Green technology on a Bio-Rad CFX96 Q-PCR instrument (Bio-Rad, United States) in duplicate. The specific primers for TLR2 were: 5′-TGATGCTGCCATTCTCATTC-3′ (forward) and 5′-CGCAGCTCTCAGATTTACCC-3′ (reverse); and for TLR4: 5′-CAGGGCTTTTCTGAGTCGTC-3′ (forward) and 5′-TGAGCAGTCGTGCTGGTATC-3′ (reverse). Each amplification reaction was run in duplicate in a final volume of 20 μL. All qPCR amplifications were optimized and performed in 0.2 mL 96-well plates with the following cycling program: initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 30 s. Negative controls lacking the template DNA were included in triplicate. The relative amount of each mRNA was normalized to *GAPDH* (forward: 5′-CCATCAATGACCCCTTCATTG-3′, reverse: 5′-CTTGACGGTGCCATGGAATT-3′) and data were analysed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All statistical analyses were performed with SPSS 22.0 for Windows (SPSS Inc., United States). To determine the statistical differences between the two groups, we used the independent *t* test and the Mann-Whitney test. Correlations were determined with Spearman's correlation test. Heat maps were generated by R-packages for TLR2 and TLR4 as environmental factors, to reveal the relationship between the diversity

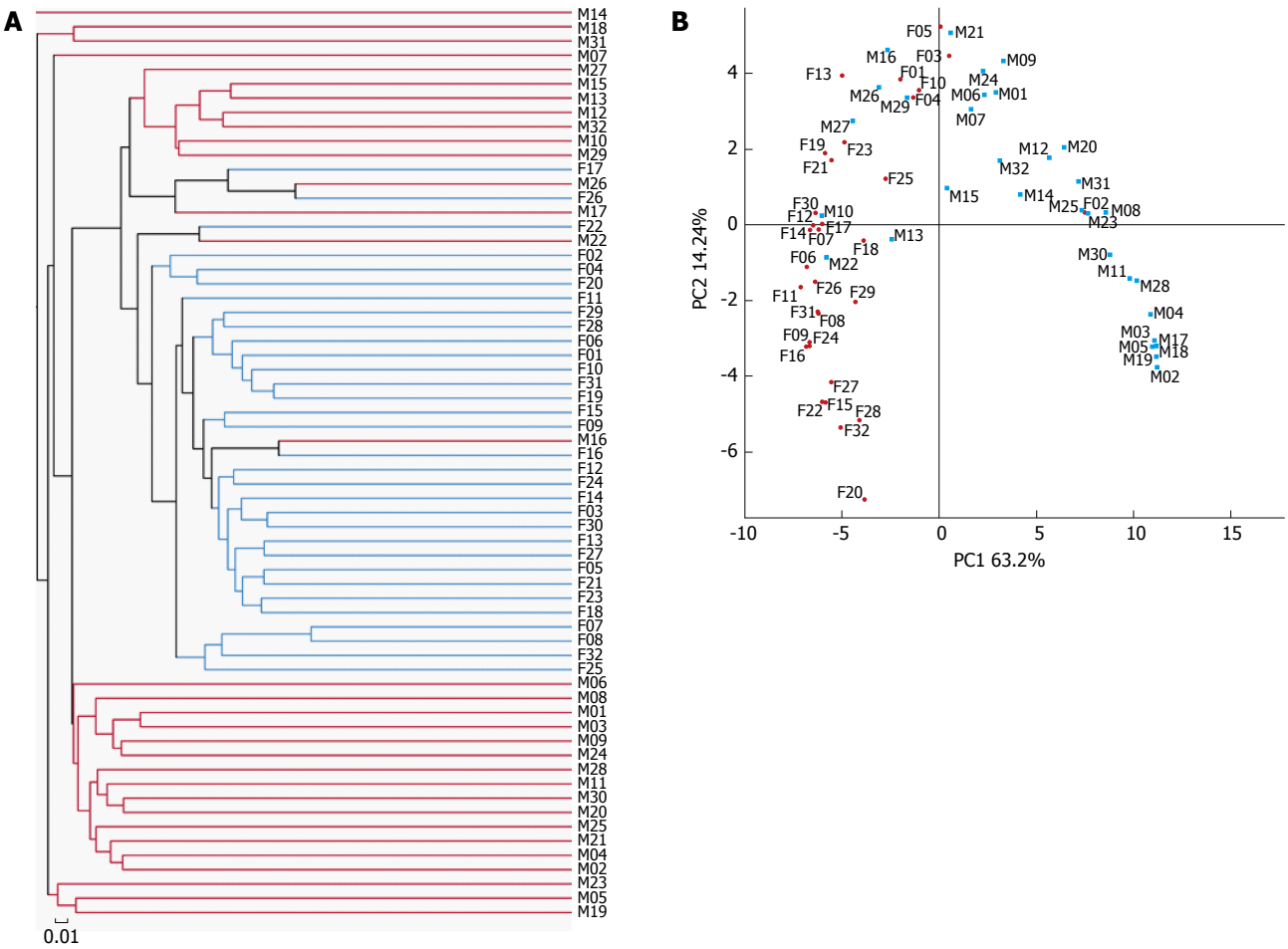

Figure 1 ***16S rRNA*** **gene surveys reveal a clear separation between luminal microbiota and mucosa-associated microbiota.** A: Dendrogram obtained from the complete linkage hierarchical clustering of the stool and mucosa samples based on the total operational taxonomic units; B: Principal coordinate analysis plot based on the weighted UniFrac metric.

Table 1 Comparison of the richness and diversity of mucosa-associated microbiota and luminal microbiota

	ACE	Chao	Shannon	Simpson
MAM	160.72 ± 51.62	151.56 ± 48.63	2.29 ± 1.24	0.33 ± 0.32
LM	195.50 ± 42.04	188.65 ± 42.70	2.901 ± 0.54	0.13 ± 0.07
P value	0.004[b]	0.002[b]	0.012[a]	0.001[b]

[a]$P < 0.05$, [b]$P < 0.01$, MAM *vs* LM. MAM: Mucosa-associated microbiota; LM: Luminal microbiota.

and environmental factors based on the Pearson's correlation analyses.

RESULTS

Study population

We investigated 64 samples from 32 subjects with FGIDs [14 with diarrhoea-predominant IBS, 14 with functional dyspepsia (FD), and 4 with other diseases]. All subjects provided both a faecal sample and a colonic mucosal sample. The study population consisted of 50% females, and had a mean age of 49 years (range, 20-65 years) and a mean body mass index of 23.24 kg/m^2 (range, 20-26 kg/m^2).

Characteristics of the pyrosequencing results

We obtained a total of 4351929 raw reads and 3545053 reads remained after filtering. The sequencing analysis of the 64 samples identified 1026 OTUs. The rarefaction curves had a tendency to approach a saturation plateau, indicating that the number of samples in this study was sufficient. The same tendency was found in the Shannon-Wiener curves, indicating that the number of *16S rRNA* gene sequences in the database was very abundant and reflected the vast majority of the microbiota. The gut faecal samples showed significantly more diversity and richness of the microbiota than the mucosal samples (Table 1) and samples of LM and MAM were significantly different (Figure 1A and B).

Significant differences between samples of LM and MAM were identified for almost all populated phyla. Bacteroidetes (44.7%) and Firmicutes (42.2%) were the most represented phyla in LM samples, followed by Proteobacteria (8.5%), whereas Proteobacteria (56.6%) was the most represented phylum in MAM samples, followed by Firmicutes (20.2%) and Bacteroidetes (12.7%) ($P < 0.05$; Figure 2).

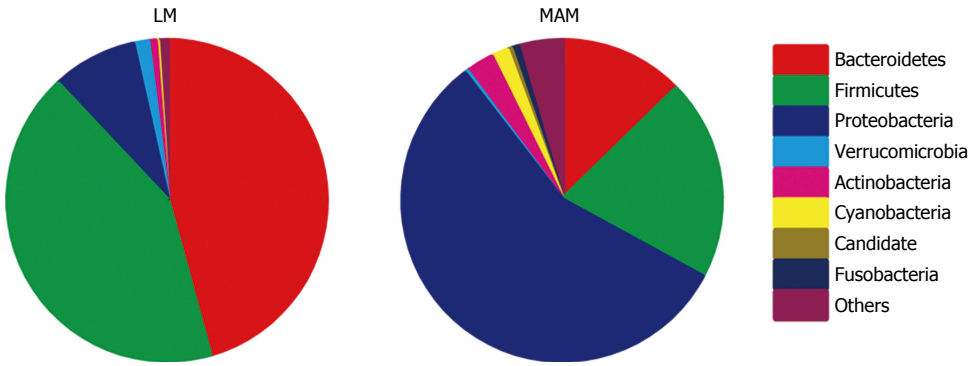

Figure 2 Relative abundance of bacterial genera in the stool and mucosa samples. Statistically significant differences were observed for all genera ($P < 0.05$). LM: Luminal microbiota; MAM: Mucosa-associated microbiota.

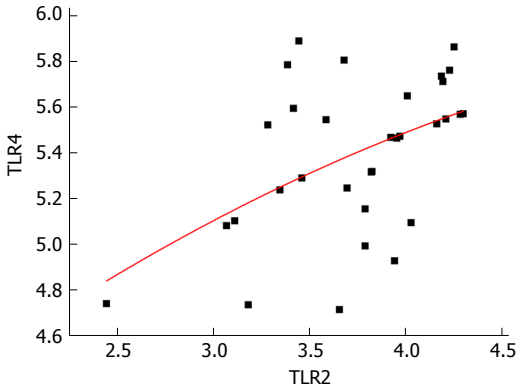

Figure 3 The expression of toll-like receptor 2 is significantly and positively correlated with toll-like receptor 4 expression (r = 0.492, P = 0.004). TLR: Toll-like receptor.

Examination at the genus level showed that the relative abundance of *Escherichia-Shigella*, *Streptococcus*, *Clostridium sensu stricto1*, *Sphingomonas*, *Acinetobacter*, *Brevundimonas*, and *Enhydrobacter* was significantly greater in samples of MAM than in LM samples, whereas abundance of *Bacteroides*, *Faecalibacterium*, *Incertae sedis*, *Subdoligranulum*, *Pseudobutyrivibrio*, *Megasphaera*, *Parasutterella*, *Akkermansia*, *Alistipes*, and *Lachnospira* was significantly lower in samples of MAM than in LM samples ($P < 0.05$).

Expression characteristics of mucosal TLR2 and TLR4

We measured the mRNA expression of TLR2 (3.7394 ± 0.43514) and TLR4 (5.3866 ± 0.33556) in all samples and expression of TLR2 was significantly and positively correlated with that of TLR4 (r = 0.492, P = 0.004) (Figure 3).

Correlation between gut microbiota and TLR4 expression

Regardless of phylum, class or order level, no bacterium was correlated with expression of TLR4 in LM samples.

At the phylum level, Firmicutes was inversely and significantly correlated with TLR4 expression in MAM samples (r = -0.4676, P = 0.0070). At the class level, Clostridia, which belongs to Firmicutes, was inversely and significantly correlated with TLR4 expression in MAM samples (r = -0.3913, P = 0.0268). At the order level among the class Clostridia, the order Clostridia was inversely and significantly correlated with TLR4 expression (r = -0.3906, P = 0.0271), as well as the families Defluviitaleaceae (r = -0.4227, P = 0.0159), Peptostreptococcaceae (r = -0.3611, P = 0.0422), and Ruminococcaceae (r = -0.4740, P = 0.0061), which belong to the order Clostridia. The genera *Faecalibacterium* and *Ruminococcus*, which belong to the family Ruminococcaceae, were also inversely and significantly correlated with TLR4 expression (r = -0.45817, P = 0.0083 and r = -0.5306, P = 0.0018, respectively) (Figure 4).

Correlation between gut microbiota and TLR2 expression

The presence of bacteria in both LM and MAM samples was correlated with TLR2 expression. However, the taxa of microbiota was very different in these two sample populations.

In LM samples, Clostridia and Bacilli at the class level were inversely and significantly correlated with TLR2 expression (r = -0.4238, P = 0.0156 and r = -0.4482, P = 0.0101, respectively), as well as Clostridiales at the order level, which belongs to Clostridia, and Lactobacillales, which belongs to Bacilli (r = -0.4482, P = 0.0156 and r = -0.4207, P = 0.0165, respectively). At the family level, Ruminococcaceae, which belongs to Clostridiales, and Streptococcaceae, which belongs to Lactobacillales, were inversely and significantly correlated with TLR2 expression (r = -0.4437, P = 0.0110 and r = -0.3839, P = 0.0300, respectively). And at the genus level, *Faecalibacterium*, which belongs to Ruminococcaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely and significantly correlated with TLR2 expression (r = -0.5743, P = 0.0058 and r = -0.3905, P = 0.0271, respectively) (Figure 4).

In MAM samples, Bacilli at the class level was inversely and significantly correlated with TLR2 expression (r = -0.4676, P = 0.0070), as well as Lactobacillales at the order level, which belongs to

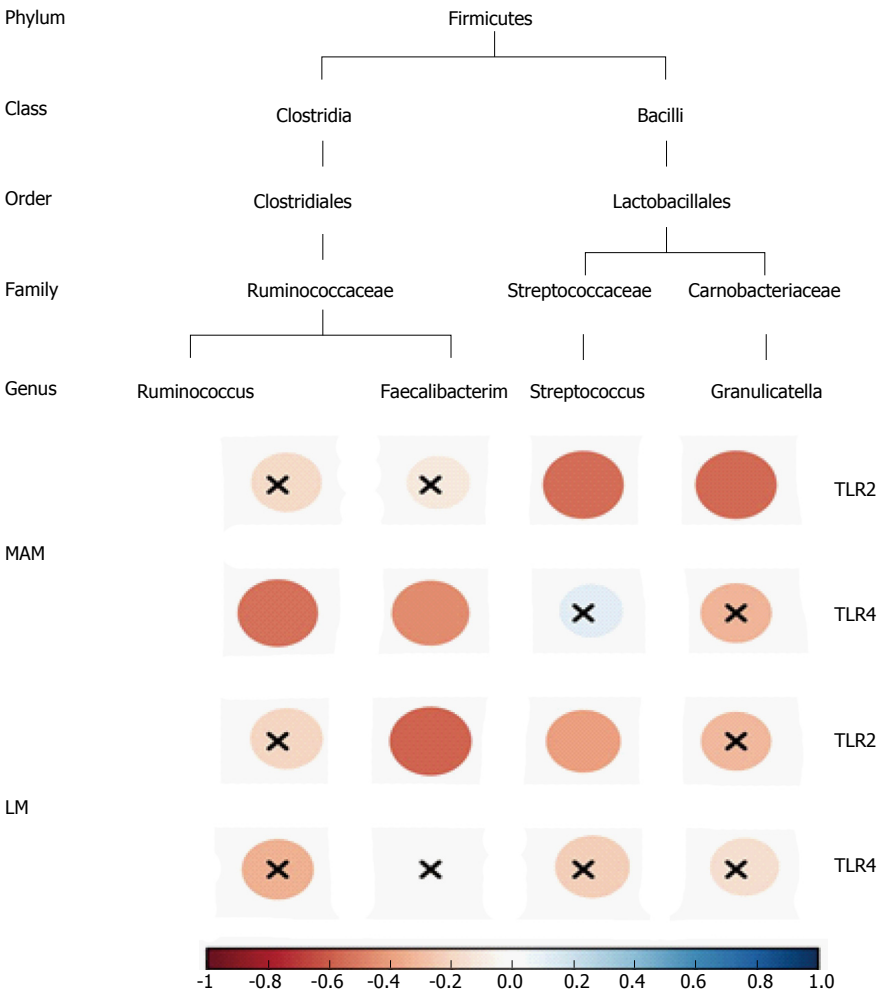

Figure 4 Correlogram showing the Pearson's correlation between key genera and toll-like receptors in both faecal and mucosa samples. Blue circles designate a positive correlation, while red ones designate a negative correlation. X means no significant result according to the significance level of 0.05. TLR: Toll-like receptor; MAM: Mucosa-associated microbiota; LM: Luminal microbiota.

Bacilli (r = -0.4574, P = 0.0085). At the family level, Carnobacteriaceae and Streptococcaceae, which belong to Lactobacillales, were inversely and significantly correlated with TLR2 expression (r = -0.5554, P = 0.0010 and r = -0.5445, P = 0.0013, respectively). And at the genus level, *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were correlated with TLR2 expression (r = -0.5573, P = 0.0010 and r = -0.5435, P = 0.0013, respectively) (Figure 4).

DISCUSSION

Deep sequencing showed that more than 95% of the sequences in all stool and mucosal samples belonged to the three most popular bacterial phyla, consisting of Firmicutes, Bacteroidetes, and Proteobacteria. This is consistent with the findings of previous studies[6], which showed that these phyla account for the majority of the gut microbiota in both stool and mucosal samples. The faecal samples displayed significantly greater bacterial diversity and richness than the mucosal samples, as in the study of Ringel *et al*[2]. Comparing the proportions of the dominant bacterial taxa in the faecal and mucosal samples revealed significant differences. In this study, Proteobacteria was the predominant phylum in MAM samples. This is different from other reports and might reflect geographical differences[7]. It is well known that the Chinese diet and genetics are very different from those in Western countries and these factors markedly influence the gut microbiota. Furthermore, MAM samples were taken from a distinct location in the colon, whereas LM samples displayed the whole intestinal microbiota. Therefore, MAM samples may reflect a stronger relationship with the host.

A correlation analysis was performed between the two bacterial populations at the phylum, class, order, family, and genus levels and the expression of TLR2 and TLR4. The results showed that although the Proteobacteria was the predominant phylum in MAM samples, the bacteria that correlated strongest with expression of TLRs were part of the phylum Firmicutes. The taxa that showed a significant correlation was selected and studied from the phylum to genus level. Finally, *Faecalibacterium, Ruminococcaceae*,

Streptococcus and *Granulicatella* genera were the bacteria that correlated strongest with TLRs expression. Although a positive correlation existed between the expression of TLR2 and TLR4, the types of bacteria associated with TLR4 expression were very different from those related to the expression of TLR2. However, this was in accordance with their distinct microbial compositions. The bacteria that correlated with TLR4 expression were only found in MAM samples. Bacteria that correlated with TLR2 expression originated from both LM and MAM. Both TLR4 and TLR2 expression were negatively correlated with MAM, however, TLR2 expression was mainly associated with bacteria belonging to the Bacilli class, whereas TLR4 expression was mainly associated with bacteria belonging to the Clostridia class.

The enteric commensal bacteria of the genus *Faecallibacterium*, which belongs to the group Clostridium, exert an anti-inflammatory effect[8]. In the present study, *Faecallibacterium* from LM samples was negatively correlated with the expression of TLR2 and *Faecallibacterium* from MAM samples was negatively correlated with the expression of TLR4. *Faecallibacterium* richness was reduced in patients with IBS and IBD[9,10]. TLR signalling, activated by pathogens, is involved in the pathogenesis of several infectious and inflammatory diseases. Imbalanced relationships among the environment, genetics, and host immunity may promote aberrant TLR signalling, thereby contributing on a large scale to acute and chronic intestinal inflammatory processes, such as IBD, colitis, and colorectal cancer[11]. In our previous study, the expression of TLR2 and TLR4 was increased in diarrhoea-predominant IBS patients and decrease of Clostridia was strongly associated with higher TLR2 and TLR4 expression[12]. In an animal study, *Faecallibacterium prausnitzii* and its supernatant (SN) had beneficial effects on intestinal epithelial barrier impairment in a chronic low-grade inflammation model[13]. Round *et al*[14] showed that unlike pathogens whose TLR ligands trigger inflammation, some commensal bacteria exploit the TLR pathway to actively suppress immune reactions. The anti-inflammatory effect of *Faecallibacterium* may be involved in the upregulation of the TLRs expression. *Ruminococcus* is another common bacterium that is decreased in patients with IBD or IBS. In addition, a reduction in the relative abundance of potentially immunomodulatory gut bacteria including *Ruminococcus* is associated with exaggerated inflammatory cytokine responses to TLR ligands and subsequent development of IgE-associated eczema[15]. In the present study, the decrease of *Faecallibacterium* and *Ruminococcus* in MAM samples was strongly associated with higher TLR4 expression, suggesting that the decrease of mucosal *Faecallibacterium* and *Ruminococcus* induced an increase in expression of TLR4 mRNA, which plays an important role in the causation of immune related gut diseases including IBD and IBS.

Streptococcus is a genus of (spherical) coccus Gram-positive bacteria belonging to the phylum Firmicutes phylum and the order Lactobacillales. Some streptococcal species, *e.g., S. pneumoniae*, are pathogenic. Tomlinson *et al*[16] suggested that leukocyte responses to bacterial lipoproteins are required for TLR2- and IL-1R-associated kinase-4-mediated inflammatory responses to *S. pneumoniae*. However, many other streptococcal species, *e.g.*, *S. thermophilus*, are not pathogenic[17] and are part of the commensal human microbiota of the mouth, skin, intestine, and upper respiratory tract. S. *thermophilus* is considered a probiotic and is a key strain of VSL#3, which is considered a therapeutic immunomodulatory tool[18]. Streptococcaceae was negatively correlated with TLR2 expression in samples of both MAM and LM in our study. Although there is no direct evidence of inhibition of TLRs by Streptococcaceae, some beneficial streptococcal species may affect disease processes *via* the TLR2 pathway. Future research will explore which species belonging to Streptococcaceae can influence the expression of TLR2. Granulicatella is part of the normal body flora and is often difficult to culture in traditional media. A few reports mention that it can induce an infection similar to Streptococci[19].

The composition of LM samples was different than that of MAM; MAM samples showed a stronger correlation with the expression of TLR2 and TLR4. The abundance of *Faecallibacterium* and *Ruminococcus* was lower in IBD and IBS, while TLRs expression was higher in patients than in healthy controls. *Faecallibacterium* and *Ruminococcus* were significantly and negatively correlated with TLR4 expression, suggesting that these two bacteria colonizing on the colonic mucosa play a key role in the pathogenesis of gut diseases by regulating the expression of mucosal TLR4. *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression.

One limitation of our study was the small biopsy from the colonic endoscopy and multiple sampling might cause bleeding, therefore we were not able to measure the expression of proteins. The other limitation of our study was the scattered distribution of the correlation between species levels, which might be explained by the restriction of pyrosequencing of species levels, therefore we did not report the results. It will be important to establish a better understanding of the relationship between TLRs and bacteria strains in future studies. Probiotics play an important role in the treatment and prevention of diseases. Further studies will have to verify the relationship between TLRs and bacteria[20,21].

COMMENTS

Background

Toll-like receptors (TLRs) detect conserved microbial products and play a

central role in the activation of innate and adaptive immune pathways. TLR2 and TLR4 are two of the best characterized TLRs that respond to microbial membrane components. Faecal samples (representing the luminal niche) were used in most studies on intestinal microbiota because their collection is easy. However, more recent studies revealed distinct microbial composition between luminal microbiota (LM) and mucosa-associated microbiota (MAM). To explore which bacteria may regulate the expression of TLRs and thereby affect intestinal functions, we performed high-throughput pyrosequencing of the bacterial *16S rRNA* gene, compared the microbial communities in the faeces and mucosa of Chinese patients with functional gastrointestinal disorders, and studied their association with the expression of colonic mucosal TLR2 and TLR4. LM differed from MAM; MAM showed a stronger association with the expression of TLR2 and TLR4.

Research frontiers

Recent advances in the understanding of intestinal immunology suggest that functional gastrointestinal disorders (FGIDs) may not all be 'functional', as considered for decades. This view was largely developed by the absence of active inflammation. Faecal microbiota and MAM play a vital role in gut immunity. It will be important to establish a better understanding of the relationship between TLRs and bacteria strains in FGIDs.

Innovations and breakthroughs

In some intestinal diseases, such as inflammatory bowel disease or irritable bowel syndrome, the expression of TLR2 and TLR4 is increased and dysbiosis is observed. However, limited data are available on the correlation between the intestinal microbiota and TLRs in humans. In this study, the abundance of *Faecallibacterium* and *Ruminococcus* was lower in many gut diseases, while the TLRs expression was higher than in healthy controls. *Faecallibacterium* and *Ruminococcus* were significantly and negatively correlated with TLR4 expression, suggesting that these two bacteria colonizing on the colonic mucosa play a key role in gut diseases by regulating the expression of mucosal TLR4. *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2.

Applications

Probiotics play an important role in the treatment and prevention of diseases. Further studies will have to verify the relationship between inflammatory proteins and bacteria. The bacteria may be used as probiotics in the treatment of diseases by regulating immunity.

Terminology

According to the literature, they described the microbiota from faecal samples as LM.

Peer-review

This is an interesting paper that investigated the intestinal LM and the MAM in Chinese patients with FGIDs and examined the association between these communities and the expression of TLR2 and TLR4. The manuscript is well written.

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ORIGINAL ARTICLE

Case Control Study

Genetic biomarkers for hepatocellular cancer risk in a caucasian population

Elena De Mattia, Erika Cecchin, Jerry Polesel, Alessia Bignucolo, Rossana Roncato, Francesco Lupo, Marina Crovatto, Angela Buonadonna, Claudio Tiribelli, Giuseppe Toffoli

Elena De Mattia, Erika Cecchin, Alessia Bignucolo, Rossana Roncato, Giuseppe Toffoli, Clinical and Experimental Pharmacology, "Centro di Riferimento Oncologico" - National Cancer Institute, 33081 Aviano, Italy

Jerry Polesel, Unit of Cancer Epidemiology, "Centro di Riferimento Oncologico" - National Cancer Institute, 33081 Aviano, Italy

Francesco Lupo, General Surgery 2U and Liver Transplantation Center, A.O.U. Città della Salute e della Scienza di Torino, University of Torino, 10124 Torino, Italy

Marina Crovatto, Cytogenetics and Molecular Biology Unit, Santa Maria degli Angeli Hospital Pordenone, 33170 Pordenone, Italy

Angela Buonadonna, Medical Oncology Unit, "Centro di Riferimento Oncologico" - National Cancer Institute, 33081 Aviano, Italy

Claudio Tiribelli, Fondazione Italiana Fegato, AREA Science Park, 34149 Trieste, Italy

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Correspondence to: Dr. Giuseppe Toffoli, MD, Director, Clinical and Experimental Pharmacology, "Centro di Riferimento Oncologico" - National Cancer Institute, Via Franco Gallini n. 2, 33081 Aviano, Italy. gtoffoli@cro.it
Telephone: 39-434-659612
Fax: 39-434-659799

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Abstract

AIM

To uncover novel genetic markers that could contribute to predicting hepatocellular carcinoma (HCC) susceptibility in Caucasians.

METHODS
The present retrospective case-control study compared genotype frequencies between a cohort of HCC cases and two, independent, HCC-free, age/sex-matched control groups. The HCC cohort comprised 192 homogeneous patients that had undergone orthotopic liver transplantation. The first control group comprised 167 patients that were matched to the HCC cohort for the percentage of hepatitis B (HBV) and/or hepatitis C (HCV) infections. A second control group included 192 virus-free, healthy individuals that were used to evaluate the generalizability of the identified predictive markers. All cases and controls were Caucasian. The three study populations were characterized with a panel of 31 markers derived from 21 genes that encoded key proteins involved in hepatocarcinogenesis-related pathways. The study end-point was to assess the association between genetic variants and HCC onset.

RESULTS
Five genetic markers were identified as risk factors for HCC in high-risk patients infected with HBV/HCV. According to a dominant model, reduced HCC risk was associated with three polymorphisms: *ERCC1* rs3212986 (OR = 0.46, 95%CI: 0.30-0.71, P = 0.0005), *GST-P1* rs1138272 (OR = 0.41, 95%CI: 0.21-0.81, P = 0.0097), and *CYP17A1* rs743572 (OR = 0.50, 95%CI: 0.31-0.79, P = 0.0032). Conversely, according to a recessive model, increased HCC risk was associated with two polymorphisms: *XRCC3* rs1799794 (OR = 3.70, 95%CI: 1.02-13.39, P = 0.0461) and *ABCB1* rs1128503 (OR = 2.06, 95%CI: 1.18-3.61, P = 0.0111). These associations remained significant in a subgroup analysis, where patients were stratified according to viral status (HBV- or HCV-positive serology). Two variants exhibited a serology-specific effect: *ABCB1* rs1128503 (OR = 4.18, 95%CI: 1.55-11.29, P = 0.0048) showed an effect in the HBV-positive subgroup; and *ERCC1* rs3212986 (OR = 0.33, 95%CI: 0.18-0.60, P = 0.0003) showed an effect in the HCV-positive subgroup. Among the five markers identified, *ERCC1* rs3212986 (OR = 0.43, P < 0.0001) and *CYP17A1* rs743572 (OR = 0.73, P = 0.0310) had a different distribution in patients with HCC compared to healthy individuals. With a recursive partitioning approach, we also demonstrated that significant gene-gene interactions between *ERCC1* rs3212986, *CYP17A1* rs743572, *GST-P1* rs1138272, and the previously described *UGT1A7**3 predictive marker, played a role in the complex trait of HCC susceptibility.

CONCLUSION
We identified five polymorphisms and interactions that contributed crucially to predicting HCC risk. These findings represented an important step towards improving HCC diagnosis and management.

Key words: ERCC1; XRCC3; GST-P1; CYP17A1; MDR-1; Polymorphisms; Hepatocellular carcinoma risk; Early diagnosis; Antiviral therapy; Hepatitis B viral/hepatitis C viral

Core tip: It is a great challenge to define new biomarkers of hepatocellular carcinoma (HCC) risk for HCC management. This work identified five genetic markers in key pathways linked to hepatocarcinogenesis (*ERCC1* rs3212986, *GSTP1* rs1138272, *CYP17A1* rs743572, *XRCC3* rs1799794, and *ABCB1* rs112850), which could predict individual HCC susceptibility, particularly in a high-risk [hepatitis B viral/hepatitis C viral (HCV)-infected] Caucasian population. We also identified potential gene-gene interactions that should be included in the definition of HCC risk. These findings could contribute to improved HCC surveillance, early cancer diagnoses, and potential curative therapies. For patients with HCV, these markers could be considered selection criteria for the personalized use of recently developed, highly expensive anti-viral therapies.

De Mattia E, Cecchin E, Polesel J, Bignucolo A, Roncato R, Lupo F, Crovatto M, Buonadonna A, Tiribelli C, Toffoli G. Genetic biomarkers for hepatocellular cancer risk in a caucasian population. *World J Gastroenterol* 2017; 23(36): 6674-6684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6674.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6674

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with a particularly high prevalence in some areas of Asia and Africa. HCC represents the second leading cause of cancer-related deaths[1]. Although liver cancer is less frequent in Western developed countries, recent data indicate that, due to dissemination of hepatitis B (HBV) and C (HCV) viral infections, the HCC incidence is also dramatically rising, in both the United States and Europe[1,2].

Unlike most malignancies, the major risk factors for HCC development are well-defined. These risk factors are chronic HCV and HBV infections, liver cirrhosis, heavy alcohol intake, tobacco smoking, exposure to environmental and dietary carcinogens (*i.e.,* aflatoxin B1), genetic and metabolic liver disease (*i.e.,* hereditary hemochromatosis, non-alcoholic steatohepatitis), and other conditions capable of inducing liver damage[2]. Moreover, the prevalence of HCC increases with age and male sex[1-3].

In addition to environmental factors, recent insights into the biology of HCC have demonstrated that accumulations of genetic and epigenetic abnormalities can also play an essential role in liver carcinogenesis. In particular, data derived from candidate-gene investigations and, more recently, genome-wide associations studies have highlighted the possible role that genetic variants might play as significant determinants of HCC susceptibility[4-6]. Additionally, host genetic factors were shown to affect the clinical course of HBV or HCV infections, because they

influence the individual's predisposition to disease progression and hepatocarcinogenesis[7,8]. However, the currently available studies are highly heterogeneous in study design, sample size, ethnicity, and clinical-demographic features of the investigated population; these differences make it difficult to draw solid conclusions[4,6]. Moreover, the majority of case-control investigations were performed with Asian populations; thus, replication studies with different ethnicities are warranted[4-8].

It is of great clinical interest to identify genetic traits that can modify liver carcinogenesis, because they can potentially be used to improve preventive, diagnostic, and therapeutic strategies in HCC management[3,9,10]. To date, the majority of patients with HCC are diagnosed at an advanced tumor stage, which precludes potentially curative therapies, including orthotopic liver transplantation (OLT), surgical resection, and local ablation[3,9,10]. The HCC surveillance programs that could lead to early cancer detection and effective treatment are currently based on highly heterogeneous scoring systems that require costly measures[3,9,10]. The identification of additional molecular biomarkers predictive of individual HCC susceptibility, particularly in a high-risk population (*i.e.,* HBV/HCV-positive), could be important in improving current guidelines for genetics-based patient screening and preventive strategies. In some countries, including the United States and Europe, HCV-related HCC accounts for most liver cancer incidents[11]. Consequently, an increased understanding of the genetic contribution to the clinical course of viral infection could be essential in optimizing the use of newly developed, but highly expensive, direct acting antiviral (DAA) treatments[12,13].

We previously reported that the low-activity alleles, *UGT1A1**28, *UGT1A7**3, *UGT1A9**22, and related haplotypes may play a protective role against HCC development in Caucasian populations. These alleles positively modulate the serum levels of beneficial molecules, like bilirubin[14]. The present study aimed to extend genetic analyses by identifying additional molecular markers that could be integrated into the HCC risk stratification algorithms used in liver cancer prevention strategies. To that end, we analyzed a set of potential candidate HCC-risk markers with low penetrance. These candidate markers were based on functionally relevant polymorphic variants in genes that encoded proteins involved in key pathways underlying carcinogenesis[6,15-17]. The pathways we investigated were: (1) membrane transporters that play an essential role in xenobiotic defense and prevent the entry of toxic environmental compounds that might damage liver tissue; (2) DNA synthesis and repair mechanisms that modulate the cell's capacity to respond to DNA damage induced by toxic agents, and thus, these mechanisms affect the accumulation of mutations in DNA; (3) detoxification systems that facilitate the elimination of detrimental endo/exogenous compounds; and (4) systems that defend against oxidative stress and related cellular damage, which can play a crucial role in the development and progression of HCC. We designed a retrospective case-control study and enrolled a homogeneous group of patients with HCC that had received OLT and two age/sex-matched control groups (a group positive for HBV and/or HCV and a healthy population). We aimed to identify novel genetic markers that could contribute to the determination of individual HCC risk, particularly in Caucasian populations.

MATERIALS AND METHODS

Study population

We studied 192 patients with histologically confirmed HCC that had undergone OLTs between May 1991 and March 2006 at the Liver Transplantation Center of the S. Giovanni Battista Hospital (Turin, Italy). All patients were infected with HBV and/or HCV and had a cirrhotic liver. The first control group included 167 subjects with HBV and/or HCV infections, but no evidence of cancer (including HCC). These subjects were followed up for hepatitis infections between November 2009 and September 2010 at the Department of Laboratory Medicine, S. Maria degli Angeli Hospital (Pordenone, Italy). This control group was matched to the HCC group to ensure equivalent percentages of patients with HBV and/or HCV and similar distributions of gender and age (in quinquennia), when possible.

The second control group included 192 healthy subjects that were matched to patients with HCC for gender and age (in quinquennia). For this control group, individuals under 70 years of age were randomly selected from a pool of blood donors that visited the Centro di Riferimento Oncologico National Cancer Institute in Aviano, Italy. This control group also included individuals aged ⩾ 70 years that were cancer-free without HBV/HCV infections. These individuals had been approached during occasional blood testing at the S. Maria degli Angeli Hospital, and they consented to provide a 5-mL blood sample for research purposes.

All cases and controls were Italian Caucasian individuals, and they all provided written informed consent for genetic analysis and for inclusion in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki, and it was approved by the Institutional Review Board of each participating institution (Ethics Committee of Azienda Ospedaliera Universitaria, S. Giovanni Battista, Torino).

Polymorphism selection and genetic analyses

Genomic DNA was extracted from peripheral blood with the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The samples underwent standard proteinase K digestion and successive template purification. The genomic DNA solution was stored at 4 ℃. Positive and negative control samples were included in each analysis.

