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REVIEW

Current and future pharmacological therapies for managing cirrhosis and its complications

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Abstract

Due to the restrictions of liver transplantation, complication-guided pharmacological therapy has become the mainstay of long-term management of cirrhosis. This article aims to provide a complete overview of pharmacotherapy options that may be commenced in the outpatient setting which are available for managing cirrhosis and its complications, together with discussion of current controversies and potential future directions. PubMed/Medline/Cochrane Library were electronically searched up to December 2018 to identify studies evaluating safety, efficacy and therapeutic mechanisms of pharmacological agents in cirrhotic adults and animal models of cirrhosis. Non-selective beta-blockers effectively reduce variceal re-bleeding risk in cirrhotic patients with moderate/large varices, but appear ineffective for primary prevention of variceal development and may compromise renal function and haemodynamic stability in advanced decompensation. Recent observational studies suggest protective, haemodynamically-independent effects of beta-blockers relating to reduced bacterial translocation. The gut-selective antibiotic rifaximin is effective for secondary prophylaxis of hepatic encephalopathy; recent small trials also indicate its potential superiority to norfloxacin for secondary prevention of spontaneous bacterial peritonitis. Diuretics remain the mainstay of uncomplicated ascites treatment, and early trials suggest alpha-adrenergic receptor agonists may improve diuretic response in refractory ascites. Vaptans have not demonstrated clinical effectiveness in treating refractory ascites and may cause detrimental complications. Despite initial hepatotoxicity concerns, safety of statin administration has been demonstrated in compensated cirrhosis. Furthermore, statins are suggested to have protective effects upon fibrosis progression, decompensation and mortality. Evidence as to whether proton pump inhibitors cause gut-liver-brain axis dysfunction is conflicting. Emerging evidence indicates that anticoagulation therapy reduces incidence and increases recanalisation rates

of non-malignant portal vein thrombosis, and may impede hepatic fibrogenesis and decompensation. Pharmacotherapy for cirrhosis should be implemented in accordance with up-to-date guidelines and in conjunction with aetiology management, nutritional optimisation and patient education.

Key words: Cirrhosis; Beta-blockers; Rifaximin; Diuretics; Statins; Proton pump inhibitors; Pharmacology

Core tip: Pharmacological therapy is central to the management of cirrhosis and its complications. Whilst there has been recent debate about the safety of beta-blockade in patients with ascites, conversely there is growing interest in potential benefits relating to a reduction in gut bacterial translocation and hepatocellular carcinoma risk. In addition to its well-established role in treating hepatic encephalopathy, rifaximin may also have a key role in preventing secondary infections. In this review, we summarise these and other uncertainties, controversies and novel developments related to pharmacotherapy in the clinical management of chronic liver disease.

INTRODUCTION

Decompensation and mortality in cirrhosis are predominantly due to the complications of portal hypertension, and prognosis in cirrhosis progressively deteriorates with the cumulative occurrence of ascites, variceal haemorrhage, hepatic encephalopathy (HE) and spontaneous bacterial peritonitis (SBP)[1]. While both cirrhosis incidence and prevalence have risen in recent decades, uptake of liver transplantation – the only definitive treatment option for cirrhosis - has stagnated at around 5500/year in Europe, primarily due to organ shortages[2]. Consequently, standardised cirrhosis mortality rates globally, and in the United Kingdom particularly, have increased significantly[3]. This development led to the United Kingdom-wide introduction of specialist cirrhosis clinics which integrate multidisciplinary services and aim to optimise supportive cirrhosis management by forestalling decompensation and facilitating recompensation. In the specialist clinic setting, one factor which has gained importance in chronic cirrhosis management is long-term, complication-guided pharmacological therapy. Whilst previous articles have addressed individual pharmacological agents and their role in treating specific complications of cirrhosis, the present article aims to provide an overview of the complete pharmacotherapy currently available for the long-term management of cirrhotic outpatients as well as an insight into emerging and future directions.

METHODS

A search of the existing literature up to December 2018 was conducted using the electronic databases PubMed, Medline and the Cochrane library, as well as relevant guidelines and reference lists. Titles and abstracts were searched for the following key terms: "Cirrhosis" and ("beta-blockers" or "lactulose" or "rifaximin" or "L-ornithine L-aspartate (LOLA)" or "acarbose" or "diuretics" or "midodrine" or "clonidine" or "vaptans" or "human serum albumin" or "anti-coagulation" or "caffeine" or "faecal microbiota transplant"). A separate search was performed for international guidelines to cirrhosis management by the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL). Additionally, reference lists of included articles were manually screened for further relevant publications. The abstracts of 2031 publications were identified and screened for studies evaluating the safety, efficacy and therapeutic mechanism of

pharmacological agents in cirrhotic adults and animal models of cirrhosis. 158 publications were considered relevant to the key question and included in the present review (Figure 1). Only articles published in English were included.

CURRENTLY-USED MEDICATIONS

Beta-blockers

Presently, non-selective beta-blockers (NSBB) are the only drug class endorsed for the long-term treatment of portal hypertension[4,5]. Along with endoscopic band ligation, NSBBs are employed for primary and secondary prophylaxis against variceal haemorrhage, as they combat the hyperkinetic portal-hypertensive syndrome by decreasing cardiac output and portal inflow (β-1 receptor blockade) and by achieving splanchnic vasoconstriction and reducing azygos blood flow (β-2 receptor blockade)[1,6-8]. Thus they efficaciously lower the risk of variceal bleeding and re-bleeding as evidenced by several randomized controlled trials (RCT)[9-11].

Recent concerns about beta-blockers: These trials, however, predominantly excluded patients with advanced decompensation, and recent studies have voiced concerns over the safety of beta-blockers in decompensated patients with refractory ascites or SBP and in cirrhotic patients with alcoholic hepatitis[12-14]. In a prospective cohort study of 151 patients with refractory ascites by Sersté *et al*[12], 1-year survival was significantly lower in patients who received propranolol (19%) compared to patients not on beta-blocker therapy (64%). In another retrospective cohort study by Sersté *et al*[13] including 139 cirrhotic patients with severe alcoholic hepatitis, cumulative incidence of acute kidney injury (AKI) was significantly higher in the group receiving beta-blockers (89.6%) *vs* the group not receiving beta-blockers (50.4%). In a retrospective analysis of 607 cirrhotic patients by Mandorfer *et al*[14], NSBB therapy was associated with increased transplant-free survival in patients without SBP, but with decreased transplant-free survival, increased length of non-elective hospitalisation and increased rates of hepatorenal syndrome (HRS) in patients with SBP. Pathophysiologically, circulating blood volume depletion during large volume paracentesis for refractory ascites and the systemic inflammatory response during SBP may threaten the already limited cardiac reserve of decompensated patients, while beta-blockade further impairs restoration of renal and systemic perfusion pressures[7]. Furthermore, patients with advanced stages of decompensation and a more amplified hyperdynamic circulation are likely to receive higher doses of beta-blockers, thus exaggerating detrimental effects on systemic haemodynamics. Accordingly, a retrospective nationwide study of 3719 Danish patients with cirrhosis found a reduction in mortality for propranolol doses < 160 mg/d but an increase in mortality for doses > 160 mg/d[15].