We considered a set of 31 molecular markers that

Table 1 Distribution of hepatocellular carcinoma patients, hepatitis B/hepatitis C -positive individuals and healthy subjects according to socio-demographic and clinical characteristics *n* (%)

Characteristics	HCC group	HBV/HCV group	Healthy group
Sex			
Men	166 (86.5)	141 (84.4)	166 (86.5)
Women	26 (13.5)	26 (15.6)	26 (13.5)
Age (yr)			
< 55	85 (44.3)	85 (50.9)	96 (50.0)
55-59	60 (31.3)	19 (11.4)	35 (18.2)
⩾ 60	47 (24.5)	63 (37.7)	61 (31.8)
Hepatitis infection			
None	0 (0.0)	0 (0.0)	192 (100.0)
HBV+ and HCV-	74 (38.5)	74 (44.3)	0 (0.0)
HBV- and HCV+	109 (56.8)	88 (52.7)	0 (0.0)
HBV+ and HCV+	9 (4.7)	5 (3.0)	0 (0.0)
Years of orthotopic liver transplantation or resection			
1991-1995	27 (14.1)		
1996-2000	60 (31.3)		
2001-2006	102 (54.7)		
Number of nodes			
1	110 (57.3)		
⩾ 2	72 (37.5)		
Unknown	10 (5.2)		
Maximal dimension of nodes (cm)			
⩽ 2	79 (41.2)		
2.1 to ⩽ 3	67 (34.9)		
> 3	37 (19.3)		
Unknown	9 (4.7)		

HCC: Hepatocellular carcinoma; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

represented functionally relevant polymorphisms (PubMed-MEDLINE search) in genes that encoded key proteins involved in hepatocarcinogenesis-related pathways. In particular, the biological processes evaluated (and related genes) were: membrane transport (*ABCB1, ABCC2*), DNA synthesis and repair mechanisms (*MTHFR, TYMS, ERCC1, ERCC2, XRCC1, XRCC3, APE1, hMLH1, hMSH2, hOOG1*), detoxifying systems (*CYP3A4, CYP3A5, CYP17A1, CYP2D6*), and oxidative stress response (*GST-M1, GST-T1, GST-P1; GSTA1, SOD2*).

All the genes, variants, and assays applied are listed in Supplementary Table 1. For pyrosequencing, we used PSQ96MA (Qiagen, Hilden, Germany). PCR amplifications were performed in an Eppendorf Mastercycler gradient, with TaqGold DNA Polymerase (AB Applied Biosystems, Foster City, CA, United States). The details of the pyrosequencing assays, primer sequences, and PCR conditions are available upon request. Pre-designed TaqMan single-nucleotide polymorphism genotyping assays were conducted with the Applera TaqMan Universal Master mix on an ABI 7900HT Real-time PCR system (AB Applied Biosystems, Foster City, CA, United States), according to the manufacturer's instructions for optimal allelic discrimination. All commercial TaqMan assays were purchased from the Applied Biosystems website (www.appliedbiosystems.com). Detailed protocols for genotyping methods, based on gel electrophoresis and enzymatic digestion, are available upon request.

Statistical analysis

In this study, we assessed associations between genetic markers and the individual risk of developing HCC. In healthy controls, deviation from Hardy-Weinberg equilibrium was tested for each polymorphism with the χ^2 test, and no deviation was found ($P < 0.05$).

The odds ratio (OR) and 95%CI were estimated with unconditional logistic regression, which was controlled for matching variables. We also investigated three genetic models (dominant, recessive, and additive) for associations, and we reported the most statistically significant one, based on the Wald χ^2-test. Statistical significance was set at $P < 0.05$ (two-sided). The analyses were performed with SAS 9.2, and a statistical review of the study was performed by a biomedical statistician.

Recursive partitioning was performed to elucidate high-order relationships among genetic factors, when stratifying patients between HCC cases and HBV/HCV group. The analysis was carried out with the five genetic variants that were identified in the present study as significant predictors of HCC risk and with the previously described *UGT1A* markers (*i.e., UGT1A1**28, *UGT1A9**22, *UGT1A7**3)[14].

RESULTS

Patient characteristics

The demographic, clinical, and serological characteristics of the 192 patients with HCC and the two control groups are reported in Table 1. The predominant gender was male (86.5% of patients with HCC, 84.4% of patients in the HBV/HCV group, and 86.5% of healthy subjects). The median ages at the time of enrolment were 56, 54 and 55 years for the HCC, HBV/HCV, and healthy groups, respectively. The HCC cohort was clinicopathologically homogeneous; all patients fulfilled the surgical intervention criteria (*i.e.,* T1/T2 primary tumor stage, evaluated with the TNM scale; well or moderately poorly differentiated grade at diagnosis; and no detectable vascular invasion).

Genetic polymorphisms and HCC risk

The average genotype call rates were 0.98 (range: 0.92-1.00), 1.00 (range: 0.99-1.00), and 0.99 (range: 0.95-1.00) for the HCC, HBV/HCV, and healthy groups, respectively. All case and control samples were included in the study, because they all reached the fixed call rate threshold of 90%.

HCC *vs* HBV/HCV group: Considering that chronic HBV and/or HCV infections represent a major risk factor for HCC development, a case/control analysis was initially performed to compare the genotype frequency distribution between patients with HCC and individuals

Table 2 Distribution of gene polymorphisms in patients with hepatocellular carcinoma, in hepatitis B/hepatitis C -positive patients, and in healthy controls *n* (%)

Gene	SNP	Genotype	HCC group	HBV/HCV group	Healthy group
ERCC1	rs3212986	GG	126 (67.7)	82 (49.1)	91 (47.4)
		GT	44 (23.7)	78 (46.7)	81 (42.2)
		TT	16 (8.6)	7 (4.2)	20 (10.4)
XRCC3	rs1799794	AA	128 (66.7)	108 (65.1)	137 (71.7)
		AG	52 (27.1)	55 (33.1)	49 (25.7)
		GG	12 (6.3)	3 (1.8)	5 (2.6)
GST-P1	rs1138272	CC	172 (92.5)	139 (83.2)	182 (94.8)
		CT	13 (7.0)	27 (16.2)	10 (5.2)
		TT	1 (0.5)	1 (0.6)	0 (0.0)
CYP17A1	rs743572	TT	72 (38.1)	39 (23.4)	54 (28.4)
		TC	84 (44.4)	88 (52.7)	91 (47.9)
		CC	33 (17.5)	40 (24.0)	45 (23.7)
ABCB1	rs1128503	CC	54 (28.1)	52 (31.1)	58 (30.2)
		CT	92 (47.9)	93 (55.7)	95 (49.5)
		TT	46 (24.0)	22 (13.2)	39 (20.3)

HCC: Hepatocellular carcinoma; SNP: Single-nucleotide polymorphism; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

without HCC, but with matched HBV/HCV infections. The frequency distributions of variant genotypes were significantly different between cases and controls (Tables 2 and 3) for five polymorphisms. No Hardy-Weinberg disequilibrium among healthy controls was detected ($P > 0.05$), which suggested that it was a representative sampling of the investigated population. The observed genotype frequency distribution in healthy controls was consistent with data published in the literature for a Caucasian population (www.ncbi.nlm.nih.gov/snp).

Specifically, *ERCC1* rs3212986 (OR = 0.46, $P = 0.0005$), *GST-P1* rs1138272 (OR = 0.41, $P = 0.0097$), and *CYP17A1* rs743572 (OR = 0.50, $P = 0.0032$) markers were observed more frequently in the HBV/HCV group than in the HCC group, and they were significantly associated with reduced liver cancer risk, according to the dominant model. In contrast, the frequency of *XRCC3* rs1799794 (OR = 3.70; $P = 0.0461$) and *ABCB1* rs1128503 (OR = 2.06; $P = 0.0111$) markers were higher in the HCC group than in the HBV/HCV group, and they were associated with increased HCC risk, according to the recessive model (Table 3).

Given the molecular and clinicopathological differences between HBV- and HCV- related hepatocarcinogenesis[18], a similar analysis was also carried out in subgroups, divided according to viral status (Table 4). In all cases, the ORs calculated for the HBV- and HCV-positive subgroups were concordant with those obtained for the entire combined group. The association was significant in both sub-groups for *CYP17A1* rs743572 (OR = 0.45, $P = 0.0315$ and OR = 0.51, $P = 0.0384$ in HBV-positive and HCV-positive subgroups, respectively). In contrast, *ABCB1* rs1128503 (OR = 4.18, $P = 0.0048$, HBV-positive subgroup) and *ERCC1* rs3212986 (OR = 0.33, $P = 0.0003$, HCV-positive subgroup) showed significant associations in only one sub-group.

HCC *vs* healthy group: We also conducted a case case/control analysis of the genotype frequency distribution in patients with HCC compared to individuals without HCC or virus infections to evaluate the extent of the predictive value of the selected five markers (Table 3). Two markers were significantly associated with HCC risk. Specifically, *ERCC1* rs3212986 (OR = 0.43, $P < 0.0001$) and *CYP17A1* rs743572 (OR = 0.73, $P = 0.0310$) were associated with reduced HCC risk, according to the dominant and additive models, respectively.

Classification and regression tree analysis: The Classification and Regression Tree (CART) method allowed us to identify 6 subgroups of subjects according to genotype features, each with a different probability of HCC occurrence. Three of the terminal nodes exhibited high-probability (59%-76%), two exhibited intermediate-probability (36% and 40%), and one exhibited low-probability (6%) of developing HCC (Figure 1). The ORs and 95%CIs were calculated for each high/intermediate-probability group, with respect to the reference low-probability group. The group with the highest percentage of HCC cases (group 5) was associated with an approximately 13-fold higher HCC risk compared to the reference low-probability group. The first node of the tree represented the *ERCC1* rs3212986 variant, which significantly interacted with *CYP17A1* rs743572, *GST-P1* rs1138272, and the *UGT1A7**3 marker in the generation of the tree (Figure 1).

DISCUSSION

HCC is a global healthcare problem. It is one of the most common malignancies worldwide, with an increasing incidence in Western developed countries. It represents the second cause of death attributable to cancer[1,2]. The genetic origin of HCC has been a focus of research in the past few years. Some studies found that genetic variants could be predictive of the clinical course of HBV or HCV and an individual's predisposition to developing liver cancer[4-8]. However, those studies were performed mainly in Asian populations; thus, few data are available on Caucasian populations. The ethnicity of the population being studied is a crucial factor in case-control studies, because polymorphism frequency varies greatly with geographical origin. This association could give rise to ethnicity-specific phenotypic effects[4,14]. The present study revealed some novel genetic markers that could be used as early diagnosis indicators for liver cancer susceptibility in Caucasians. The availability of these markers could improve HCC risk stratification algorithms and surveillance programs.

Table 3 Odds ratios and corresponding 95%CI for hepatocellular carcinoma compared to hepatitis B/hepatitis C infected patients and blood donors according to genetic polymorphisms

Gene	SNP	Allelic change	HCC *vs* HBV/HCV			HCC *vs* Healthy		
			Mod	OR (95%CI)[1]	*P* value	Mod	OR (95%CI)[2]	*P* value
ERCC1	rs3212986	G>T	Dom	0.46 (0.30-0.71)	0.0005[b]	Dom	0.43 (0.28-0.65)	< 0.0001[b]
XRCC3	rs1799794	A>G	Rec	3.70 (1.02-13.39)	0.0461[a]	Rec	2.44 (0.84-7.08)	0.1003
GST-P1	rs1138272	C>T	Dom	0.41 (0.21-0.81)	0.0097[b]	Add	1.58 (0.70-3.57)	0.2699
CYP17A1	rs743572	T>C	Dom	0.50 (0.31-0.79)	0.0032[b]	Add	0.73 (0.55-0.97)	0.0310[a]
ABCB1	rs1128503	C>T	Rec	2.06 (1.18-3.61)	0.0111[a]	Dom	1.22 (0.75-1.98)	0.4194

[1]Estimated through logistic regression model, adjusted for sex, age and HBV and/or HCV infection; [2]Estimated through logistic regression model, adjusted for sex and age. [a]$P < 0.05$, [b]$P < 0.01$. HCC: Hepatocellular carcinoma; SNP: Single-nucleotide polymorphism; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

Table 4 Odds ratios and corresponding 95%CI for hepatocellular carcinoma compared to hepatitis B/hepatitis C infected patients according to the viral status

Gene	SNP	Allelic change	Mod	HCC *vs* HBV/HCV			
				HBV-positive		HCV-positive	
				OR (95%CI)[1]	*P* value	OR (95%CI)[1]	*P* value
ERCC1	rs3212986	G>T	Dom	0.59 (0.30-1.15)	0.1231	0.33 (0.18-0.60)	0.0003[b]
XRCC3	rs1799794	A>G	Rec	2.23 (0.39-12.82)	0.3695	6.73 (0.82-55.03)	0.0753
GST-P1	rs1138272	C>T	Dom	0.40 (0.14-1.15)	0.0881	0.41 (0.16-1.02)	0.0558
CYP17A1	rs743572	T>C	Dom	0.45 (0.22-0.93)	0.0315[a]	0.51 (0.27-0.97)	0.0384[a]
ABCB1	rs1128503	C>T	Rec	4.18 (1.55-11.29)	0.0048[b]	1.49 (0.71-3.11)	0.2893

[1]Estimated through logistic regression model, adjusted for sex, age and HBV and/or HCV infection. [a]$P < 0.05$, [b]$P < 0.01$. HCC: Hepatocellular carcinoma; SNP: Single-nucleotide polymorphism; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

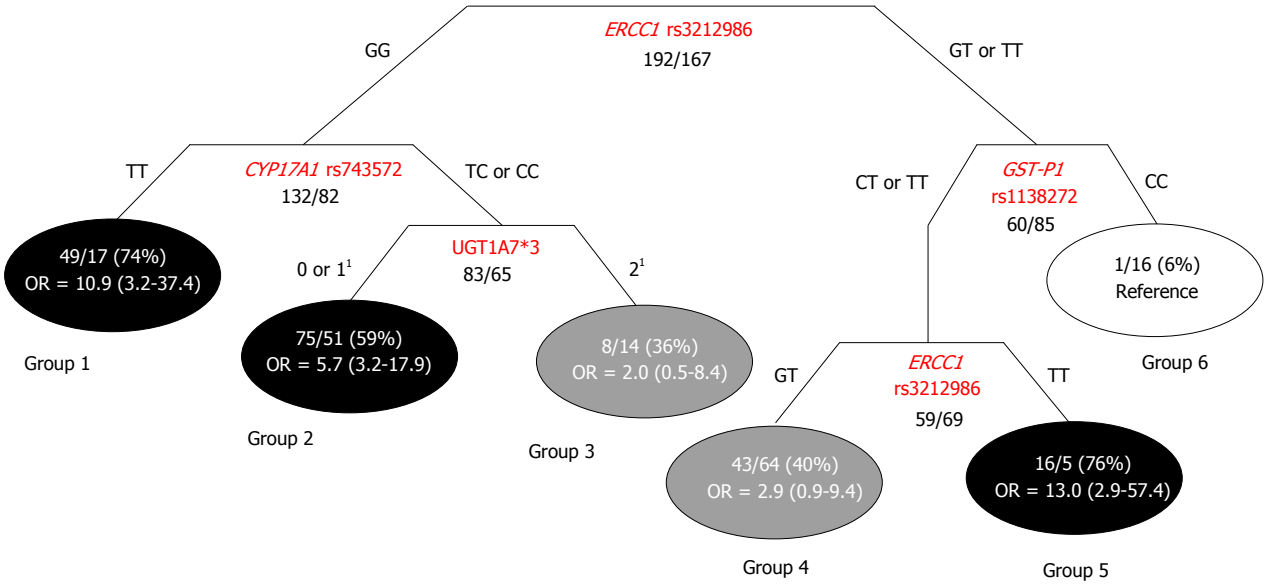

Figure 1 Classification and regression tree representation of the markers combination significantly predictive of hepatocellular carcinoma risk. Fractions indicate the number of HCC cases patients *vs* number of HBV/HCV infected patients (percentage of cases in parenthesis). Black circles represent terminal nodes with high probability to develop hepatocellular carcinoma (HCC) (ratio ⩾ 70%); gray circles represent terminal nodes with intermediate probability to develop HCC (30 ⩽ ratio < 70%); white circles represent terminal nodes with low probability to develop HCC (ratio < 30%). Odds ratios (OR) and 95%CI were calculated for each group in respect to the reference group (lower HCC risk) through logistic regression model, adjusted for sex, age and viral status. [1]Number of alleles carried by the patient. CART: Classification and regression tree; HCC: Hepatocellular carcinoma; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

The main result of this work was the identification of five polymorphisms (*ERCC1* rs3212986, *GST-P1* rs1138272, *CYP17A1* rs743572, *XRCC3* rs1799794, and *ABCB1* rs1128503) that could identify sub-populations of patients among high-risk Caucasian individuals (HBV/HCV-positive), which had differential predispositions for HCC development. The *ABCB1* rs1128503 and *ERCC1* rs3212986 variants displayed different effects, based on the viral status of the patient (HBV- or HCV-positive serology). This result was consistent with the

molecular and clinical-pathological differences between HBV- and HCV-related hepatocarcinogenesis[18]. Two polymorphisms (*ERCC1* rs3212986, and *CYP17A1* rs743572) could also predict HCC risk among the healthy Caucasian population. A CART analysis provided evidence of gene-gene interactions between *ERCC1* rs3212986, *CYP17A1* rs743572, *GST-P1* rs1138272, and the previously described *UGT1A7**3 marker. These interactions defined subgroups of patients with genetic combinations that could further increase the risk of HCC.

The *ERCC1* rs3212986 (8092G>T) polymorphism was demonstrated to be a strong protective factor against liver cancer development in both high-risk (HCV/HBV-positive) and healthy individuals. Furthermore, in an analysis of subgroups with different viral statuses, a particularly significant effect was detected in the HCV-positive subgroup.

ERCC1 is one of several key rate-limiting enzymes in the nucleotide excision repair (NER) pathway. The NER mechanism of the DNA repair system maintains genomic integrity by removing inter-strand DNA crosslinks, and thus, it influences the cell's sensitivity to DNA-adducting carcinogens[19]. A NER-related reduction in DNA repair capacity has been associated with some types of tumors (*i.e.,* lung, head and neck, prostate, glioma, ovarian cancers). Accordingly, *ERCC1* gene variants were shown to influence susceptibility to cancer[20,21]. However, few studies have investigated the implication of the NER biological pathway and *ERCC1* polymorphisms, including rs3212986[20,21], in hepatocarcinogenesis, and no data are available in Caucasian populations. The *ERCC1* rs3212986 variant, located in the 3' untranslated region (3'UTR) of the gene, is thought to increase transcript stability, although further biochemical studies are required to clarify the effect of this polymorphism on gene transcription and protein translation[22,23]. Based on our data, we inferred that the rs3212986 variant allele was associated with elevated ERCC1 expression and/or activity, which could lead to enhanced protection against the accumulation of mutagenic DNA lesions, and consequently, against hepatocarcinogenesis. However, we could not exclude that this marker might also be closely linked to other functionally relevant genetic variants[21] that might alter ERCC activity and/or HCC onset.

The *XRCC3* rs1799794 (4541A>G) marker was associated with elevated HCC susceptibility in a high-risk (HCV/HBV-positive) population. XRCC3 is another DNA repair protein that participates in the DNA double-strand break/recombination repair machinery. XRCC3 was shown to be involved in the pathogenesis of many cancers, including HCC[24,25]. Reduced XRCC3 activity was associated with significantly elevated levels of bulky DNA adducts[26]. Furthermore, epidemiological evidence, derived mainly from studies in an Asian population, have shown that XRCC3 contributed to the repair of DNA damage induced by environmental toxins, such as aflatoxin B1, and that this effect depended on viral status and ethnicity[25,27]. The *XRCC3* rs1799794 variant, located in a regulatory region in the 5'UTR of the gene, was predicted to have a potential effect on protein expression levels[28]. The results of the present study suggested that this polymorphism could reduce the ability to repair DNA adducts, which would contribute to the accumulation of mutagenic lesions, and thus, increase the risk of HCC onset. However, the exact molecular mechanism of the rs1799794 variant is currently unknown, and we could not exclude a linkage with another functional marker[29].

The *GST-P1* rs1138272 (341C>T) polymorphism was found to have a protective effect on liver cancer development in a high-risk HCV/HBV-positive population. GST-P1 belongs to the GST multigene family. It is a phase Ⅱ enzyme involved in the inactivation of electrophilic xenobiotics by conjugation with glutathione[30], which facilitates the excretion of molecules that are potentially toxic to the liver[31]. This enzyme also contributes to protecting liver cells from damage related to reactive oxygen species generated, for instance, by chronic alcohol and tobacco abuse[32]. Several studies have clearly demonstrated that silencing GST-P1 expression by promoter methylation was positively correlated with the incidence of HCC[31] and with virus-related hepatocarcinogenesis[33]. In contrast, in meta-analyses, *GST-P1* genetic polymorphisms (*i.e.,* Ile105Val, rs1695) were not associated with HCC risk[34,35] although the few data available were insufficient for drawing any definite conclusions. The less investigated *GST-P1* rs1138272 polymorphism is a missense variant (Val114Ala) that lies in the electron binding, active domain of the protein. This variant was reported to affect substrate specificity by altering its ability to distinguish between planar and non-planar substrates[36,37]. Our results suggested that this functional change in GST-P1 activity may result in a lower risk of HCC development; further clinical experimental studies are required to clarify the role of the rs1138272 polymorphism in the response of GST-P1 to environmental xenobiotics and oxidative stress, particularly in a virus-infected liver.

The *CYP17A1* rs743572 (-34T>C) polymorphism was found to be a strong protective factor against HCC development in both the high-risk HCV/HBV-positive group and healthy individuals. CYP17A1 is an enzyme that plays a critical role in the biosynthesis of steroid hormones by catalyzing both 17a-hydroxylase and 17, 20-lyase activities[38]. Some data derived from animal models and human epidemiologic studies have demonstrated that estrogens play an essential role in hepatocarcinogenesis; however, the exact mechanism underlying the effect of estrogen levels on HCC development is currently controversial[39]. It was initially suggested that the *CYP17A1* rs743572 variant, located in the 5'UTR of the gene, served as a new Sp1-type (CCACC box) binding site, which provided an additional promoter, and thus, it could increase estrogen biosynthesis[40]. However, subsequent studies could not replicate the phenotypic impact of this

variant on CYP17A1 mRNA expression or estrogen levels[41]. Moreover, the involvement of the rs743572 marker on HCC development was not well defined[42,43]. From the present data, it could be speculated that the rs743572 variant might contribute to changing CYP17A1 activity, and its dependent steroid levels, to provide a protective effect against liver cancer development. Estrogen is known to participate in various biological functions. For example, an increase in sex hormone levels was suggested to suppress the inflammatory processes mediated by pro-inflammatory cytokines[44,45]. Additional work will be required to clarify the functional significance of the rs743572 marker and the molecular mechanism of CYP17A1 in hepatocellular carcinogenesis.

Finally, A*BCB1* rs1128503 (1236C>T) was associated with elevated HCC susceptibility in a high-risk (HCV/HBV-positive) population. Furthermore, an analysis of subgroups with different viral statuses showed that a particularly significant effect was detected in the HBV-positive subgroup. The *ABCB1* gene encodes MDR-1, also known as P-glycoprotein (P-gp). MDR-1 belongs to the ATP-binding cassette (ABC) transporter superfamily[46,47]. This membrane efflux pump is widely expressed in various organs, including the liver, and it is physiologically involved in the protection of normal tissue from environmental and endogenous toxins[46-48]. MDR1 is implicated in hepatic clearance of oxidative and other genotoxic products generated by chronic inflammation induced by HCC risk factors. Thus, it contributes to preventing the initiation of hepatocarcinogenesis[49,50]. Many studies have described MDR1 over-expression in various tumor tissues, including liver cancer[51]; thus, this membrane carrier and its functional genetic variants are good candidates for influencing liver susceptibility to HCC. *ABCB1* rs1128503 is a synonymous variant (Gly412Gly) of a residue located in the cytoplasmic domain of the transporter. It is reported to affect mRNA stability[52], but the exact phenotypic impact of this variant on protein expression/activity remains to be defined[53,54]. Several studies, performed in Asian populations, have investigated associations between *ABCB1* polymorphisms (including rs1128503) and the risk of developing HCC. A recent meta-analysis[55] showed that polymorphic alleles of the *ABCB1* gene significantly increased HCC risk. In analyses of subgroups with different types of variations, both synonymous and non-synonymous polymorphisms were associated with elevated liver cancer susceptibility. In particular, synonymous variants (*i.e.*, rs1128503) were suggested to be correlated with changes in transcriptional and translational processes. In addition, genetic markers located in the cytoplasmic domain of the transporter, like rs1128503, were shown to have the greatest predictive power for HCC risk. These data, although obtained in Asian cohorts, were consistent with the result of the present study, which provided the first evidence of a role of the *ABCB1* rs1128503 marker in HCC risk in a Caucasian population.

Due to the multifactorial nature of hepatocarcinogenesis, an exploratory CART analysis was applied to investigate interactions between the genetic factors identified as predictors of liver cancer susceptibility in the high-risk population (HBV/HCV-infected group). The polymorphism in the gene of the DNA repair system (*i.e., ERCC1* rs3212986) was demonstrated to significantly interact with variants of genes that encoded phase Ⅰ (*CYP17A1* rs74357) and Ⅱ (*GST-P1* rs1138272, *UGT1A7**3) metabolic enzymes; these interactions were important in defining individual HCC risk. The CART analysis generated a distinctive clustering of subjects, according to different probabilities of developing liver cancer. These data pointed out that previously acknowledged predictive markers, such as the *UGT1A*3* polymorphism[14], should be considered in association with novel identified genetic variants for properly defining the complex trait of HCC susceptibility. In particular, *ERCC1* rs3212986 was shown to be the most relevant polymorphism for HCC risk stratification, because it appeared twice in the generated tree. This marker significantly interacted with three additional genetic variants (*CYP17A1* rs74357, *GST-P1* rs1138272, and *UGT1A7**3) in defining individual susceptibility. The analysis of gene-gene interactions and the network shape uncovered specific genetic-context effects that could not be detected in evaluations of single markers. These findings highlighted the importance of combining genetic markers to obtain a better representation of the biological cooperation among multiple pathways in hepatocarcinogenesis.

The present study had some limitations. First, our case-cohort comprised a highly specific group of patients with HCC (patients that received OLT). These patients had definite clinical features that could limit the generalizability of the present findings. The validity of the present results was also limited to Caucasian individuals; the results remain to be verified in other populations, like Asians or Africans. Second, our work did not evaluate other confounding etiological factors, such as smoking and alcohol intake. However, the use of a control group matched to HCC cases for HBV/HCV viral status permitted us to control for this crucial confounding variable. Third, the exact functional and phenotypic properties of some of the genetic markers that we identified as predictors of HCC risk have not been well established. Moreover, we could not exclude any linkages with other functionally relevant genetic variants. Finally, considering the retrospective and exploratory nature of our case-control analysis, the results obtained should be interpreted with caution. Our findings will require independent validation in future prospective, large-scale epidemiological investigations that are performed in well-defined, homogeneous populations.

The validation of the present findings could have important clinical implications for improving HCC

management. The novel identified genetic markers and their gene-gene interactions, in combination with other well-known serum biomarkers (*e.g.,* alphafetoprotein) and diagnostic imaging (*e.g.*, ultrasonography)[9], could contribute to improving the HCC risk stratification algorithms and surveillance programs, particularly for patients at risk. Those improvements might facilitate early cancer detection and might lead to potentially curative therapies. Furthermore, an enhanced understanding of the genetic factors associated with the evolution of HCV infection could be essential for the optimized use of the recently developed, but highly expensive, anti-HCV DAAs[12,13]. Thus, improved screening could assist the health care system in containing costs and ensuring the most clinically appropriate treatments.

The present study identified five variants in genes that encode DNA repair system proteins (*i.e.,* ERCC1, XRCC3), phase Ⅰ (*i.e.,* CYP17A1) and Ⅱ (*i.e.,* GST-P1) enzymes, and a membrane transporter (*i.e.,* MDR-1). Our data suggested that these genes might play a role in predicting HCC susceptibility in a high-risk (HBV-HCV infected) population and in a healthy Caucasian population. The CART analysis pointed out the potential value of studying of gene-gene interactions for the stratification of individuals at different risks of developing HCC. Although validation is required, these preliminary results might have important clinical implications in the management of HCC prevention and patient care.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) represents a global health problem, due to its high incidence and mortality rates. Although the etiologies of this neoplasia are well-defined, the majority of patients are diagnosed at advanced-stage disease, which excludes them from receiving a potentially curative therapy. Hence, they urgently need the definition of new biomarkers of HCC susceptibility that can permit early cancer detection, particularly for individuals in a high-risk population [hepatitis B (HBV)/hepatitis C (HCV)-positive]. Moreover, in developed countries (*i.e.,* United States and Europe), HCV-related HCC can account for most liver cancers; therefore, increasing our knowledge of the genetic contribution to the clinical course of viral infections could be essential in optimizing the use of the newly developed, but highly expensive, direct acting antiviral treatments.

Research frontiers

Accumulating data derived from candidate-gene investigations and genome-wide association studies have highlighted the possible role of genetic variants as significant predictors of HCC susceptibility and as determinants of the clinical course of HBV or HCV infections. However, the high heterogeneity among published works has prevented drawing definite conclusions. Furthermore, the majority of case-control investigations were performed in Asian populations; thus, replication studies with different ethnicities are warranted. Consequently, there is a critical need to intensify research efforts in identifying effective molecular biomarkers predictive of individual HCC risk in a homogeneous, well-defined cohort study, with particular attention to the (currently) poorly investigated Caucasian population.

Innovations and breakthroughs

This work uncovered five markers in genes that encode key proteins involved in pathways underlying hepatocarcinogenesis (*ERCC1*-rs3212986, *GSTP1*-rs1138272, *CYP17A1*-rs743572, *XRCC3*- rs1799794, and *ABCB1*-rs112850). These markers were predictive of individual HCC susceptibility in a high-risk Caucasian population (HBV/HCV-positive). Two variants also exhibited serology-specific effects when a subgroup analysis was performed in patients stratified according to HBV or HCV positive serology. That analysis showed that *ABCB1* rs1128503 specifically affected the HBV-positive subgroup and *ERCC1* rs3212986 specifically affected the HCV-positive subgroup. Among the five markers identified, *ERCC1* rs3212986 and *CYP17A1* rs743572 retained predictive value for HCC risk among healthy individuals. In addition, they found gene-gene interactions between *ERCC1* rs3212986, *CYP17A1* rs743572, *GST-P1* rs1138272, and the previously described *UGT1A7**3 marker, which were included in the definition of the complex trait of HCC susceptibility.

Applications

The definition of genetic traits that confer susceptibility to liver carcinogenesis is of potential clinical interest. This information can facilitate improvements in preventive, diagnostic, and therapeutic strategies for HCC management. If validated, these markers and their interactions should be considered for improving HCC risk stratification algorithms and surveillance programs. Their inclusion in screening could facilitate early cancer detection and might lead to potentially curative therapies. For patients with HCV infections, the biomarkers we discovered could also contribute to optimizing the use of recently developed, but highly expensive, anti-viral therapies. These findings address a great challenge in HCC management and might help enhance care for patients with HCC.

Terminology

Polymorphism: a variation in the DNA sequence that occurs in a population at a frequency of 1% or higher; CART: classification and regression tree analysis; this is a statistical method, based on recursive partitioning, that is applied to elucidate potential high-order gene-gene and gene-environmental relationships among discriminating factors that contribute to a specific end-point; ERCC1: a key rate-limiting enzyme that acts in the nucleotide excision repair pathway. This pathway represents a DNA repair mechanism designed to maintain genomic integrity by removing inter-strand DNA crosslinks; XRCC3: a DNA repair protein that participates in DNA double-strand break/recombination repair machinery; GST-P1: a phase Ⅱ enzyme involved in the inactivation of electrophilic xenobiotics; it acts by conjugating with glutathione, which facilitates excretion of xenobiotics from the body; CYP17A1: an enzyme that plays a critical role in the in the biosynthesis of steroid hormones; MDR-1 (encoded by *ABCB1*): A widely expressed membrane efflux pump, physiologically involved in the protection of normal tissue from environmental and endogenous toxins.

Peer-review

The aim of this study was to find novel genetic markers that could contribute in predicting HCC susceptibility in Caucasians. The authors identified five genetic markers in key pathways linked to hepatocarcinogenesis (ERCC1-rs3212986, GSTP1-rs1138272, CYP17A1-rs743572, XRCC3- rs1799794, ABCB1-rs112850) that could predict the individual HCC susceptibility, especially in high-risk (HBV/HCV-infected) Caucasians population. The article provides the novel informations that could contribute to improving HCC surveillance, allowing early cancer diagnosis and possible curative therapy.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prognostic value of lymphovascular invasion in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma

Bei Li, Xian-Ze Xiong, Yong Zhou, Si-Jia Wu, Zhen You, Jiong Lu, Nan-Sheng Cheng

Bei Li, Xian-Ze Xiong, Yong Zhou, Si-Jia Wu, Zhen You, Jiong Lu, Nan-Sheng Cheng, Department of Biliary Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

ORCID number: Bei Li (0000-0002-5310-5370); Xian-Ze Xiong (0000-0001-6699-3787); Yong Zhou (0000-0003-2412-3745); Si-Jia Wu (0000-0002-4236-0800); Zhen You (0000-0002-1196-8901); Jiong Lu (0000-0002-5541-9808); Nan-Sheng Cheng (0000-0002-9334-5715).

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Correspondence to: Nan-Sheng Cheng, MD, PhD, Department of Biliary Surgery, West China Hospital of Sichuan University, No. 37, Guoxue Alley, Chengdu 610041, Sichuan Province, China. nanshengcheng@yeah.net

Telephone: +86-28-85422465

Fax: +86-28-85422465

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Abstract

AIM

To assess the prognostic value of lymphovascular invasion (LVI) in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma (HC) patients.

METHODS

A retrospective analysis was performed on 142 consecutively recruited type Ⅳ HC patients undergoing radical resection with at least 5 years of follow-up. Survival analysis was performed by the Kaplan-Meier method, and the association between the clinicopathologic variables and survival was evaluated by log-rank test. Multivariate analysis was adopted to identify the independent prognostic factors for overall survival (OS) and disease-free survival (DFS). Multiple logistic regression analysis was performed to determine the association between LVI and potential variables.

RESULTS

LVI was confirmed histopathologically in 29 (20.4%) patients. Multivariate analysis showed that positive resection margin (HR = 6.255, 95%CI: 3.485-11.229, $P < 0.001$), N1 stage (HR = 2.902, 95%CI: 1.132-7.439, $P = 0.027$), tumor size > 30 mm (HR = 1.942, 95%CI: 1.176-3.209, $P = 0.010$) and LVI positivity (HR = 2.799, 95%CI: 1.588-4.935, $P < 0.001$) were adverse

prognostic factors for DFS. The independent risk factors for OS were positive resection margin (HR = 6.776, 95%CI: 3.988-11.479, $P < 0.001$), N1 stage (HR = 2.827, 95%CI: 1.243-6.429, $P = 0.013$), tumor size > 30 mm (HR = 1.739, 95%CI: 1.101-2.745, $P = 0.018$) and LVI positivity (HR = 2.908, 95%CI: 1.712-4.938, $P < 0.001$). LVI was associated with N1 stage and tumor size > 30 mm. Multiple logistic regression analysis indicated that N1 stage (HR = 3.312, 95%CI: 1.338-8.198, $P = 0.026$) and tumor size > 30 mm (HR = 3.258, 95%CI: 1.288-8.236, $P = 0.013$) were associated with LVI.

CONCLUSION

LVI is associated with N1 stage and tumor size > 30 mm and adversely influences DFS and OS in type Ⅳ HC patients.

Key words: Bismuth-Corlette classification; Disease-free survival; Lymphovascular invasion; Overall survival; Hilar cholangiocarcinoma

Core tip: Previous studies have reported that lymphovascular invasion (LVI) provokes an adverse impact on the long-term survival of several malignances, including breast, gastric, and esophageal carcinoma, among many others. However, the correlation between LVI and hilar cholangiocarcinoma remains unclear. In our study, LVI was found to be an independent risk factor for overall survival and disease-free survival in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma patients. To our knowledge, this report indicates for the first time that LVI is an adverse predictor of long-term survival in the setting of type Ⅳ hilar cholangiocarcinoma.

Li B, Xiong XZ, Zhou Y, Wu SJ, You Z, Lu J, Cheng NS. Prognostic value of lymphovascular invasion in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma. *World J Gastroenterol* 2017; 23(36): 6685-6693 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6685.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6685

INTRODUCTION

Hilar cholangiocarcinoma (HC), also known as Klatskin tumor, is a neoplasia arising from the biliary epithelium at the common hepatic duct bifurcation and may extend to the intrahepatic biliary tree and liver[1,2]. The only clinical approach that is considered to provide patients with an opportunity for a curative outcome and importantly long-term survival is radical surgical resection[2-6]. The Bismuth-Corlette classification is the most widely used preoperative system of evaluation that can predict resectability of the lesion and assist in the design of an appropriate surgical approach. A Bismuth type Ⅳ lesion is defined as a tumor that can invade the secondary biliary radicals of both hepatic ducts. During the past decades, accompanied liver resection has been increasingly but gradually recognized as the mainstay of surgical approaches for targeting a Bismuth type Ⅳ tumor[7-9], however, there are relatively few studies that have reported in any detail the factors that might affect long-term survival of type Ⅳ HC patients. The over-arching aim of the current study was to identify prognostic factors for long-term survival of patients following radical surgery for type Ⅳ HC patients, especially in the setting of lymphovascular invasion (LVI).