The 'window hypothesis': Consequently, the 'window hypothesis' by Krag *et al*[16] proposes the existence of a specific time frame of opportunity during the natural course of cirrhosis, only within the bounds of which NSBB therapy exerts beneficial effects on survival. It is propositioned that the window opens with the development of moderate/large varices and closes during advanced cirrhosis with the advent of refractory ascites, SBP, HRS or with the occurrence of alcoholic hepatitis[6,16]. An elegantly-designed randomized controlled trial by Groszmann *et al*[17] demonstrated that NSBBs are ineffective in the pre-primary prophylaxis of variceal development and even result in adverse effects in patients without varices, since the absence of a hyperdynamic circulation in patients with subclinical portal hypertension (Hepato-Venous Pressure Gradient (HVPG) < 10 mmHg) attenuates the portal pressure lowering effects of beta-blockers[18,19]. A more recent meta-analysis by Kumar *et al*[20] also found no difference in incidence of first variceal haemorrhage and development of large varices comparing patients receiving beta-blockers and patients not receiving beta-blockers, yet a significantly higher rate of adverse events [relative risk (RR) 4.66, 95% confidence interval (CI) 1.36–15.91] was observed in patients receiving beta-blockers. With cirrhosis progression, portal pressure heightens while effective circulating volume decreases as a result of splanchnic vasodilatation and raised portal inflow. At this level of disease progression, the drop in effective circulating blood volume is compensated for by enhanced sympathetic stimulation of the cardiocirculatory system in the context of preserved cardiac reserves[4,21]. It is precisely at this stage that NSBBs are proposed to effectively counteract portal hypertension and abrogate the hyperdynamic state, thus improving patient survival[4]. With further advancement of cirrhosis and portal hypertension, however, the cardiac response to stimuli such as volume depletion by variceal haemorrhage becomes limited and beta-blockade impedes restoration of systemic and renal perfusion pressures, thus

Figure 1 Flow diagram of study inclusion.

negatively impacting patient survival[7,21]. In line with the 'window hypothesis', Kim *et al*[22] conducted a nested case-control study in patients awaiting liver transplantation, matching 205 patients with AKI to 205 patients without AKI. On multivariate analysis, patients with ascites receiving beta-blockers had a 3-fold increased [Hazard ratio (HR) 3.31] risk of developing AKI, while patients without ascites receiving beta-blockers had a 5-fold reduced (HR 0.19) risk of developing AKI[21,22].

As a consequence of these concerns, widespread withholding of NSBB therapy in patients with advanced cirrhosis ensued. Yet, data from additional studies, performed after this initial apprehension had surfaced, provide counteracting evidence. A recent post-hoc analysis of 1188 patients with cirrhotic ascites from three satavaptan RCTs showed no association between NSBB therapy and increased mortality[23]. Similarly, a retrospective study by Leithead *et al*[24] (105 beta-blocker users propensity risk score matched to 105 beta-blocker non-users) correlated NSBB use with improved survival in patients with ascites awaiting orthotopic liver transplantation (HR 0.55, 95% CI 0.32-0.95), and even on subgroup analysis of patients with refractory ascites, beta-blockers remained independently associated with improved survival (HR 0.35, 95% CI 0.14-0.86). Furthermore, Onali *et al*[25] evaluated 316 consecutive cirrhotic patients with ascites undergoing assessment for liver transplantation. Beta-blockers were associated with reduced overall mortality (HR 0.55, 95% CI 0.33-0.94) and on sub-group analysis of patients with refractory ascites no difference in mortality was observed (HR 0.43, 95% CI 0.20-1.11). The numerous observational studies investigating the risks and benefits of NSBBs in advanced cirrhosis are summarised in Table 1. A recent meta-analysis of three RCTs and 13 observational studies summarising the effect of NSBBs on mortality in cirrhotic patients with ascites found that survival was comparable between NSBB and control groups for both the overall population (HR 0.86, 95% CI 0.71-1.03, P = 0.11) and the refractory ascites subgroup (HR 0.90, 95% CI 0.45-1.79, P = 0.76) with significant heterogeneity between included studies[26].

Carvedilol: With the hypothesis that these discrepancies regarding the effect of beta-blockers in patients with refractory and non-refractory ascites may be related to the type of beta-blocker administered, a meta-analysis of nine observational studies in cirrhotic patients with ascites by Njei *et al*[27] found that the traditional NSBBs propranolol and nadolol were not associated with increased mortality, but that carvedilol, a NSBB with additional anti-alpha-1-adrenergic receptor activity, demonstrated a statistically significant association with increased all-cause mortality (RR 1.75, 95% CI 1.06-2.90)[27]. Previous studies had shown that carvedilol is more potent in reducing HVPG than traditional NSBBs[28] and is able to achieve a haemodynamic response in a high percentage (56%) of non-responders to propranolol[29]. In rodents, carvedilol administration was shown to modulate inflammatory cytokine generation and augment antioxidant production, resulting in diminished liver fibrogenesis[30,31]. The anti-alpha-1 mediated reduction in intrahepatic resistance brought about by carvedilol may be of benefit to patients with less advanced portal hypertension, where intrahepatic resistance still constitutes a major factor in portal pressure[32]. While traditional beta-blockers were shown to be ineffective in the primary prevention of variceal development, carvedilol achieved a delay in progression of small to large varices from 18.7 to 20.8 mo in a RCT by Bhardwaj *et al*[33]. However, carvedilol also shows a trend towards a more potent reduction in systemic arterial pressure compared to traditional beta-blockers[19,28] and could thus further destabilise the delicately-balanced haemodynamic state in cirrhotic patients with ascites, as Njei *et al*[27] hypothesise in view of the detrimental effect of carvedilol on their cohort. In contrast, Sinha *et al* recently investigated the effects of

Table 1 Observational studies investigating the effects of non-selective beta-blockers in advanced cirrhosis

Study	Study type	Study population	Type of NSBB	Outcomes
Sersté *et al*[12]	Single-centre, prospective cohort study	Cirrhotic inpatients with refractory ascites (*n* = 151; mean MELD 18.8 ± 4.1; 69% diuretic-intractable, 31% diuretic-resistant), all treated with large-volume paracentesis + IV albumin Median follow-up: 8 mo (range 1-47)	Propranolol (40-160 mg per day)	Median survival was 5.0 mo (95% CI 3.5-6.5 mo) for patients on propranolol compared to 20.0 mo (95% CI 4.8-35.2 mo) for patients not on propranolol (*P* < 0.0001)
Sersté *et al*[13]	Single centre, retrospective cohort study	Cirrhotic inpatients with alcoholic hepatitis (*n* = 139; mean MELD score 27.3 ± 7.6; mean Maddrey score 71.0 ± 34.4), all treated with methylprednisolone	Propranolol (40-160 mg per day)	At 168-d follow-up: AKI incidence was 89.6% (95% CI 74.9%-95.9%) for patients on propranolol compared to 50.4% (95% CI 39.0%-60.7%) for patients not on propranolol (*P* < 0.0001) Transplant-free mortality was 56.8% (95% CI 41.3%-69.7%) in NSBB users compared to 46.7% (95% CI 35.0%-57.6%) in non-users (*P* = 0.25)
Mandorfer *et al*[14]	Single-centre retrospective observational study	Cirrhotic outpatients with ascites (*n* = 607; mean MELD 17.5 ± 10.6), all treated with large-volume paracentesis + IV albumin Follow-up: 660 person-years	Propranolol (20-120 mg per day); Carvedilol (6.25-25 mg per day)	In patients without SBP: NSBB use was associated with higher transplant-free survival (HR 0.75, 95% CI 0.581-0.968) and with reduced length of hospitalisation In patients with SBP: NSBB use was associated with reduced transplant free survival (HR 1.58, 95% CI 1.098-2.274), development of HRS (24% *vs* 11%, *P* = 0.03), and increased length of hospitalisation
Bang *et al*[15]	Multicentre, retrospective, propensity-adjusted, longitudinal study of Danish databases	Decompensated cirrhotic in- and outpatients (*n* = 702 propranolol-users matched to *n* = 702 non-users). Stratified into mild decompensation (1-4 previous paracenteses) and severe decompensation (> 4 paracenteses)	Propranolol (< 80 mg, 80-160 or > 160 mg per day)	At 2-year follow-up: Propranolol use was associated with lower mortality in patients with mildly decompensated cirrhosis (HR 0.7, 95% CI 0.6-0.9) and severely decompensated cirrhosis (HR 0.6, 95% CI 0.4-0.9). Survival benefit was only found for propranolol doses < 160 mg/d.
Kim *et al*[22]	Single-centre, retrospective, nested case-control study	Cirrhotic patients listed for liver transplantation who developed AKI (*n* = 205 patients with AKI matched to *n* = 205 patients without AKI) Median follow-up: 18.2 mo (range 1-198 mo)	Propranolol and nadolol (propranolol equivalent 40 mg per day, IQR 30.0–60.0 mg)	In patients with ascites: NSBB use was associated with an increased risk of AKI (HR 3.31, 95% CI 1.57-6.95) In patients without ascites: NSBB use was associated with a reduced risk of AKI (HR 0.19, 95% CI 0.06-0.60)
Bossen *et al*[23]	Post-hoc observational analysis of three multicentre RCTs (satavaptan *vs* placebo)	Cirrhotic patients with diuretic-responsive (*n* = 600) and refractory (*n* = 588) ascites (*n* = 559 NSBB users, *n* = 629 non-users)	Propranolol and carvedilol (doses not specified)	At 52-wk follow-up: In patients with refractory ascites, the cumulative mortality in NSBB users was 30.5% compared to 30.9% in non-users (HR 1.02, 95% CI 0.74-1.39). In patients with diuretic-responsive ascites, the cumulative mortality in NSBB users was 17.0% compared to 19.5% in non-users (HR 0.78, 95% CI 0.53-1.16)

Leithead *et al*[24]	Single-centre, retrospective, propensity-adjusted, observational study	Consecutive cirrhotic patients with ascites listed for liver transplantation (*n* = 105 NSBB users matched to *n* = 105 non-users) Median follow-up: 72 d (IQR 27-162 d)	Propranolol (median dose 80mg per day, range 10-240 mg); Carvedilol (median dose 6.25 mg per day, range 3.125-12.5)	In patients with diuretic-responsive ascites: NSBB users showed lower waitlist mortality compared to non-users (HR 0.55, 95% CI 0.32-0.95) In patients with refractory ascites: NSBB users showed lower waitlist mortality compared to non-users (HR 0.35, 95% CI 0.14-0.85)
Onali *et al*[25]	Single-centre, retrospective audit	Consecutive cirrhotic patients with ascites assessed for liver transplant suitability (*n* = 316, median MELD score 15, range 6-40) Median follow-up: 7 mo (± 12)	Propranolol (median dose 80 mg per day, IQR 40); Carvedilol (median dose 6.25 mg per day, IQR not specified)	In the whole population, NSBB use was associated with lower mortality (HR 0.55, 95% CI 0.33-0.94). In patients with refractory ascites, there was no difference in survival in NSBB users compared to non-users (HR 0.43, 95% CI 0.20-1.11)

AKI: Acute kidney injury; CI: Confidence interval; HR: Hazard ratio; HRS: Hepatorenal syndrome; IQR: Interquartile range; IV: Intravenous; MELD: Model for end stage liver disease; NSBB: Non-selective beta-blocker; RCT: Randomised controlled trial; SBP: Spontaneous bacterial peritonitis.

long-term, low-dose (12.5 mg) carvedilol treatment in a retrospective, propensity score matched cohort of 264 patients with ascites. After a median follow-up of 2.3 years, carvedilol therapy was associated with a hazard ratio of 0.47 (95% CI 0.29-0.77) in patients with mild ascites and was not associated with increased mortality in patients with moderate to severe ascites[30]. Zacharias *et al*[34] recently conducted a Cochrane systematic review of 10 RCTs and 810 patients comparing the safety and efficacy of carvedilol versus traditional NSBBs in the primary and secondary prevention of variceal haemorrhage; they identified no differences in the incidence of mortality, variceal haemorrhage and serious adverse events between both groups despite greater reductions in HVPG for the carvedilol group. Due to the low quality of assessed evidence, these findings were associated with substantial uncertainty.

Haemodynamically-independent potential of beta-blockers: Additional observational studies have proposed the existence of haemodynamically independent effects of beta-blockers. Senzolo *et al*[35] and Bang *et al*[15] observed protective effects of NSBB administration on SBP occurrence in patients with cirrhotic ascites. Similarly, Merli *et al*[36] prospectively followed 400 cirrhotic inpatients, finding that beta-blockade generally protected against infection and that infected patients showed lower morbidity and mortality when receiving beta-blocker therapy. Mookerjee *et al*[37] investigated the immunomodulatory effect of beta-blockers in 349 patients with acute-on-chronic liver failure (AoCLF) and observed that beta-blockade favourably affected 28-d mortality (24% *vs* 34%, *P* = 0.048). Patients on beta-blockers demonstrated reduced severity of AoCLF, slower progression of AoCLF and lower white blood cell counts on admission[37]. Facciorusso *et al*[38] analysed 107 cirrhotics admitted to a single centre with sepsis, noting that NSBB users had significantly reduced in-hospital mortality (18.7% *vs* 42.3%, *P* = 0.01), hospital stay duration (15.0 *vs* 18.5 d, *P* = 0.03), and white blood cell counts (9.2 *vs* 12.1, *P* = 0.004) compared to non-users.

A potential pathophysiological mechanism underlying these observations was provided by Reiberger *et al*[39] who measured reduced serum levels of interleukin 6 and lipopolysaccharide binding protein in cirrhotic patients receiving beta-blockers, indicative of decreased bacterial translocation. Bacterial translocation secondary to intestinal permeability and hypomotility is augmented in liver cirrhosis and appears to play a pivotal role in causing systemic immune dysfunction in these patients[21]. In animal models with ascites, cirrhotic rats were observed to have significantly reduced intestinal permeability as well as faster intestinal transit following propranolol administration[40]. Recently, Gimenez *et al*[41] observed that monocytes and granulocytes of cirrhotic patients on long-term NSBB therapy displayed significantly raised phagocytic capacity in the presence of bacterial DNA compared to NSBB-naïve patients.

Other potential effects of beta-blockers upon cirrhotic patients: To further investigate the haemodynamically independent effects of beta-blockade in patients with cirrhosis, Thiele *et al*[42] performed a meta-analysis of 23 RCTs on 2618 patients finding a reduced incidence of hepatocellular carcinoma (HCC) in patients receiving NSBBs [risk difference 0.026, 95% CI 0.052-0.001, number needed to treat (NNT) 38

patients]. Recently, Herrera *et al*[43] prospectively evaluated 173 patients included in the early HCC detection program for a median follow-up time of 11 years. The cumulative incidence of HCC was significantly lower in NSBB users compared to non-users (6% *vs* 19% at 10 years, 16% *vs* 24% at 15 years), with beta-blockade being the only parameter inversely correlated with HCC development on multivariate analysis. Regarding a pathophysiological mechanism underlying this finding, the authors propose that the reduction in bacterial translocation effected by beta-adrenergic blockade may diminish the portal load of pathogen-associated molecular patterns and thus hepatic inflammation[44]. Secondly, beta-adrenergic blockade may impede angiogenesis through inhibition of vascular endothelial growth factor production. Both hepatic inflammation and neo-angiogenesis are critical drivers in the pathogenesis of HCC[44].

Clinical guidelines and future directions: Presently, the only effective surrogate marker for assessing response to beta-blocker administration is HVPG. HVPG reduction by 20% or to < 12 mmHg was demonstrated to significantly decrease the risk of variceal bleeding and improve survival[9,45], yet the clinical utility of HVPG testing is limited by its invasiveness and availability[32]. Clinical practitioners predominantly titrate beta-blockers to reach a specific target heart rate (50-55 beats per minute) despite insufficient evidence that reductions in heart rate correlate with reductions in HVPG[32]. It is imperative that future research focuses on the identification of novel, non-invasive biomarkers for the assessment of beta-blocker response, since it is critical to identify the change from benefit to detriment of beta-blocker treatment during the natural progression of cirrhosis, so that beta-blocker treatment can be individualised and patient benefit maximised.