MATERIALS AND METHODS

Patient selection

One hundred and forty-two consecutive patients who underwent radical resection for a pathological diagnosis of type Ⅳ HC at the West China Hospital between January 2000 and February 2012 were enrolled in this study and then reviewed retrospectively. The inclusion criteria included the following: (1) patients who were confirmed with Bismuth type Ⅳ HC by pathological examination; and (2) patients who had undergone radical resection (R0 and R1 resection). The exclusion criteria included the following: (1) patients with gallbladder or intrahepatic cholangiocarcinoma extending to the hilum; (2) presence of a recurrent or metastatic tumor; and (3) R2 resection.

Preoperative workup

Preoperative assessment consisted of acquiring a medical history, physical examination, laboratory tests and radiographic analyses. All patients were evaluated by contrast-enhanced ultrasound, contrast-enhanced computed tomography or magnetic resonance cholangiography with magnetic resonance cholangiopancreatography with the intention of determining the Bismuth type, the location and extent of the tumor. Biliary drainage, including endoscopic retrograde cholangiopancreatography (ENBD) and percutaneous transhepatic cholangiodrainage (PTCD), was applied in the setting of patients presenting with obstructive jaundice that exceeded 85 μmol/L total bilirubin.

Surgical characteristics of the patients

Based on the relative location and extent of the tumor, different types of resection were performed, which included extrahepatic bile duct resection and *en bloc* resection of the caudate lobe combined with left hemihepatectomy, right hemihepatectomy and trisectionectomy. In addition, standard regional lymph node dissection should be performed. However, under conditions where tumor metastases to the distant lymph nodes was confirmed during surgery, the surgical intervention was abandoned. According to

the American Joint Committee on Cancer (AJCC, 7th edition), the location of regional lymph nodes was defined as follows: along the common bile duct, cystic duct, portal vein and proper hepatic artery[10]. Vascular resection and reconstruction was only performed when vessels could not be detached from the tumor.

Pathological examination

The pathological evidence of cancer was determined by examination of paraffin sections. All included Bismuth type Ⅳ HC cases were histopathologically confirmed by an experienced pathologist. The presence of tumor emboli within peritumoural endothelial lined spaces was defined as LVI. Resection margin was defined as ductal (*i.e*., proximal and distal ducts) and with evidence of radial margins. The radial margin was defined as the vertical margin between the tumor edge and dissected periductal structures (*e.g*., liver parenchyma, blood vessels and adjacent fat tissues). An R0 resection was defined as the presence of a microscopically tumor-free resection margin. An R1 resection was defined as microscopic evidence of tumor tissue at the resection margin, and an R2 resection was defined as macroscopic evidence of tumor tissue at the resection margin. In this study, radical resection was defined as an R0 and R1 resection, a negative resection margin indicated R0 resection, and a positive resection margin indicated an R1 resection.

Follow-up

Whether or not chemotherapy and radiotherapy can benefit HC patients was controversial. None of the patients received postoperative routine chemotherapy or radiotherapy. All enrolled patients had routine follow-ups every 3 mo in the first year and every 6 mo subsequently until at least 5 years after the surgery. The tumor markers [serum levels of carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen], liver functions and ultrasonography were conducted. If there was a suspicion of recurrence, contrast-enhanced computed tomography or magnetic resonance imaging was further performed. Tumor recurrence was diagnosed on the basis of the combined findings of typical radiological appearance, quantification of CA19-9 levels, and clinical presentation. The date of the first suspicious radiological finding was recorded as the date of initial disease recurrence.

Statistical analysis

Patient data were retrospectively collected and statistical analyses were performed with SPSS version 19.0 (SPSS Inc. Chicago, IL, United States). Survival was described using the Kaplan-Meier method and differences between subgroups were reviewed by the log-rank test. Multivariate analysis for prognostic factors was performed using a Cox proportional hazards model to analyze variables whose *P*-value was less than 0.1 in the univariate analysis. Multiple logistical regression analysis was performed to determine the association between LVI and potential variables. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Patients' characteristics and operative outcomes

Patients' characteristics and operative outcomes are shown in Table 1. Altogether 142 patients had a radical resection for type Ⅳ HC, including 75 men and 67 women with a median age of 59 years (range: 23-78). Pre-operative biliary drainage was carried out in 105 of the 123 obstructive jaundice (total bilirubin > 85 μmol/L) patients, wherein 71 patients underwent PCTD and 34 patients underwent ENBD. Preoperative portal vein embolization was performed in 6 patients.

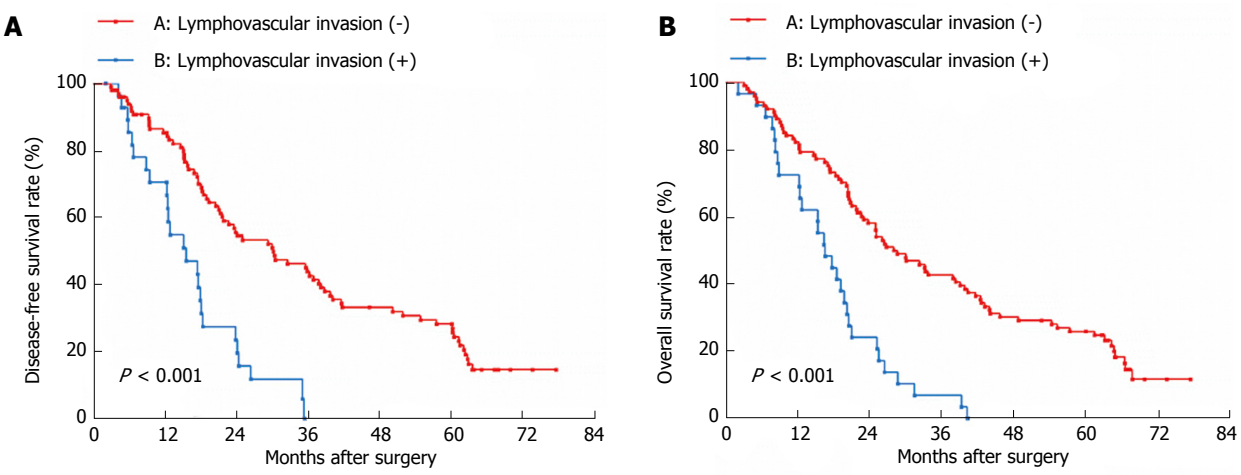
Radical resection included extrahepatic bile duct resection and *en bloc* resection of the caudate lobe combined with left hemihepatectomy (n = 73, 51.4%), extended left hemihepatectomy (n = 5, 3.5%), left trisectionectomy (n = 6, 4.2%), right hemihepatectomy (n = 51, 35.9%), extended right hemihepatectomy (n = 5, 3.5%), and right trisectionectomy (n = 2, 1.4%). Regional lymph node dissection was conventionally performed. The R0 resection rate was 75.6%.

Clinicopathological variables influencing DFS and OS

As shown in Table 2, potential factors that might influence the DFS and OS were analyzed. Univariate analysis demonstrated that age (P = 0.039), preoperative ALB (P = 0.005), resection margin (P < 0.001), histologic grade (P = 0.023), T stage (P = 0.004), N stage (P < 0.001), AJCC stage (P < 0.001), LVI (P < 0.001), tumor size (P < 0.001), portal vein invasion (P = 0.003) and hepatic artery invasion (P = 0.008) significantly influenced DFS. By contrast, patient gender, preoperative CA19-9, surgical method, perineural invasion and transfusion did not significantly influence DFS. Preoperative ALB (P = 0.009), resection margin (P < 0.001), histologic grade (P = 0.026), T stage (P = 0.001), N stage (P < 0.001), AJCC stage (P < 0.001), LVI (P < 0.001), tumor size (P < 0.001), portal vein invasion (P = 0.002) and hepatic artery invasion (P = 0.013), but not patient age, gender, preoperative CA19-9, surgical methods, perineural invasion or transfusion, significantly influenced the OS. Multivariate analysis indicated that positive resection margin, higher N stage, tumor size > 30 mm and LVI were adverse factors that influenced DFS and OS.

Association between LVI and N stage and tumor size

LVI was confirmed histologically in 29 (20.4%) of the 142 patients. Table 3 shows the association of LVI with tumor size, N stage and AJCC stage. On univariate analysis, N stage (P < 0.001) and tumor size (P = 0.001), but not AJCC stage, were significantly associated with LVI. Multivariate analysis using a



No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	101	79	49	37	27	23
B	29	19	6	0	0	0

No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	101	83	49	41	29	24
B	29	21	7	2	0	0

Figure 1 Disease-free survival and overall survival based on lymphovascular invasion in type Ⅳ hilar cholangiocarcinoma patients with radical resection. A: The 5-yr DFS rate in the LVI negative group was significantly higher than that in the LVI positive group; B: The 5-yr OS rate in the negative group was significantly higher than that in the LVI positive group. LVI: Lymphovascular invasion; DFS: Disease-free survival; OS: Overall survival.

Table 1 Demographics and operative outcomes

Characteristic	*n* (%)
Age (yr)[1]	59 (23-78)
Gender	
Male	75 (52.8)
Female	67 (47.2)
Albumin level (g/L)[2]	36.74 ± 4.93
CA19-9	
> 200	82 (57.7)
< 200	60 (42.3)
Radiological examination	
Contrast-enhanced US	8 (5.6)
Contrast-enhanced CT	43 (30.3)
MRI + MRCP	91 (64.1)
Operative time (min)[2]	429.47 ± 134.19
Blood loss (ml)[1]	600 (180-4000)
Transfusion	76 (53.5)
R0 resection	107 (75.4)
Differentiation degree	
Well differentiated	14 (9.9)
Moderately differentiated	90 (63.4)
Poorly differentiated	38 (26.8)
Perineutral invasion positive	69 (48.6)
N stage	
N0	89 (62.7)
N1	53 (37.3)
Tumor diameter (mm)[1]	30 (12-55
Hospital stay[1]	19 (5-115)

[1]Parameters are presented as median and range; [2]Parameters are presented as mean ± SD.

logistic regression model indicated that N1 stage (P = 0.026) and tumor size > 30 mm were significant factors that were associated with LVI in Bismuth type Ⅳ HC. DFS and OS based on LVI status are shown in Figure 1. The 5-year DFS rate in the LVI negative group was significantly higher than that in the LVI positive group (22.8% *vs* 0%, P < 0.001) as was the OS (23.8% *vs* 0%, P < 0.001). Based on the LVI status, N0 stage patients and patients with tumor size ≤ 30 mm were divided into two subgroups, respectively. In the N0 stage subgroups, the 5-year DFS rate in the LVI negative group was significantly higher than that in the LVI positive group (31.0% *vs* 0%, P = 0.002), and the 5-year OS rate in the LVI negative group was also significantly higher than that in the LVI positive group (32.4% *vs* 0%, P = 0.001). In the tumor size ≤ 30 mm subgroups, the 5-year DFS rate in the LVI negative group was significantly higher than that in the LVI positive group (31.8% *vs* 0%, P = 0.003), and the 5-year OS rate in the LVI negative group was significantly higher than that in the LVI positive group (34.3% *vs* 0%, P = 0.006).

DISCUSSION

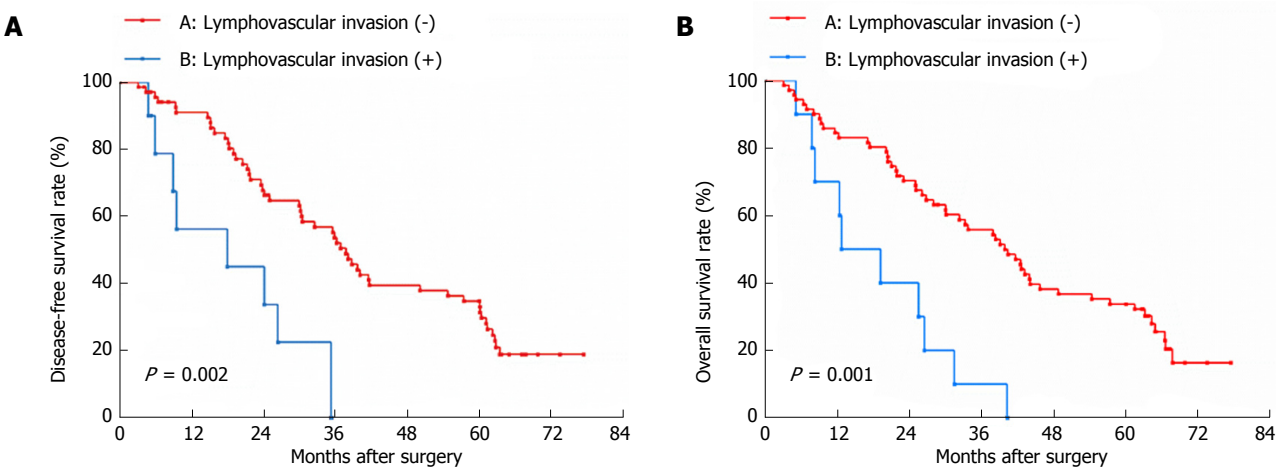
LVI is recognized as a dismal prognostic factor for OS in patients with breast cancer, colorectal cancer, and esophageal cancer[11-14]. However, no studies have yet been published on whether LVI affects the prognosis of Bismuth type Ⅳ HC. Thus, the current study was undertaken to clarify the significance of LVI in type Ⅳ HC patients who had a radical resection. LVI is defined as the involvement of arterial vessels, venules and lymphatic channels[11], but it is histologically difficult to distinguish, and the American Joint Committee on Cancer/Union Internationale Contre Cancer staging guidelines use the term lymphovascular to refer to those structures[11]. Furthermore, LVI can be confirmed specifically on HE-stained specimens[13]. In our series, LVI was confirmed histopathologically in 29 patients.

It is well recognized that resection margin status is

Table 2 Potential prognostic factors for disease-free survival and overall survival after radical resection for type Ⅳ hilar cholangiocarcinoma patients excluding operative mortality *n* (%)

Variable	Disease-free survival				Overall survival			
	5-yr survival	Univariate analysis	Multivariate analysis		5-yr survival	Univariate analysis	Multivariate analysis	
		P value	HR (95%CI)	*P* value		*P* value	HR (95%CI)	*P* value
Gender		0.682				0.965		
Male	13 (18.8)				14 (20.3)			
Female	10 (16.4)				10 (16.4)			
Age (yr)		0.039	1.291 (0.680-2.453)	0.435		0.230		
< 65	19 (19.2)				20 (20.2)			
> 65	4 (12.9)				4 (12.9)			
CA19-9		0.287				0.301		
< 200	13 (23.2)				14 (25)			
> 200	10 (13.5)				10 (13.5)			
ALB (g/L)		0.005	0.669 (0.413-1.085)	0.103		0.009	0.760 (0.492-1.173)	0.215
< 35	3 (7.1)				3 (7.1)			
> 35	21 (23.9)				20 (22.7)			
Surgical method		0.847				0.684		
Left-sided hepatectomy	13 (15.1)				13 (15.1)			
Right-sided hepatectomy	10 (17.2)				11 (19.0)			
Resection margin		< 0.001	6.255 (3.485-11.229)	< 0.001		< 0.001	6.776 (3.988-11.479)	< 0.001
Positive	0				0			
Negative	23 (23.7)				24 (24.7)			
Histologic grade		0.023	1.594 (0.994-2.554)	0.053		0.026	1.294 (0.830-2.017)	0.256
Well/moderate	20 (21.5)				21 (22.6)			
poor					3 (8.1)			
Perineural invasion		0.211				0.417		
Present	14 (22.6)				15 (24.2)			
Absent	9 (13.2)				9 (13.2)			
T stage		0.004	1.582 (0.390-6.415)	0.521		0.001	2.399 (0.734-7.836)	0.147
T1/2	22 (22)				21 (21)			
T3/4	2 (6.7)				2 (6.7)			
N stage		< 0.001	2.902 (1.132-7.439)	0.027		< 0.001	2.827 (1.243-6.429)	0.013
N0	22 (27.2)				23 (28.4)			
N1	1 (2.0)				1 (2.0)			
AJCC stage		< 0.001	0.673 (0.289-1.567)	0.358		< 0.001	0.844 (0.351-2.028)	0.704
Stage Ⅰ/Ⅱ	20 (32.8)				21 (34.4)			
Stage Ⅲ/Ⅳ	3 (4.9)				3 (4.9)			
Lymphovascular invasion		< 0.001	2.799 (1.588-4.935)	< 0.001		< 0.001	2.908 (1.712-4.938)	< 0.001
Present	0					0.000		
Absent	23 (22.8)				24 (24.8)			
Tumor size (mm)		< 0.001	1.942 (1.176-3.209)	0.010		< 0.001	1.739 (1.101-2.745)	0.018
≤ 30	15 (20)				16 (21.3)			
> 30	8 (14.5)				8 (14.5)			
Portal vein invasion		0.003	1.759 (0.534-5.800)	0.353		0.002	1.130 (0.408-3.127)	0.815
Present	1 (4.2)				1 (4.2)			
Absent	22 (20.8)				23 (21.7)			
Hepatic invasion		0.008	1.499 (0.612-3.668)	0.376		0.013	1.196 (0.526-2.719)	0.669
Present	1 (9.1)	1 (9.1)			1 (9.1)			
Absent	22 (18.5)	22 (18.5)			23 (19.3)			
Transfusion		0.445				0.199		
Yes	11 (16.4)				11 (16.4)			
No	12 (19.0)				13 (20.6)			

the most important factor affecting long-term survival outcomes of HC patients[14,15], and positive resection margin remains a major dismal prognostic factor for type Ⅳ HC patients. Furthermore, since type Ⅳ HC had been considered unresectable[16], radical resection performed in such patients is technically challenging. Bracingly, in our center the R0 resection rate is 75.4%. Regional lymph node involvement represents another important dismal prognostic factor in HC patients who had undergone radical resection[17,18]. Concordantly, the multivariate analysis showed that lymph node metastasis was an adverse factor affecting DFS and OS in type Ⅳ HC. Furthermore, DeOliveira *et al*[19] proposed a new staging system, in which tumor size > 30 mm was defined as the T3 stage. The choice of 30 mm as a cutoff value for T3 is based on increasing evidence that the smaller the tumor, the better the prognosis[20,21]. Our results indicated that tumor size > 30 mm had an unfavorable impact on both DFS and OS. More importantly, we found that LVI is a significant adverse prognostic factor influencing DFS and OS in multivariate analysis. To our knowledge, no other



No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	71	59	44	34	25	22
B	10	5	4	0	0	0

No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	71	60	49	39	26	23
B	10	7	4	0	0	0

Figure 2 Disease-free survival and overall survival based on lymphovascular invasion in type Ⅳ hilar cholangiocarcinoma patients without lymph node metastasis. A: The 5-yr DFS rate in the LVI negative group was significantly higher than that in the LVI positive group; B: The 5-yr OS rate in the negative group was significantly higher than that in the LVI positive group. LVI: Lymphovascular invasion; DFS: Disease-free survival; OS: Overall survival.

reports have shown the correlation between LVI and the prognosis of type Ⅳ HC.

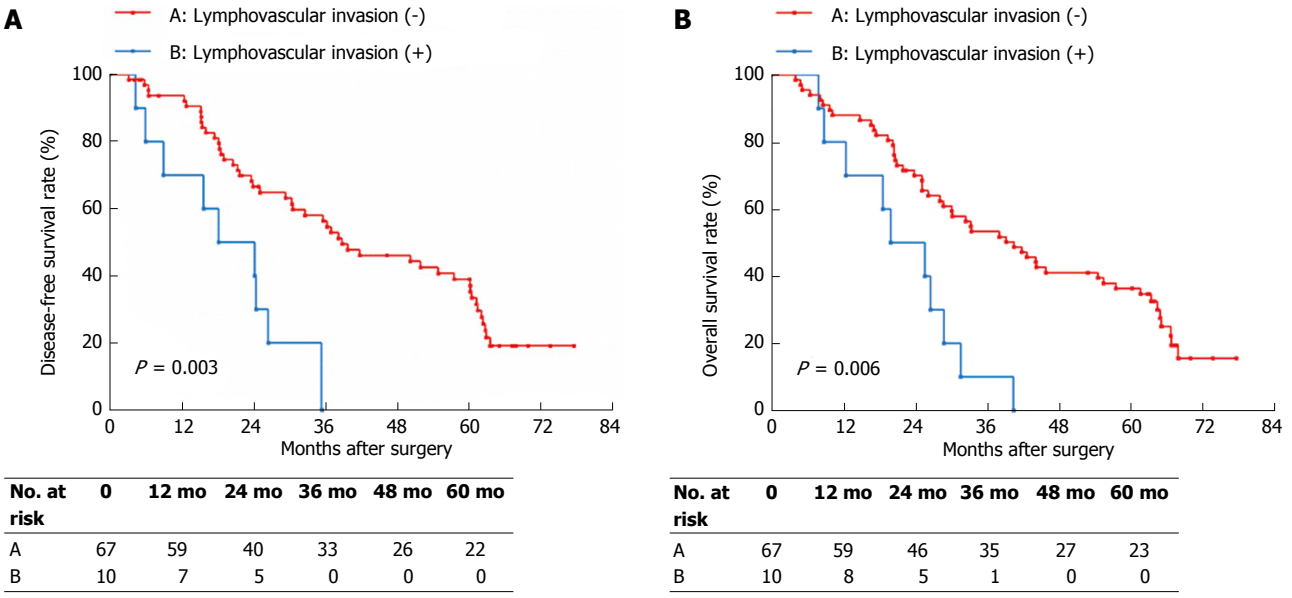
Previous studies suggested that LVI may interact with other adverse risk factors, which then have a dismal impact on the OS of esophageal cancer patients[22,23]. Lee *et al*[24] found that the presence of LVI correlated with the presence of lymph node metastasis in patients with gastric carcinoma. For different types of cholangiocarcinoma, the effect of LVI on prognosis was also different. Kim *et al*[25] reported that LVI did not influence the survival of patients with distal cholangiocarcinoma. Fisher *et al*[26] reported that LVI had an adverse influence on survival of patients with intrahepatic cholangiocarcinoma. Both of studies found that LVI was associated with lymph node metastasis[25,26]. In our analysis, N1 stage was a significant factor that was associated with LVI in type Ⅳ HC patients. It is generally known that tumor cells and tumor stromal cells (such as macrophages and thrombocytes) can produce pro-lymphangiogenic factors, which increase lymphovascular density in and around the tumors[27,28], mainly peritumoral regions. Lymph node metastases often occurred in tumors lacking intratumoral functional lymphatics, suggesting that functional lymphatics at the peritumoral regions are the route of lymphatic dissemination. Increased peritumoral lymphovascular density is considered to increase the flow of lymphatic fluid and provide an opportunity for invasive tumor cells to access the lymphatic vessels[29]. This may elucidate our results that LVI is closely related to lymph node metastasis. Moreover, we speculated that LVI may be the precursor of lymph node metastasis. Thus, we divided patients without lymph nodes metastases into LVI positive and LVI negative subgroups, and found that the 5-year DFS and OS rates in the LVI negative group were significantly higher than those in the LVI positive group (Figure 2A and B). This result indicates that LVI is an admirable prognostic predictor for patients with type Ⅳ HC when lymph node metastasis is absent.

Additionally, tumor size > 30 mm was another significant factor correlated with LVI in our series. Tumor size is recognized as a staging basis for many malignant tumors, including thyroid carcinoma, breast carcinoma, and liver carcinoma among others. Gurleyik *et al*[30] reported that LVI correlated with tumor size, and the rate of LVI positive increased with tumor size in patients with breast carcinoma. The significant correlation between LVI and tumor size could be explained through two potential aspects: (1) as the tumor size increases, the peritumoral areas increase. Thus, the tumor is endowed with the potential to make contact with an increasing lymphovasculature, and thus the possibility of LVI increases; and (2) tumor size is proportional to the time of growth: the larger the tumor, the greater the duration of time that the tumor can continue developing and growing in size. During a relatively longer growth time, a tumor has increasing opportunities to develop LVI. In our study, patients with tumor size ≤ 30 mm were also divided into LVI positive and LVI negative subgroups. Here, the 5-year DFS and OS rates in the LVI negative group were significantly higher than those found in the LVI positive group (Figure 3A and B). This result indicates that LVI is also an excellent prognostic predictor in type Ⅳ HC patients with smaller tumor size.

Some limitations of the study should also be taken into account when interpreting the results. First, our study was retrospective with inherent limitations in its design. Thus, some clinical bias was inevitable. Next, LVI

Table 3 Logistic regression analysis for factors associated with lymphovascular invasion

Variable	Lymphovascualr invasion (+), *n* = 29	Lymphovascualr invasion (-), *n* = 113	Univariate analysis (*P* value)	OR (95%CI)	Multivariate analysis (*P* value)
AJCC stage			0.063	0.223 (0.026-1.927)	0.173
Ⅰ/Ⅱ	8	27			
Ⅲ/Ⅳ	21	86			
Tumor diameter (mm)			0.001	3.258 (1.288-8.236)	0.013
≤ 30	10	77			
> 30	19	36			
N stage			< 0.001	3.312 (1.338-8.198)	0.026
N0	10	79			
N1	19	34			



No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	67	59	40	33	26	22
B	10	7	5	0	0	0

No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	67	59	46	35	27	23
B	10	8	5	1	0	0

Figure 3 Disease-free survival and overall survival based on lymphovascular invasion in type IV hilar cholangiocarcinoma patients with tumor size ≤ 30 mm. A: The 5-yr DFS rate in the LVI negative group was significantly higher than that in the LVI positive group; B: The 5-yr OS rate in the negative group was significantly higher than that in the LVI positive group. LVI: Lymphovascular invasion; DFS: Disease-free survival; OS: Overall survival.

was confirmed by hematoxylin and eosin (HE) staining alone without application of an immunohistochemical staining with D2-40 antibody, which may improve the detection rate of LVI[31]. Third, N1 stage and tumor size > 30 mm might serve as potential confounding factors that could affect the association between LVI and the eventual prognosis. Finally, we did not carry out preclinical medical experiments to elaborate the specific molecular mechanism that could play a key role in the capacity of LVI to affect the prognosis of type Ⅳ HC patients. Future research should take into account this topic with a greater sample size. Prospective studies, even randomized controlled trials, are also urgently needed. Of course, the specific molecular mechanism responsible for LVI affecting the prognosis of type Ⅳ HC patients will be determined by empirical research.

In conclusion, the presence of LVI may be regarded as an indicator of biologically aggressive behavior, metastatic ability, and regional and systemic risk of metastasizing the primary malignancy. LVI is associated with N1 stage and tumor size > 30 mm and imparts an adverse influence on OS and DFS in type Ⅳ HC patients who received radical resection.

COMMENTS

Background

Despite advances in surgical techniques and that resection rates for Bismuth type Ⅳ hilar cholangiocarcinoma (HC) continue to increase, the prognosis of patients with type Ⅳ HC remains unsatisfactory. The reasons for this remain unclear and seem to be complex and multifactorial. Lymphovascular invasion (LVI) is associated with a poorer prognosis in patients with various malignances. This study sought to investigate whether LVI could predict type Ⅳ HC prognosis.

Research frontiers

This study estimates prognostic factors that might be associated with overall survival (OS) and disease-free survival (DFS) after radical resection in type Ⅳ HC patients. To our knowledge, this study represents the first clinical insight indicating that LVI is associated with the prognosis of type Ⅳ HC.

Innovations and breakthroughs

This findings confirmed that R1 resection, N1 stage, presence of LVI and tumor size > 30 mm were adverse prognostic predictors for type Ⅳ HC patients after radical resection. Further, LVI was found to be associated with N1 stage and tumor size > 30 mm and adversely influence OS and DFS.

Applications

Observations from the present study might formally indicate novel factors for predicting post-surgical survival in HC. Moreover, LVI might present a potentially novel target for developing anti-cancer strategies.

Terminology

HC is a neoplasia arising from the biliary epithelium at the common hepatic duct bifurcation that might extend to the intrahepatic biliary tree and liver. Bismuth-Corlette classification is the most commonly used HC typing system, which is often used by surgeons to develop preliminary surgical protocols.

Peer-review

This is a retrospective study evaluating the effect of LVI on the prognosis of Bismuth-Corlette type Ⅳ HC. The authors concluded that LVI had an adverse influence on the prognosis of patients with Bismuth-Corlette type Ⅳ HC. This manuscript is very interesting and well written.

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ORIGINAL ARTICLE

Retrospective Study

Gastrointestinal symptom prevalence depends on disease duration and gastrointestinal region in type 2 diabetes mellitus

Midori Fujishiro, Akifumi Kushiyama, Hiroki Yamazaki, Sunao Kaneko, Yuko Koketsu, Takeshi Yamamotoya, Takako Kikuchi, Hideyuki Sakoda, Ryo Suzuki, Takashi Kadowaki

Midori Fujishiro, Hiroki Yamazaki, Sunao Kaneko, Ryo Suzuki, Takashi Kadowaki, Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, the University of Tokyo, Tokyo 113-8655, Japan

Akifumi Kushiyama, Takako Kikuchi, Division of Diabetes and Metabolism, Institute for Adult Diseases, Asahi Life Foundation, Tokyo 103-0002, Japan

Yuko Koketsu, Department of Diabetes and Metabolic Diseases, Shinko Hospital, Kobe 651-0072, Japan

Takeshi Yamamotoya, Department of Medical Science, Graduate School of Medicine, University of Hiroshima, Hiroshima 734-8553, Japan

Hideyuki Sakoda, Division of Neurology, Respirology, Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kiyotakecho Kihara, Miyazaki 889-1692, Japan

Author contributions: Fujishiro M and Kushiyama A contributed equally to this work; Fujishiro M designed the study, collected and analyzed the data, and drafted the manuscript; Kushiyama A provided analytical oversight and revised the manuscript for important intellectual content; Yamazaki H, Kaneko S, Koketsu Y and Yamamotoya T collected and analyzed the data; Kikuchi T analyzed the data; Sakoda H designed the study and collected the data; Suzuki R and Kadowaki T supervised the study; all authors have read and approved the final version to be published.

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Correspondence to: Midori Fujishiro, MD, PhD, Assistant Professor, Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. midori-tky@umin.ac.jp
Telephone: +81-3-3815-5411
Fax: +81-3-5802-5679

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Abstract

AIM

To unravel relationships between gastrointestinal (GI) symptoms impairing quality of life (QOL) and clinical profiles of diabetes mellitus (DM) patients.

METHODS

We enrolled 134 outpatients with type 2 DM. Mean

age was 64.7 years, mean body mass index was 24.7 kg/m^2, mean glycated hemoglobin was 7.1%, and mean DM duration was 13.7 years. GI symptom-related QOL was determined using the Izumo scale, based on five factors, *i.e.*, heartburn, gastralgia, postprandial fullness, constipation and diarrhea. The sum of scores obtained for the three questions in each domain was calculated, and subjects with a score of 5 or higher were considered to be symptomatic with impaired QOL. JMP Clinical version 5.0 was used for all statistical analyses.

RESULTS

Lower abdominal symptoms were found to be more frequent than those affecting the upper abdomen. Diabetic duration and medications showed associations with GI symptoms. We identified differences in peak prevalences of the five symptoms. Gastralgia (P = 0.02 *vs* 10-14 years) and total GI symptoms (P = 0.01 and P = 0.02 *vs* 5-9 years and 10-14 years, respectively) peaked at a diabetes duration of 15-19 years. Heartburn (P = 0.004) and postprandial fullness (P = 0.03) tended to increase with disease duration. Constipation and diarrhea showed bimodal peaks, with the first early and the second late (*e.g.*, P = 0.03 at 15-19 years *vs* 10-14 years for diarrhea) in the disease course. Finally, GI symptoms showed clustering that reflected the region of the GI tract affected, *i.e.*, constipation and diarrhea had similar frequencies (P < 0.0001).

CONCLUSION

Our study highlights the importance of questioning patients about QOL impairment due to abdominal symptoms, especially in the early and the late periods of diabetes.

Key words: Gastrointestinal symptoms; Questionnaire survey; Disease duration; Type 2 diabetes; Quality of life; Gastrointestinal tract regions

Core tip: We describe the results of a questionnaire survey of 134 type 2 diabetes mellitus outpatients experiencing gastrointestinal symptoms. The novel finding is that symptom frequencies differed among disease durations and according to affected gastrointestinal regions. Lower abdominal symptoms not only manifested during the late but also in the early stage of diabetes when there were no organ complications related to this disease. Our study highlights the importance of not underestimating gastrointestinal symptoms and of questioning patients about quality of life impairment due to abdominal symptoms, especially in both the early period and after a diabetes duration of 10 or even 15 years.

Fujishiro M, Kushiyama A, Yamazaki H, Kaneko S, Koketsu Y, Yamamotoya T, Kikuchi T, Sakoda H, Suzuki R, Kadowaki T. Gastrointestinal symptom prevalence depends on disease duration and gastrointestinal region in type 2 diabetes mellitus. *World J Gastroenterol* 2017; 23(36): 6694-6704 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6694.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6694

INTRODUCTION

The dramatic increase in the incidence of type (T)2 diabetes mellitus (DM) over the last century has become a major public health concern worldwide[1]. Both micro- and macrovascular complications of diabetes damage a variety of organs, leading to various symptoms, and impair quality of life (QOL) in patients with T2DM.

Among these symptoms, those affecting the gastrointestinal (GI) tract occur frequently in patients with diabetes[2]. Various GI symptoms are associated with this disease[3-5], especially diabetic neuropathy which develops with longstanding diabetes[6,7]. GI hyper- and hypofunction lead to delayed gastric or esophageal emptying, diabetic gastroparesis, constipation and diarrhea[6,8,9]. Other GI-related disorders and symptoms are also known to accompany diabetes, such as obesity with gastroesophageal reflux disease[10,11], and aging with gastric mucosal injury[12].

GI disorders in diabetes have thus far been poorly characterized, despite their high frequencies in both T1DM and T2DM patients[13]. Hyperglycemia-associated neuropathy was long considered to be a major mechanism underlying the pathogenesis of GI symptoms, probably *via* oxidative stress and inflammation, as with other microangiopathies. Levels of enteral hormones, such as incretin-related peptides [*e.g.*, glucagon-like peptide-1 (GLP-1), GLP-2, pancreatic polypeptide (PPY)][14] and serotonin[15], and enteric neurotransmitters, such as vasoactive intestinal peptide (VIP)[16], are altered in patients with DM, and affect GI motility and neural fiber growth. Furthermore, smooth muscle cells, interstitial cells of Cajal[17], gut microbiota[18] and intestinal stem cells[19], may be altered in DM and these changes are potentially related to GI symptoms. The relevance of these mechanisms in the development of human GI symptoms in T2DM remains uncertain.

As for the pathophysiological mechanisms underlying the development of complications, hyperglycemia and the resultant glycation products[20], oxidative stress[21] and inflammation[22], are the main candidates. GI symptoms in DM are associated with both poor glycemic control and diabetic complications, as shown in a large number of subjects[23], although we found no relationships between GI symptoms and glycemic control, probably due to the long and varied disease durations of our subjects.

There has been little systematic and comprehensive research focusing on GI conditions in patients with

T2DM. The Izumo Scale, a validated and useful tool for QOL assessment, is a scale for assessing GI symptoms[13,14]. Applying this scale, we investigated actual GI symptoms employing a questionnaire and determined whether the symptoms identified are related to the clinical profiles of patients with T2DM.

MATERIALS AND METHODS

Patients

Patients who visited the department of diabetes and metabolic diseases in our hospital, including those with T1DM and T2DM, from December 2011 to March 2014, were enrolled in this study. We asked 200 consecutive patients to answer the questionnaire. Of the 170 patients who gave informed consent and responded to the questionnaire, data from 134 were analyzed after exclusion of 31 non-T2DM patients and 5 with a past history of GI tract surgery. This protocol was approved by our institutional ethics review committees (approval number 3643). Patient QOL was assessed by asking the subjects if they suffered from GI symptoms and then scoring the symptoms according to the Izumo scale, as described below.

Patient profiles, including diabetic microangiopathy, were collected from medical records. We considered subjects to have "diabetic autonomic neuropathy (DAN)" if they had any autonomic signs or symptoms. Distal symmetric polyneuropathy (DSP) includes symmetric neurosensory signs and symptoms, such as tingling, numbness or cramps in the legs, based on the attending physician's assessment. Diabetic retinopathy (DR) includes non-proliferative DR diagnosed by the presence of microaneurysms and retinal hemorrhages[24], and all DR cases were confirmed by an ophthalmologist. Diabetic nephropathy was clinically diagnosed by attending physicians based on microalbuminuria or overt proteinuria with no evidence of other kidney or urological disease[25].

Izumo Scale

The Izumo Scale was developed and validated by Furuta *et al*[26]. The Izumo Scale is a self-administered questionnaire designed to assess the effects of abdominal symptoms on QOL and includes 15 items in five domains with three items in each domain: heartburn (questions 1-3), gastralgia (questions 4-6), postprandial fullness (questions 7-9), constipation (questions 10-12), and diarrhea (questions 13-15)[27]. Each question is rated on a 6-point Likert scale from 0 to 5, with higher values indicating more severe symptoms. This scale has been shown to have good internal consistency, reproducibility, as well as good correlations with the visual analogue scale of abdominal symptoms and the Gastrointestinal Symptoms Rating Scale (GSRS), and is thus widely utilized in Japan[28-31]. The sum of scores obtained for the three questions in each domain was calculated, and subjects with a score of 5 or higher were considered to be symptomatic with impaired QOL[30]. When the five symptoms were classified by GI region, heartburn was considered to involve the esophagus, gastralgia and postprandial fullness the upper GI tract, and constipation and diarrhea the lower GI tract.