While beta-blockers continue to occupy a pivotal role in the treatment of portal hypertension, recent evidence has not only outlined additional, haemodynamically-independent beneficial effects of beta-blockers in cirrhosis, but also described potentially debilitating effects in advanced cirrhotics. Concerning clinical practice, D'Amico *et al*[46] recommend that beta-blocker therapy should: (1) Not be used in compensated cirrhotics with no evidence of varices; (2) be used in cirrhotic patients with varices at risk of bleeding or re-bleeding independent of the absence/presence of ascites; and (3) be used with caution in cirrhotic patients with refractory ascites and discontinued if haemodynamic or renal compromise arises. Currently, the Braveno VI consensus and 2017 AASLD guidelines recommend temporarily reducing or withholding beta-blockers in patients with refractory ascites and circulatory dysfunction (serum sodium < 130 mEq/L, systolic BP < 90 mmHg)[5,47,48]. Similarly, the 2018 EASL guidelines recommend discontinuation of beta-blockers in patients who develop hypotension (systolic BP < 90 mmHg), sepsis, bleeding, AKI or SBP, followed by an attempt at re-introduction of beta-blocker therapy after recovery. Presently, EASL does not recommend the use of carvedilol[49].

Overall, the evidence-base for the use of NSBBs in cirrhotic patients remains disputed with the requirement for further assessment of safety and efficacy. A large body of evidence regarding the use of beta-blockers in advanced cirrhosis comes from observational studies which are at risk of indication bias as patients receiving beta-blockers are likely to have relatively severe liver disease with clinically significant portal hypertension and large varices[4]. This disparity in liver disease severity between patient cohorts is difficult to account for without randomisation[4].

Lactulose and rifaximin

Lactulose: From the 1980s onwards, non-absorbable disaccharides (lactulose and lactitol) have been the mainstay of treatment for HE and have been recommended as first line therapy ever since lactulose was shown to be equally as effective but safer than neomycin[50-52]. The adverse effect profile of non-absorbable disaccharides is well-characterised, and is predominantly characterised by non-serious gastrointestinal side effects such as bloating, flatulence and diarrhoea[52]. Lactulose exerts its main effect through reducing production of ammonia. Ammonia has been implicated in the pathogenesis of HE by causing direct neurotoxicity and astrocytic swelling. Lactulose is metabolised to lactic acid by colonic bacteria, resulting in intestinal acidification. The acidic environment both promotes the transfer of ammonia from tissues into the intestinal lumen and impedes the growth of ammoniagenic coliforms. Additionally, the cathartic effects of lactulose aid in decreasing intestinal bacterial load[53].

In 2004 a systematic review of 22 RCTs demonstrated that non-absorbable disaccharides had no significant effect on mortality or HE incidence after exclusion of trials with high risk of bias, and concluded that evidence to support the use of non-absorbable disaccharides in the treatment of HE was insufficient[51]. As a consequence, further trials assessing the efficacy of lactulose in the management of HE were performed. A RCT of 140 patients by Sharma *et al*[54] investigating the efficacy of

lactulose for secondary prophylaxis of HE found that, after 14-mo follow-up, a significantly lower proportion of patients receiving lactulose (19.6%) redeveloped HE compared to patients receiving placebo (46.8%, *P* = 0.001). Similarly, Agrawal *et al*[55] also investigated the efficacy of lactulose for secondary prevention of HE in a RCT (*n* = 235), finding that lactulose therapy was associated with significantly reduced HE recurrence compared to no intervention (26.2% *vs* 56.9%, *P* = 0.001). Another RCT by Sharma *et al*[56], this time investigating the efficacy of lactulose for primary prophylaxis of HE, found that, after 12-mo follow-up, a significantly lower proportion of patients receiving lactulose (11%) developed overt HE compared to patients receiving placebo (28%, *P* = 0.02). Furthermore, lactulose therapy was associated with improvement in both cognitive function and health-related quality of life in patients with minimal HE[57]. Mittal *et al*[58] then assessed the effectiveness of lactulose in the treatment of minimal HE in the RCT setting (*n* = 322), finding significant improvements in minimal HE in the lactulose group (47.5%) compared to the no intervention group (10%, *P* = 0.006) as well as significant reductions in arterial ammonia levels (- 8.57 *vs* - 0.52, *P* = 0.0001). Incorporating the new evidence generated after 2004, a recent Cochrane review including 38 RCTs and 1828 patients found moderate quality evidence for beneficial effects of non-absorbable disaccharides on mortality (RR 0.59, 95% CI 0.40-0.87), HE (RR 0.58, 95% CI 0.50-0.69), and other complications of cirrhosis such as liver failure, variceal haemorrhage and HRS (RR 0.47,95% CI 0.36-0.60), when compared to placebo or no intervention[52]. In view of the current evidence-base for non-absorbable disaccharides, EASL and AASLD recommend lactulose as the first-choice agent for the treatment of episodic overt HE and prevention of recurrent HE[59].

Rifaximin: As described above, the cathartic and ammonia-lowering effects of the non-absorbable disaccharide lactulose have been the cornerstone for treating acute, overt HE and maintaining remission in recurrent HE for decades[60,61]. However, its long-term therapeutic value has been limited as a consequence of treatment non-adherence due to its numerous, unpleasant side effects[62]. Lately, the semi-synthetic, non-absorbable antibiotic rifaximin surged in popularity as an alternative or additive to lactulose based on its gut-selective antimicrobial activity favouring non-pathogenic species and its favourable safety and tolerability profile[61]. A randomized, double-blinded trial in 103 patients found the impact of rifaximin on the portosystemic encephalopathy index to be superior to the effect of lactitol[60,63]. A further placebo-controlled RCT by Bass *et al*[64] established the safety and efficacy of rifaximin therapy in the maintenance of remission in 299 patients with recurrent HE over a follow-up period of 6 mo, finding that a lower proportion of patients receiving rifaximin experienced an overt episode of HE (22.1%) and were hospitalised with HE (13.6%) compared to patients receiving placebo (45.9% and 22.6%, respectively)[60,64]. As a sequel to this trial, Mullen *et al*[62] performed an open-label, 24-mo maintenance study in 326 patients to investigate the long-term safety profile and treatment effect durability of rifaximin, finding that long-term rifaximin administration achieves a persistent reduction in HE-related and all-cause hospitalisation without a corresponding increase in infection or antibiotic resistance rates. A meta-analysis of 21 RCTs and 2258 patients demonstrated that rifaximin reduced mortality in overt HE, but not minimal HE, when compared to placebo, and had no effect on mortality when compared to non-absorbable disaccharides. In this study, rifaximin also decreased the risk of serious adverse events and had a potential beneficial effect on quality of life[65]. However, the evidence analysed in this meta-analysis was of low-to-moderate quality[65]. Another meta-analysis by Wu *et al*[66] incorporating 8 RCTs outlined that there was no significant difference in the efficacy of preventing and treating HE between rifaximin and lactulose, but that rifaximin therapy was correlated with less adverse events. A recent retrospective analysis of 1042 patients with HE by Kang *et al*[67] correlated rifaximin-lactulose combination therapy with significantly improved mortality (HR 0.67, *P* = 0.024) and reduced risk of recurrent HE (HR 0.45, *P* < 0.001), variceal haemorrhage (HR 0.43, *P* = 0.011), and SBP (HR 0.21, *P* < 0.001), compared to lactulose alone. This was only evident in the non-HCC cohort (*n* = 421), however. Kabeshova *et al*[68] evaluated the cost-effectiveness of long-term rifaximin-lactulose combination therapy compared to lactulose monotherapy for recurrent HE in the French healthcare system. This study found that rifaximin-lactulose combination therapy incurs a cost of 18517 EUR (approximately 16000 GBP or 21000 USD) to gain one additional QALY compared to lactulose monotherapy, concluding that rifaximin is a cost-effective treatment in the context of the cost-effectiveness threshold range of 23000-34000 EUR (20000-30000 GBP) adopted by NICE. In view of the current evidence, EASL and AASLD state that rifaximin is effective as an add-on therapy to lactulose for secondary prevention of HE recurrence[59].

The potential uses of rifaximin beyond hepatic encephalopathy: Additionally,

recent evidence has indicated that the therapeutic effects of rifaximin may extend beyond its original indication of treating HE. An initial, prospective, observational study conducted by Vlachogiannakos *et al*[69] observed reduced incidence of variceal haemorrhage, HE, SBP and HRS in 23 patients receiving rifaximin compared to controls. Similarly, Hanouneh *et al*[70] investigated 404 patients with cirrhotic ascites and demonstrated a 72% reduction in SBP incidence with rifaximin therapy compared to placebo. Norfloxacin is a systemic antibiotic, currently recommended as the first line agent in the prevention of SBP by both AASLD and EASL[71,72]. Recently, Praharaj *et al*[73] conducted a RCT (*n* = 117) comparing rifaximin and norfloxacin for both primary and secondary prevention of SBP. Praharaj *et al*[73] established that patients on norfloxacin had a significantly higher rate of SBP development compared to patients on rifaximin in secondary prophylaxis (39% *vs* 7%, *P* = 0.007), while no significant difference (20% *vs* 14%, *P* = 0.73) was manifested in primary prophylaxis. A meta-analysis performed by Sidhu *et al*[74]—incorporating three RCTs and one prospective observational study - compared norfloxacin and rifaximin with the primary outcome of SBP occurrence and secondary outcomes of mortality and adverse events. The included studies, of moderate quality evidence, either demonstrated superior efficacy of rifaximin or no significant difference between rifaximin and norfloxacin in SBP prevention, leading the authors to conclude that rifaximin could be a safe and efficacious alternative to norfloxacin in SBP prophylaxis for patients with hepatitis C virus (HCV) cirrhosis. Furthermore, a small prospective study in 13 patients with advanced cirrhosis suggests improvements in renal function after intestinal decontamination by rifaximin[75]. Baik *et al*[76] compared propranolol monotherapy to propranolol-rifaximin combination therapy in 65 patients with advanced cirrhosis, finding amplification of mean HVPG reduction with combination therapy. However, a recent RCT investigating the haemodynamic effect of rifaximin in 54 patients with cirrhotic ascites observed no difference in HVPG, cardiac output or glomerular filtration rate compared to placebo[77]. Due to its relative novelty, the evidence-base for the impact of rifaximin on outcomes in cirrhotic patients, particularly regarding its effects outside HE treatment, is lacking in robustness, with the majority of conducted studies featuring very limited sample sizes. A United Kingdom based, multicentre RCT evaluating the role of rifaximin in preventing secondary infections in cirrhotic patients, including both community and hospital acquired infections, is currently being undertaken and its results are awaited[78].

L-ornithine L-aspartate and acarbose: As outlined, ammonia has been identified as the pivotal neurotoxin implicated in the pathogenesis of HE and its reduction is a central objective in the therapeutic approach to HE management. LOLA has demonstrated ammonia-lowering properties by enhancing residual hepatic urea cycle activity and skeletal muscle glutamine synthesis[79,80]. Goh *et al*[79] performed a recent Cochrane systematic review of 36 RCTs and 2377 patients summarising the evidence of LOLA in the prevention and treatment of HE. The authors found very low quality evidence that LOLA had beneficial effects on mortality, HE and serious adverse events compared to placebo. However, these findings were not upheld when only trials with low risk of bias were considered. On subgroup analysis, there was no difference between intravenous and oral LOLA administration or between minimal and overt HE. In comparison to lactulose and rifaximin, LOLA demonstrated no effect on mortality, HE and serious adverse events. The uncertainty stemming from data quality concerns led the authors to conclude that new, high-quality RCTs are required for the definitive evaluation of evidence[79]. A randomised, placebo-controlled, quadruple blinded, phase IV trial investigating the efficacy of LOLA in treating overt HE is currently in progress and its results are awaited[81].

One randomised, double-blinded, placebo-controlled trial in 107 cirrhotic patients with HE and type 2 diabetes mellitus provided encouraging data for the safety and efficacy of acarbose in treating HE with the intervention group demonstrating lower blood ammonia levels, improved encephalopathy global score and reduced Child-Pugh score[82]. However, the generalisability of these findings is diminished by the highly selective study population of compensated Child-Pugh A cirrhotics with predominantly Grade 2 encephalopathy, as well as the scarcity of further studies investigating the efficacy of acarbose in treating HE[83]. Acarbose is not mentioned in current EASL and AASLD guidelines for HE management.

Diuretics

The most common complication of cirrhosis is ascites[84]. Diuretics are the mainstay of treatment for moderate ascites (Grade 2)[72,85]. Excessive aldosterone generation secondary to splanchnic vasodilatation and systemic hypotension is considered the primary causative factor for increased sodium reabsorption in the kidneys of patients with cirrhotic ascites[86]. Consequently, spironolactone monotherapy was shown

superior to loop diuretics for initial ascites management, with furosemide frequently used as an addition to potentiate diuresis in recurrent ascites[72]. Side-effects may result from excessive diuresis causing hypovolaemia and renal dysfunction along with electrolyte disturbances such as hyponatraemia, hypokalaemia and hyperkalaemia[72,87,88]. Furthermore, diuretic therapy may precipitate HE; although the underlying mechanism remains unknown[72,89,90], this is likely related to a combination of electrolyte disturbance and hypovolaemia. To forestall these adverse effects, it is recommended to adjust diuretic dosage so that daily weight loss does not exceed 800 g[91]. In cirrhotic patients who develop ascites, one-year survival ranges from 60 to 85%[92]. Sodium restriction and diuretic therapy enable mobilisation of ascites in approximately 90% of those patients.

α-1 and α-2 adrenergic agonists: One-year survival substantially declines to 25% with the development of refractory ascites (diuretic-resistant or diuretic-intractable), when ascites mobilisation fails due to a lack of effect or advent of complications such as SBP, hyponatraemia and renal impairment[84,93]. First-line management for refractory ascites is serial large-volume paracentesis[49]. However, recent evidence has proposed a role for α1 and α2-adrenergic agonists in managing refractory ascites. A group of heterogenous studies with differing dosing and follow-up regimes demonstrated that midodrine, a peripheral α1-adrenergic agonist acting as a splanchnic vasoconstrictor, increases mean arterial pressure, urine output as well as urine sodium output, and decreases plasma renin and aldosterone activity in patients with refractory ascites[84]. Two small randomised pilot studies found that midodrine plus standard medical therapy transiently (3 mo) improves the mobilisation of refractory ascites compared to diuretic therapy alone[94,95]. Guo *et al*[96] performed a meta-analysis of 10 RCTs (*n* = 462) finding that midodrine enhances response rates to diuretics, but does not increase survival in patients with refractory ascites. A recent small-scale prospective observational study also suggests that oral midodrine and subcutaneous octreotide combination therapy could ameliorate cirrhosis-induced hyponatraemia (pre-treatment serum Na: 124 mmol/L *vs* post-treatment serum Na: 130 mmol/L, *P* = 0.00001)[97]. AASLD advise to consider the administration of midodrine in patients with refractory ascites, while EASL have not recommended its use in view of limited sample size of existing trials[49,71]. Clonidine, a central α2-adrenergic agonist with sympatholytic activity, is thought to ameliorate sympathetic nervous system overstimulation which leads to renal hypoperfusion and excessive renin-angiotensin system activation in refractory ascites[84,98]. Similar to midodrine, clonidine has been shown to improve urine output and urine sodium output, while lowering plasma aldosterone and renin activity in patients with refractory ascites. Multiple small-scale RCTs with limited follow-up investigated the clinical efficacy of clonidine in such patients, demonstrating that clonidine reduced diuretic requirements and induced earlier diuretic response[94,98]. In animal models of cirrhosis, clonidine improved renal function at low doses, but negatively influenced mean arterial pressure and urine sodium output at high doses[99]. Clonidine is currently not recommended by AASLD or EASL as an adjunct to diuretics in refractory ascites management due to the non-existence of sufficiently powered, long-term studies[49,71].