Statistical analysis

JMP Clinical version 5.0 (SAS Institute, Cary, NC, United States) was used for all statistical analyses. ANOVA was used to compare scores among different groups. Frequencies were compared using Fisher's exact test. Trend comparisons for frequencies among disease duration, age, body mass index (BMI), and glycated hemoglobin (HbA1c) were performed using a Cochran-Armitage test. Hierarchical cluster analysis was performed to assess the relationships among GI symptoms. All P values are two-sided, and $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Characteristics of the enrolled patients

The characteristics of enrolled patients are listed in Table 1. We enrolled 134 patients with T2DM (87 males and 47 females, mean age: 64.7 years, range: 29-88 years). Mean diabetes duration was 13.7 (0.3-33.0) years, BMI was 24.7 (16.5-42.5) kg/m^2 and HbA1c was 7.1 (5.2-11.6)%. As to incidences of diabetic microangiopathy, 32 patients (24%) had DAN, 20 (15%) had DSP, 31 (23%) had DR and 64 (48%) had nephropathy. As to antidiabetic drugs, 8 patients (6%) received no medications, 34 (25%) used various forms of insulin including 22 (16%) also taking oral antidiabetic drugs (OADs), the details of which are presented in Table 1.

Eighty-six (64%) patients received OAD only, including 63 (47%) with one OAD, 30 (22%) with 2 OADs, 22 (16%) with three OADs, and 11 (8%) with four or more OADs. In detail, 38 patients (28%) used α-glucosidase inhibitors, 67 (50%) used biguanides, 26 (19%) used thiazolidinediones, 54 (40%) used sulfonylureas, 3 (2%) used glinides and 53 (40%) used dipeptidyl peptidase-4 (DPP4) inhibitors, at various daily doses, as indicated in Table 1. Six patients (4%) used GLP-1 agonists, 3 of whom were also taking an OAD. Sixty-two patients (46%) were receiving diet therapy with daily intakes ranging from 1200-1800 kcal (21-29 kcal/IBW) and, in 51 patients (38%), salt was restricted to less than 6 g/d.

As to antithrombotic or anti-inflammatory agents, 29 patients (22%) used antiplatelet agents, 7 (5%) used anticoagulants, 8 (6%) used steroids or non-steroidal anti-inflammatory drugs and 7 (5%) used other antithrombotic agents such as prostaglandin E1 derivatives, prostaglandin I2 derivatives, or ethyl esters of eicosapentaenoic acid. As to GI agents, 29 patients (22%) used antacids, 13 (10%) used mucosal protectives, 11 (8%) used antimicrobials, and 18

Table 1 Characteristics of the study population

Variable	*n* (%)	mean (95%CI)
Sex, male/female	87/47 (65/35)	
Age of 65 yr or older	72 (54)	64.7 (62.8-66.6)
BMI of 25 kg/m^2 or more	56 (42)	24.7 (24.0-25.4)
HbA1c of 7% or more	69 (51)	7.1 (6.9-7.3)
Duration of diabetes, yr		13.7 (12.4-14.9)
⩽ 4	12 (9)	
5-9	27 (20)	
10-14	35 (26)	
15-19	31 (23)	
⩾ 20	29 (22)	
Incidence of diabetic microangiopathy		
Autonomic neuropathy	32 (24)	
Distal symmetric polyneuropathy	20 (15)	
Retinopathy	31 (23)	
Nephropathy	64 (48)	
Use of antidiabetic drugs		
None	8 (6)	
Insulins	34 (25)	
Insulins with OAD	22 (16)	
OAD only	86 (64)	
1 OAD	63 (47)	
2 OAD	30 (22)	
3 OAD	22 (16)	
⩾ 4 OAD	11 (8)	
GLP-1	6 (4)	
GLP-1 with OAD	3 (2)	
Insulins	34 (25)	
Range of injections per day	1-5	
Range of total daily insulin doses, IU	2-114	
Insulin glargine	12	
Insulin detemir	7	
Human NPH insulin	14	
Insulin aspart	2	
Insulin glulisine	4	
Insulin lispro	2	
Premixed human insulin 30/70	9	
Biphasic insulin aspart 30/70	1	
α-Glucosidase inhibitors	38 (28)	
Acarbose (150/200/300 mg)	3 (1/1/1)	
Miglitol (50/75/100/150 mg)	22 (1/12/18)	
Voglibose (0.6/0.9 mg)	13 (5/8)	
Biguanides	67 (50)	
Buformin 150 mg	1	
Metformin (500/750/1000/1500/2250 mg)	66 (12/20/14/19/1)	
Thiazolidinediones	26 (19)	
Pioglitazone (15/30 mg)	26 (18/8)	
Sulfonylureas	54 (40)	
Glimepiride (0.5/1/2/3/4 mg)	26 (10/10/2/1/3/)	
Gliclazide (20/40/120 mg)	21 (15/5/1)	
Glibenclamide (1.25/2.5/5 mg)	7 (1/4/2)	
Glinides	3 (2)	
Repaglinide 0.75 mg	1	
Mitiglinide (10/30 mg)	2 (1/1)	
Dipeptidyl peptidase-4 inhibitors	53 (40)	
Alogliptin 25 mg	12	
Sitagliptin (50/100 mg)	17 (15/2)	
Vildagliptin (50/100 mg)	24 (5/19)	
Glucagon-like peptide-1 agonists	6 (4)	
Liraglutide (0.6/0.9 mg)	4 (1/3)	
Exenatide 20 mg	2	
Dietary information		
Receiving dietary therapy	62 (46)	
With salt restriction to < 6 g per day	51 (38)	
Range of total energy intake, kcal	1200-1800	
Range of total energy intake per IBW, kcal/IBW	21-29	
Use of antithrombotic or anti-inflammatory agents		
Antiplatelet agents	29 (22)	
Anticoagulants	7 (5)	

Steroids/NSAIDs	8 (6)
Others[1]	7 (5)
Use of GI agents	
Antacids	29 (22)
Mucosal protectives	13 (10)
Antimicrobials	11 (8)
GI stimulants	18 (13)
Anti-diarrheals	0 (0)
Baseline blood parameters, mean ± SD	
WBC, × 1000/μL	6.1 ± 1.9
RBC, × 10000/μL	439 ± 78.1
Hb, g/dL	13.4 ± 2.4
Hct, %	40.8 ± 6.9
Plt, × 10000/μL	21.9 ± 6.6
TP, g/dL	7.1 ± 0.5
Alb, g/dL	3.8 ± 0.3
AST(GOT), U/L	24.4 ± 13.4
ALT(GPT), U/L	24.5 ± 17.7
γ-GTP, U/L	34.1 ± 24.3
CK, U/L	113 ± 63.6
T-Cho, mg/dL	180 ± 39.2
HDL-C, mg/dL	60.3 ± 18.3
TG, mg/dL	128 ± 67.2
cLDL, mg/dL	95.4 ± 26.2
BUN, mg/dL	17.6 ± 11.9
Cre, mg/dL	0.9 ± 0.8
eGFR, mL/min per 1.73 m^2	66.6 ± 22.8
UA, mg/dL	5.5 ± 1.4

[1]Others' includes other antithrombotic agents such as prostaglandin E1 derivatives, prostaglandin I2 derivatives, or ethyl esters of eicosapentaenoic acid. Data are expressed as means with the 95%CI in parentheses, with numbers for each sex or numbers with the percentage of the total in parentheses. BMI: Body mass index; GI: Gastrointestinal; HbA1c: Hemoglobin A1c; IBW: Ideal body weight; NPH: Neutral protamine Hagedorn; NSAIDs: Nonsteroidal anti-inflammatory drugs; OAD: Oral antidiabetic drug.

(13%) used GI stimulants, but none were taking anti-diarrheal agents. Baseline blood parameter findings were unremarkable.

GI symptoms and diabetes duration

In the QOL assessment employing the Izumo Scale, the scores of total GI symptoms peaked during the 15- to 19-year diabetes duration period (Figure 1). When GI symptoms were analyzed separately, lower GI symptoms were found to be most frequent. Prevalence patterns differed between the esophagus/upper GI tract and lower GI tract regions. Scores for heartburn (P = 0.004) and postprandial fullness (P = 0.03) consistently and significantly rose as disease duration increased (Figure 2A).

Scores for gastralgia, like total GI symptoms, peaked during the 15- to 19-year diabetes duration period. Interestingly, only the scores for constipation and diarrhea showed bimodal peaks, the first in the early period of diabetes and the second late in the disease course. Age showed a similar but weaker relationship to diabetes duration. There were no apparent relationships of GI symptoms with age or BMI, but postprandial fullness, alone among the five symptoms, was slightly associated with high HbA1c levels (Figure 2B-D).

Relationships of GI symptoms with diabetic complications and medications

Next, we examined whether there were any relationships between GI symptoms and patient characteristics such as diabetic microangiopathy and the drugs administered (Figure 3A-D). None of the GI symptoms showed significant relationships with any of the forms of diabetic microangiopathy (Figure 3A), though symptoms tended to be more frequent in patients with than in those without DAN and/or DSP. As shown in Figure 3B, the rate of DPP4 inhibitor use was lower in patients suffering from constipation (P = 0.04). Insulin therapy and oral hypoglycemic agents other than DPP4 inhibitors were not related to GI symptoms. The numbers of patients using glinides or GLP-1 agonists were too small to allow statistically meaningful evaluation. As shown in Figure 3C, of the 29 patients taking antiplatelet agents, 38% (n = 11) had constipation (P = 0.046) and 62% (n = 18) had diarrhea (P = 0.03). Of the 18 patients using GI stimulants, 44% (n = 8) had constipation (P = 0.03) (Figure 3D).

We next investigated the effect of diabetic duration on constipation, excluding users of DPP4 inhibitors, antiplatelet agents or GI stimulants. We focused on the association of diabetes duration with constipation (Figure 4A), and on that with diarrhea, except in antiplatelet agent users (Figure 4B). When users of these medications were excluded, the early and late peaks in constipation and diarrhea remained.

Clustering and overlap of GI symptoms

A cluster analysis tree is presented in Figure 5. Gastralgia and postprandial fullness are more similar to

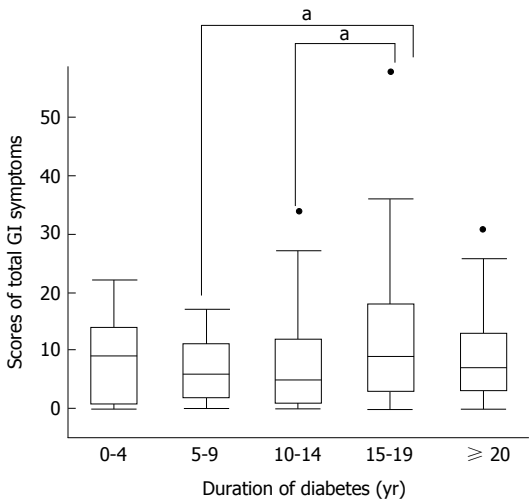

Figure 1 Distribution of the sum of scores of symptoms related to all GI areas by duration of diabetes. [a]*P* < 0.05, significantly different. Sums of symptom scores peaked during the 15- to 19-yr diabetes duration period, compared by Student's *t*-tests for paired periods (*P* = 0.01 and *P* = 0.02 when compared with 5-9 yr and 10-14 yr disease durations, respectively).

each other than to constipation and diarrhea. Heartburn is closer to upper than to lower GI symptoms. The relationship between constipation and diarrhea was found to be closer than the other associations identified in this study. The overlaps between two different symptoms on the Izumo Scale are shown in Table 2. Significant differences, determined by Fisher's exact test, between the presence and absence of two distinct symptoms, were demonstrated.

As in the cluster analysis, heartburn was associated with gastralgia (*P* = 0.03) or postprandial fullness (*P* = 0.002). Gastralgia and postprandial fullness (*P* < 0.0001) were strongly related to each other, as were constipation and diarrhea (*P* < 0.0001). Postprandial fullness was found to be related to all GI tract regions, showing modest interactions with both constipation (*P* = 0.004) and diarrhea (*P* = 0.001). Furthermore, all 31 patients with constipation also had episodes of diarrhea, while only 52.5% of those complaining mainly of diarrhea also had constipation (Table 2).

DISCUSSION

We investigated whether outpatients with T2DM actually have GI symptoms and, if present, the clinical severity of such symptoms. However, patients do not usually complain of GI symptoms. Overall, 10%-20% of adults have functional GI disorders[32-35]. In patients with diabetes, symptoms involving all portions of the GI tract are reportedly more common than in the general population[36]. However, we found that total symptom scores did not increase linearly with disease duration. In fact, rates peaked during different periods, according to GI regions. Lower abdominal symptoms were prominent early in the disease course, while diabetic complications were still mild, as well as in the late stage.

Significant confounding factors include aging, glycemic control and BMI reduction[5,8-10,37]. Japanese patients have become increasingly obese over the past 20 years and those in the younger generation have poor glycemic control at the diagnosis of T2DM, independent of hyperglycemic symptoms[38]. Therefore, symptom prevalence may change in the future.

We found no significant relationships between GI symptoms and either DAN or DSP. There might, however, be a detection bias or low sensitivity for DAN and DSP, since a previous report noted that symptoms tend to be related to lifestyle factors, rather than to either glycemic control or peripheral neuropathy[36]. Another study found no association of GI symptoms with either diabetic neuropathy or psychiatric illnesses[39]. Early symptoms might well be related to psychiatric illnesses and/or lifestyle factors but we did not obtain data pertaining to such factors in the present study.

Among anti-hyperglycemic medications, only the relationship between DPP4 inhibitors and constipation was significant. Constipation is a well-known side effect of these drugs[40], and physicians often discontinue or do not start DPP4 inhibitors due to this adverse effect[41]. Oral medications are generally prescribed to patients with poor glycemic control[42] and are frequently used to manage longstanding diabetes[43]. Administration of anti-platelet agents was associated with a variety of symptoms, as expected. These agents were still preferentially used despite apparently being harmful to the upper, middle and lower GI tracts[44,45], since the treatment of cardiovascular diseases has the highest priority. However, the bimodal peaks for constipation and diarrhea late and early in the course of diabetes were maintained and were independent of medication use.

The mechanisms underlying rapid development of lower GI complications, such as diarrhea and constipation, are unknown. In our cluster analysis, the affected GI tract region was found to be an important factor. Furthermore, the mechanisms underlying the bimodal early and late disturbances are suggested to not be the same. Early diabetic neuropathy is reportedly multifactorial, being triggered by impairment of insulin signaling, abnormal blood flow, and oxidative stress including N-acetylglucosamine, activation of protein kinase C, activation of the polyol sugar pathway and glucose autoxidation, as well as non-enzymatic protein glycation[46]. Insulin growth factors (IGFs) possess multiple neurotrophic functions, including neuronal survival, neurite outgrowth and regeneration. C-peptide also exerts IGF-like activity, such that endogenous proinsulin production might be related to the pathogenesis of GI symptoms[47]. One possible explanation of mechanistic differences between early lower GI symptoms and upper GI symptoms might involve cellular composition. The lower GI tract contains colonic stem cells and their functions are disrupted in states of diabetic enteropathy[19].

Table 2 Overlapping of gastrointestinal symptoms

Overlapping symptom	Heartburn, n = 9	Gastralgia, n = 11	Postprandial fullness, n = 17	Constipation, n = 31	Diarrhea, n = 59
Heartburn	-	3/11[a]	5/17[b]	5/31	6/59
Gastralgia	3/9[a]	-	8/17[b]	4/31	7/59
Postprandial fullness	5/9[b]	8/11[a]	-	9/31[b]	14/59[b]
Constipation	5/9	4/11	9/17[b]	-	31/59[b]
Diarrhea	6/9	7/11	13/17[b]	31/31[b]	-

[a]P < 0.05 or [b]P < 0.01, significantly different.

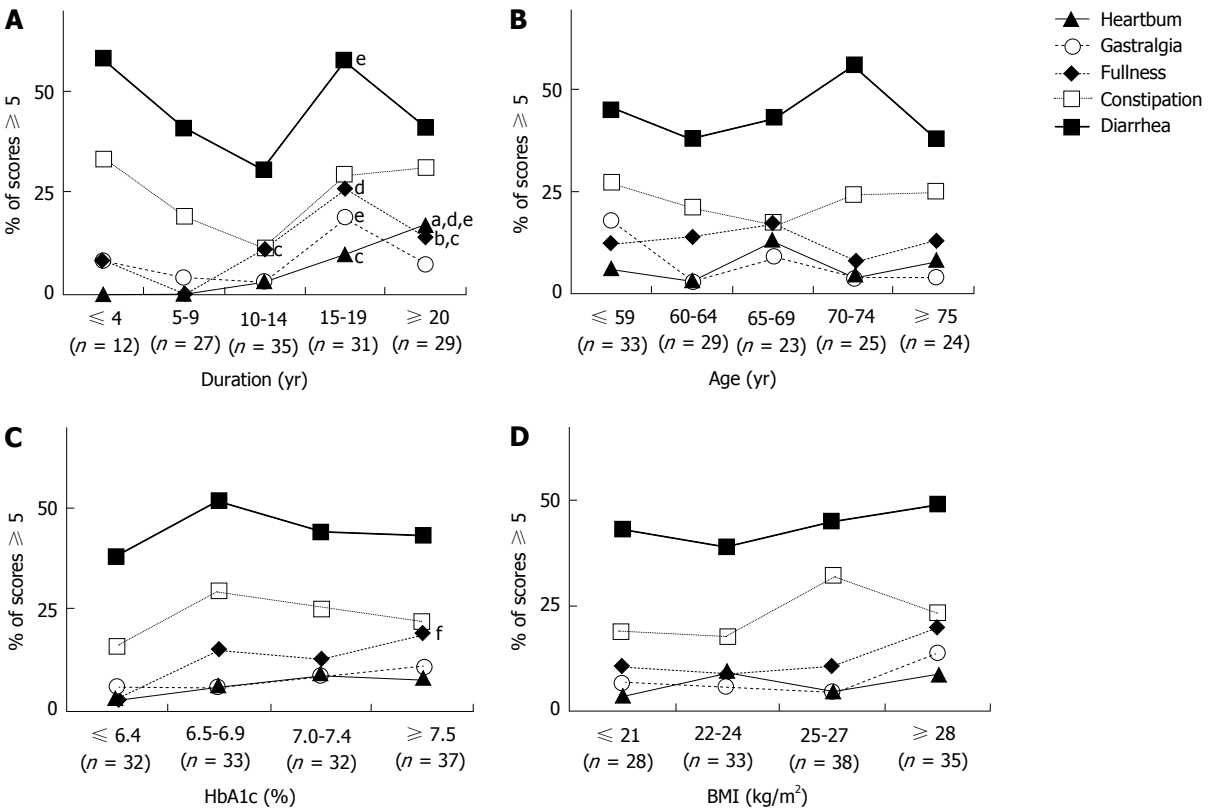

Figure 2 Relationships of abdominal symptoms with duration of diabetes, age, body mass index and hemoglobin A1c. The lines show the percentages of subjects with a score of 5 or higher, for each GI region, among those considered to be sufficiently symptomatic to impair quality of life. Comparisons among groups with five disease duration periods (A), five age ranges (B), four HbA1c levels (C) and four BMI levels (D) were performed using a Cochran-Armitage-trend-test. [a]P < 0.05 or [b]P < 0.01, significantly different. As shown in the figure (A), scores for heartburn (P = 0.004) and postprandial fullness (P = 0.029) consistently and significantly increased with disease duration. Student's t-tests for paired samples were performed to compare percentages of subjects with a score of 5 or higher, for each GI region, among five durations of diabetes (A), five age ranges (B), four HbA1c levels (C) and four BMI levels (D). [c]P < 0.05 *vs* 5-9 years, [d]P < 0.01 *vs* 5-9 years, or [e]P < 0.05 *vs* 10-14 years when compared with other disease duration periods and [f]P < 0.05 *vs* 6.4% or less which indicate significant differences. As shown in the figure (A), percentages of subjects with a score of 5 or higher for all symptoms except constipation in the disease duration period of 15-19 years were significantly higher than those in the 5-9 years and 10-14 years periods; P = 0.048 for heartburn, P = 0.024 for gastralgia, P = 0.008 for fullness and P = 0.03 for diarrhea. Likewise, percentages of subjects with a score of 5 or higher for heartburn and fullness in the disease duration period of 20 years or more were significantly higher than those in the 5-9 years and 10-14 years periods; P = 0.01 and P = 0.04 for heartburn and P = 0.02 for fullness. Furthermore, the percentage of subjects with a score of 5 or higher for fullness in the disease duration period of 10-14 yr was significantly higher than that in the 5-9 years period (P = 0.029). As shown in the figure (C), the percentage of subjects with a score of 5 or higher for fullness in those with HbA1c levels of 7.5% or above was significantly higher than that in those whose HbA1c was 6.4% or less (P = 0.030). BMI: Body mass index; HbA1c: Hemoglobin A1c.

Lower GI tract symptoms were found to be most frequent and cluster analysis demonstrated these symptoms to be related more to the affected region of the GI tract than to its functions, such as acid secretion and motility. In fact, a high concordance between constipation and diarrhea is unusual in general populations[48]. Upper and lower GI symptoms reportedly overlap and early satiety more frequently overlaps with constipation than diarrhea in general populations[49,50].

This study has several limitations. First, it is difficult to determine the causes of symptoms with a cross-sectional study design. There is diagnostic uncertainty regarding the GI disorders studied, because the reporting of symptoms was mainly subjective. Endoscopy can be used to exclude organic diseases with

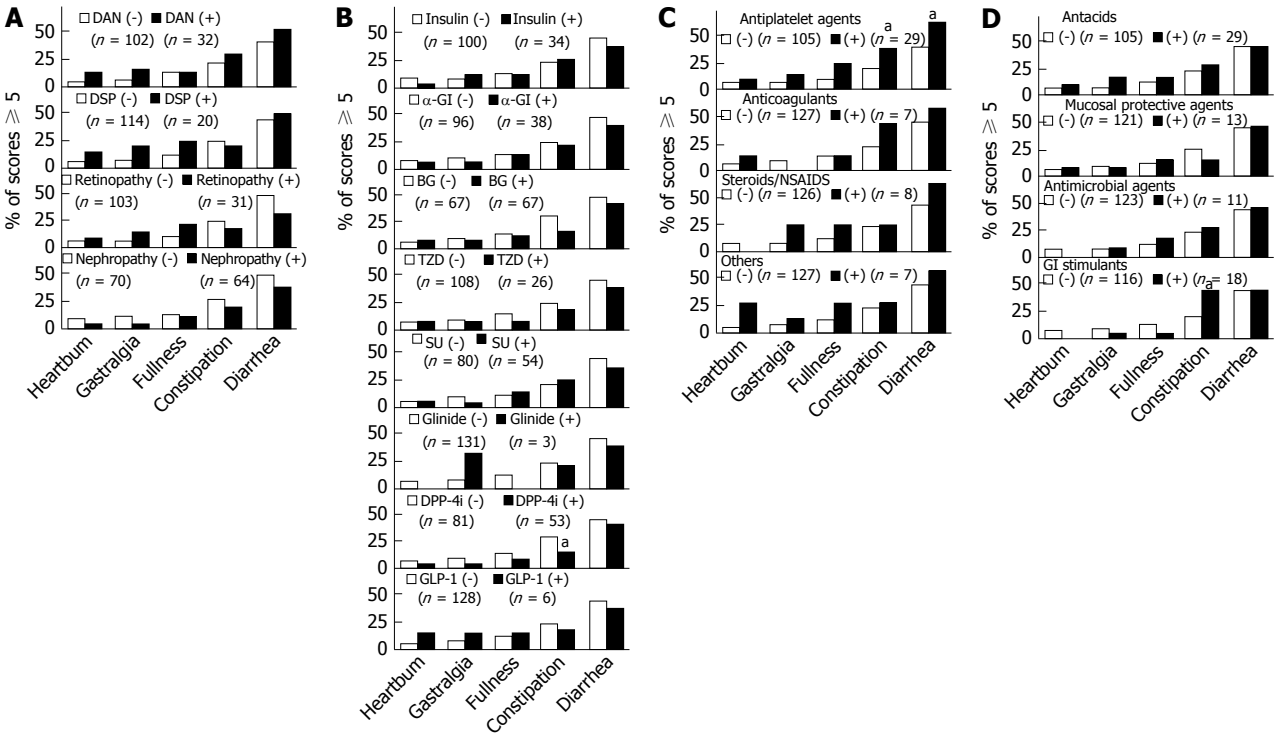

Figure 3 Relationships between various parameters and gastrointestinal symptoms involving each gastrointestinal region ("Heartburn" for esophagus, "Gastralgia" and "Postprandial Fullness" for upper abdomen, and "Constipation" and "Diarrhea" for lower abdomen). Bars: White indicates the absence and black the presence of each parameter. The percentages of subjects with a score of 5 or higher, for each GI region, considered to be sufficiently symptomatic to cause impaired quality of life, are shown. [a]$P < 0.05$, significant difference between presence and absence of each parameter, as analyzed by Fisher's exact test. A: Comparison between subjects with and without diabetic microangiopathy. The indicated percentages were obtained by dividing the number of subjects with a score of 5 or higher by the total number of patients in the same group (with or without diabetic microangiopathy); B: Comparisons between groups of patients with and without antidiabetic agent administration; C: Comparisons between groups of patients with and without antithrombotic or anti-inflammatory agents. 'Others' includes other antithrombotic agents such as prostaglandin E1 derivatives, prostaglandin I2 derivatives, or ethyl esters of eicosapentaenoic acid; D: Comparisons between groups of patients with and without GI agents. αGI: Alpha-glucosidase inhibitors; DAN: Diabetic autonomic neuropathy; DPP-4i: Dipeptidyl-peptidase 4 inhibitor; DSP: Distal symmetric polyneuropathy; GI: Gastrointestinal; GLP-1: Glucagon-like peptide-1; NSAIDs: Nonsteroidal anti-inflammatory drugs; SU: Sulphonyl urea; TZD: Thiazolidinedione.

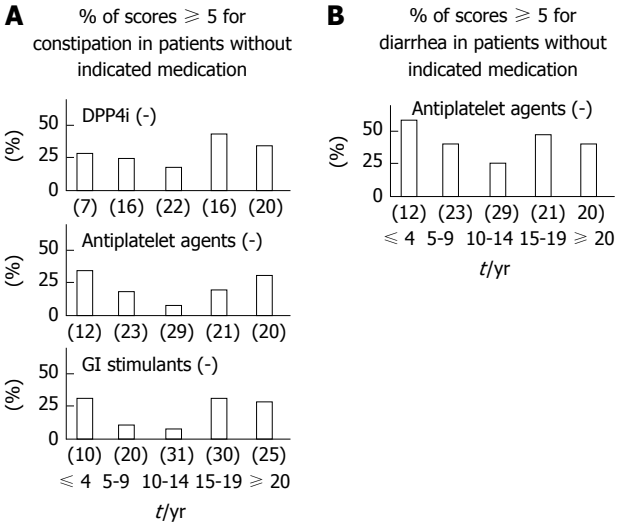

Figure 4 Relationships between various medications, which demonstrated significance as shown in Figure 3, and diabetes duration in terms of the presence of related gastrointestinal symptoms. White bars show the percentages of subjects with a score of 5 or higher for the indicated GI symptoms (A: Constipation, B: Diarrhea) without use of the indicated medications. Numbers under each bar within brackets are the number of patients not using the indicated medications. DPP-4i: Dipeptidyl-peptidase 4 inhibitor; GI: Gastrointestinal.

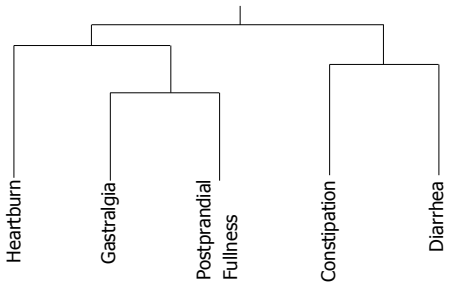

Figure 5 Cluster analysis was conducted for various gastrointestinal symptoms. The treeplot shows relationships among the five symptoms. Lengths of branches between symptoms indicate symptom similarity.

symptoms similar to those of diabetic GI disorders (*e.g.*, inflammatory bowel disease, colonic polyposis, and celiac disease)[51]. Few therapeutic or diagnostic approaches are available to assess the GI disorders possibly associated with diabetes. Organic diseases not requiring surgery and functional disorders could not be precisely distinguished because we did not perform upper and lower GI endoscopy or gastric emptying scintigraphy at the time of this study, since it was retrospective. Our results might have low sensitivity due to the small sample sizes in some groups.

In conclusion, our study highlights the importance of questioning patients about QOL impairment due to abdominal symptoms, especially lower GI symptoms in the early period and both lower and upper GI symptoms in the late period of diabetes. The heterogeneous nature of the underlying pathophysiological mechanisms underlying GI symptoms, including medication usage, should be taken into consideration when managing patients with T2DM.

COMMENTS

Background

Type 2 diabetes mellitus (T2DM) incidence is dramatically increasing, and both micro- and macrovascular complications of diabetes lead to various symptoms and impair quality of life (QOL) in T2DM patients. Among these symptoms, those affecting the gastrointestinal (GI) tract are frequent in T2DM patients. GI disorders in diabetes have been poorly characterized. Hyperglycemia-associated neuropathy was considered to be related to GI symptoms, probably *via* oxidative stress and inflammation. Levels of enteral hormones are altered in patients with DM, affecting both GI motility and neural fiber growth. Furthermore, smooth muscle cells, interstitial cells of Cajal, gut microbiota and intestinal stem cells may also be altered in DM and these changes might be related to GI symptoms. The relevance of these mechanisms in human GI symptoms affecting T2DM patients have yet to be clarified. The Izumo Scale, a validated and useful tool for QOL assessment, is a scale for assessing GI symptoms. Applying this scale, we investigated actual GI symptoms employing a questionnaire and determined whether the symptoms identified are related to the clinical profiles of patients with T2DM.

Research frontiers

This study highlights the importance of not underestimating gastrointestinal symptoms and of questioning patients about QOL impairment due to abdominal symptoms, especially in both the early period and after a diabetes duration of 10 or even 15 years.

Innovations and breakthroughs

The novel finding of this study is that symptom frequencies differed among disease durations and according to affected gastrointestinal regions. Lower abdominal symptoms not only manifested during the late but also in the early stage of diabetes when there were no organ complications related to this disease. This results are apparently inconsistent with the previously suggested mechanisms of GI symptoms related to DM, especially diabetic neuropathy which develops with longstanding diabetes.

Applications

This study highlights the importance of questioning patients about QOL impairment due to abdominal symptoms, especially lower GI symptoms in the early period and both lower and upper GI symptoms in the late period of diabetes, while underscoring the need for systematic and comprehensive research focusing on GI conditions in patients with T2DM.

Terminology

Izumo Scale: This self-administered questionnaire designed to assess the effects of abdominal symptoms on QOL includes 15 items in five domains with three items in each domain: heartburn, gastralgia, postprandial fullness, constipation, and diarrhea. This scale was developed and validated by Furuta *et al*. Each question is rated on a 6-point Likert scale from 0 to 5, with higher values indicating more severe symptoms.

Peer-review

Considering the paucity of data available in the literature on this topic, this study may reinforce the clinical relevance of assessing intestinal disorders in diabetes and increase the interest of the scientific community in this topic. Overall, this report describes relevant novel findings that may be further addressed and confirmed with detailed experimental studies.

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ORIGINAL ARTICLE

Observational Study

Serum angiotensin-converting enzyme level for evaluating significant fibrosis in chronic hepatitis B

Ryuichi Noguchi, Kosuke Kaji, Tadashi Namisaki, Kei Moriya, Mitsuteru Kitade, Kosuke Takeda, Hideto Kawaratani, Yasushi Okura, Yosuke Aihara, Masanori Furukawa, Akira Mitoro, Hitoshi Yoshiji

Ryuichi Noguchi, Kosuke Kaji, Tadashi Namisaki, Kei Moriya, Mitsuteru Kitade, Kosuke Takeda, Hideto Kawaratani, Yasushi Okura, Yosuke Aihara, Masanori Furukawa, Akira Mitoro, Hitoshi Yoshiji, Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara 634-8522, Japan

Author contributions: Noguchi R and Kaji K contributed equally to this work; Noguchi R and Kaji K collected and analyzed the data, and drafted the manuscript; Namisaki T, Moriya K, Kitade M, Takeda T, Kawaratani H, Okura Y, Aihara Y, Furukawa M and Mitoro A offered the technical or material support; Yoshiji H designed and supervised the study; all authors have read and approved the final version to be published.

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Correspondence to: Kosuke Kaji, MD, PhD, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. kajik@naramed-u.ac.jp
Telephone: +81-744-223051
Fax: +81-744-247122

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Abstract

AIM

To evaluate the diagnostic performance of angiotensin-converting enzyme (ACE) on significant liver fibrosis in patients with chronic hepatitis B (CHB).

METHODS

In total, 100 patients with CHB who underwent liver biopsy in our hospital were enrolled, and 70 patients except for 30 patients with hypertension, fatty liver or habitual alcoholic consumption were analyzed. We compared histological liver fibrosis and serum ACE levels and evaluated the predictive potential to diagnose significant liver fibrosis by comparison with several biochemical marker-based indexes such as the aspartate aminotransferase (AST)-to-platelet ratio index (APRI), the fibrosis index based on four factors (FIB-4), the Mac-2 binding protein glycosylation isomer (M2BPGi) level and the number of platelets (Plt).

RESULTS

Serum ACE levels showed moderately positive correlation with liver fibrotic stages (R^2 = 0.181). Patients with significant, advanced fibrosis and cirrhosis (F2-4) had significantly higher serum ACE levels than those with

early-stage fibrosis and cirrhosis (F0-1). For significant fibrosis (≥ F2), the 12.8 U/L cut-off value of ACE showed 91.7% sensitivity and 75.0% specificity. The receiver-operating characteristic (ROC) curves analysis revealed that the area under the curve (AUC) value of ACE was 0.871, which was higher than that of APRI, FIB-4, M2BPGi and Plt.

CONCLUSION

The serum ACE level could be a novel noninvasive, easy, accurate, and inexpensive marker of significant fibrosis stage in patients with CHB.

Key words: Angiotensin-converting enzyme; Hepatitis B virus; Liver fibrosis; Noninvasive fibrosis marker; Aspartate aminotransferase-to-platelet ratio index; Fibrosis index based on four factors; Mac-2 binding protein glycosylation isomer

Core tip: Liver fibrosis is one of key factors to determine therapeutic intervention for patients with chronic hepatitis B (CHB). However, the noninvasive prediction of CHB-related liver fibrosis is difficult. Angiotensin-converting enzyme (ACE) is reportedly involved in liver fibrogenesis. In this paper, we demonstrate that serum ACE levels are elevated in patients with CHB and show the predictive potential to diagnose significant fibrosis (≥ F2), which is the therapeutically adapted stage, with higher accuracy as compared with other fibrotic markers including APRI, FIB-4, M2BPGi and Plt. The serum ACE level could be a novel noninvasive marker of significant fibrosis stage in CHB.

Noguchi R, Kaji K, Namisaki T, Moriya K, Kitade M, Takeda K, Kawaratani H, Okura Y, Aihara Y, Furukawa M, Mitoro A, Yoshiji H. Serum angiotensin-converting enzyme level for evaluating significant fibrosis in chronic hepatitis B. *World J Gastroenterol* 2017; 23(36): 6705-6714 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6705.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6705

INTRODUCTION

Hepatitis B virus (HBV) annually affects 350-400 million people and causes 1 million deaths worldwide[1,2]. Chronic HBV infection results in a risk of progressive liver fibrosis, leading to cirrhosis with decreased liver reserve and hepatocellular carcinoma (HCC)[3]. The annual incidence of HCC is reported to be 10%-17% in HBV-induced liver cirrhosis, and the fibrotic status stepwisely increases the risk of HCC, as in case of chronic infection of hepatitis C virus (HCV)[4]. Therefore, an early assessment of liver fibrosis is required for not only the prevention of disease progression but also the judgment of therapeutic intervention in patients with HBV infection. Liver biopsy is currently the gold standard for estimating fibrosis progression, although frequent application of this procedure is limited because of sampling error, invasiveness, and some other complications[5]. These reasons have prompted many investigators to explore noninvasive predictors for liver fibrosis. Of several noninvasive methods, two tests, namely the aspartate aminotransferase (AST)-to-platelet index (APRI) and the fibrosis index based on four factors (FIB-4), are utilized for relatively accurate detection of liver fibrosis especially induced by HCV infection[6-8]. At present, the accuracy of these models has been externally validated in patients with chronic viral hepatitis, nonalcoholic fatty liver disease (NAFLD), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). With regard to chronic hepatitis B (CHB), a recent meta-analysis suggested that APRI and FIB-4 could identify CHB-related fibrosis with moderate sensitivity and accuracy[9]. However, more methods are required to circumstantially classify liver fibrosis.