Vaptans: To avoid the increased incidence of adverse effects with high-dose conventional diuretic therapy, the so-called vaptans, selective antagonists to the vasopressin V2 receptors in principal cells of collecting ducts, have gained interest in recent decades as they are able to achieve a highly hypotonic diuresis without impacting on the electrolyte balance[100]. Thus vaptans might be valuable in the correction of hyponatraemia, an important predictor of mortality in cirrhotic patients with ascites. However, controversies exist with regards to their expense and their effect on clinically relevant outcomes and prognosis in patients with cirrhosis[100]. In their meta-analysis (16 RCTs, *n* = 2620) Yan *et al*[100] found that vaptans showed a significant aquaretic effect and increased serum sodium significantly both when used alone and when used in combination with traditional diuretics, and concluded that vaptans could play a major role in the pharmacological treatment of refractory ascites with insufficient response to traditional diuretics in order to alleviate ascites volume and the need for paracentesis without incurring a higher rate of adverse events. Yet, despite the substantial beneficial impact of vaptans on diuresis and hyponatraemia, vaptan therapy made no significant difference to short-term or long-term survival in these cirrhotic patients. An adverse effect of vaptan treatment observed in the included trials was excessive correction of serum sodium, which may lead to osmotic demyelination, thus necessitating monitoring when administering vaptans[100]. Furthermore, the Food and Drug Administration has recommended to avoid the use of vaptans in patients with chronic liver disease, as tolvaptan has the potential to induce serious liver injury[101]. In a phase 3, placebo-controlled, randomized trial (*n* =

1200), satavaptan therapy showed no difference to placebo regarding ascites control, but was superior to placebo in improving serum sodium of hyponatraemic patients. However, in one of the trial groups, satavaptan therapy was associated with increased mortality (HR 1.47; 95% CI 1.01-2.15). No specific causes for this mortality increase were identified, but the majority of deaths were attributed to known complications of cirrhosis. The authors concluded that vaptan therapy is ineffective in controlling ascites, but may have a role in managing hypervolaemic hyponatraemia in cirrhotic patients[102]. Current EASL and AASLD guidelines do not endorse the use of vaptans in cirrhotic patients in light of their costs, risks and lacking efficacy in clinical settings[49,71].

Statins

Although statins are the mainstay for preventing atherosclerosis and have well-defined beneficial effects on cardiovascular health, they are under-prescribed in cirrhotic patients due to concerns over hepatotoxicity[103]. However, recent evidence points towards an unexpected, potentially beneficial impact of statins on cirrhosis resulting from their pleiotropic properties which comprise antioxidant, anti-fibrotic, anti-infective and anti-inflammatory effects[103,104]. Endothelial dysfunction and diminished nitric oxide generation play essential roles in the establishment of the pro-inflammatory and pro-fibrotic microenvironment of cirrhosis[104]. Numerous pre-clinical studies *in vitro* and *in vivo* have demonstrated that statins upregulate Kruppel-like factor 2 and nitric oxide (NO) availability as well as down-regulate hepatic stellate cell activation, thus exerting favourable effects on fibrogenesis, endothelial function and portal hypertension[103]. In addition to findings from pre-clinical studies, simvastatin enhanced hepatosplanchnic NO output and reduced intrahepatic vascular resistance in a study involving 30 cirrhotic patients[105]. A multicentre RCT conducted by Abraldes *et al*[106] in 59 cirrhotic patients with portal hypertension described that simvastatin significantly decreased HVPG by 8.3% and improved hepatocyte perfusion without discernible harmful effects on the systemic circulation[103,104,106].

In a retrospective study of patients with predominantly Child-Pugh A cirrhosis (81 statin users and 162 controls), statin use was associated with lower mortality (HR 0.56) and decompensation (HR 0.55) risk on multivariate analysis after 36-mo follow-up[107]. Similarly, another recent retrospective analysis of patients with compensated HCV cirrhosis (685 statin users and 2062 controls) also described statin use to be associated with lower mortality (HR 0.56) and decompensation rates (HR 0.55) at 10-year follow-up after adjusting for age, serum albumin, model for end stage liver disease (MELD) and Child-Pugh scores[108]. Using the Taiwanese National Health Insurance database, Chang *et al*[109] conducted a nested case-control study with 13-year follow-up, matching 675 statin users with 675 statin non-users. Following a dose-response relationship, statin use correlated with a significantly decreased risk of decompensation (HR 0.39), HCC development (HR 0.52), and mortality (HR 0.46). The most robust evidence regarding the therapeutic benefit of statins in cirrhosis comes from a multicentre RCT of 158 cirrhotic patients who received either standard therapy (beta-blocker and band ligation) after variceal haemorrhage, or standard therapy plus simvastatin. After 24-mo follow-up, addition of simvastatin had no effect on the rate of re-bleeding but significantly reduced all-cause mortality compared to the control group (9% *vs* 22%, *P* = 0.03). However, this survival benefit was only observed in the Child-Pugh A/B cohort, not in Child-Pugh C patients, and no difference was found in the rate of cirrhosis-related complications between the intervention and control groups. Notably, two patients with decompensated cirrhosis developed rhabdomyolysis, thus again raising questions about the safety of statin use in advanced cirrhosis[103,104,110].

Recently, the evidence regarding the impact of statin use on cirrhosis and its complications was summarized by three meta-analyses. Kamal *et al*[111] performed one meta-analysis of 9 studies (2 RCTs, 7 observational studies) and 166000 patients (40950 statin users) finding moderate-quality evidence that statin use is associated with reduced fibrosis progression (HR 0.55, 95% CI 0.49-0.61) and rate of decompensation (HR 0.54, 95% CI 0.46-0.65) as well as low-quality evidence that statin use is associated with reduced mortality (0.62, 95% CI 0.43-0.91). The second meta-analysis by Kim *et al*[112] included 121000 patients (46% statin users) and found moderate-quality evidence of an association between statin use and decreased risk of decompensation (RR 0.54, 95% CI 0.47-0.61), mortality (RR 0.54, 95% CI 0.47-0.61) and variceal bleeding (RR 0.73, 95% CI 0.59-0.91). Thirdly, Singh *et al*[113] performed a large meta-analysis including 1.4 million patients with 4298 cases of HCC, associating statin use with a 37% risk reduction [odds ratio (OR) 0.63, 95% CI 0.52-0.76] of HCC development. This risk reduction was significantly more pronounced in the Asian population, in which chronic hepatitis B virus infection is the predominant risk factor for HCC development. In this population, the NNT to prevent one case of HCC per year was

estimated at 5209. As a consequence of this high NNT in combination with the significant heterogeneity between included studies, the authors conclude that they cannot recommend statins for chemoprevention of HCC[103,113]. In the context of increasingly stretched healthcare budgets, the inexpensiveness of statins and the encouraging evidence presented above calls for the implementation of a phase 3 RCT in patients with Child-Pugh A/B cirrhosis with the primary endpoint of decompensation or death[114].

While the use of statins in cirrhotic patients remains disputed due to safety concerns, multiple studies have demonstrated that statins are safe to use in patients with compensated non-alcoholic fatty liver disease (NAFLD) irrespective of liver enzyme elevations[115,116]. Due to the strong association between NAFLD and cardiovascular morbidity and mortality, both EASL and AASLD guidelines recommend the initiation of cholesterol-lowering therapy with statins in patients with compensated NAFLD[117,118]. However, current AASLD guidelines advise against the use of statins in patients with decompensated cirrhosis in view of the potentially deleterious complications in this population of patients[117]. Since the majority of evidence regarding the pleiotropic effects of statins in chronic liver disease is derived from observational studies and small-scale trials, adequately powered RCTs are required to assess whether the pleiotropic properties of statins can modulate clinical endpoints in cirrhotic patients[119].