The renin-angiotensin-aldosterone system (RAAS) is a key mediator in the regulation of arterial blood pressure and body fluid homeostasis[10]. RAAS also reportedly plays an important role in the hemodynamics of several organs[11,12]. RAAS is frequently activated in patients with chronic liver diseases such as cirrhosis[13,14]. Angiotensin-I-converting enzyme (ACE), a central component of RAAS, converts the inactive decapeptide angiotensin Ⅰ (AT-Ⅰ) into the octapeptide angiotensin Ⅱ (AT-Ⅱ), which shows many physiological activities, including vascular hormonal secretion and tissue growth[15]. AT-Ⅱ has been considered to be a potential mediator of portal hypertension because its plasma level is markedly increased in patients with cirrhosis and its administration induces the elevation of portal pressure[13,16-18]. AT-Ⅱ is pathologically recognized to induce the contractility and proliferation of hepatic stellate cells (HSCs), which play a pivotal role in the progression of liver fibrosis[19-21]. The aim of the present study was to evaluate the behavior of circulating ACE in patients with CHB and assess the relationship between CHB-related liver fibrosis and serum levels of ACE.

MATERIALS AND METHODS

Patients

In total, 100 patients who were diagnosed with serologically and histologically confirmed CHB at Nara Medical University between 2013 and 2015 were enrolled. Chronic HBV infection was diagnosed in patients according to the following criteria: (1) detectable hepatitis B surface antigen (HBsAg) for ≥ 6 mo; and (2) serum HBV-DNA ≥ 1.3 log IU/mL. The status of other HBV markers such as HB envelope antigen (HBeAg), anti-HBe, and anti-HB core (HBc) IgG was not considered as a criterion for the current assessment. All the patients who fulfilled these criteria underwent

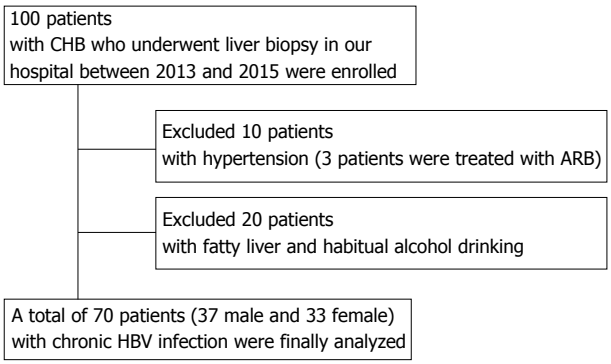

Figure 1 The selection of the study population. 70 patients except for 10 patients with hypertension and 20 patients with fatty liver or habitual alcoholic consumption were finally analyzed.

routine liver biopsies before therapeutic intervention. All pathological specimens were evaluated by at least two experienced pathologists. The degree of hepatic fibrosis was assessed and graded according to the METAVIR score for chronic hepatitis graded 0-4: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis[22,23]. Hepatic steatosis is histologically defined with more than 5% of accumulated lipid droplets in the hepatic parenchyma, and habitual alcohol consumption is defined with a daily ethanol intake of > 20 g (female) or > 30 g (male). Patients were excluded if they had other concomitant chronic liver diseases, including chronic hepatitis C (CHC), autoimmune hepatitis (AIH), PBC, PSC, hemochromatosis, or Wilson's disease as well as cancer, severe cardiopulmonary or renal diseases, diabetes mellitus (DM), sarcoidosis, dysthyroidism, or a previous history of liver transplantation. 10 patients treated with antihypertensive agents were also excluded regardless of the type of drug because high blood pressure was considered to affect serum ACE levels (Figure 1). The study was conducted in accordance with the standards of the Helsinki Declaration, and written informed consents were provided by all the study subjects. The protocols used were approved by the Ethics Committee of Nara Medical University (Nara, Japan; Approval number 1077) and other facilities.

Laboratory analysis and measurement of angiotensin-converting enzyme levels

Serum samples from all patients were collected when liver biopsy was performed and were stored and used for the present study. The following laboratory parameters were routinely measured: complete blood count, AST, alanine aminotransferase (ALT), and albumin (Alb). To assess liver fibrosis, we evaluated four serum fibrotic markers, namely, hyaluronic acid, type 4 collagen 7S, type 3 procollagen-N-peptide (P-Ⅲ-P), and Mac-2-binding protein glycosylation isomer (M2BPGi), as well as the number of platelets (Plt). APRI and FIB-4 were also used as noninvasive tests for the assessment of liver fibrosis. In these tests, evaluation was performed using the following formula: APRI = [(AST of the sample/reference AST) × 100]/platelets; FIB-4 = (age × AST)/[(platelets) × (ALT)$^{1/2}$]. Serum ACE levels were measured using Kasahara's colorimetry-based methods[24]. A serum ACE level between 8.3 and 21.4 IU/L was considered to be within the normal range according to the manufacturer's instruction.

Statistical analysis

Continuous variables of patients with discordance and those without discordance were compared using independent *t*-tests or Mann-Whitney *U* tests, as appropriate. The χ^2 or Fisher's exact tests were used for categorical variables. Area under the receiver operating characteristic (AUROC) curves and other statistical analyses were performed using R software as described previously[25].

RESULTS

Serum ACE levels in patients with fatty liver and alcohol abuse

ACE activity is susceptible to the presence of fatty liver and/or habitual alcoholic consumption (FL/AL), as reported previously[26]. Thus, we initially evaluated the serum ACE activity in patients histologically diagnosed with hepatic steatosis to exclude patients with FL/AL from the current subjects. Histological analysis demonstrated that 20 patients showed hepatic steatosis. Of these patients, 8 patients habitually drank alcohol. Similar to several reports, serum ACE levels were significantly higher in patients with CHB with FL/AL than in those without FL/AL (Figure 2A), although other fibrotic markers, including APRI, FIB-4, and M2BPGi, were not affected by the coexistence of FL/AL (Figure 2B-2D). Interestingly, a fibrosis stage-matched comparison showed that this difference in serum ACE levels was prevalently observed in patients with early-stage (F0 and F1) liver fibrosis (Figure 2E). Consequently, 70 patients with CHB and without hypertension and/or FL/AL were included as subjects in the present study.

Characteristic features of patients

The demographic and clinicopathological characteristics of the patients in the final analysis are presented in Table 1. In total, 70 patients with CHB without FL/AL (37 males and 33 females) with a median age of 48.6 ± 14.1 years were included in the present study. The fibrosis stages were F0, F1, F2, F3 and F4 in nine (12.8%), 25 (35.7%), 17 (24.3%), 13 (18.6%), and six (8.6%) patients, respectively. All the patients in F4 were classified as Child-Pugh A. There were no differences in serum Alb levels and HBV-DNA and HBsAg values among each stage of fibrosis.

Elevated serum ACE level above F2 stage in liver fibrosis

In patients with CHB, the mean serum ACE level was

Table 1 The clinicopathological characteristics of the patients with hepatitis B

Variables	Patients with HB (n = 70)
Sex (males/females)	37/33
Age	48.6 ± 14.1
Fibrosis stage (F0/F1/F2/F3/F4)	9/25/17/13/6
Platelet (× 10^4 μL) (F0/F1/F2/F3/F4)	17.9 ± 5.0 (23.2 ± 4.7/18.4 ± 3.6/18.7 ± 3.8/15.1 ± 5.4/18.9 ± 3.3)
Alb (g/dL)	4.1 ± 0.4
AST (IU/L)	35.8 ± 26.7
ALT (IU/L)	42.6 ± 39.4
HBV DNA (Log IU/mL)	4.04 ± 2.24
HBsAg (IU/mL)	22697.1 ± 52927.9
Hyaluronic acid (ng/mL)	62.4 ± 92.1
Type 4 collagen 7S (ng/mL)	4.1 ± 2.0
Serum ACE (U/L) (F0/F1/F2/F3/F4)	14.1 ± 5.1 (10.8 ± 2.3/11.1 ± 4.7/17.9 ± 4.4/6.4 ± 3.9/15.0 ± 2.9)
APRI (F0/F1/F2/F3/F4)	1.0 ± 1.3 (0.3 ± 0.1/0.7 ± 0.5/0.9 ± 0.6/2.3 ± 2.1/1.0 ± 0.9)
FIB4 (F0/F1/F2/F3/F4)	2.0 ± 1.9 (1.3 ± 0.6/1.6 ± 1.0/1.3 ± 0.8/2.8 ± 1.7/1.7 ± 0.7)
M2BPGi (COI) (F0/F1/F2/F3/F4)	1.2 ± 1.1 (0.9 ± 0.9/0.9 ± 0.6/.1 ± 1.0/2.2 ± 1.8/1.3 ± 0.6)

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; Alb: Albumin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ACE: Angiotensin-I-converting enzyme; APRI: Aspartate aminotransferase-to-platelet index; FIB4: Fibrosis index based on four factors; M2BPGi: Mac-2-binding protein glycosylation isomer.

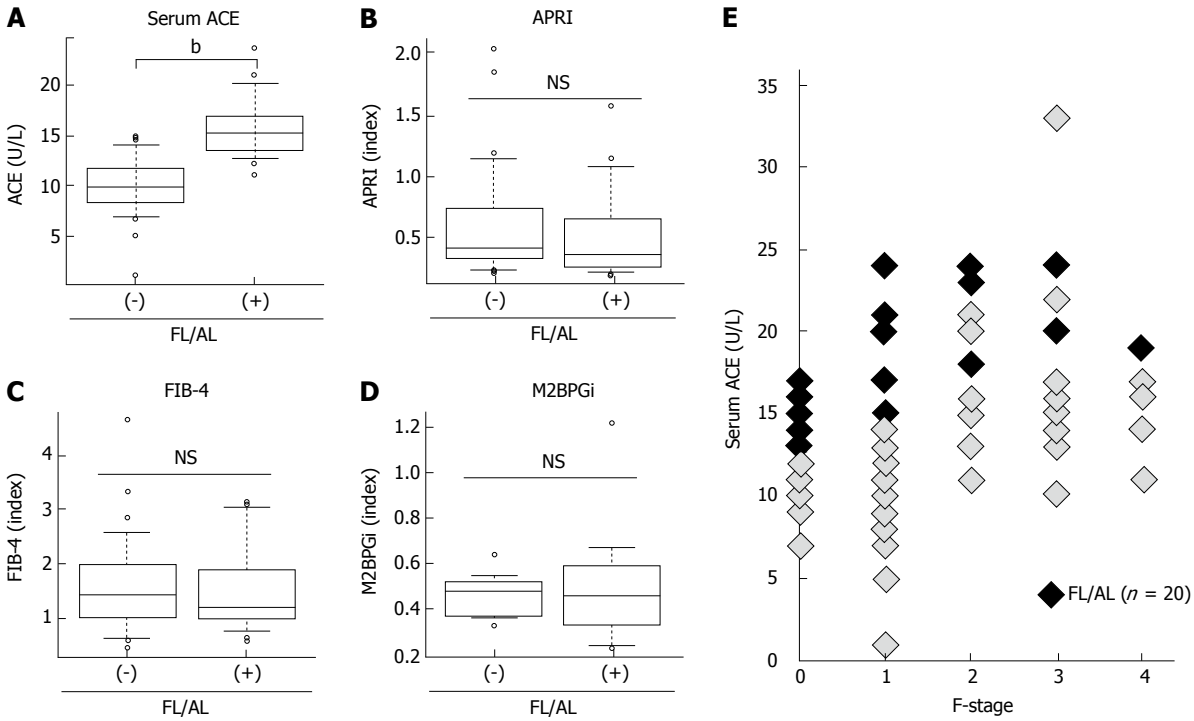

Figure 2 Serum levels of angiotensin-converting enzyme and fibrotic markers in patients with fatty liver and/or habitual alcoholic drinking. A: Serum angiotensin-converting enzyme (ACE) level; B: Aspartate aminotransferase to platelet index (APRI); C: Fibrosis index based on the four factors (FIB-4), D: Serum Mac-2 binding protein glycosylation isomer (M2BPGi) level in chronic hepatitis B patients with and without fatty liver and/or habitual alcoholic drinking (FL/AL). Serum ACE levels were significantly higher in the patients with FL/AL than those without FL/AL; E: Fibrosis stage-matched comparison showed that this difference in serum ACE levels between with and without FL/AL was prevalently observed in early stages (F0 and F1) of liver fibrosis. Data are means ± SD, $^bP < 0.01$.

14.1 ± 5.1 U/L and serum ACE levels at F0, F1, F2, F3, and F4 were 10.8 ± 2.3 U/L, 11.1 ± 4.7 U/L, 17.9 ± 4.4 U/L, 16.4 ± 3.9 U/L, and 15.0 ± 2.9 U/L, respectively. Pearson's correlation coefficient showed moderately positive correlation between serum ACE levels and fibrotic stages (R^2 = 0.181), and Mann-Whitney U tests demonstrated that patients with significant, advanced and cirrhotic stages (F2-4) showed markedly higher serum ACE levels than those with early-stage fibrosis and cirrhosis (F0-1) (Figure 3A). To validate the diagnostic performance of the serum ACE level in predicting significant liver fibrosis, we next performed receiver-operating characteristic (ROC) curve analysis. For significant fibrosis (⩾ F2), ROC curve analysis revealed that the optimal ACE level cut-off point was 12.8 U/L (sensitivity, 91.7%; specificity, 75.0%; PPV, 80.5%; NPV, 89.7%; accuracy, 84.3%), and the area under the curve (AUC) value for significant fibrosis (⩾

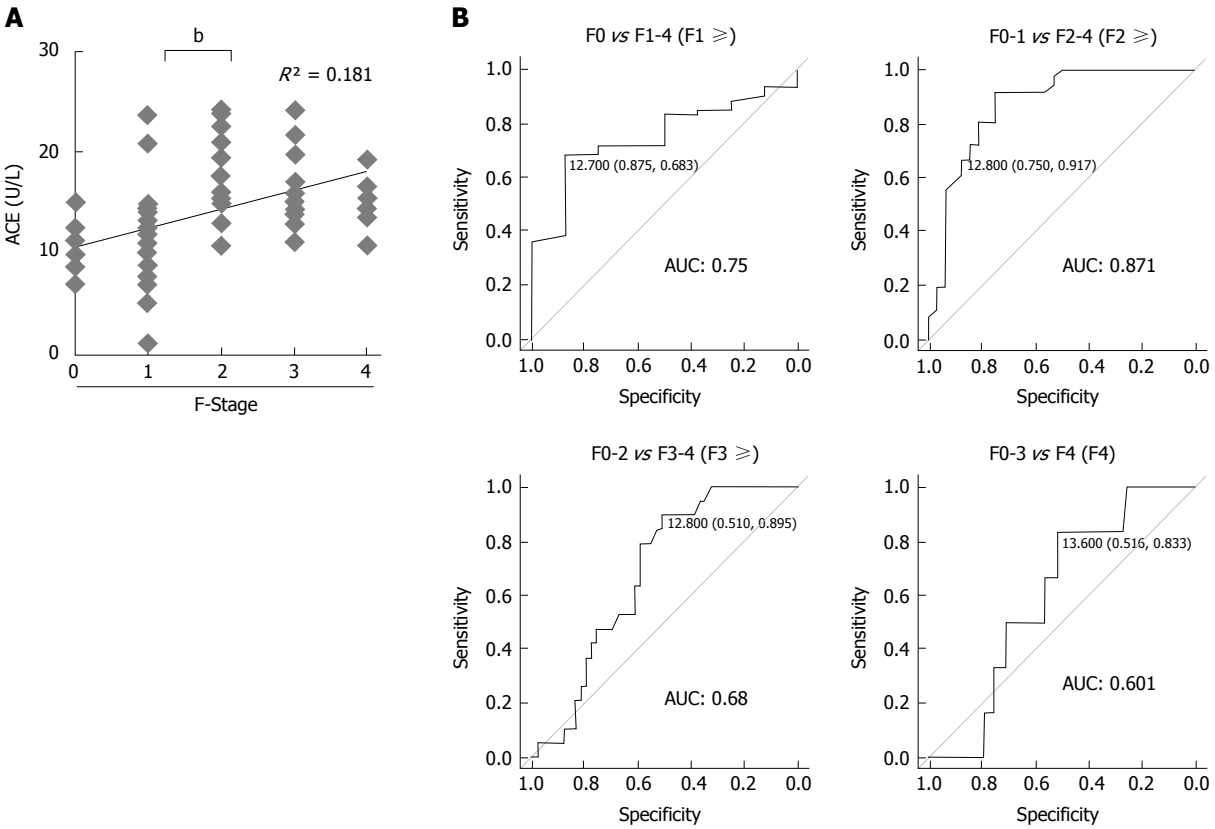

Figure 3 Serum angiotensin-converting enzyme levels and liver fibrosis development in patients with chronic hepatitis B. A: Correlation between serum angiotensin-converting enzyme (ACE) level and liver fibrosis stage (F-Stage) (R = 0.42, R^2 = 0.181); B: Receiver operating characteristic curve analysis and area under curve (AUC) value for diagnostic performance of serum ACE level for predicting each stage of liver fibrosis. The optimal ACE level cut-off point for significant fibrosis was 12.8 U/L. Data are means ± SD, ^bP < 0.01.

F2) was 0.871 (Figure 3B). Meanwhile, the AUC values to predict mild (≥ F1), advanced (≥ F3) and cirrhotic (F4) stage of fibrosis were 0.75 (sensitivity, 68.3%; specificity, 87.5%), 0.68 (sensitivity, 89.5%; specificity, 51.0%) and 0.601 (sensitivity, 83.3%; specificity, 51.6%), respectively which were remarkably lower than significant fibrosis (Figure 3B). These findings suggest that serum ACE level manifests its efficient performance in diagnosing significant fibrosis (≥ F2).

Diagnostic performance in other markers to predict significant stage liver fibrosis

Next, to evaluate predictive potential for significant fibrosis in other markers, we performed similar analysis for well-known fibrotic parameters, including APRI, FIB-4, M2BPGi and Plt. Unlike the ACE level, there was a significant difference below and above the F3 stage in these parameters, APRI (F2, 0.86 ± 0.6 *vs* F3, 2.27 ± 2.0; P < 0.05), FIB-4 (F2, 1.34 ± 0.6 *vs* F3, 2.76 ± 1.7; P < 0.05), M2BPGi (F2, 1.1 ± 1.0 *vs* F3, 2.2 ± 1.8; P < 0.05) and Plt (F2, 18.7 ± 3.8 *vs* F3, 15.1 ± 5.4 × 10^4/μL; P = 0.0616) (Figure 4A-D). Similarly, the established fibrosis markers such as hyaluronic acid, type 4 collagen 7S, and P-Ⅲ-P indicated an advanced fibrosis stage (≥ F3) (Figure 4E-G). Furthermore, we assessed the diagnostic performance of APRI, FIB-4, M2BPGi and Plt to predict significant fibrosis (≥ F2) by ROC curve analysis. For significant fibrosis (≥ F2), the APRI cut-off point was 0.57 (sensitivity, 83.3%; specificity, 69.7%; PPV, 76.0%; NPV, 71.1%; accuracy, 73%), FIB-4 cut-off point was 2.23 (sensitivity, 40.0%; specificity, 84.8%; PPV, 70.6%; NPV, 39.1%; accuracy, 47.6%), M2BPGi cut-off point was 0.81 (sensitivity, 50.0%; specificity, 48.9%; PPV, 69.7%; NPV, 29.4%; accuracy, 49.3%), and Plt cut-off point was 17.8 × 10^4/μL (sensitivity, 68.8%; specificity, 71.9%; PPV, 33.3%; NPV, 72.4%; accuracy, 50.8%) (Supplementary Figure 1). The AUC value of serum ACE (0.871) was higher than those of APRI (0.83, P = 0.224), FIB-4 (0.641, P = 0.0012), M2BPGi (0.717, P = 0.0239), and Plt (0.70, P = 0.016) (Figure 5A-D), indicating that compared with other noninvasive markers, the serum ACE level is distinctively capable of the enclosure of significant liver fibrosis (≥ F2) in patients with CHB.

APRI is a beneficial marker in predicting advanced liver fibrosis with the highest accuracy

As the next step to closely diagnose significant fibrosis in patients with CHB, patients with > F2 fibrosis need to be further classified into significant (F2) and advanced (F3) fibrosis. Therefore, we compared the AUC values to predict advanced fibrosis stages (≥ F3) among APRI, FIB-4, M2BPGi, and Plt to evaluate diagnostic

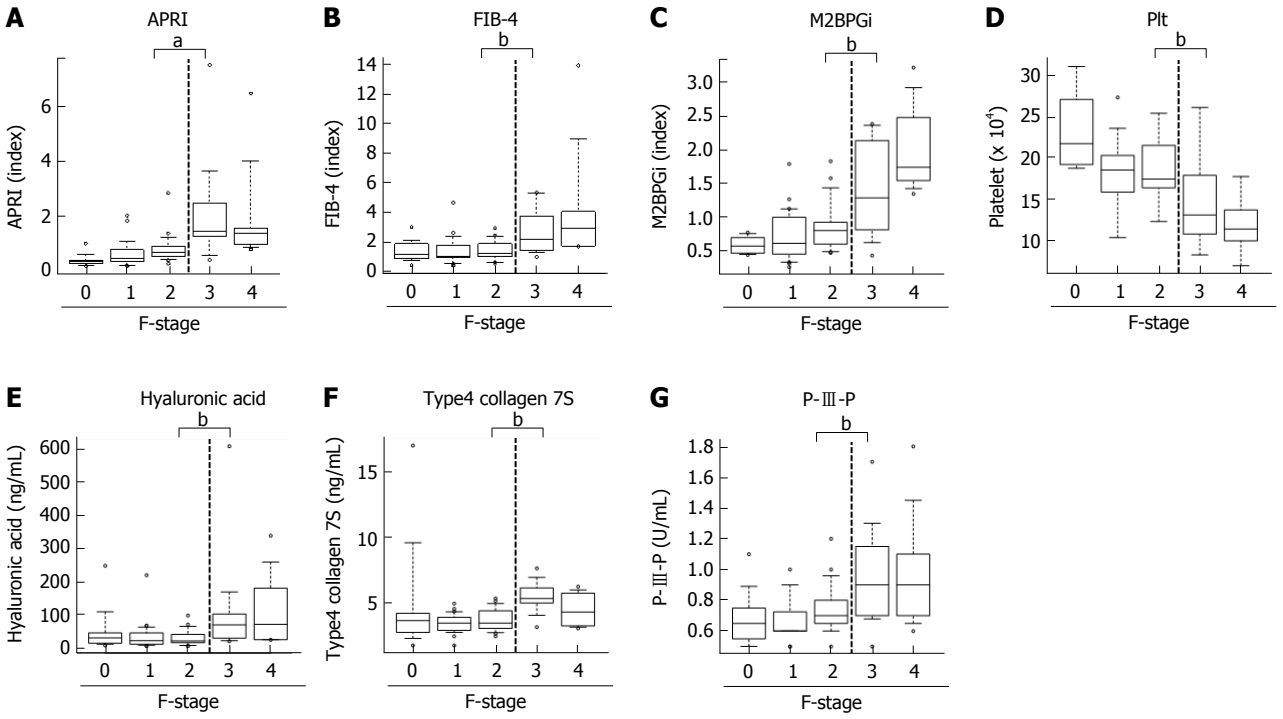

Figure 4 Serum levels of other markers and liver fibrosis in patients with chronic hepatitis B. A: Aspartate aminotransferase to platelet index (APRI); B: Fibrosis index based on the four factors (FIB-4); C: Serum levels of Mac-2 binding protein glycosylation isomer (M2BPGi); D: The number of platelets (Plt); E: Serum hyaluronic acid level; F: Type4 collagen 7S; and G: P-Ⅲ-P levels in patients with each liver fibrotic stage (F-Stage). Data are means ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$.

performance of these parameters for advanced fibrosis. The ROC curve analysis demonstrated that the optimum cut-off points for advanced fibrosis (≥ F3) are APRI; 1.27 (sensitivity, 75%; specificity, 91.5%), FIB-4; 2.51 (sensitivity, 56.2%; specificity, 93.6%), M2BPGi; 0.82 (sensitivity, 87.5%; specificity, 64.0%), and Plt; 15.0 × 10^4/μL (sensitivity, 70.6%; specificity, 87.2%) (Supplementary Figure 1). The AUC value is the highest in APRI (0.879) as compared with FIB-4 (0.803), M2BPGi (0.791), and Plt (0.813).

DISCUSSION

Identifying the degree of liver fibrosis is a clinical requisite for the treatment of chronic liver diseases regardless of the etiology because it plays a key role in predicting therapy responses and long-term outcomes of patients. In the case of CHB, nucleos(t)ide analogs (NAs) inhibit HBV-DNA replication and reduce the serum HBV level to achieve therapeutic improvement. Meanwhile, NAs do not play any role in the complete elimination of HBV and do not provide HBsAg clearance or persistent HBeAg seroconversion. Moreover, NAs frequently cause drug resistance and relapse after the termination of therapy. These pharmacological properties of NAs require long-term administration for patients to avoid the risk of liver decompensation due to cirrhosis and HCC progression, suggesting that the ideal time to start the therapy should be carefully decided considering the fibrotic stage as well as HBV-DNA and ALT levels which are extensively recognized as guideline for treatment. At present, APRI and FIB-4 indexes are widely known as noninvasive predictors to evaluate liver fibrosis[7,8]. Xiao *et al*[9] systematically reviewed the performance of two indexes in HBV-associated liver fibrosis. The total AUC values of APRI/FIB-4 for the diagnosis of significant fibrosis, advanced fibrosis, and cirrhosis were 0.7407, 0.7844 and 0.7347/0.8165, and 0.7268 and 0.8448, respectively. These data indicate that APRI and FIB-4 can identify CHB-related fibrosis with moderate sensitivity and accuracy. However, additional markers are required for diagnosis with higher accuracy and sensitivity. Recent evidences demonstrated that several serum markers are possibly beneficial for prediction of earlier fibrosis. Oztas *et al*[27] reported that soluble ST2, a receptor for the Th2 cytokine IL-33, could be used for differentiating significant fibrosis from mild fibrosis in CHB patients, and Deng *et al*[27] showed that serum complement 5a concentration significantly decreased in severe HBV fibrosis stages and earlier cirrhosis[28].

Our results show that serum ACE levels were markedly higher in patients with CHB who were histologically diagnosed with significant, advanced fibrosis and cirrhosis than in those with early fibrosis, while other predictors distinguished advanced fibrosis and cirrhosis (≥ F3) but not significant fibrosis (F2). These results indicate that the serum ACE level has a distinctive potential to enclose significant fibrosis (≥ F2), which is the therapeutically adapted stage for NAs[23]. As confirmed by AUC, the diagnostic value of serum ACE for detecting significant fibrosis above F2 was 87.1%,

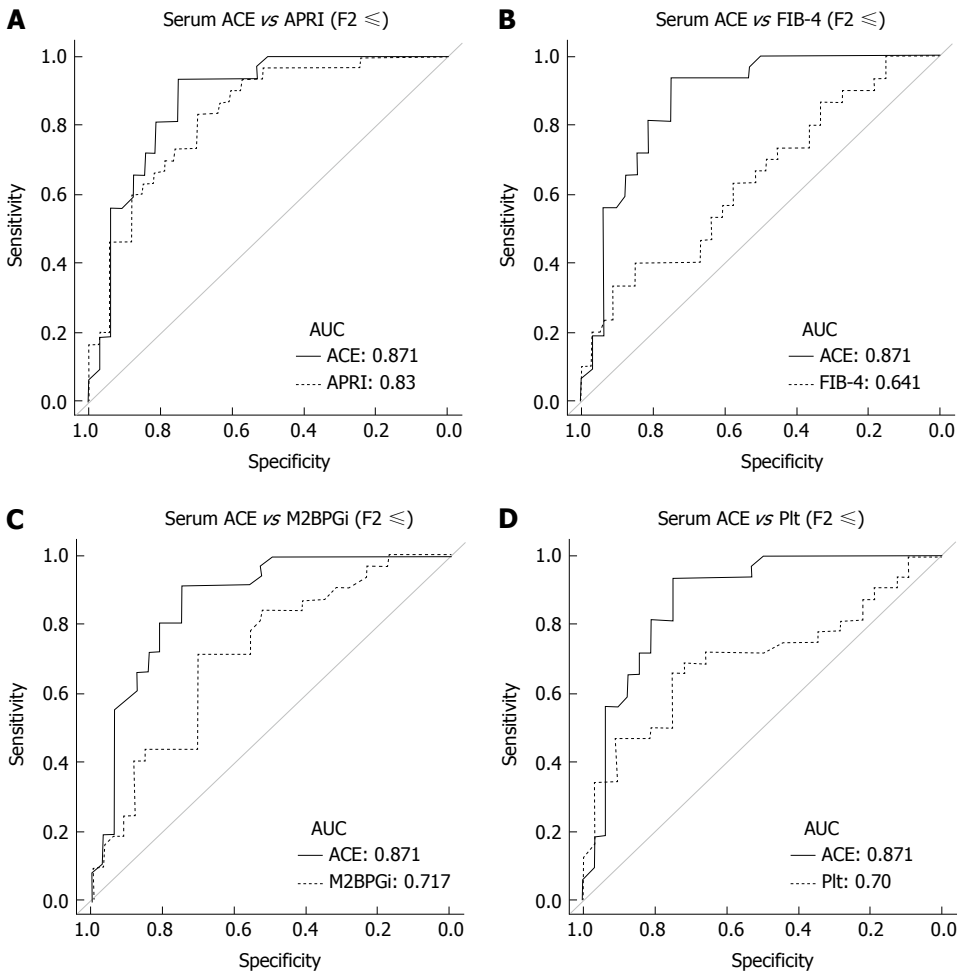

Figure 5 Receiver operating characteristic curve analysis for diagnostic performance for predicting significant liver fibrosis. Compared to other markers, the area under curve (AUC) value in serum angiotensin-converting enzyme (ACE) level, 0.871, was higher than that in A: Aspartate aminotransferase to platelet index (APRI), 0.83 (P = 0.224); B: Fibrosis index based on the four factors (FIB-4), 0.641 (P = 0.0012); C: Serum levels of Mac-2 binding protein glycosylation isomer (M2BPGi), 0.717 (P = 0.0239); D: The number of platelets (Plt), 0.70 (P = 0.016).

which was higher than other that of fibrotic markers, including APRI, FIB-4, M2BPGi and Plt.

As described in previous reports, AT-Ⅱ, which is produced by ACE converted from AT-Ⅰ, plays an important role in liver fibrosis development[21,29,30]. AT-Ⅱ induces HSC proliferation, upregulates transforming growth factor-β and collagen-Ⅰ gene expression, and promotes extracellular matrix synthesis[19,20]. Additionally, previous animal study demonstrated that ACE gene was up-regulated in the bile duct ligation-induced fibrotic liver[31]. Recent evidence also suggested that upregulation of hepatic ACE is accelerated particularly during fibrogenesis and is dampened in the developed fibrosis state[32]. Correspondingly, our results showed that serum ACE level was increased with moderately positive correlation with liver fibrosis development, and reached a threshold in the significant fibrosis (F2) (Figure 3A). These findings suggest that the suppression of RAAS is an inevitable strategy for preventing liver fibrosis progression. Our recent animal studies have revealed that ACE inhibitor (ACE-I) and AT receptor blocker (ARB) show significant antifibrotic effects on experimental liver fibrosis along with the suppression of activated HSC[33-36]. A clinical study also demonstrated that the group of patients with HCV treated using angiotensin-blocking agents exhibited lesser fibrosis than those without hypertension[37]. Another report showed that RAAS blocker-treated hypertensive patients with NAFLD prevalently had a mild degree of liver fibrosis[38]. Liver fibrogenesis progresses through multiple processes that are dependent on the etiology. Therefore, the clinical findings in patients with HCV and NAFLD may provide a basis for the assessment of the antifibrotic properties of RAAS blockers in HBV. A previous report actually showed that circulating ACE levels were elevated in patients with chronic HBV and more prominent elevation was observed in patients with advanced fibrosis[39].

Other reports have validated whether the serum ACE level has the clinical potential to predict liver fibrosis induced by other chronic liver diseases. Efe *et al*[40] suggested that ACE sustains hepatic fibrogenesis in AIH. They demonstrated that serum ACE levels increased for each fibrosis score in 73 patients with AIH, and AUC values of serum ACE for the diagnosis of significant fibrosis (≥ F2), advanced fibrosis

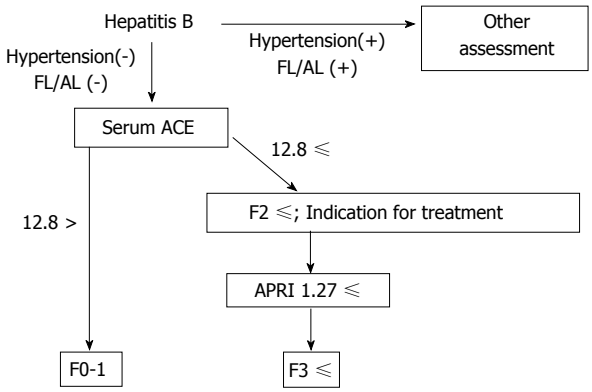

Figure 6 Schematic algorithm for noninvasive diagnosis of chronic hepatitis B. ACE: Angiotensin-converting enzyme; FL: Fatty liver; AL: Habitual alcoholic drinking; APRI: Aspartate aminotransferase to platelet index.

(≥ F3), and cirrhosis (F4) were 0.89, 0.91, and 0.95, respectively. The diagnostic performance for HCV-related fibrosis is controversial. Raslan *et al*[41] showed that ACE polymorphism was associated with the progression of hepatic fibrosis in chronic HCV infection. In contrast, Forrest *et al*[42] reported that no association was identified between four RAAS polymorphisms and fibrosis in chronic HCV infection. Our other assessment also suggested that serum ACE levels in patients with CHC did not correlate with the fibrosis stage (data not shown). This discrepancy may be explained by the interaction between HCV infection and steatosis. HCV infection induces metabolic abnormalities such as insulin resistance, leading to hepatic steatosis[43,44]. Interestingly, our data show that serum ACE levels were elevated in patients with hepatic steatosis (Figure 2). The evidence indicates that hepatic steatosis induced by HCV infection may destabilize serum ACE levels.

In our results, we also found that APRI, FIB-4, M2BPGi and Plt might be good predictive markers for advanced fibrosis in patients with CHB, and APRI showed a potential with the highest accuracy in these parameters. Based on these data, we can propose a strategy to noninvasively evaluate CHB-related liver fibrosis in combination with serum ACE and APRI (Figure 6). This algorithm may be utilized to enclose significant fibrosis, particularly in patients with CHB without fatty liver and/or habitual alcoholic consumption.

Our data have some limitations such as the lack of clinicopathological and prognostic data and a small sample size. Moreover, our study was not performed prospectively. Sequential measurements of serum ACE are required in longitudinal studies to assess the change in the serum ACE level associated with the degree of fibrosis. In addition, the optimum cut-off value for significant fibrosis of 12.8 U/L deviates from the value of 52.5 U/L in the previous study because a different method is used to measure the serum ACE level[39,40]. Therefore, comparison with data from other reports to validate the integration is difficult.

In conclusion, we indicated that the serum ACE level is a beneficial noninvasive marker to evaluate significant fibrosis and to further determine whether therapeutic intervention with NAs is necessary for patients with CHB. Higher accuracy will be expected by combination with other markers, including APRI, FIB4, M2BPGi and Plt. Moreover, our results suggest that blockade of RAAS is an effective new therapeutic strategy against CHB-related fibrosis.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection results in a risk of progressive liver fibrosis, leading to cirrhosis with decreased liver reserve and hepatocellular carcinoma. Therefore, an early assessment of liver fibrosis is required for not only the prevention of disease progression but also the judgment of therapeutic intervention. Currently, several noninvasive markers are explored for estimating early stage of HBV-related fibrosis alternatively to liver biopsy. However, it is required to identify both easier and more beneficial methods.

Research frontiers

Angiotensin-I-converting enzyme (ACE), a central component of renin-angiotensin-aldosterone system, plays a key role in the progression of liver fibrosis, however, its diagnostic performance to evaluate HBV-related earlier fibrosis.

Innovations and breakthroughs

The present study indicates that the patients with significant, advanced fibrosis and cirrhosis (F2-4) have significantly higher serum ACE levels than those with early-stage fibrosis and cirrhosis (F0-1), and serum ACE shows higher accuracy than other markers including APRI, FIB-4, M2BPGi and Plt in diagnostic performance to differentiate significant fibrosis (F2) from mild fibrosis (F1).

Applications

The serum ACE level is a beneficial noninvasive marker to evaluate significant fibrosis and to further determine whether therapeutic intervention is necessary for patients with chronic HBV.

Terminology

ACE converts the inactive decapeptide angiotensin Ⅰ (AT-Ⅰ) into the octapeptide angiotensin Ⅱ (AT-Ⅱ), which shows many physiological activities, including vascular hormonal secretion and tissue growth. AT-Ⅱ has been pathologically recognized to induce the contractility and proliferation of hepatic stellate cells, which play a pivotal role in the progression of liver fibrosis.

Peer-review

The authors report an interesting approach about the diagnostic value evaluation of serum ACE for the detection of earlier HBV-related fibrosis. The sensitivity of the test discriminating F0-F1 from F2-F4 proved to be higher than that of other tests. Overall, the study is well planned and results are presented clearly.

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ORIGINAL ARTICLE

Observational Study

Wilson's disease in Lebanon and regional countries: Homozygosity and hepatic phenotype predominance

Kassem Barada, Aline El Haddad, Meghri Katerji, Mustapha Jomaa, Julnar Usta

Kassem Barada, Aline El Haddad, Department of Internal Medicine, American University of Beirut Medical Center, Beirut 110236, Lebanon

Meghri Katerji, Mustapha Jomaa, Julnar Usta, Department of Biochemistry and Molecular Genetics, Faculty of Medicine, American University of Beirut, Beirut 110236, Lebanon

ORCID number: Kassem Barada (0000-0002-9629-1708); Aline El Haddad (0000-0001-7527-0935); Meghri Katerji (0000-0003-0971-8177); Mustapha Jomaa (0000-0003-3807-6839); Julnar Usta (0000-0002-6191-6763).

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Correspondence to: Julnar Usta, PhD, Professor, Department of Biochemistry and Molecular Genetics, American University of Beirut, Riad el Solh, Beirut 110236, Lebanon. justa@aub.edu.lb
Telephone: +961-1-350000

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Abstract

AIM

To determine the phenotypes and predominant disease-causing mutations in Lebanese patients with Wilson's disease, as compared to regional non-European data.

METHODS

The clinical profile of 36 patients diagnosed in Lebanon was studied and their mutations were determined by molecular testing. All patients underwent full physical exam, including ophthalmologic slit-lamp examination ultrasound imaging of the liver, as well as measurement of serum ceruloplasmin and 24-h urinary-Cu levels. In addition, genetic screening using PCR followed by sequencing to determine disease-causing mutations and polymorphisms in the *ATP7B* gene was carried on extracted DNA from patients and immediate family members. Our phenotypic-genotypic findings were then compared to reported mutations in Wilson's disease patients from regional Arab and non-European countries.

RESULTS

Patients belonged to extended consanguineous families. The majority were homozygous for the disease-causing mutation, with no predominant mutation identified.

The most common mutation, detected in 4 out of 13 families, involved the ATP hinge region and was present in patients from Lebanon, Egypt, Iran and Turkey. Otherwise, mutations in Lebanese patients and those of the region were scattered over 17 exons of *ATP7B*. While the homozygous exon 12 mutation Trp939Cys was only detected in patients from Lebanon but none from the regional countries, the worldwide common mutation H1069Q was not present in the Lebanese and was rare in the region. Pure hepatic phenotype was predominant in patients from both Lebanon and the region (25%-65%). Furthermore, the majority of patients, including those who were asymptomatic, had evidence of some hepatic dysfunction. Pure neurologic phenotype was rare.

CONCLUSION

Findings do not support presence of a founder effect. Clinical and genetic screening is recommended for family members with index patients and unexplained hepatic dysfunction.

Key words: Wilson Disease; Cu-metabolism; Phenotype; Genotype; ATP7B; Hepatic manifestations

Core tip: We report on the genotype-phenotype of 36 Lebanese patients with Wilson's disease from 13 different families. The majority were homozygous for disease-causing mutations. The most common mutation worldwide, His1069Trp, was absent in our patients. The ATP hinge region may comprise a hot spot for mutations, as it was detected in 4 families. Hepatic phenotypes were predominant in both symptomatic and asymptomatic patients. Neurologic phenotypes were rare. Compared to findings reported in regional Arab and non-European countries, our results do not support a founder effect. Mutations are scattered over 17 exons, with no common or frequent mutation characterizing the region.

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INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism, resulting from defects in the *ATP7B* gene protein. It is characterized by failure of Cu incorporation into ceruloplasmin (Cp) and decreased biliary Cu excretion. As a consequence, Cu accumulates in various organs, primarily liver and brain. The clinical presentations of WD are characterized by substantial diversity. Patients can present at any age in variable combinations of liver impairment, neurologic dysfunction and/or osseomuscular symptom. Hepatic manifestations include asymptomatic transaminitis, acute or chronic hepatitis, fulminant hepatic failure and/or cirrhosis, while neurologic symptoms vary from mild tremors, articulating problems, dysarthria, Parkinson-like features, seizures and cognitive dysfunction. Some patients have mixed hepato-neurologic presentation[1]. Ophthalmologic involvement with Kaiser-Fleischer (KF) rings is common.

Traditionally, the diagnosis of WD is based on low serum-Cp (< 20 mg/dL), high 24-h urinary Cu and high hepatic Cu content (250 μg/g dry tissue)[2,3]. Recent guidelines for the diagnosis of WD were published by the European Association for the Study of the Liver (EASL)[4]. Nonetheless, the diagnosis of WD may be difficult based on clinical and laboratory criteria, and in some patients it is delayed, leading to detrimental consequences[5]. This is why molecular testing and genotypic analysis may be warranted for confirming and/or supporting a diagnosis of WD, particularly in asymptomatic patients[3].

More than 500 mutations have been identified in WD with a very high allelic heterogeneity. Most patients are compound heterozygous, rendering it difficult to ascribe a phenotype to a specific genotype[6]. Furthermore, a large number of mutations are rare, making it impractical to screen populations for all WD-causing mutations[7]. Some mutations, however, are relatively frequent and population-specific, like the p.His1069Gln on exon 14 in Northern and Eastern European patients[8], the p.Arg778Leu and the p.Arg778Gly mutations on exon 8 among Chinese and Taiwanese patients respectively[9], and the deletion in the 5' regulatory region in Sardinian patients[10]. These findings facilitate molecular diagnosis based on patients' ethnic background. In the Arab World, consanguinity and marriage among individuals belonging to the same ethnic background is very common, thereby increasing the prevalence of genetic disorders, including WD[11]. However, it is not known whether there is a predominant WD mutation in the Arab world, and if so what its phenotypic associations are.

In a cohort of Egyptian patients, genotypic and phenotypic profiles were described, but no prevalent mutation was identified[12]. Moreover, previous reports from Lebanon on a limited number of families suggested an association of liver presentation with homozygous missense mutations: Gly691Arg and non-His1069Trp in exons 7 and 14 of the *ATP7B* gene respectively[13,14]. Whether a specific WD mutation prevails in Lebanon is not known.

In this study, we described the spectrum and frequency of mutations and phenotypes in 36 Lebanese WD patients. We also conducted a comprehensive

literature search for regional studies on WD in Arab and non-European countries in the Middle East. In order to determine whether there is a frequent mutation characterizing the region, a comparative study was undertaken to identify common mutations in the region, and to compare them to our data. We also determined if common mutations in the region were associated with similar clinical phenotypes.

MATERIALS AND METHODS

A total of 36 patients (P_1-P_{36}) from 13 unrelated Lebanese families (U, Or, S, Ah, T, B, H, Ha, Is, Z, Ri, Sc and Gh) were enrolled in the study. Most patients were diagnosed at the American University of Beirut (AUB) Medical Center, a major tertiary referral center in Lebanon. All participating subjects were asked to sign a written consent form (Protocol No. BioCh.JU.01) that was approved by both the Institutional Review Board and Research Committee at AUB.

Clinical testing

Patients' data for evaluation included: history, date of birth, age of onset of symptoms, age at diagnosis, and findings from full physical exam, ophthalmologic slit-lamp examination, and biochemical tests, including liver function tests, serum-Cp and 24-h urinary-Cu levels. Abdominal ultrasound imaging of the liver was performed on all patients and, when necessary, brain magnetic resonance imaging was done. Phenotypic classifications were designated following Ferenci's classification as hepatic, neurologic, mixed or asymptomatic[3]. Diagnosis was further established by computing total WD score developed at the 8th international meeting[15]. Family members (siblings, parents) of all WD-confirmed index patients were also subjected to physical, biochemical and genotypic testing.

Genotypic screening

DNA screening for disease-causing mutations and single nucleotide polymorphisms was performed on all recruited subjects and their immediate family members. Extraction of DNA from blood samples followed by amplification (using PCR) of the 21 *ATP7B* exons was carried out as described[6,14]. Amplified PCR products were purified, sequenced and compared to published normal sequences in the following databanks: Blat at University of California Santa-Cruz, Genome Bio-informatics (http://www.Genome.ucsc.edu/cgi-bin/hgBlat) or Blast at National Center for Biotechnology Information (http://www.ncbi.nih.gov/blast).

WD in regional countries

After identifying the disease-causing mutations in our patients, we compared them to reported mutations in WD patients from regional Arab and Non-European countries. A comprehensive literature search of PubMed and Medline, as well as of the University of Alberta database (http://www.wilsondisease.med.ualberta.ca/database.asp), was conducted for articles published from the regional Arab and non-European countries. Index terms used were Wilson Disease, genotype, phenotype, and each of the following countries: Lebanon, Syria, Jordan, Egypt, Iraq, Saudi Arabia, Kuwait, Bahrain, Qatar, UAE, Yemen, Tunisia, Morocco, Libya, Mauritania, Turkey, Iran and Oman. We included studies in which both the genotype and phenotype were identified. In some studies, it was not clearly indicated whether patients presenting to one medical center with a certain mutation belonged to the same family or to different ethnic groups[16,17]. This made it difficult to estimate the most frequent genotype. We, therefore, opted to identify common mutations between Lebanon and the region, and to determine the frequent regional mutations as indicated by the authors of the various reports.

RESULTS

Clinical presentation

In this study, 36 Lebanese WD patients, including 15 females and 21 males, were recruited from different regions in Lebanon. Patients belonged to 13 unrelated families, referred to as: U (P_1-P_9); Or (P_{10}); S (P_{11}-P_{19}); Ah (P_{20}); T (P_{21}-P_{23}); B (P_{24}-P_{25}); H (P_{26}-P_{28}); Ha (P_{29}-P_{30}); Is (P_{31}); Z (P_{32}-P_{33}); Ri (P_{34}); Sc (P_{35}); and Gh (P_{36}) families. Consanguinity was present in the parents of 27 of the patients (75%), who belonged to the U, S, B, H, Ha, and Z families (Table 1). WD scores computed following EASL guidelines ranged between 4 and 12 (Table 2), confirming the diagnosis.

The clinical profiles of affected subjects are summarized in Table 2. Age at diagnosis ranged between 1 and 39 years. All patients had low Cp level (< 0.2 g/L), except for P_4 and P_{14}. Out of 31 patients, 18 (58%) had KF rings (5/36 were NAV). Out of the 36 WD patients, 24 were symptomatic (67%; 16 males and 8 females) and presented clinically at an average age of 14.5 years. Data on P_6-P_9 were not available. Twelve patients were asymptomatic (33%), diagnosed by genetic screening of family members of index patients. Their average age was 7.6 years.

Pure hepatic phenotype was the most common in our symptomatic patients [9/32: P_1, P_{16}-P_{17}, P_{18}, P_{20}, P_{25}, P_{27}-P_{28} and P_{32}]. Neurologic presentation was noted in 12.5% of patients (4/32: P_{12}, P_{14}, P_{34} and P_{36}). Mixed presentation was observed in 25% of patients (7/32: P_2, P_{10}, P_{24}, P_{26}, P_{29}, P_{31} and P_{35}), two of whom had suicidal attempts/disposition (P_{10} and P_{26}). Notably, liver cirrhosis was present in 12 symptomatic (38%) patients (P_1, P_2, P_{10}, P_{16}-P_{18}, P_{20}, P_{24}, P_{26}-P_{28} and P_{32}), including 4 patients with mixed presentation.

Of the asymptomatic subjects who were diagnosed by screening, 10/12 patients had evidence of liver disease, ranging from transaminitis (P_3-P_5, P_{11}, P_{13},

Table 1 Spectrum of mutations in the *ATP7B* gene of Lebanese patients with Wilson's disease

Family	ID	Sex	Birth date	AD	Exon	Mutation(s)	Region of protein
U	P_1	M	1966	7	7	Gly691Arg	TM2
	P_2	M	1985	9	7	Gly691Arg	TM2
	P_3	F	1986	13[1]	7	Gly691Arg	TM2
	P_4	F	1990	9[1]	7	Gly691Arg	TM2
	P_5	M	1996	3[1]	7	Gly691Arg	TM2
	P_6	M	NAV	3[2]	7	Gly691Arg	TM2
	P_7	M	NAV	7[2]	7	Gly691Arg	TM2
	P_8	M	NAV	12[2]	7	Gly691Arg	TM2
	P_9	F	NAV	NAV	7	Gly691Arg	TM2
Or	P_{10}	F	1986	21	7/10	Gly691Arg/Val845Ser	TM2/Td
S	P_{11}	F	1993	5[1]	8	2299insC/2299insC	TM4
	P_{12}	M	1973	12	8	2299insC/2299insC	TM4
	P_{13}	F	1997	10[1]	8	2299insC/2299insC	TM4
	P_{14}	M	1980	16	8	2299insC/2299insC	TM4
	P_{15}	M	2007	1[1]	8	2299insC/2299insC	TM4
	P_{16}	M	1981	16	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
	P_{17}	F	1983	14	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
	P_{18}	F	1993	12	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
	P_{19}	F	1989	15[1]	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
Ah	P_{20}	M	1992	15	8	2299insC/2299insC	TM4
T	P_{21}	F	1998	7	12	Trp939Cys	Td
	P_{22}	M	2001	8[1]	12	Trp939Cys	Td
	P_{23}	M	2006	3[1]	12	Trp939Cys	Td
B	P_{24}	M	1992	13	12	Trp939Cys	Td
	P_{25}	M	2002	5[3]	12	Trp939Cys	Td
H	P_{26}	F	1985	18	18	Asn1270Ser	ATP hinge
	P_{27}	F	1987	18[1]	18	Asn1270Ser	ATP hinge
	P_{28}	F	1991	8	18	Asn1270Ser	ATP hinge
Ha	P_{29}	F	1998	14	18	Asn1270Ser	ATP hinge
	P_{30}	F	2002	11	18	Asn1270Ser	ATP hinge
Is	P_{31}	M	1995	13	18	Asn1270Ser	ATP hinge
Z	P_{32}	M	1990	15	18	Pro1273Leu	ATP hinge
	P_{33}	M	2000	6[1]	18	Pro1273Leu	ATP hinge
Ri	P_{34}	M	2009	3	19	Arg1319stop	TM7
Sc	P_{35}	M	1979	22	15/19	Thr1092Met/Arg1319stop	ATP loop/TM7
Gh	P_{36}	M	1970	39	-	None identified	-

[1]Screening; [2]Died at age; [3]Deceased. AD: Age at diagnosis; NAV: Not available.

P_{21}-P_{23}, P_{30} and P_{33}) and hepatomegaly detected by abdominal ultrasound (P_{11}, P_{13} and P_{21}) to full blown cirrhosis (P_{22}-P_{23}). Overall, 27/32 patients (84%) on whom we had clinical information presented with some form of hepatic dysfunction.

Patients with the neurologic phenotype presented at an average age of 22.3 years, while those with hepatic and mixed phenotypes presented at 12.2 and 14 years, respectively. KF rings were present in 17 symptomatic patients (5 symptomatic were NAV) and absent in two (P_{25}, P_{34}). They were not identified in the asymptomatic patients, except for patient P_{19} who had KF rings with no evidence of hepatic or neurologic dysfunctions.

Mutation analysis

Sequencing of the *ATP7B* gene revealed (Table 1) 9 different disease-causing mutations in 70 chromosomes (35 patients), which were distributed as: 7 missense (exons: 7, 12, 10, 13, 15 and 18), 1 non-sense (exon 19), and 1 frame-shift (exon 8). Out of 70 chromosomes, missense/frameshift and/non-sense mutation(s) were detected in 51:16:3 chromosomes at 72.8%:22.8%:4.3% frequency respectively. No mutation was identified in P_{36}, who had been diagnosed based on KF rings, and clinical and biochemical testing.

Out of 35 patients, 29 were homozygous (82.8%) for a disease-causing mutation and 6 were compound heterozygous (17.1%). Parents of our index patients were carriers for the disease-causing mutations. Mutations were most frequent in the exon 18 motif encoding the conserved ATP hinge region of WD gene product. Four out of the 13 unrelated families (H, Ha, Is and Z) had, in this motif, missense mutations in the homozygous state; these were Asn1270Ser in 6 patients (P_{26}-P_{31}) and Pro1273Leu in 2 patients (P_{32}-P_{33}), accounting for 17% and 5.7% of chromosomes respectively. Other identified mutations (Table 1) included missense mutations in exon 7 (Gly691Arg; 10 patients: U and Or) and exon 12 (Trp939Cys; 5 patients: T and B), frameshift in exon 8 (2299insC; 10 patients: S and Ah), and nonsense mutation in exon 19 (Arg1319stop; 2 patients: Ri and Sc), accounting for a chromosome frequency of 27%, 14%, 23% and

Table 2 Phenotypic and genotypic profiles of Lebanese patients with Wilson's disease

ID	Mutation(s)	GI manifestation(s)	Neurological manifestations	KF rings	Cp	Urinary Cu	Score
P_1	Gly691Arg	Liver cirrhosis	Absent	Present	NAV	718.8	8
P_2	Gly691Arg	Liver cirrhosis	Change in school performance	Present	0.11	1998	10
P_3	Gly691Arg	Asymptomatic[1]	Absent	Absent	0.03	148.5	8
P_4	Gly691Arg	Asymptomatic[1]	Absent	Absent	0.22	304	6
P_5	Gly691Arg	Asymptomatic[1]	Absent	Absent	0.02	65.9	7
P_6	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_7	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_8	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_9	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_{10}	Gly691Arg/ Val845Ser	Liver cirrhosis	Suicidal attempts	Present	0.08	2184	12
P_{11}	2299insC	Asymptomatic	Absent	Absent	0.04	99	7
P_{12}	2299insC	Absent	Slurred speech, ataxia, tremors	Present	0.072	512	12
P_{13}	2299insC	Asymptomatic	Absent	Absent	0.03	152.8	8
P_{14}	2299insC	Absent	Choreoathetosis, tremors, rigidity	Present	0.423	2300	10
P_{15}	2299insC	Asymptomatic	Absent	Absent	0.019	10	6
P_{16}	2299insC/ p.Ala1003Thr	Liver cirrhosis	Absent	Present	0.096	775	10
P_{17}	2299insC/ p.Ala1003Thr	Liver cirrhosis	Absent	Present	0.096	590	10
P_{18}	2299insC/ p.Ala1003Thr	Liver cirrhosis	Absent	Present	0.17	645	9
P_{19}	2299insC/ p.Ala1003Thr	Absent	Absent	Present	0.12	487	9
P_{20}	2299insC	Liver cirrhosis	Absent	NAV	0.023	651	8
P_{21}	Trp939Cys	Asymptomatic	Absent	Absent	0.02	77.6	7
P_{22}	Trp939Cys	Asymptomatic	Absent	Absent	0.02	20	6
P_{23}	Trp939Cys	Asymptomatic	Absent	Absent	0.02	41.5	6
P_{24}	Trp939Cys	Liver cirrhosis, ascites	Jaw drooping, hypersalivation, slurred speech, narrow based gait, intention tremors	Present	0.021	744	12
P_{25}	Trp939Cys	Liver cirrhosis, Hepatic encephalopathy, Hepatomegaly, Mild to moderate ascites	Absent	Absent	0.04	NAV	6
P_{26}	Asn1270Ser	Liver cirrhosis	Psychiatric symptoms and suicidal attempts	Present	0.03	27.6	10
P_{27}	Asn1270Ser	Liver cirrhosis	Absent	Present	0.03	65.1	9
P_{28}	Asn1270Ser	Ascites, liver cirrhosis	Absent	Present	0.04	55	9
P_{29}	Asn1270Ser	Transaminitis	Neurodevelopmental	Present	0.078	171	11
P_{30}	Asn1270Ser	Asymptomatic	Absent	Absent	0.03	116	8
P_{31}	Asn1270Ser	Chronic liver parenchymal disease	Dysarthria and left-sided dystonia	Present	0.029	402.3	12
P_{32}	Pro1273Leu	Ascites, Liver cirrhosis, Hepatic encephalopathy	Absent	Present	0.17	1041.1	9
P_{33}	Pro1273Leu	Asymptomatic	Absent	Absent	0.19	89.7	6
P_{34}	Arg1319stop	Asymptomatic	Delay in speech	Absent	0.02	92	8
P_{35}	Thr1092Met/ Arg1319stop	Chronic liver disease and early portal hypertension	Clenching of mandible, left side dystonia, sialorrhea, dysarthia, head tremors	Present	0.025	199	12
P_{36}	None identified	Absent	Drooling, dysathria, difficulty concentrating, dysphagia	Present	0.085	NAV	6

[1]Developed later. Normal range: Serum ceruloplasmin: 0.2 to 0.6 g/L; Urine copper: 15 to 50 μg/24 h. Score = Ferrenci Score of diagnosis. 2 or less: Very unlikely; 3: Possible, more tests needed; 4 or more: Established[4]. Cp: Ceruloplasmin (g/L); KF: Keiser-Fleischer; NAV: Not available; Urinary Cu: 24-h urine copper (μg/24 h).

4.3% respectively. Compound heterozygous mutations were identified in exons 10 (Or: P_{10}), 13 (S: P_{16}-P_{19}) and 15 (P_{35}).

Eight polymorphisms were detected in exons 2, 3, 10, 12, 13 and 16 (Table 3) in patients and normal chromosomes obtained from related and unrelated individuals. Three polymorphisms (Lys832Arg, Arg952Lys and Val1140Ala) were present in the homozygous state in 94% (34/36) of patients and in the heterozygous state in 5% (P_{11} and P_{19}), in addition to others in exons 2, 3 and 13 (Table 3).

WD patients: Lebanon vs regional countries

A search of the literature for population studies on the spectrum of mutations in WD patients in the region, including Arab and non-European countries,

Table 3 Identified polymorphisms in the *ATP7B* gene of Lebanese patients with Wilson's disease

Polymorphism	Asp96Gly	Ser406Ala	Val456Leu	Lys832Arg	Arg952Lys	Ala1003 Ala	Val1140Ala	Ser1166Ser
Exon	2	2	3	10	12	13	16	16
Base change	GAC → GGC	TCT → GCT	GTG → CTG	AAG → AGG	AGA → AAA	GCG → GCA	GTC → GCC	AGC → AGT
Domain	Cu1-4	Cu4 binding	Cu4/Cu5	Td	Tm5	ATP binding/Tm6	ATP loop	ATP loop
Family								
U				HM	HM		HM	
Or		HM	HM	HM	HM		HM	
S								
P1, P2, P31, P41, P59				HM	HM		HM	
P7, P8				HT	HT		HT	
P3, P4					HM		HM	
AH			HM	HM	HM		HM	
TF				HM	HM	HM	HM	HM
B				HM	HM	HM	HM	
H		HM	HM	HM	HM		HM	
Ha								HM
Is			HM	HM	HM		HM	
Z		HM	HM	HM	HM		HM	
Ri	HM			HM			HM	
Sc			HT	HM	HM		HM	
Gh		HM	HM	HM	HM		HM	
Ah			HM	HM	HM		HM	

was conducted. A total of 77 articles on WD patients were initially identified, but only those reporting the genotypes and/or the phenotypes were considered. Consanguinity, homozygosity and frequency of mutation were also noted when indicated.

Seventeen articles were included and distributed as follows: Saudi Arabia[18-21], Egypt[12,22-24], Turkey[25,26], Iran[27,28], Oman[29] and Lebanon[6,13,14,30]. Two reports on WD from Iraq were not included, as they had no genotypic information. There were no reports on WD from Jordan, Libya, Tunisia, Morocco or Syria.

Homozygosity was highly prevalent in Lebanese WD patients (83%), and ranged between 68%-85.7% and 50%-53% in Egyptian and Saudi Arabian patients respectively. This finding is attributed to high consanguinity (Table 4) that is common in our societies, or the high prevalence of the same mutation in carriers. Frequency of asymptomatic cases was relatively similar in Lebanon, Egypt and Saudi Arabia. Similar to Lebanese patients, many of asymptomatic patients had evidence of hepatic dysfunction on laboratory and/or imaging studies. Hepatic phenotype was more common than neurologic phenotype in patients from Lebanon, Egypt, Turkey, Iran and Saudi Arabia. Taking into account patients who are asymptomatic and those with mixed phenotype, the vast majority of patients in those countries have some form of hepatic dysfunction. A minority of patients had pure neurologic phenotype. Also, the frequency of patients having KF rings was high and was similar in the 5 countries (Table 4). In a report on a single family from Oman, 78% of patients were asymptomatic and 21% had neurologic phenotype. No patients had a hepatic phenotype in that study.

In conducting our analysis of genotypes, we considered a mutation to be frequent if it was present in multiple unrelated families. We compared genotypic changes in the *ATP7B* gene of Lebanese patients with those from regional Arab and non-European patients. In our patients, the conserved ATP hinge region (exon 18) was the most frequently mutated region identified in 4 unrelated families (Table 1).

Table 3 shows that Lebanese patients share in common with: (1) Egypt, Iran and Turkey, the Val845Ser and Asp1270Ser mutations in exons 10 and 18 respectively; (2) Egypt, the Pro1273Leu mutation in exon 18; (3) Egypt and Turkey, the Arg1319X mutation in exon 19; and (4) Turkey, the Ala1003Thr mutation in exon 13 and the exon 7 mutation (Gly691Arg) reported in one Turkish patient[26]. More interestingly, the mutation in exon 12 (Trp939Cys) was only detected in Lebanese patients and in none of the searched/listed countries. Whereas the worldwide exon 14 mutation (His1069Gln) was detected in some patients from Egypt, Iran and Turkey, it was not identified in Lebanese or in Saudi Arabian patients.

DISCUSSION

The diagnosis of WD based on clinical grounds alone is often difficult. Thus, it may be necessary to resort to genetic testing. In this study, involving more than 500 patients from Lebanon and the region, we found a great deal of genetic heterogeneity with no common or population specific mutation. This reflects the extensive ethnic diversity of people in this part of the world and argues against the presence of a founder gene, even in highly consanguineous populations. It also implies that patients suspected to have WD without a family history, *i.e.*, without a known mutation

Table 4 Lebanese *vs* regional Arab and non-European Wilson's disease patients: Genotype-phenotype

	Lebanon	Egypt	Iran	Turkey	Saudi Arabia	Oman
Number of patients	36	198	88	46	152[1]	14
Number of families	13	135	-	46	53	1
% Homozygosity	83%	68.4% - 85.7%	NAV	NAV	50%-53%	NAV
% Consanguinity	75%	39.5% - 78.9%	NAV	NAV	36.6%-88.8%	NAV
% Hepatic manifestation	28%	45.5% - 84.2%	65.20%	43.50%	25%-54.9%	0%
% Neurologic manifestation	12.50%	4.2%-15.8%	4.30%	34.80%	0%-25%	21.40%
% Mixed manifestation	21.80%	0%-20.9%	21.70%	21.70%	19.6%-55.6%	0%
% Asymptomatic	37%	0%-35.1%	-	0%	30.35%	78.60%
% KF rings	58%	26.3%-69.2%	65.20%	67.40%	50.7%-59%	NAV
Mutation						
E2		Glu396stop				
E3				Gly457stop		
E4-6			No common mutations identified			
E7	Gly691Arg			Gly691Arg		
E8	2299insC	c. 2304-5insC	Trp779Gly	Gly710Ser	Ser744Pro	
		Cys703Tyr		Pro767Arg		
E9			No common or frequent mutations identified			
E10	Val845Ser	Val845Ser	Val845Ser	Val845Ser		
E11			No common or frequent mutations identified			
E12	Trp939Cys					
E13	Ala1003Thr		3061-1G>A sp	Ala1003Thr		Deletion of E13
E14		Thr1076Ile				
		His1069Gln	His1069Gln	His1069Gln		
E15	Thr1092Met	His1126fs	Ile1102Thr			
E16-17			No common or frequent mutations identified			
E18	Asn1270Ser	Asn1270Ser	Asn1270Ser	Asn1270Ser		
	Pro1273Leu	Pro1273Leu				
		IVS18-2A>G				
E19	Arg1319stop	Arg1319stop		Arg1319stop		
E20					Gly1341Ser	
E21					Gln1399Arg	
Ref.	Barada *et al*[13,30]	Abdelghaffar *et al*[12,22]	Dastsooz *et al*[27]	Simsek Papur *et al*[25]	Al Jumah *et al*[18]	Al-Tobi *et al*[29]
					Al Fadda *et al*[19]	
	Usta *et al*[6,14]	El-Karaksy *et al*[23]	Zali *et al*[28]	Loudianos *et al*[26]	Majumdar *et al*[20,21]	
		El-Mougy *et al*[24]				

[1]152 patients described in articles from Saudi Arabia, of which 5 patients were from yemen and syria.

in their family, may need to be screened for mutations in all exons of the *ATP7B* gene. In view of clustering of WD patients within families, their members should be screened for mutations identified in index patients. This is important as it could prevent the silent progression of WD, which may occur as early as 1 year of age, and facilitate management.

Based on the recently published EASL criteria for diagnosis, all our symptomatic and asymptomatic patients had a composite score > 4 (range: 6-12), confirming

the diagnosis beyond doubt. In many of our patients, confirmation of the diagnosis required mutation analysis. Traditionally WD was diagnosed on the basis of low Cp level, KF ring presence and increased 24-h urine Cu level in the context of hepatic and/or neurologic manifestations[31]. In our experience, many patients with WD do not satisfy all these criteria. For example, patients P_4 and P_{14} had normal Cp, 13 did not have KF rings and 4 had normal 24-h urinary Cu. This highlights the difficulties and challenges of making a diagnosis of WD based on clinical grounds alone, particularly in asymptomatic patients.

Worldwide, the majority of WD patients are compound heterozygous[32]. In contrast, in our community, the high rate of consanguinity increases the chance of homozygosity, which is present in 83% of our patients. Only 17% of our patients were compound heterozygous. Missense mutations were the most predominant in Lebanese patients, as worldwide[33]. These occurred in 8 exons of *ATP7B*. One possible hot spot of the WD gene in our patients is that of the conserved ATP hinge region in exon 18. Two mutations in the homozygous state, Pro1273Leu and Asn1270Ser, were the most frequent, being identified in 8 patients from 4 unrelated families. None of the possible hot spot mutations in Lebanon were shared with those of Asia, Latin America or Europe.

One of our WD patients had no identifiable mutations in the coding region of the *ATP7B* gene. Mutations may be present in the promoter or the transcription factor regions which control protein translation and function. In such cases, detailed clinical testing and family history may be of help in diagnosis, such as P_{36}, in whom the diagnosis was based on clinical assessment showing low Cp and presence of KF rings in the context of neurologic manifestation. Finally, all our patients had multiple genetic polymorphisms that may influence the final folded conformation, affinity and/or the function of the Wilson protein and possibly the phenotype of WD patients[34].

Remarkable differences in phenotypes and age at diagnosis were noted among patients and even among family members carrying the same genotype. The age of onset of the disease varied between 1-22 years, with one (P_{36}) diagnosed at 39 years. In the B family, patient P_{25} was diagnosed at 5 and passed away before the onset of his brother's symptoms at the age of 13 (P_{24}). Variation in age at diagnosis was also observed in asymptomatic cases. During a checkup at the age of 7, the female index patient in family T (P_{21}) was found to have transaminitis and hepatomegaly. She was confirmed to have WD and was homozygous for a mutation in exon 12. Genetic screening of her 2 brothers, P_{22} (8 years) and P_{23} (3 years), confirmed WD. Though they were asymptomatic, it was surprising to find that both already had evidence of liver cirrhosis on liver imaging. This raises the question as to whether sex plays a role in the clinical manifestations of disease[15]. Verification of this, however, requires a cohort study with a larger number of patients. Such phenotypic diversity has been reported even among monozygotic twins[35], suggesting a role for epigenetic and/or environmental factors in the expression of WD[36-38].

Diversity in clinical presentation introduces yet another obstacle in the diagnosis of WD, regardless of whether the patient is symptomatic or asymptomatic. A patient, at age of diagnosis, may have mild to severe hepatic and/or neurologic symptoms with or without KF rings. This emphasizes, again, the limitations of pure clinical evaluation and argues for genetic testing of all family members of an affected sibling. In our patients, 28% had pure hepatic manifestations ranging from transaminitis and hepatomegaly to clinically unapparent or overt cirrhosis and portal hypertension. On the other hand, only 9% of our patients had pure neurologic symptoms ranging from weak school performance, slurred speech, tremors, drooling, dysarthria, dysphagia and ataxia to suicidal attempts in some (P_{10} and P_{26}).

Our asymptomatic patients (36%) were found to have liver involvement (transaminitis, fatty liver and cirrhosis) with no KF rings, except for P_{19}. Changes such as fatty liver were detected at the age of 1 year in P_{15}, diagnosed by genetic screening. Therefore, early diagnosis is important in families with index patient(s), to mitigate against progression of the disease. This is in line with the EASL recommendations to perform genetic testing for WD, in individuals with liver disease or neurologic movement disorders of unclear etiology. Whether genetic testing for WD in patients with unexplained hepatic dysfunction will turn out to be cost effective or not in this part of the world is unclear.

Few studies from the Arab world on WD from Lebanon, Egypt, Saudi Arabia and Oman have been published. Similar to Lebanon, the majority of patients from Egypt and Saudi Arabia have consistently shown a high prevalence of consanguinity and homozygosity, with a great deal of genetic heterogeneity, and no mutation characteristic of the region identified. The predominant phenotype of WD in the region was also hepatic, suggesting the benefits of screening for WD in patients with unexplained hepatic dysfunction.

Lebanese and Egyptian patients share missense mutations in exons 8, 10, 18 and 19 (Table 4). However, mutations Gly691Arg and Trp939Cys were identified in Lebanese patients but not in Saudi Arabian or Egyptian ones. There were also common mutations with Turkish WD patients, including exon 7 (Gly691Arg), exon 10 (Val845Ser), exon 13 (Ala1003Thr), exon 18 (Asp1270Ser) and exon 19 (Arg1319stop). Only exon 10 (Val845Ser), and exon 18 (Asp1270Ser) were shared with Iranian patients. Interestingly, the exon 12 mutation of Trp939Cys was detected in Lebanon but not in any regional country. We reported this mutation in the homozygous state in 5 Lebanese patients, while worldwide it was only detected in 1 Hungarian patient in the heterozygous state[39]. This extensive genotypic diversity argues for testing patients suspected to have WD for mutations in all exons of ATP7B. The shared

mutations with the region may be attributed to common ancestors (Turkey and Egypt) who ruled Lebanon in the past. The origin of the Trp939Cys mutation, however, remains undetermined.

To our surprise, the His1069Gln mutation which is common in diverse populations in North America, Europe and several Mediterranean countries[40] was not present in Lebanese patients, but was reported in a minority of patients from Egypt, Iran and Turkey. We did not identify a predominant mutation in Lebanon or the region. Whether mutations in the ATP hinge region in exon 18 may turn out to be a hot spot in this part of the world requires further studies on larger numbers of WD patients.

One major strength of our study is that it involves more than 500 patients from Lebanon and the region. It includes a comprehensive clinical and genetic assessment of WD patients in Lebanon, as well as studies from the region clearly stating the genotype and phenotype. Our patients belonged to extended consanguineous families having similar environmental exposures and dietary habits, which helped in reducing the effects of compounding factors on the genotype and phenotype of patients. In addition, our study has some limitations including the absence of true population studies and the lack of long-term follow-up to determine reliably the true phenotype of patients. It is possible that many WD patients in Lebanon and the region remain undiagnosed or unreported, hence missing new mutations and other genotype-phenotype associations.

In conclusion, WD in Lebanon and the region is characterized by extensive genotypic and phenotypic diversity, and by high rates of consanguinity and homozygosity. No predominant mutation has been identified in the region, while the predominant phenotype seems to be hepatic. Clinical and/or genetic testing of all family members for WD, as well as those with unexplained hepatic dysfunction may increase the detection rate of the disease. This could facilitate early institution of therapy and reduce the mortality and morbidity of this condition.

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COMMENTS

Background

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism, characterized by extensive phenotypic diversity. Most of the patients are compound heterozygotes, having different mutation on each of the *ATP7B* alleles. Attempts to establish genotype-phenotype correlations was hampered by the large number of mutations in the *ATP7B* gene and difficulty in ascribing a phenotype to one allele. This, however, may be overcome by examining WD in homozygous patients. In Lebanon, consanguinity is quite prevalent, increasing the probability of homozygosity and possibility of establishing a phenotype-genotype correlation. They hereby report the spectrum of mutations and phenotypes of 36 Lebanese patients diagnosed with WD. In addition, we examine if a frequent mutation characterizing the region exists by comparing our findings with those reported from regional studies on WD in Arab and non-European countries.