Proton pump inhibitors

Despite suboptimal evidence for their efficacy in cirrhosis and growing concerns over their promotion of bacterial overgrowth and translocation, proton pump inhibitors (PPI) are frequently prescribed to patients with cirrhosis receiving multidrug treatment for variceal haemorrhage or portal hypertensive gastropathy in order to forestall peptic complications[120]. In fact, 46%-78% of cirrhotic patients are reported to use PPIs[121]. Initially, concerns about the safety of PPI use in cirrhosis were raised by a variety of low-powered observational studies. A prospective study of 272 patients with overall survival as the primary endpoint demonstrated that PPI treatment was associated with higher MELD scores, ascites and mortality[122]. In addition, a meta-analysis of four studies and 772 patients identified a significant correlation between PPI use and SBP development (OR 2.77)[123]. A prospective cohort study of 400 patients hospitalized with cirrhosis established that PPI use was an independent risk factor for the development of infection (OR 2.0), while beta-blocker use was a protective factor (OR 0.46)[36].

Recently, two large, retrospective analyses fuelled the established fear over the impact of PPI use on SBP and HE development. Dam *et al*[121] retrospectively analysed data from three large, multicentre RCTs (*n* = 865) for the use of satavaptan in cirrhotic ascites, describing that current PPI use was associated with an increased risk of HE (HR 1.36, 95% CI 1.01-1.84) and SBP development (HR 1.72, 95% CI 1.10-2.69) compared to non-users. Additionally, the authors described that only 56% of PPI users had a valid indication for PPI prescription[121,124]. Using data from the Taiwan National Health Insurance database, Tsai *et al*[125] conducted a nested case-control study of 1166 cirrhotic patients with HE matched to 1166 cirrhotic patients without HE, observing a dose-response relationship between PPI use and risk of HE development. The pathophysiological mechanism hypothesized to underlie this observed association between PPI use and SBP/HE development is based on PPIs weakening the stomach acid barrier by increasing gastric pH, thus promoting bacterial dysbiosis, translocation and small intestinal bacterial overgrowth (SIBO)[121,124]. Since the majority of hepatic blood supply is derived from the gut, there is an intimate relationship between the liver and the intestinal microbiome. Gut dysbiosis with consequent translocation of pathogenic bacterial taxa and endotoxins into the portal and systemic circulation promotes immune system dysfunction as well as hepatic inflammation and injury[126]. In line with this hypothesis, Bajaj *et al*[127] recently conducted a cohort study, observing the readmission rates in 343 cirrhotic inpatients and conducting stool microbiota analyses in 137 cirrhotic outpatients. In the inpatient cohort, PPI use was independently associated with higher readmission rates, while in the outpatient cohort, the microbiota composition of PPI users showed increased oral-origin taxa and decreased beneficial autochthonous taxa. In contrast, a large (*n* = 1191), retrospective analysis by Ratuapli *et al*[128] described no significant difference in the frequency of SIBO diagnosis, as established by glucose hydrogen breath testing, between PPI users and non-users. Alternatively, Assaraf *et al*[129] hypothesise that PPI use and HE development may be linked to a cerebral build-up of toxic metabolites as PPIs act as agonists to ATP-binding cassette efflux transporters found on the blood-brain barrier.

However, this negative trend for PPI use in cirrhosis has recently come under question. Khan and colleagues published two articles highlighting the limitations of

the retrospective analyses by Dam *et al*[121] and Tsai *et al*[125]. In both studies, the ORs achieved were below 3, an indication that the observed associations may be due to residual confounding rather than causality[124,130]. In the Tsai study, patients receiving PPIs had higher rates of comorbidities, thus representing a potential source of channelling bias[130]. The Dam study analysed data not collected specifically for the purpose of assessing the impact of PPIs on SBP and HE incidence and could thus not account for all potential confounders[121]. Therefore, Khan *et al*[124,130] concluded that the current evidence-base against PPIs is not of sufficient quality to withhold PPIs from cirrhotic patients with a reasonable indication for PPI treatment. A large (*n* = 519), prospective, multicentre study by Terg *et al*[131] observed no significant difference in PPI use between infected and non-infected patients (44.3% *vs* 42.8%) or between patients with SBP and patients with ascites and no SBP (46% *vs* 42%). Furthermore, no difference was observed in the causative species or infection origin between PPI users and non-users who developed SBP[131]. The largest meta-analysis (16 studies, 10 case-control/6 cohort, *n* = 8145) on this issue by Yu *et al*[132] found that the harmful association between PPI use and SBP incidence was present in case-control studies, but not cohort studies, and that there was no association between PPI use and mortality during hospitalisation in both case-control and cohort studies. The authors therefore concluded that causality between PPI administration and increased SBP incidence and mortality could not be established[132]. In view of the conflicting evidence and substantial limitations of the conducted studies outlined above, there is the need for a randomized, placebo-controlled, adequately powered trial investigating the effects of long-term PPI administration in cirrhotic patients with the primary outcomes of HE and SBP occurrence.

Anticoagulation

Historically, cirrhotic patients were viewed as 'auto-anticoagulated', since cirrhosis is associated with a global impairment in clotting factor synthesis excluding factor VII and von Willebrand factor. More recently, it has been recognized that the relationship between cirrhosis and the coagulation cascade is more complex and that routine haemostatic tests, such as the international normalized ratio or activated partial thromboplastin time, lack accuracy in predicting coagulation status in these patients. In light of a coexisting reduction in anticoagulant factors (namely antithrombin III, protein S and C), recent studies have highlighted the importance of procoagulant complications in cirrhotic patients, with higher rates of venous thromboembolism (VTE) and splanchnic vein thrombosis being observed. These findings have suggested the potential role for anticoagulation in cirrhosis. Furthermore, there is emerging evidence that coagulation proteins may activate hepatic myofibroblasts to accelerate fibrogenesis. As such, there has been the suggestion that anticoagulant medication may thus have a further role in impeding fibrosis progression[133-135].

Retrospective studies investigating the role of prophylactic anticoagulation in cirrhotic patients at risk of VTE have provided heterogenous results with some studies failing to demonstrate a reduction in VTE incidence with anticoagulative treatment[133,136]. In a single-centre, non-blinded RCT of 70 cirrhotic outpatients, Villa *et al*[137] investigated the safety and efficacy of low-molecular-weight heparin in preventing portal vein thrombosis (PVT). The major findings were that subcutaneous enoxaparin not only reduced PVT incidence (0% *vs* 27.7%, *P* = 0.001) but also protected against hepatic decompensation (11.7% *vs* 59.4%, *P* < 0.001) with no apparent increase in haemorrhagic complications. The authors attribute the observed reduction in decompensating events to improvements in hepatic microcirculation with consequent diminution of bacterial translocation and immune dysfunction[134,137]. Further prospective and randomised studies are required to delineate the role of prophylactic anticoagulation in cirrhosis.

Regarding patients with established non-malignant PVT, numerous studies have demonstrated a favourable effect of low-molecular weight heparin therapy on portal vein recanalization rates, achieving re-permeation in 40%-90% of treated patients compared to 0% of untreated controls. Early initiation of anticoagulative therapy was the most important factor associated with successful re-permeation. Furthermore, these studies indicate that anticoagulation therapy has an acceptable safety profile in cirrhotic patients with no significant increase in bleeding rates provided that oesophageal varices are adequately screened for and managed[134,138-141]. At present, however, there is a need for detailed guidelines regarding the use of anticoagulation therapy in patients with cirrhosis.

Further treatments

A number of different agents with potentially therapeutic effects upon patients with cirrhosis are currently at different stages of pre-clinical and clinical assessment. These are summarised in Table 2.