Research frontiers

This manuscript examines whether genotype-phenotype correlation exists in Lebanese patients diagnosed with WD. It also determines if a frequent mutation characteristic of the Lebanese patients and/or the region occurs.

Innovations and breakthroughs

This is the first comparative study that attempts to identify a frequent mutation characterizing WD patients from Lebanon and regional Arab and non-European countries. Although this region is characterized by high rates of consanguinity and homozygosity, no frequent mutation has been identified in the region but predominance of the hepatic phenotype was noted.

Applications

This study improves our understanding of WD pathogenesis and the genetic determinants of patients' phenotype. It emphasizes the importance of genetic screening for WD in family members with index patients, as well in patients with unexplained hepatic dysfunction. This would surely facilitate diagnosis and early management prior to onset of symptoms, thereby preventing the progressive clinical deterioration of the patient.

Terminology

WD is a rare disease of copper homeostasis that results from a defect in the *ATP7B* gene encoding a copper transporter. Ceruloplasmin is the major copper carrying protein in blood with ferroxidase activity. Kaiser-Fleischer rings refer to copper deposition circumscribing the iris of the eye, diagnostic of WD.

Peer-review

It is a very interesting manuscript.

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ORIGINAL ARTICLE

Observational Study

Short-term outcomes of overlapped delta-shaped anastomosis, an innovative intracorporeal anastomosis technique, in totally laparoscopic colectomy for colon cancer

Hai-Tao Zhou, Peng Wang, Jian-Wei Liang, Hao Su, Zhi-Xiang Zhou

Hai-Tao Zhou, Peng Wang, Jian-Wei Liang, Hao Su, Zhi-Xiang Zhou, Department of Colorectal Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

ORCID number: Hai-Tao Zhou (0000-0003-0239-4966); Peng Wang (0000-0001-5537-6281); Jian-Wei Liang (0000-0002-2216-8870); Hao Su (0000-0002-9021-7160); Zhi-Xiang Zhou (0000-0003-3810-1715).

Author contributions: Zhou HT and Wang P contributed equally to this work; Zhou HT, Wang P and Zhou ZX designed the research; Zhou HT, Liang JW and Zhou ZX performed the research; Su H analyzed the data; Zhou HT and Wang P wrote the paper; Zhou ZX revised the paper.

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Correspondence to: Zhi-Xiang Zhou, MD, Professor, Department of Colorectal Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 17 Panjiayuan Nanli, Chaoyang District, Beijing 100021, China. zhouzx001@163.com
Telephone: +86-10-87787110
Fax: +86-10-87787110

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Abstract

AIM

To introduce an innovative intracorporeal anastomosis technique named overlapped delta-shaped anastomosis (ODA) for colon cancer cases undergoing totally laparoscopic colectomy (TLC) and to assess its feasibility and safety.

METHODS

From January 2016 to March 2017, a total of 20 consecutive patients with colon cancer accepted TLC and the ODA technique at our medical center. Patient demographics, operative outcomes, perioperative complications, and pathological results were collected and analyzed.

RESULTS
We successfully completed TLC and the ODA procedure in all 20 cases, including 6 (30%) males and 14 (70%) females. In total, 11 (55%), 2 (10%), and 7 (35%) cases accepted right hemicolectomy, transverse hemicolectomy, and left hemicolectomy, respectively. None of the surgeries were converted to an open operation. Mean operative time was 178.5 min, and mean estimated blood loss was 58.5 mL. Mean time to first flatus was 2.5 d, and mean postoperative hospitalization duration was 6.8 d. No severe complications occurred, such as anastomotic leakage, snastomotic stenosis, anastomotic bleeding, and wound infection, except for one case who suffered from an abdominal infection and another case who suffered from gastric paralysis syndrome. Tumor recurrence was not observed in any patient during the follow-up period.

CONCLUSION
The ODA technique for colon cancer cases undergoing TLC appears to be safe and feasible, although our current results need to be verified in further studies.

Key words: Overlapped delta-shaped anastomosis; Safety; Totally laparoscopic colectomy; Intracorporeal anastomosis; Colon cancer

Core tip: Intracorporeal anastomosis technique is one of the biggest difficulties encountered by surgeons during the totally laparoscopic colectomy procedure. In this paper, we introduce an innovative intracorporeal anastomosis technique named overlapped delta-shaped anastomosis and assess its feasibility and safety.

Zhou HT, Wang P, Liang JW, Su H, Zhou ZX. Short-term outcomes of overlapped delta-shaped anastomosis, an innovative intracorporeal anastomosis technique, in totally laparoscopic colectomy for colon cancer. *World J Gastroenterol* 2017; 23(36): 6726-6732 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6726.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6726

INTRODUCTION

With the improvement of living standards and the extension of life expectancy, the incidence of colon cancer is increasingly rising and will continue to rise in China[1,2]. Surgery is the mainstay of treatment for colon cancer. Over the past three decades, minimally invasive surgery for colon cancer has drawn more and more attention[3]. Nowadays, laparoscopic assisted colectomy (LAC) is widely used for cases with colon cancer and the advantages of this procedure have been widely verified[4-6].

With the advances in surgical devices and the improvements in surgical performance, totally laparoscopic colectomy (TLC) has gradually been adopted by experienced surgeons[7-8]. In theory, the TLC procedure conforms more to the concept of minimally invasive surgery and the principle of the tumor-free technique, while the intracorporeal anastomosis (IA) technique still represents one of the biggest difficulties for surgeons during TLC.

As a new technique, delta-shaped anastomosis was first presented by a Japanese scholar named Kanaya in 2002 after he completed the first case of totally laparoscopic gastroenterostomy (TLG)[9]. This procedure has been widely adopted for TLG and has proven to be both safe and feasible[10-12]. However, delta-shaped anastomosis for TLC is rarely reported. Since January 2016, an innovative technique named "overlapped delta-shaped anastomosis (ODA)" has been applied to colon cancer cases undergoing TLC at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. Herein, we introduce this surgical innovation and assess its feasibility and safety.

MATERIALS AND METHODS

Methods

Prior to surgery, the advantages and disadvantages of TLC and ODA were explained to all patients in detail, and then preoperative informed content was acquired. All patients underwent the same preoperative examinations including routine blood tests, chest X-ray, electrocardiogram examination, and computerized tomography (CT) of the abdominal and pelvic cavity to exclude cases with surgical contraindications and distant organ metastasis. Patients with intestinal obstruction were excluded from our study. The American Joint Committee on Cancer (AJCC) staging system (the seventh edition) was used for tumor staging. Postoperative pain was evaluated by the patients on a subjective visual pain scale ranging from 0 to 10, with 0 representing no pain and 10 representing the worst pain imaginable. The ethics committee at our institution approved this study, and this retrospective study conformed to the ethical standards of the World Medical Association Declaration of Helsinki.

Patients

From January 2016 to March 2017, a total of 20 consecutive patients with colon cancer underwent TLC and the ODA procedure. All of these cases accepted colonoscopy examination, and colon cancer was diagnosed by pathology. Preoperative mechanical bowel preparation was performed using polyethylene glycol electrolyte powder. Preventative antibiotics were administered by intravenous drip 30 min preoperatively and continued for 24 h after the operation.

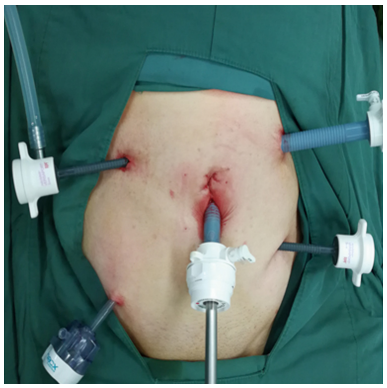

Figure 1 Five-port technique.

Surgical procedure (taking right hemicolectomy as an example)

Step 1: Positioning the patient and placing trocars: The patient was placed in the modified lithotomy position. A five-port technique was used: a 12 mm sub-umbilical port as the observation port, a 12 mm port located in the left upper quadrant as the primary operating port, and three 5 mm ports located in the left lower quadrant, the right lower quadrant and the right upper quadrant, respectively, as the secondary operating ports (Figure 1). Abdominal pressure was maintained at approximately 15 mmHg, and then the patient was placed in the Trendelenburg position and left tilt applied in order to expose the mesenteric root, ileocolic vessels, and superior mesenteric vessels.

Step 2: Clearing lymph nodes and tailoring the mesentery: Along the surface of the superior mesenteric vessels, the mesocolon was dissected in a bottom-up fashion, and then the ileocolic vessels, ascending colon vessels, and the right branch of the transverse colon vessels were exposed and carefully severed (Figure 2A). During this process, attention should be paid to the gastrocolic trunk, pancreatic head, duodenum, and right gastroepiploic vessels, such that these structures are protected. Next, adhesions between the right abdominal wall and the ascending colon were dissected in a similar bottom-up fashion. Finally, the mesentery of the terminal ileum approximately 15 cm away from the ileocecal region, and the right half of the transverse colon, was tailored, respectively.

Step 3: Transecting and anastomosing the bowel: The terminal ileum and the right half of the transverse colon were transected using two endoscopic linear cutter staplers (Johnson and Johnson, PSE60A and ECR60B) (Figure 2B). The broken ends were sterilized using alcohol gauze swabs. Then, the proximal ileum and the distal transverse colon were fixed using a piece of absorbable suture; this was performed in an overlapped fashion in order to facilitate anastomosis (Figure 2C). Two small openings located at the ileum and the transverse colon were created using an ultrasound scalpel. After imbedding the lumens with another endoscopic linear cutter stapler, intestinal walls with no mesentery were got through (Figure 2D). Finally, the common opening was closed using an endoscopic linear cutter stapler (Figure 2E). The specimen was removed from the abdominal cavity using a transverse incision above the symphysis pubis (Figure 2F).

The ways the transverse and left colectomy was performed were similar to the method above mentioned in the right hemicolectomy, including the number of trocars, type of staplers, and same anisoperistaltic anastomosis.

Follow-up

The first day after surgery represented the beginning of the follow-up period. Patients were asked to visit doctors every three months after leaving hospital until two years after surgery, biannually for the next three years and then annually, and the deadline of follow-up period was March 31, 2017.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software (21.0 version for Windows; SPSS Inc. Chicago, IL, United States) was used for data analyses. Quantitative data following a normal distribution are provided as mean and its range. Qualitative data are provided as number and its percentage. The statistical methods of this study were reviewed by Wang M from our institution.

RESULTS

We successfully completed TLC and the ODA procedure in all 20 cases, including 6 (30%) males and 14 (70%) females. None of the surgeries were converted to an open operation. Mean patient age was 52.6 years (range: 38-67 years), and mean body mass index (BMI) was 22.9 kg/m^2 (range: 20.2-25.5 kg/m^2). Our study included 10 (50%) cases with ascending colon cancer, 4 (20%) with transverse colon cancer, and 6 (30%) with descending colon cancer. In total, 11 (55%), 2 (10%), and 7 (35%) cases accepted right hemicolectomy, transverse hemicolectomy, and left hemicolectomy, respectively. Four (20%) out of the 20 cases accepted preoperative chemotherapy (Table 1).

The mean operative time was 178.5 min (range: 155-225 min), and mean estimated blood loss was 58.5 mL (range: 30-100 mL). For all 20 cases, the mean time to first flatus was 2.5 d (range: 1-3 d), and mean first time to oral intake was 3.0 d (range: 2-4 d). Mean postoperative hospitalization period was 6.8 d (range 5-8 d). Patients only reported slight pain (scoring 2.8 on average) on the first day after the operation, and there was almost no pain (scoring 0.7 on average)

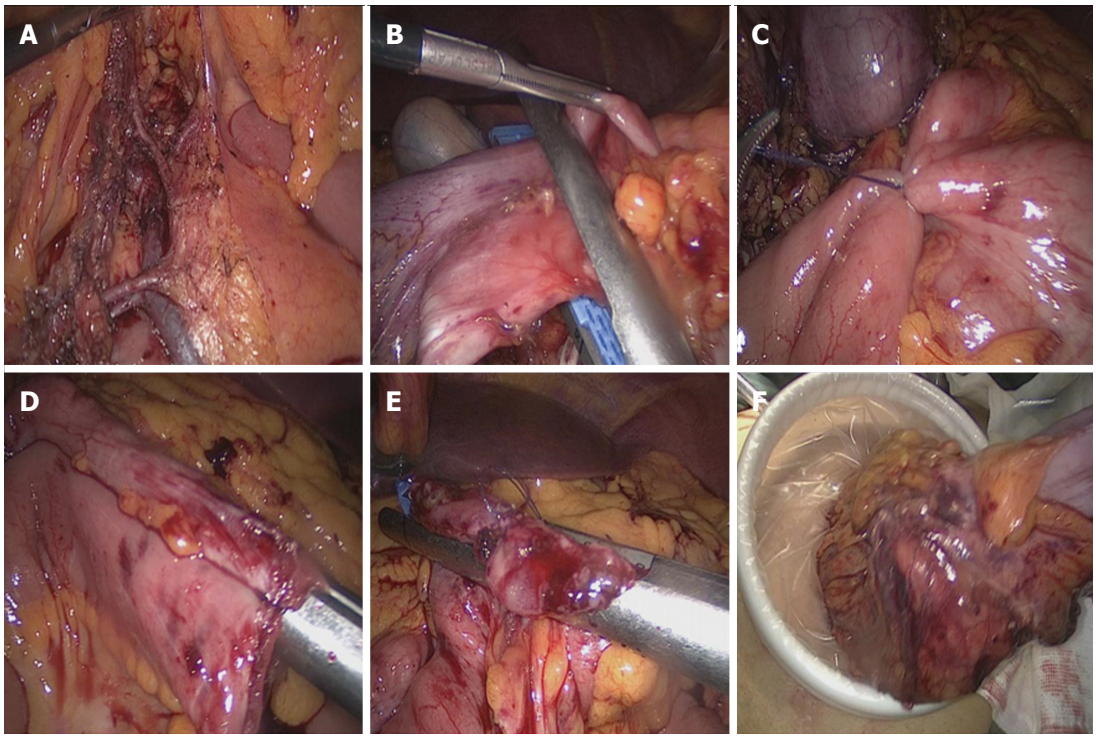

Figure 2 Surgical procedure. A: The ileocolic vessels, ascending colon vessels, and the right branch of the transverse colon vessels were exposed; B: The right half of the transverse colon was transected using endoscopic linear cutter staplers; C: The proximal ileum and the distal transverse colon were fixed in an overlapped fashion using a piece of absorbable suture to facilitate anastomosis; D: After imbedding the lumens with an endoscopic linear cutter stapler, intestinal walls with no mesentery were got through; E: The common opening was then closed using an endoscopic linear cutter stapler; F: Finally, the specimen was removed from the abdominal cavity using a transverse incision above the symphysis pubis.

Table 1 Patient demographics *n* (%)

Parameter	
Gender	
Male	6 (30)
Female	14 (70)
Age, yr, mean (range)	52.6 (38-67)
BMI, kg/m^2, mean (range)	22.9 (20.2-25.5)
ASA score	
1	10 (50)
2	7 (35)
3	3 (15)
Tumor site	
Ascending colon	10 (50)
Transverse colon	4 (20)
Descending colon	6 (30)
Surgical procedure	
Right hemicolectomy	11 (55)
Transverse hemicolectomy	2 (10)
Left hemicolectomy	7 (35)
Preoperative chemotherapy	4 (20)

BMI: Body mass index; ASA: American Society of Anesthesiologists.

by the third day after the operation. No severe complications were encountered, such as anastomotic leakage, anastomotic stenosis, anastomotic bleeding, deep vein thrombosis, or intestinal obstruction, except one case who suffered from abdominal infection due to chylous fistula, and another case who suffered from gastric paralysis syndrome. However, both of these patients made a heathy recovery with conservative treatments. There were no deaths during the perioperative period (Table 2).

Pathological results are shown in Table 3. Mean tumor size was 4.2 cm (range: 2.8-6.3 cm) and mean proximal and distal resection margins were 19.5 cm (range: 13.8-23.5 cm) and 17.8 cm (range: 12.2-21.6 cm), respectively. The mean number of lymph nodes harvested was 32.4 (range: 23-45). Among these cases, there were five (25%) cases of stage Ⅰ, eight (40%) cases of stage Ⅱ, and seven (35%) cases of stage Ⅲ disease. A typical specimen and the transverse incision above the symphysis pubis are shown in Figure 3. The mean follow-up time was 8.5 mo (range: 1-15 mo). No patient was lost to follow-up and no cases experienced recurrence during the follow-up period.

DISCUSSION

Surgery plays an important role in the treatment of colon cancer and the goal of every surgeon is to create as little trauma as necessary and encourage a quick recovery. With recent advances in surgical devices and improvements in surgical performance, surgeons have achieved a significant breakthrough in the treatment of colon cancer by changing from open surgery to laparoscopic surgery[3]. Minimally invasive surgery and quick recovery following laparoscopic surgery have

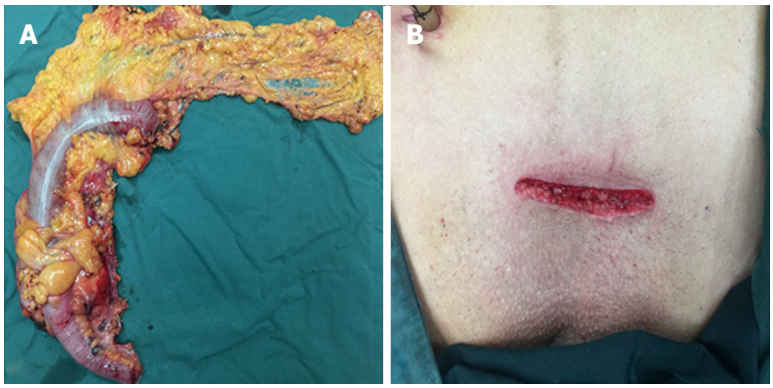

Figure 3 Typical specimen (A) and a transverse incision above the symphysis pubis (B).

Table 2 Operative outcomes and perioperative complications

Parameter	
Operative outcomes	
Operative time, min, mean (range)	178.5 (155-225)
Estimated blood loss, mL, mean (range)	58.5 (30-100)
Time to first flatus, d, mean (range)	2.5 (1-3)
Time to first oral intake, d, mean (range)	3.0 (2-4)
Postoperative hospitalization, d, mean (range)	6.8 (5-8)
Length of transverse incision, cm, mean (range)	4.8 (4-6)
Postoperative pain score	
The first day, mean (range)	2.8 (2-4)
The second day, mean (range)	1.5 (1-3)
The third day, mean (range)	0.7 (0-1)
Perioperative complications (%)	
Anastomotic leakage	0 (0)
Anastomotic stenosis	0 (0)
Anastomotic bleeding	0 (0)
Abdominal infection	1 (5)
Deep-vein thrombosis	0 (0)
Wound infection	0 (0)
Intestinal obstruction	0 (0)
Gastric paralysis syndrome	1 (5)
Reoperation (%)	0 (0)

Table 3 Pathological results

Parameter	
Tumor size, cm, mean (range)	4.2 (2.8-6.3)
Proximal resection margin, cm, mean (range)	19.5 (13.8-23.5)
Distal resection margin, cm, mean (range)	17.8 (12.2-21.6)
No. of lymph nodes harvested, mean (range)	32.4 (23-45)
pTNM stage (%)	
Ⅰ	5 (25)
Ⅱ	8 (40)
Ⅲ	7 (35)

pTNM: Pathological tumor node metastasis.

been well verified and documented. As a consequence, laparoscopic surgery has become incredibly popular over the last three decades and several new laparoscopic techniques, including robotic-assisted colectomy, single incision laparoscopic surgery, natural orifice specimen extraction surgery, and natural orifice transluminal endoscopic surgery, have also been reported[3,13-17]. Although, totally laparoscopic surgery has been carried out by experienced surgeons[7-8], IA technique still represents a significant difficulty during TLC. Therefore, an easy and feasible anastomosis technique is very important.

In 2002, the Japanese scholar Kanaya first reported the delta-shaped anastomosis of TLG[9]. In 2011, he summarized 100 cases of delta-shaped anastomosis performed by eight surgeons, and the results showed that, on average, only 13 min were needed to complete anastomosis, and that all eight surgeons experienced a short learning curve. In addition, this earlier study reported that patients had an early time of first oral intake[18]. Subsequently, more and more studies confirmed the advantages of this procedure[10-12].

In recent years, several studies have described IA for patients with colon cancer. Wang *et al*[17] reported that 11 patients with sigmoid cancer undergoing totally laparoscopic sigmoid colectomy with delta-shaped anastomosis and transvaginal specimen extraction, and these procedures were successful without serious complications. Jian-Cheng *et al*[19] reported 56 cases with colon cancer, who had accepted total laparoscopic right hemicolectomy (LRH) with 3-step stapled intracorporeal isoperistaltic ileocolicanastomosis (TLG group). Compared to cases in the extracorporeal anastomosis group (LG group), cases in the TLG group had shorter IA time (9.9-15.5 min *vs* 13.5-18.2 min, $P < 0.001$), lower mean intraoperative blood loss (83.2 mL *vs* 93.3 mL, $P < 0.001$), faster recovery of bowel function ($P < 0.001$), and lower postoperative pain score ($P < 0.001$). van Oostendorp *et al*[20] conducted a meta-analysis including 12 non-randomized comparative studies and the results showed that IA in the LRH group was associated with reduced short-term morbidity and decreased length of hospital stay suggesting faster recovery. In addition, Wu *et al*[21] also reported that the IA for LRH could improve cosmetic effect and result in better postoperative recovery outcomes without increasing intraoperative and postoperative complications. However, the difficulties of IA procedure behind the satisfactory results were all reported by above-mentioned studies.

In the present study, we performed TLC in 20

colon cancer cases using a new IA method named the ODA procedure; all surgeries were carried out smoothly and none of the cases were converted to open operation. Patients had slight pain after surgery and showed early time of first flatus and oral intake. The results also showed that the ODA procedure did not increase postoperative complications and that radical resection was also guaranteed. In particular, the transverse incision above the symphysis pubis was only 4.8 cm in length, much shorter and more covert than that required for LAC. Collectively, these observations provide promising results with regard to its feasibility and safety. It thus appears that TLC and the ODA procedure are more in line with the concept of minimally invasive surgery and enhance recovery following surgery.

This was a retrospective study and only 20 patients were included; these factors may, therefore, represent potential limitations. However, all operations were successfully accomplished and no severe complications occurred. Prospective randomized controlled trials with larger sample sizes and longer follow-up periods are now needed to confirm our results.

The ODA technique for cases with TLC appears to be safe and feasible for suitable patients when performed by experienced surgeons. This promising result now allows us to cater for colon cancer patients who require medical cosmetology.

COMMENTS

Background

With the advances in surgical devices and the improvements in surgical performance, totally laparoscopic colectomy (TLC) has gradually been adopted by experienced surgeons in large medical centers. However, the intracorporeal anastomosis (IA) technique is one of the biggest difficulties encountered by surgeons during the TLC procedure, and the currently adopted delta-shaped anastomotis technique is difficult to operate.

Research frontiers

Now, the mainstream of colectomy for colon cancer is still laparoscopic assisted colectomy. TLC is only adopted by experienced surgeons in large medical centers for the difficulties of IA technique.

Innovations and breakthroughs

In this study, a modified delta-shaped anastomotis technique named overlapped delta-shaped anastomosis (ODA) has been applied to colon cancer cases undergoing TLC in this medical institution. The results show that the ODA technique is a feasible and safe procedure.

Applications

In the present study, we have reported, for the first time, the ODA technique adopted during the TLC procedure. The perfect result has proven its feasibility and safety. Therefore, the ODA procedure can be applied to colon cancer patients who have no contraindications for laparoscopic surgery.

Terminology

The ODA procedure is currently used for the anastomosis between the proximal and distal intestinal canals for colon cancer cases who accepted TLC.

Peer-review

IA technique is one of the biggest difficulties during the TLC procedure. The authors report an innovative IA technique named the "ODA technique". The short-term outcomes are exciting and it deserves further promotion.

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ORIGINAL ARTICLE

Prospective Study

Effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy

Jing-Xian Sun, Ke-Yun Bai, Yan-Feng Liu, Gang Du, Zhi-Hao Fu, Hao Zhang, Jin-Huan Yang, Ben Wang, Xiu-Yu Wang, Bin Jin

Jing-Xian Sun, Ke-Yun Bai, Department of Anorectal Surgery, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250011, Shandong Province, China

Yan-Feng Liu, Gang Du, Zhi-Hao Fu, Hao Zhang, Jin-Huan Yang, Ben Wang, Bin Jin, Department of Hepatobiliary Surgery, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Xiu-Yu Wang, Department of Anesthesiology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

ORCID number: Jing-Xian Sun (0000-0002-8376-4764); Ke-Yun Bai (0000-0003-3437-1574); Yan-Feng Liu (0000-0001-8540-3631); Gang Du (0000-0001-5891-6167); Zhi-Hao Fu (0000-0001-5891-6169); Hao Zhang (0000-0001-5891-6345); Jin-Huan Yang (0000-0001-5841-6345); Ben Wang (0000-0002-8731-4840); Xiu-Yu Wang (0000-0001-4591-6167); Bin Jin (0000-0001-9790-9795).

Author contributions: Sun JX collected the data and drafted the manuscript; Liu YF, Fu ZH, Zhang H, Yang JH and Wang XY contributed to data collection and statistical analysis; Du G and Wang B helped revise the manuscript; Jin B and Bai KY contributed to the study design and revised the manuscript; all authors read and approved the final manuscript.

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Correspondence to: Bin Jin, MD, PhD, Associate Professor, Department of Hepatobiliary Surgery, Qilu Hospital of Shandong University, 107 Wenhua Xi Road, Jinan 250012, Shandong Province, China. jinglewei@sdu.edu.cn
Telephone: +86-531-82169203
Fax: +86-531-82169243

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Abstract

AIM

To prospectively evaluate the effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy.

METHODS

A total of 56 patients undergoing open hepatectomy

were randomly divided into two groups: a ropivacaine group (wound infiltration with ropivacaine solution) and a control group (infiltration with isotonic saline solution). A visual analog scale (VAS) at rest and on movement was used to measure postoperative pain for the first 48 h after surgery. Mean arterial pressure (MAP), heart rate (HR), time to bowel recovery, length of hospitalization after surgery, cumulative sufentanil consumption, and incidence of nausea and vomiting were compared between the two groups. Surgical stress hormones (epinephrine, norepinephrine, and cortisol) were detected using enzyme-linked immunosorbent assay, and the results were compared.

RESULTS

VAS scores both at rest and on movement at 24 h and 48 h were similar between the two groups. Significantly lower VAS scores were detected at 0, 6, and 12 h in the ropivacaine group compared with the control group (P < 0.05 for all). MAP was significantly lower at 6, 12, and 24 h (P < 0.05 for all); HR was significantly lower at 0, 6, 12, and 24 h (P < 0.05 for all); time to bowel recovery and length of hospitalization after surgery (P < 0.05 for both) were significantly shortened; and cumulative sufentanil consumption was significantly lower at 6, 12, 24, and 36 h (P < 0.05 for all) in the ropivacaine group than in the control group, although the incidence of nausea and vomiting showed no significant difference between the two groups. The levels of epinephrine, norepinephrine, and cortisol were significantly lower in the ropivacaine group than in the control group at 24 and 48 h (P < 0.01 for all).

CONCLUSION

Local wound infiltration with ropivacaine after open hepatectomy can improve postoperative pain relief, reduce surgical stress response, and accelerate postoperative recovery.

Key words: Local wound infiltration; Ropivacaine; Open hepatectomy; Postoperative pain; Surgical stress

Core tip: This study prospectively evaluated the effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy. Wound infiltration with ropivacaine could provide more effective analgesia both at rest and on movement in the first 48 h after surgery, with lower mean arterial pressure, heart rate and sufentanil consumption, accelerated postoperative recovery, and reduced stress response. These results suggest that local wound infiltration with ropivacaine is a simple, convenient and effective analgesic method that can provide postoperative analgesia and short-term benefits after open hepatectomy.

Sun JX, Bai KY, Liu YF, Du G, Fu ZH, Zhang H, Yang JH, Wang B, Wang XY, Jin B. Effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy. *World J Gastroenterol* 2017; 23(36): 6733-6740 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6733.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6733

INTRODUCTION

Postoperative pain is an important issue for surgeons, anesthetists, patients, and other related personnel. The intensive pain caused by upper abdominal laparotomy may influence postoperative recovery, prolong hospitalization, and cause stress response and complications, including respiratory and cardiovascular depression and gastrointestinal and neuroendocrine dysfunction[1,2]. Currently, intravenous analgesia or epidural analgesia (EA) with a patient-controlled analgesia pump is the most common analgesic approach for controlling postoperative pain after laparotomy[3]. Although favored after major laparotomy, patient-controlled intravenous analgesia (PCIA) can delay postoperative recovery because of nausea and vomiting, excessive sedation, and dizziness; moreover, this analgesic mode involves the risk of addiction with large opioid dosages over long periods[4,5]. As to EA, which provides better analgesia than PCIA, it is restricted by contraindications, epidural puncture failure, and side effects[6,7]. Therefore, finding other analgesic strategies with fewer potentially serious adverse effects will be beneficial for patients suffering from postoperative pain.

Postoperative analgesia is a crucial section of perioperative management, and local anesthetic methods are more effective than systemic analgesia regardless of the operation type[8]. Currently, wound infiltration with local anesthetics, which is a simple, effective, and inexpensive method, is performed in various surgical procedures and provides satisfactory analgesia without major side effects[9]. Ropivacaine and bupivacaine, as long-acting local anesthetics, are commonly used for local anesthesia and pain management in China[10]. Ropivacaine has the same analgesic effects as bupivacaine and levobupivacaine, but it is associated with a low incidence of motor block[11]. Thus, ropivacaine appears to be an important component for local anesthesia and postoperative analgesia. Meanwhile, surgical pain frequently increases the systemic stress response during the perioperative period, which can induce the excessive release of catecholamines (epinephrine and norepinephrine) and cortisol. Optimistically, local anesthesia provides considerable advantages over general anesthesia by suppressing catecholamines and cortisol levels[12].

In this study, we aimed to assess the effect of local wound infiltration with ropivacaine on postoperative pain control, mean arterial pressure (MAP), heart rate

(HR), cumulative sufentanil consumption, incidence of nausea and vomiting, time to bowel recovery, and length of hospitalization after open hepatectomy. The changes of three stress hormones, namely, epinephrine, norepinephrine, and cortisol, were evaluated in patients undergoing wound infiltration with and without ropivacaine.

MATERIALS AND METHODS

A total of 56 patients undergoing open hepatectomy, which was performed by the same experienced surgical team at the Department of Hepatobiliary Surgery of Qilu Hospital of Shandong University from January 2016 to March 2017, participated in this study. The study was approved by the Medical Ethics Committee of Qilu Hospital of Shandong University (No. 2017052), and written informed consent was obtained from all patients. The inclusion criteria included adult patients (aged 18-75 years) who would undergo open hepatectomy and were classified as grades Ⅰ-Ⅲ according to the American Society of Anesthesiologists (ASA) Physical Status Classification System. Patients with a history of known allergy to local anesthetics, chronic preoperative opioid consumption, or any other analgesic treatment for chronic pain before surgery, psychiatric or neurological diseases, or acquired or genetic hemostatic abnormality were excluded from the study.

On the day of surgery, the patients were randomly divided into two groups with a table of random numbers. Surgeons and patients were kept blinded to the assigned treatment groups throughout the study. Wound infiltration was performed with a 7.5 mg/mL ropivacaine solution in the ropivacaine group and with an isotonic saline solution in the control group. Solutions were prepared and provided by the anesthetist, and surgeons were blinded to patient allocation. When closing the abdomen at the end of the surgical procedure, 20 mL of the prearranged solution was used to infiltrate the subcutaneous tissues, deep muscular fascia, and parietal peritoneum. Moreover, one or two drainage tubes were routinely placed near the cutting surface of the liver and then pulled out and fixed on the abdominal skin. In the presence of tube incision or pulling of the tube during movement or when turning over, the surrounding tissues of the tube were also infiltrated with the solution. Infiltration was performed under direct vision by the surgeon. All patients were given unrestricted access to sufentanil through a 100 mL disposable patient-controlled analgesic (PCA) device containing 1 μg/mL sufentanil that was delivered at a rate of 2 μg/h and a bolus of 0.5 μg with a 15 min lockout time. When the skin was closed, the PCA pump was connected to the venous catheter and routinely removed 36 h after the operation.

The intensity of postoperative pain at rest was measured on a visual analogue scale (VAS) graded from 0 (no pain) to 10 (very severe pain) for the first 48 h after surgery. Movement pain was scored using VAS when coughing or turning over. Pain scores were recorded both by nurses and surgeons blinded to patient allocation. Pain measurements were performed at 0, 6, 12, 24, and 48 h after the surgery. Other variables were recorded, including time to bowel recovery, length of hospitalization after surgery, hemodynamic data represented by MAP and HR, cumulative sufentanil consumption, and postoperative nausea and vomiting (PONV). The three surgical stress hormones, namely, epinephrine, norepinephrine, and cortisol, were detected using commercial enzyme-linked immunosorbent assay kits (Cusabio Biotech Co., Ltd., Wuhan, China). Time to bowel recovery was defined as the time to first anal exhaust. PONV was recorded with a three-point rating scale: 1, no nausea and vomiting; 2, nausea without vomiting; 3, nausea with vomiting.

Statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, United States). All data were checked for normal distribution and the results are expressed as mean ± SD for continuous variables. The *t* test, χ^2 test, Fisher's exact test, or analysis of variance was carried out where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

All patients successfully received the surgical procedure, including the wound infiltration with a prearranged solution. However, three patients (two in the ropivacaine group and one in the control group) were dropped from the study for postoperative bleeding and bile leakage; finally, 26 patients were enrolled in the ropivacaine group and 27 enrolled in the control group. The demographic characteristics of the patients assigned to the two groups were comparable in terms of age, gender, weight, ASA grade, incision length, and postoperative pathology, except for operation type, which showed a statistical difference but had no clinical significance (Table 1).

The VAS scores both at rest and on movement were similar between the two groups at 24 h and 48 h after open hepatectomy (Figure 1A and B). Significant differences in VAS scores at rest were detected at 0 h ($P = 0.0106$), 6 h ($P = 0.0032$), and 12 h ($P = 0.0002$). Moreover, significant differences in VAS scores on movement were observed at 0 h ($P = 0.0208$), 6 h ($P = 0.0043$), and 12 h ($P = 0.0089$). The details are shown in Table 2.

Hemodynamic data are presented in Figure 1C and D and Table 2. MAP was significantly lower in the ropivacaine group than in the control group at 6 h ($P = 0.0241$), 12 h ($P = 0.0001$), and 24 h ($P = 0.002$). In the ropivacaine group, HR was significantly lower at 0 h ($P = 0.0103$), 6 h ($P = 0.0087$), 12 h ($P < 0.0001$), and

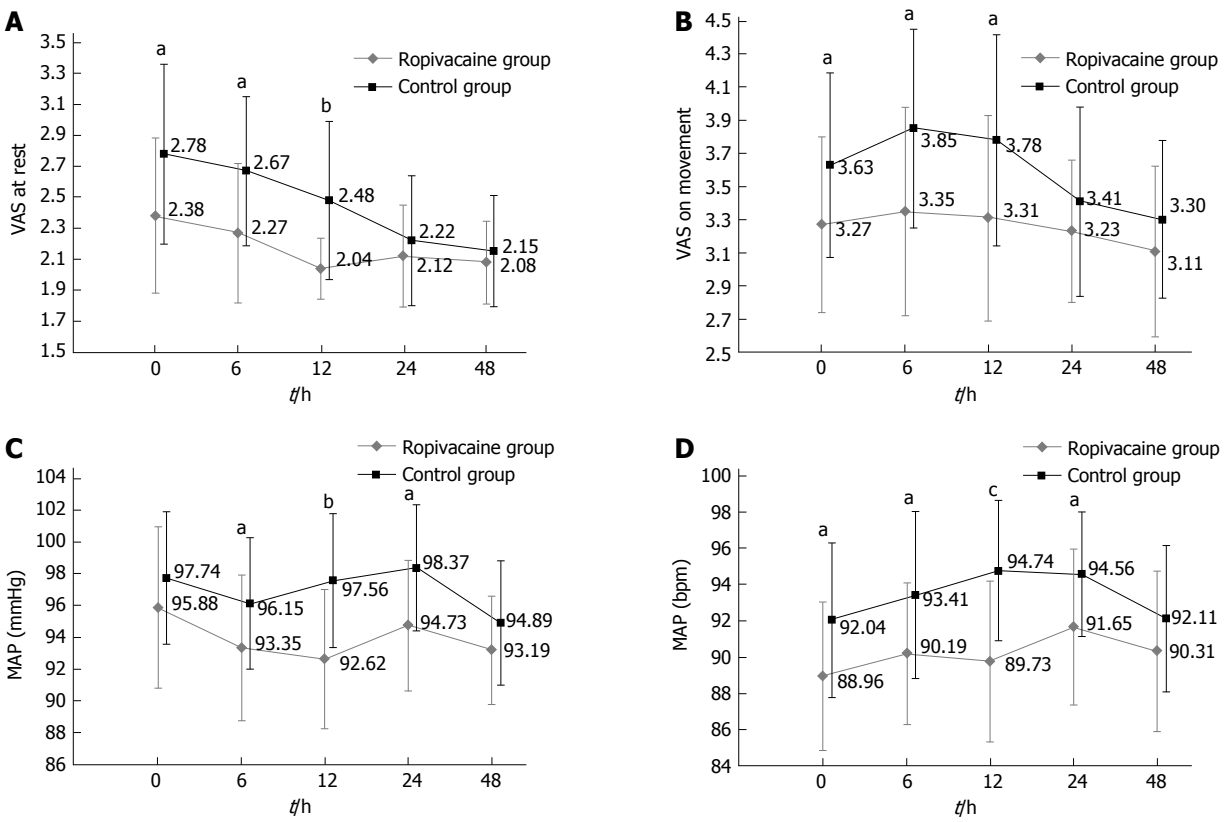

Figure 1 Visual analogue scale scores at rest and on movement, mean arterial pressure, and heart rate during the first 48 h after surgery. A: VAS scores at rest; B: VAS scores on movement; C: MAP; D: HR. [a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0001$. VAS: Visual analogue scale; MAP: Mean arterial pressure; HR: Heart rate.

Table 1 Demographic characteristics of the patients studied (mean ± SD)

Characteristic	Ropivacaine group	Control group	t/χ^2	*P* value
Age (yr)	48.38 ± 11.74	49.59 ± 12.42	-0.36	0.7176
Gender				
Male/female	18/8	18/9	0.04	0.8415
Weight (kg)	63.04 ± 9.21	66.04 ± 9.86	-1.14	0.2583
ASA grade				
I/II/III	4/17/5	7/15/5	0.92	0.6298
Incision length (cm)	24.65 ± 1.83	24.22 ± 2.76	0.67	0.5075
Operation type				
Left hepatectomy	8	5		0.0086
Right hepatectomy	13	8		
Mesohepatectomy	1	0		
Caudate lobectomy	2	1		
Irregular hepatectomy	2	13		
Postoperative pathology				
Hepatocellular carcinoma	22	18		0.3292
Intra-and extrahepatic cholangiolithiasis	3	7		
Hepatic focal nodular hyperplasia	1	1		
Hepatocellular adenoma	0	1		

ASA: American Society of Anesthesiologists.

24 h ($P = 0.0089$).

No statistically significant difference was observed in baseline levels (0 h) of epinephrine, norepinephrine, or cortisol between the two groups. The levels of epinephrine at 24 and 48 h were significantly lower in the ropivacaine group than in the control group ($P = 0.0064$, $P = 0.0078$). Similarly, the values of norepinephrine and cortisol at 24 and 48 h were significantly reduced in the ropivacaine group ($P < 0.0001$ for all), as shown in Figure 2A-C and Table 3.

Cumulative sufentanil consumption at 36 h after surgery is presented in Figure 2D and Table 2. The consumption was significantly lower in the ropivacaine group than in the control group at 6 h ($P = 0.022$),

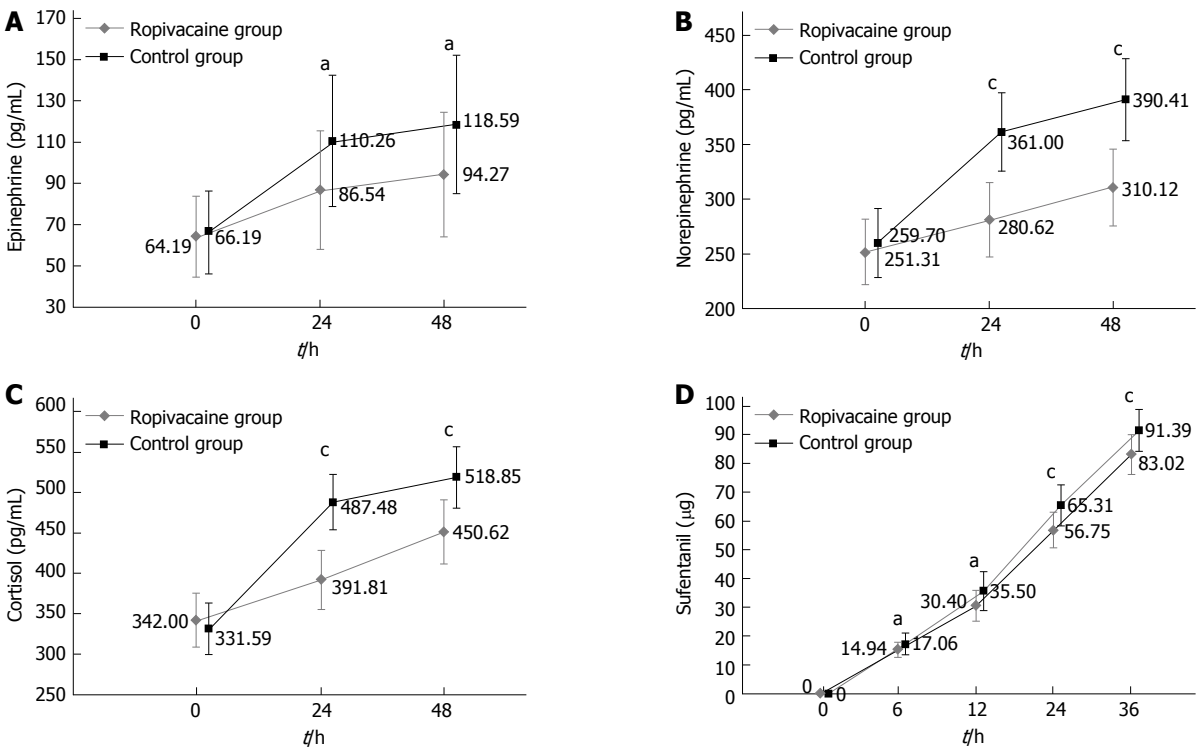

Figure 2 Plasma levels of epinephrine, norepinephrine and cortisol and cumulative sufentanil consumption during the first 48 h after surgery. A: Epinephrine; B: Norepinephrine; C: Cortisol; and D: Cumulative sufentanil consumption. $^{a}P < 0.05$; $^{c}P < 0.0001$.

Table 2 Visual analog scale scores at rest and on movement, mean arterial pressure, heart rate, and cumulative sufentanil consumption

Characteristic	0 h	6 h	12 h	24 h	48 h[1]
VAS at rest					
Ropivacaine group	2.38 ± 0.50	2.27 ± 0.45	2.04 ± 0.20	2.12 ± 0.33	2.08 ± 0.27
Control group	2.78 ± 0.58	2.67 ± 0.48	2.48 ± 0.51	2.22 ± 0.42	2.15 ± 0.36
t	-2.65	-3.17	-4.21	-1.03	-0.81
P value	0.0106	0.0032	0.0002	0.3096	0.4230
VAS on movement					
Ropivacaine group	3.27 ± 0.53	3.35 ± 0.63	3.31 ± 0.62	3.23 ± 0.43	3.11 ± 0.52
Control group	3.63 ± 0.56	3.85 ± 0.60	3.78 ± 0.64	3.41 ± 0.57	3.30 ± 0.47
t	-2.39	-2.99	-2.72	-1.27	-1.34
P value	0.0208	0.0043	0.0089	0.2110	0.1857
MAP (mmHg)					
Ropivacaine group	95.88 ± 5.08	93.35 ± 4.63	92.62 ± 4.43	94.73 ± 4.16	93.19 ± 3.41
Control group	97.74 ± 4.17	96.15 ± 4.14	97.56 ± 4.23	98.37 ± 3.99	94.89 ± 3.90
t	-1.46	-2.32	-4.16	-3.25	-1.69
P value	0.1515	0.0241	0.0001	0.0020	0.0981
HR (bpm)					
Ropivacaine group	88.96 ± 4.12	90.19 ± 3.92	89.73 ± 4.45	91.65 ± 4.30	90.31 ± 4.45
Control group	92.04 ± 4.27	93.41 ± 4.62	94.74 ± 3.88	94.56 ± 3.43	92.11 ± 4.05
t	-2.66	-2.73	-4.37	-2.72	-1.54
P value	0.0103	0.0087	< 0.0001	0.0089	0.1289
Cumulative sufentanil consumption (μg)					
Ropivacaine group	0	14.94 ± 2.56	30.40 ± 5.39	56.75 ± 6.20	83.02 ± 7.05
Control group	0	17.06 ± 3.81	35.50 ± 6.91	65.31 ± 7.09	91.39 ± 7.34
t		-2.36	-2.99	-4.67	-4.23
P value		0.0220	0.0043	< 0.0001	< 0.0001

[1]As to cumulative sufentanil consumption, the time point was set at 36 h. VAS: Visual analog scale; MAP: Mean arterial pressure; HR: Heart rate.

12 h (P = 0.0043), 24 h (P < 0.0001), and 36 h (P < 0.0001). Even so, the incidence of nausea and vomiting, the side effects of sufentanil, between the two groups had no significant difference (Table 4). Moreover, in the ropivacaine group, time to bowel recovery (P = 0.0133) and hospitalization after surgery

Table 3 Plasma concentrations of epinephrine, norepinephrine and cortisol (pg/mL)

Stress hormone	0 h	24 h	48 h
Epinephrine			
Ropivacaine group	64.19 ± 19.62	86.54 ± 28.64	94.27 ± 30.10
Control group	66.19 ± 20.30	110.26 ± 31.88	118.59 ± 33.65
t	-0.36	-2.85	-2.77
P value	0.7180	0.0064	0.0078
Norepinephrine			
Ropivacaine group	251.31 ± 30.19	280.62 ± 34.22	310.12 ± 35.15
Control group	259.70 ± 31.72	361.00 ± 36.06	390.41 ± 37.73
t	-0.99	-8.32	-8.01
P value	0.3286	< 0.0001	< 0.0001
Cortisol			
Ropivacaine group	342.00 ± 33.72	391.81 ± 36.53	450.62 ± 39.39
Control group	331.59 ± 31.92	487.48 ± 34.36	518.85 ± 38.21
t	1.15	-9.82	-6.40
P value	0.2537	< 0.0001	< 0.0001

Table 4 Time to bowel recovery, postoperative nausea and vomiting, and hospitalization length in the two groups

Characteristic	Ropivacaine group	Control group	t/χ^2	*P* value
Time to bowel recovery (d)	3.15 ± 1.01	3.93 ± 1.17	-2.56	0.0133
PONA				
No PONA	7	3		0.2729
Nausea without vomiting	16	18		
Nausea with vomiting	3	6		
Hospitalization (d)	8.65 ± 2.43	10.52 ± 3.49	-2.25	0.0289

PONA: Postoperative nausea and vomiting.

(P = 0.0289) were significantly shortened (Table 4).

DISCUSSION

Laparoscopic hepatectomy is commonly adopted in clinical settings because of its many advantages, including little trauma, low pain, fast recovery, and short hospitalization; however, open hepatectomy remains irreplaceable, especially in the presence of lesions close to or invading the root of the hepatic veins or the inferior vena cava, history of previous hepatectomy or any previous surgery potentially causing severe adhesion around the liver, and concomitant cardiopulmonary disease[13]. A right subcostal incision or reversed L-shaped incision (> 20 cm) is often made for open hepatectomy, and either of these two incision types is the most important source of postoperative pain. Thus, finding an effective way to reduce postoperative pain is urgent and necessary.

Local anesthetic wound infiltration is a useful and important component of a multimodality approach to postoperative pain control, and it can be applied in many types of surgery, including lumbar spine surgery, breast surgery, and inguinal hernia repair[9,14,15]. Local anesthetics used in the wound can block parietal afferents, reduce the sensitization of spinal dorsal horn neurons, and provide analgesia by inhibiting the transmission of noxious impulses from the incision[16]. Moreover, local anesthetics can suppress local inflammatory responses to incision injury that could sensitize nociceptive receptors and contribute to hyperalgesia[17]. Ropivacaine, a pure levorotatory stereoisomer and long-acting amide local anesthetic agent, has been widely used for local anesthesia and postoperative analgesia, and its reduced lipophilicity is associated with decreased incidence of central nervous system toxicity and cardiotoxicity[18]. Postoperative pain comes from superficial structures and deep muscular-peritoneal components; therefore, ropivacaine infiltrated not only the subcutaneous tissues but also the parietal peritoneum and deep muscular fasciae in our study. Our results showed that in the first 12 h after surgery, the local anesthetic ropivacaine significantly relieved the pain intensity at rest and on movement, demonstrating the potential of local wound infiltration with ropivacaine as a reliable analgesic strategy after open hepatectomy.

Surgical stress could cause a spectrum of changes in the body, involving the neuroendocrine, metabolic, immunological, and hematological systems[12]. The body's surgical stress response is mainly determined by the surgical wound severity, including the length of the incision in the abdominal wall from the skin to the parietal peritoneum[19]. The incision of open hepatectomy often exceeds 20 cm, and the surgical stress is thought to be high. Therefore, using local anesthetics to block surgical stress is feasible. Surgical stress response to injury causes a series of hormone changes; moreover, catecholamines (epinephrine and norepinephrine) and cortisol, as the main and most reliable peripheral hormones, correlate well with the extent of surgical stress. In this study, surgical stress was significantly reduced in the first 48 h after surgery as revealed by the levels of epinephrine, norepinephrine, and cortisol. Changes in MAP and HR were recorded, and the results demonstrated that the indexes of the ropivacaine group were obviously decreased. These results indicate that local wound infiltration with ropivacaine could also reduce surgical stress responses.

Opioids are commonly used for postoperative analgesia *via* venous access. However, they are associated with a potential risk of addiction, especially in large doses over long periods. Moreover, opioids possess potentially serious side effects, such as nausea, vomiting, constipation, respiratory depression, excessive sedation, and liver function impairment; hence, sparing opioids may reduce the incidence of the above side effects[20]. Reducing the dosage and duration of opioid usage is regarded suitable for avoiding

potentially serious adverse effects. Wound infiltration, as part of an opioid-sparing, multimodal analgesic regime, should therefore be recommended. Our current study showed that cumulative sufentanil consumption was significantly reduced in the ropivacaine group. Moreover, the time to bowel recovery was shorter in the ropivacaine group than in the control group. This may be caused by a combination of several reasons. First, sufentanil inhibits gut motility and propulsive activity by combining the μ-2 and κ receptors in the digestive tract[21]. Second, a previous animal study demonstrated that catecholamines reduce gut motility[22] and that the level of catecholamines in the ropivacaine group was reduced. Finally, ropivacaine could accelerate postoperative intestinal motility by reducing the inflammatory response.

The current study may have some limitations. The sample size was relatively small, and thus, more patients are needed in future studies to confirm our results. Compared with a previous study using catheters as a continuous wound infiltration method to deliver ropivacaine into the wound[23], we used single-shot ropivacaine infiltration into the superficial and deep muscular-peritoneal layers to achieve an analgesic effect. In our study, the drainage tube was routinely placed beside the liver resection surface and fixed outside. Movement and turning over could drag the tube and cause intensive pain, and thus, infiltration around the tube was highly effective. Moreover, the catheter under the wound could bring potential risks, such as infection and delayed wound healing, and the delivery rates and volumes of local anesthetics remain unidentified. Thus, single-shot infiltration with ropivacaine is a simple, convenient, and effective analgesic method that can bring short-term benefits for patients who underwent open hepatectomy. A previous study suggested that local anesthesia and stress response reduction could decrease cancer formation and that local anesthesia and analgesia may improve overall patient survival after oncologic surgery[24]. Thus, future research about local anesthesia, tumor recurrence, and patient survival after open hepatectomy is required.

In conclusion, local wound infiltration with ropivacaine after open hepatectomy can decrease acute postoperative pain and surgical stress response. This simple, convenient, and effective analgesic method provides postoperative analgesia and short-term benefits after open hepatectomy.

COMMENTS

Background

The postoperative pain caused by laparotomy delays patients' recovery and incurs stress response. Although commonly used to control pain, intravenous analgesia and epidural analgesia still have their contraindications and side effects. Local wound infiltration is a simple and effective method that can provide satisfactory analgesia without major side effects. The current study was designed to evaluate the effect of local wound infiltration with ropivacaine on postoperative pain and stress response after open hepatectomy.

Research frontiers

Postoperative analgesia is an indispensable component of fast track surgery for surgical patients, especially those who undergo laparotomy. Local anesthetics can effectively provide analgesia by inhibiting the transmission of noxious impulses from the wound and suppress local inflammatory responses to wound injury.

Innovations and breakthroughs

Wound infiltration with ropivacaine could provide effective analgesia in the first 48 h after open hepatectomy, with lower mean arterial pressure, heart rate and sufentanil consumption, accelerated postoperative recovery, and reduced stress response. These results suggest that this method is a simple, convenient and effective analgesic method that can provide postoperative analgesia and short-term benefits after open hepatectomy.

Applications

This study provides additional evidence supporting that local wound infiltration with ropivacaine after open hepatectomy can improve postoperative pain relief, reduce surgical stress response, and accelerate postoperative recovery.

Terminology

The intensity of postoperative pain at rest was measured on a visual analogue scale graded from 0 (no pain) to 10 (very severe pain) after surgery. Postoperative nausea and vomiting was recorded with a three-point rating scale: 1, no nausea and vomiting; 2, nausea without vomiting; 3, nausea with vomiting.

Peer-review

The study was well written and its findings are informative. Local wound infiltration with ropivacaine has good effects for pain relief and stress response reduction after open hepatectomy.

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CASE REPORT

Pedicled omental patch as a bridging procedure for iatrogenic bile duct injury

Jun Jie Ng, Alfred Wei Chieh Kow

Jun Jie Ng, Alfred Wei Chieh Kow, Department of Surgery, National University Hospital, Singapore 119074, Singapore

ORCID Number: Jun Jie Ng (0000-0003-0779-8468); Alfred Kow (0000-0001-8942-8262).

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Correspondence to: Jun Jie Ng, Senior Resident, Department of Surgery, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074, Singapore. jun_jie_ng@nuhs.edu.sg
Telephone:+65-97375930
Fax: +65-67795678

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Abstract

Iatrogenic bile duct injuries during cholecystectomy can present as fulminant intra-abdominal sepsis which precludes immediate repair or biliary reconstruction. We report the case of a 29-year-old female patient who sustained a bile duct injury after an open cholecystectomy in a neighboring country. She presented to our institution 22 d after initial surgery with septic shock and multiple intra-abdominal collections. Endoscopic retrograde cholangiography revealed a large common hepatic duct defect corresponding to a Strasberg type D bile duct injury. Definitive reconstruction such as a hepaticojejunostomy cannot be performed due to the presence of dense adhesions with infected and friable tissues. She underwent a combination of endoscopic biliary stenting and pedicled omental patch repair of the bile duct to control bile leak and sepsis as a bridging procedure to definite hepaticojejunostomy three months later.

Key words: Cholecystectomy; Endoscopic retrograde cholangiopancreatography; Case reports; Bile ducts; Abdominal abscess

Core tip: Iatrogenic bile duct injury is a challenging condition to treat. This case report describes a novel and innovative surgical technique, whereby a pedicled omental patch repair was performed as a bridging procedure to definitive repair such as a hepaticojejunostomy, in a patient who presented in a delayed fashion with severe intra-abdominal sepsis.

Ng JJ, Kow AWC. Pedicled omental patch as a bridging procedure for iatrogenic bile duct injury. *World J Gastroenterol* 2017; 23(36): 6741-6746 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6741.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6741

INTRODUCTION

Iatrogenic bile duct injuries (BDIs) are uncommon but can lead to significant morbidity and mortality in patients who had undergone cholecystectomy. Although the incidence of BDIs in LC was reported to be almost tenfold that of OC during the 1990s, LC has now become the gold standard in the treatment of gallstone disease, with a low and declining incidence of BDIs[1,2]. Several studies have reported the rate of BDIs during LC to be between 0.4% to 0.7%, but a more recent retrospective review of more than 10000 patients who had undergone LC showed that the incidence of BDIs was 0.2%, which was comparable to that of OC[3-5]. Approximately one-third of BDIs is diagnosed intra-operatively and are amenable to immediate repair or reconstruction. However, the majority of BDIs are recognized only in the post-operative period with varying clinical presentations such as abdominal pain, jaundice, bile peritonitis or shock[6-8]. BDIs can be classified using the Strasberg classification, with most BDIs manifesting as biliary leaks (Strasberg type A) that can be managed using endoscopic techniques[6]. The other large group of BDIs are common bile duct or common hepatic duct transections (Strasberg type E1 to E3) which usually warrants surgical repair[9]. A tension-free Roux-en-Y hepaticojejunostomy is the preferred surgical procedure for reconstitution of bilio-enteric continuity in major common bile duct or common hepatic duct transections, but rarely, in some patients, the presence of severe intra-abdominal sepsis precludes immediate biliary reconstruction[8]. We report a novel method of using a pedicled omental patch, combined with endoscopic biliary stenting, as a bridging procedure to control bile leak, in a patient with severe intra-abdominal sepsis from a large common hepatic duct transection after OC, before attempting definitive hepaticojejunostomy.

CASE REPORT

A 29-year-old female underwent an open cholecystectomy for acute calculous cholecystitis in a hospital from a neighbouring country. Exact intra-operative findings and details of her post-operative recovery were not made available to us. She developed obstructive jaundice on post-operative day three with bilious effluent from her abdominal drain on post-operative day seven. She was initially treated expectantly but due to persistent high bilious output from her abdominal drain, she underwent an exploratory laparotomy on post-operative day 16. The surgeons noted a collapsed common bile duct, but were not able to locate the exact site of bile leak. She was subsequently transferred to our institution on post-operative day 22 for further management for further management of her bile leak.

Upon presentation to our institution, the patient was in septic shock. There was active leakage of bilious fluid from her a right subcostal incision. Her abdomen was distended with generalized tenderness. She was resuscitated and started on broad spectrum antibiotics. Laboratory investigations revealed elevated infective markers with a white cell count of 19 × 10^9/L and a C-reactive protein level of 48 mg/L. Serum bilirubin was normal. Computed tomography scan of the abdomen revealed a 3 cm biloma at the gallbladder fossa (Figure 1A) with multiple rim-enhancing abdominal collections around the upper abdomen (Figure 1B) - the largest being a 9.3 cm × 8.5 cm perisplenic collection (Figure 1C). Endoscopic retrograde cholangiography performed on post-operative day 23 revealed a large common hepatic duct defect just below the bifurcation, corresponding to a Strasberg type D BDI with contrast seen extravasating into the sub-hepatic recess (Figure 2A). After much difficulty, guide-wires were placed across the bile duct defect and plastic biliary stents were deployed into the left and right hepatic ducts respectively (Figure 2B).

In view of persistent sepsis with the presence of multiple intra-abdominal collections, a decision to perform damage control surgery was taken. At the exploratory laparotomy performed on post-operative day 24, a large 2 cm defect at the anterolateral aspect of the common hepatic duct with biliary stents visible from within was found (Figure 3A). There was a large perisplenic abscess with presence of turbid bile and debris in the sub-hepatic recess which was drained and washed out thoroughly. Neither primary repair of the biliary defect nor biliary reconstruction with a hepaticojejunostomy were suitable options due to dense adhesions around the hepatic hilum with infected and friable surrounding tissues. The biliary drains were unable to completely exclude the biliary system entirely due to the size of the biliary defect. Hence, a well-vascularized and healthy pedicled omental patch was harvested and tagged down circumferentially to the biliary defect using absorbable sutures (Figure 3B). An illustration of the pedicled omental patch is as demonstrated (Figure 4). Drains were placed and the abdomen was closed primarily. The patient improved after surgery with resolution of pyrexia and sepsis. Abdominal drains were removed within eight days of surgery. Laboratory infective markers normalized and serum bilirubin levels remained normal. Intra-abdominal cultures grew Pseudomonas aeruginosa and Klebsiella pneumoniae and the patient was sent home to complete four weeks of intravenous antibiotics.

A repeat computed tomography scan of the abdomen three months later revealed complete resolution of the intra-abdominal collections. Although the biliary stents were in-situ, there was mild intrahepatic biliary dilatation suggesting the possibility of a common hepatic duct stricture. The patient subsequently underwent creation of a hepaticojejunostomy one week later. Intra-operatively, there were dense adhesions around the porta hepatis with evidence of early stricture

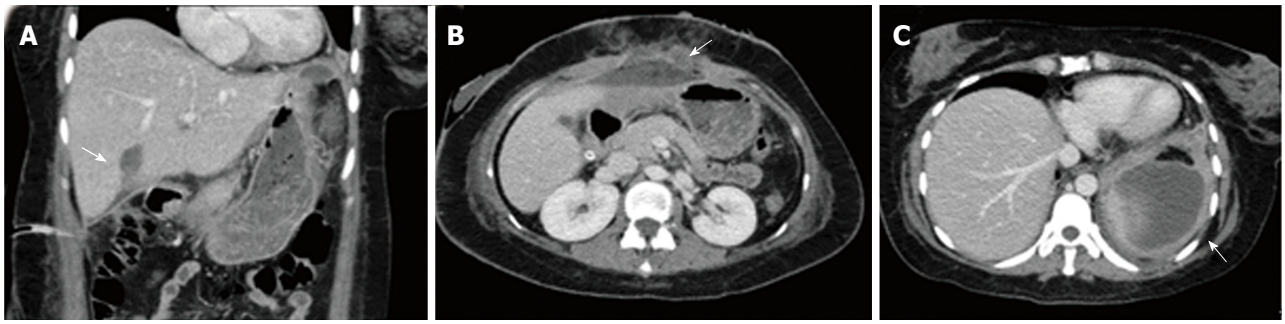

Figure 1 Computed tomography scan. A: Initial computed tomography scan of the abdomen revealing a 3 cm biloma at the gallbladder fossa with bile tracking into the sub-hepatic recess; B: Multiple rim-enhancing smaller intra-abdominal collections were also present in the upper abdomen; C: The largest intra-abdominal collection was a 9.3 cm × 8.5 cm perisplenic collection.

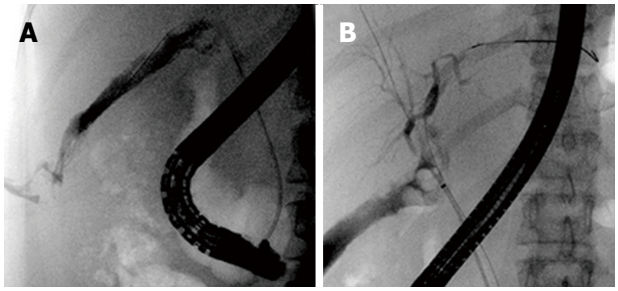

Figure 2 Endoscopic retrograde cholangiography. A: Endoscopic retrograde cholangiography revealed a large Strasberg Type D common hepatic duct defect with contrast seen immediately in the sub-hepatic recess; B: Plastic biliary stents inserted across the biliary defect into the left and right hepatic ducts.

formation at the previous site of BDI. The patient had an uneventful recovery after surgery and was discharged on post-operative day five.

DISCUSSION

BDIs rarely present in such a delayed fashion with fulminant intra-abdominal sepsis. The initial management of BDIs especially for patients that present late in the post-operative period is directed at delineating the type and extent of BDI, controlling sepsis and ongoing bile leak[8]. We describe a technique that can be utilized as a bridge to definitive bilioenteric reconstruction such as a hepaticojejunostomy when prevailing conditions such as severe intra-abdominal sepsis renders immediate or early reconstruction unfavourable and not ideal. Our technique fulfils the above-mentioned principles of BDI management - first by using endoscopic retrograde cholangiogram to characterize the BDI with placement of biliary stents endoscopically to help control the bile leak. The stents will negate the function of the sphincter of Oddi as well as direct bile into the duodenum. Subsequent laparotomy will allow source control of sepsis *via* drainage of intra-abdominal abscesses whilst the placement of a pedicled omental patch will ensure proper sealing of the biliary defect and exclusion of the biliary system from the peritoneal cavity. This restores continuity of bile flow, allows for resolution of intra-abdominal sepsis, inflammation and fibrosis, after which definitive bilioenteric reconstruction can be performed at an elective setting as the presence of peritonitis has been reported to confer a poorer outcome in patients undergoing biliary reconstruction[10].

The use of omentum as an autologous graft to seal, patch or reinforce tissues during surgery is widely practiced, and its ability to support and adhere to local tissue is due to its abundant blood supply, angiogenic activity, innate immune function and high concentration of tissue factor[11,12]. Moreover, animal studies have shown that the use of a pedicled omental flap in tissue reconstruction confers an anti-inflammatory effect, which can lead to an improved healing response[13].

Omentum has been commonly used as a patch to close perforations in the gastro-intestinal tract such as a perforated duodenal ulcer. There have been several reports describing the use of omentum in biliary reconstruction. Meissner described his technique of "T-tube stented omentoplasty" used to successfully treat a common hepatic duct stricture. After incising the strictured segment of bile duct resulting in a 4 cm non-circumferential biliary defect, a large bore T-tube was placed within and a pedicled omental flap was then sutured down over the horizontal limb of the T-tube to bridge any remnant biliary defect as well as to envelop the vertical limb. The T-tube was removed after 6 mo with no sequelae and repeat ERC 19 years later showed normal biliary anatomy[14]. Ebata *et al*[15] also described a similar technique of "hilar cholangioplasty" where he used a pedicled omental flap with a 16F T-tube to seal a 2 cm × 3 cm ductal defect due to a biliobiliary fistula. T-tube cholangioscopy 1 mo after surgery revealed a yellowish polypoid mass at the previous ductal defect and a biopsy taken revealed normal biliary epithelium lining the surface of the grafted omentum. The patient was followed up for 3 years without evidence of cholangitis or abnormal liver function. Another similar technique was described

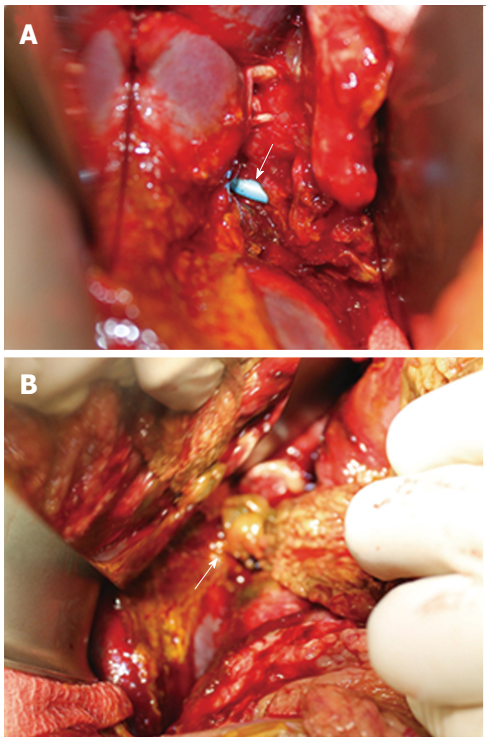

Figure 3 Damage control surgery. A: At the time of exploratory laparotomy, endoscopically placed biliary stents were visible within a large 2 cm anterolateral common hepatic duct defect; B: A pedicled omental patch was harvested and secured to the biliary defect using absorbable sutures.

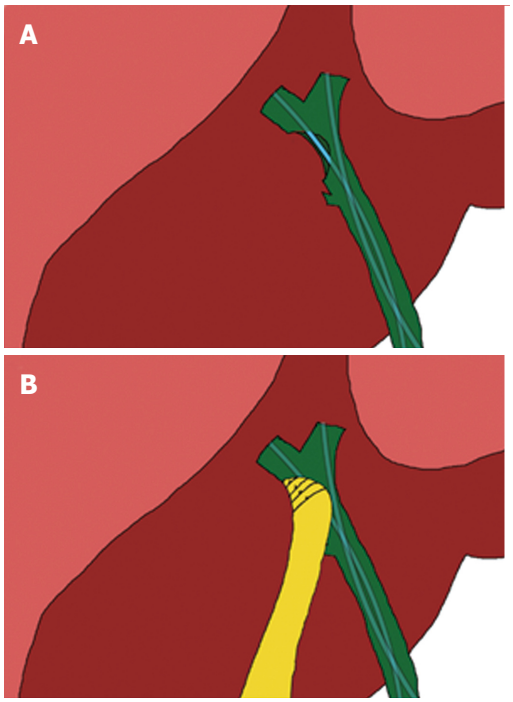

Figure 4 An illustration of the pedicled omental patch. A: An illustration showing the location of the common hepatic duct defect and endoscopic biliary stents placed across; B: The harvested pedicled omental patch was placed over the biliary defect and secured using absorbable sutures that run through the anterior and posterior margins of the biliary defect.

by Chang but he used the falciform ligament instead of the omentum to patch a large bile duct defect due clip necrosis after laparoscopic cholecystectomy[16]. Some authors have also used pedicled omental flaps to reinforce high risk biliary anastomoses in liver transplantation with good results[17].These examples, although limited to individual case reports reaffirms the ideal properties of omentum that make it a suitable autologous graft to aid in biliary reconstruction.

We postulate that our technique could also have been used as a stand-alone procedure without the need of a subsequent hepaticojejunostomy. The biliary stents could then be retrieved endoscopically after 10 to 12 wk. The above-mentioned case reports have shown good long-term patency of the reconstructed bile duct but established data is lacking. A hepaticojejunostomy would still confer the best long-term outcomes, especially in our patient who is young[18]. Using our technique as a stand-alone procedure remains experimental and may be suitable in patients who cannot tolerate longer periods of general anaesthesia needed in a hepaticojejunostomy. Intensive follow-up would be necessary for such patients due to the risk of biliary strictures. We performed a definitive hepaticojejunostomy for our patient as she was due to return to a rural area with no access to regular follow-up for the development of biliary strictures.

Alternative management strategies that could be deployed in our patient would be the placement of percutaneous trans-hepatic biliary drainage tubes or endoscopic biliary stents, combined with either percutaneous or surgically inserted intra-abdominal drains. This might be feasible if the ductal defect was small. However, if the ductal defect is large, the bile leak not be controlled leading to persistent biliary peritonitis and the development of intra-abdominal collections. Moreover, the drains would have to be placed on active suction for a significant period of time to be able to successfully divert and control the bile leak. More often than not, these drains would have to be kept for weeks to months until the patient undergoes definitive bilioenteric reconstruction. In a patient like ours who present with uncontrolled bile leak, intra-abdominal collections and sepsis, the utilization of our technique would allow for control of bile leak without any other additional percutaneous procedures or prolonged drain placement. Another management strategy that could be considered would be use of a T-tube in combination with an omental pedicled flap similar to techniques described above. We were, however, with the assistance of an experienced operator, able to cannulate the left and right hepatic ducts individually and place biliary stents across the biliary defect obviating the need of a T-tube.

Lastly, and most importantly, our case highlights the

need for early referral to an institution with experienced hepatobiliary surgeons, endoscopists, or interventional radiologists once a BDI is diagnosed. It is irrefutable evidence that best outcomes are obtained when first repair is performed in experienced tertiary centres[18,19].

This case highlights the need for early referral of BDIs to specialist tertiary centres for further management once detected. Immediate repair or biliary reconstruction is contraindicated in patients with BDIs who present in a delayed fashion with severe intra-abdominal sepsis. We describe an alternative technique which combines endoscopic biliary stenting with a pedicled omental patch as a bridging procedure to definitive hepaticojejunostomy in this group of patients.

COMMENTS

Case characteristics

A 29-year-old female presented with septic shock and generalized abdominal pain following an open cholecystectomy performed three weeks ago in another hospital.

Clinical diagnosis

Physical examination revealed generalized abdominal tenderness and leakage of bile stained fluid from a right subcostal incision.

Differential diagnosis

Bile duct injury, duodenal injury, small bowel injury.

Laboratory diagnosis

Initial laboratory investigations revealed an elevated total white cell count and C-reactive protein levels.

Imaging diagnosis

Computed tomography scan of the abdomen revealed multiple large intra-abdominal collections. Endoscopic retrograde cholangiography revealed a large common hepatic duct defect (Strasberg type D bile duct injury).

Treatment

An exploratory laparotomy with a pedicled omental patch repair of the common hepatic duct was performed initially, followed by a definitive hepaticojejunostomy three months later.

Related reports

This is the first report describing the use of a pedicled omental patch combined with biliary stenting for treatment of a large bile duct injury. Two other case reports have described the use of a pedicled omental patch in conjunction with a T tube for similar bile duct injuries.

Experiences and lessons

In patients who present with severe intra-abdominal sepsis after bile duct injury, up-front creation of a definitive hepaticojejunostomy may not be possible. Instead, a pedicled omental patch repair of the biliary defect may be performed as a bridging procedure to a hepaticojejunostomy, or even as a stand-alone procedure.

Peer-review

The authors presented a novel and alternative technique by using a pedicled omental patch repair as a bridge to a definitive procedure in bile duct injuries.

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