Table 2 Summary of further pharmacological agents with potentially therapeutic effects

Treatment	Potential mechanism of action	Indications and Evidence
Human Serum Albumin (HAS)	Oncotic properties: Acts as a plasma expander to counteract splanchnic arterial vasodilatation in cirrhosis; Non-oncotic properties: Modulation of the inflammatory response through binding of reactive oxygen species and NO. Also affects capillary integrity. Furthermore, cirrhosis affects the capacity of albumin to bind endogenous and exogenous substances, which may be compensated for by albumin infusion.	A recent meta-analysis found that HAS infusion in combination with antibiotics decreases the incidence of renal failure and mortality in patients with SBP; Several studies demonstrate that HAS infusion together with vasoconstrictors reduces mortality in patients with type 1 HRS; A meta-analysis by Bernardi *et al* showed that HAS infusion was effective in preventing paracentesis-induced circulatory dysfunction; A multicentre RCT by Caraceni *et al*[148] (*n* = 440) in cirrhotic patients with uncomplicated ascites showed that long-term HAS plus diuretics prolonged survival compared to diuretics alone[142-148].
Faecal microbiota transplant (FMT)	As described previously, cirrhosis and its progression have been closely linked to gut dysbiosis with a predominance of pathogenic bacterial taxa and SIBO. Amelioration or even reversion of dysbiosis may be achieved through direct manipulation of the intestinal microbiome using FMT.	Although FMT has been extremely successful in repopulating the healthy intestinal microbiome of patients with *C. difficile* diarrhoea and has even been endorsed in guidelines for the treatment of recurrent *C. difficile* diarrhoea, studies of FMT in patients with liver disease are smaller and more limited. A pilot study suggested a reduced burden of HE in patients given a single FMT enema compared to standard-of-care therapy. Currently, Woodhouse *et al*[126] are in the process of conducting a randomised, single-blinded, placebo-controlled trial in 32 cirrhotic patients in order to assess the safety and tolerability of FMT delivered by upper GI endoscopy[126,149-151].
Caffeine/ Coffee	Caffeine antagonizes the A2a adenosine receptor on hepatic stellate cells, the effector cells of fibrogenesis. Activation of the A2a adenosine receptor has been directly associated with matrix production in rodent models. However, decaffeinated coffee has also been shown to lower transaminases. Hence there may be additional anti-fibrotic constituents of coffee such as polyphenols which are potent anti-inflammatories and anti-oxidants.	Both retrospective and prospective observational studies have indicated that an inverse dose-response relationship exists between coffee consumption and cirrhosis risk as well as cirrhosis-related complications. Corrao *et al*[155] retrospectively analysed 274 decompensated cirrhotic patients and 458 matched controls with chronic liver disease, finding that daily consumption of one cup of coffee conferred an odds ratio of 0.47, while daily consumption of four cups of coffee even conferred an odds ratio of 0.16 for cirrhosis risk. Similarly, a prospective cohort study of patients with advanced hepatitis C induced liver disease found that liver-related mortality and complication rates declined with increasing coffee consumption (12.1/100 person years for > 1 cup/d; 8.2/100 for 1-3 cups/d; 6.3/100 for > 3 cups/d; p-trend = 0.001). A recent prospective cohort study analysed non-invasive liver stiffness measurements in the general Dutch population (*n* = 2424), observing that coffee consumption was inversely correlated with liver stiffness. Similarly, recent data from a meta-analysis showed a favourable effect of coffee consumption on risk of HCC development (RR 0.55, 95% CI 0.44-0.67). At present, no guidelines exist regarding the prescription of coffee to patients with cirrhosis or chronic liver disease[152-158].

CI: Confidence interval; FMT: Faecal microbiota transplant; GI: Gastrointestinal; HAS: Human Serum Albumin; HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; HRS: Hepatorenal syndrome; NO: Nitric oxide; RCT: Randomised controlled trial; RR: Relative risk; SBP: Spontaneous bacterial peritonitis; SIBO: Small intestinal bacterial overgrowth.

CONCLUSION

The evidence presented above clearly demonstrates that, while pharmacotherapy plays an important role for the long-term supportive management of the cirrhotic outpatient, its application is also highly complex and controversial. Many questions regarding the effect of the individual agents in the different stages of cirrhosis remain unanswered and require further research, particularly in a randomized, controlled setting with well-defined cohorts of cirrhotic patients. In this article we have provided an update with regards to the latest studies and international consensus on specific treatments. To maximize benefits and minimize drawbacks of chronic

pharmacotherapy in cirrhosis, it is essential that these drugs are prescribed and administered only in close accordance with up-to-date guidelines and that patients are reviewed frequently, so that adverse effects can be recognized early. To heighten the effectiveness of pharmacotherapy in cirrhosis, drug administration needs to be individualized with respect to the following criteria: current complications of cirrhosis, stage of cirrhosis, type of drug used (especially for beta-blockers) and dose of drug used. Pharmacotherapy is only one part of the holistic management of the cirrhotic outpatient and will only achieve its full effectiveness if used in conjunction with treatment of the underlying aetiology of liver disease, nutritional management and patient education in a specialist clinic setting.

REFERENCES

1   **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

2   **Blachier M**, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: A review of available epidemiological data. *J Hepatol* 2013; **58**: 593-608 [PMID: 23419824 DOI: 10.1016/j.jhep.2012.12.005]

3   **Williams R**, Aspinall R, Bellis M, Camps-Walsh G, Cramp M, Dhawan A, Ferguson J, Forton D, Foster G, Gilmore I, Hickman M, Hudson M, Kelly D, Langford A, Lombard M, Longworth L, Martin N, Moriarty K, Newsome P, O'Grady J, Pryke R, Rutter H, Ryder S, Sheron N, Smith T. Addressing liver disease in the UK: A blueprint for attaining excellence in health care and reducing premature mortality from lifestyle issues of excess consumption of alcohol, obesity, and viral hepatitis. *Lancet* 2014; **384**: 1953-1997 [PMID: 25433429 DOI: 10.1016/S0140-6736(14)61838-9]

4   **Moctezuma-Velazquez C**, Kalainy S, Abraldes JG. Beta-blockers in patients with advanced liver disease: Has the dust settled? *Liver Transpl* 2017; **23**: 1058-1069 [PMID: 28590564 DOI: 10.1002/lt.24794]

5   **de Franchis R**; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]

6   **Ge PS**, Runyon BA. Treatment of Patients with Cirrhosis. *N Engl J Med* 2016; **375**: 767-777 [PMID: 27557303 DOI: 10.1056/NEJMra1504367]

7   **Reiberger T**, Mandorfer M. Beta adrenergic blockade and decompensated cirrhosis. *J Hepatol* 2017; **66**: 849-859 [PMID: 27864004 DOI: 10.1016/j.jhep.2016.11.001]

8   **Gluud LL**, Morgan MY. Endoscopic therapy and beta-blockers for secondary prevention in adults with cirrhosis and oesophageal varices Cochrane Database of systematic Reviews 2017; (6): Art. No.: CD012694. [DOI: 10.1002/14651858.CD012694]

9   **Groszmann RJ**, Bosch J, Grace ND, Conn HO, Garcia-Tsao G, Navasa M, Alberts J, Rodes J, Fischer R, Bermann M. Hemodynamic events in a prospective randomized trial of propranolol versus placebo in the prevention of a first variceal hemorrhage. *Gastroenterology* 1990; **99**: 1401-1407 [PMID: 2210246 DOI: 10.1016/0016-5085(90)91168-6]

10 **Poynard T**, Calès P, Pasta L, Ideo G, Pascal JP, Pagliaro L, Lebrec D. Beta-adrenergic-antagonist drugs in the prevention of gastrointestinal bleeding in patients with cirrhosis and esophageal varices. An analysis of data and prognostic factors in 589 patients from four randomized clinical trials. Franco-Italian Multicenter Study Group. *N Engl J Med* 1991; **324**: 1532-1538 [PMID: 1674104 DOI: 10.1056/NEJM199105303242202]

11 **Lebrec D**, Poynard T, Hillon P, Benhamou JP. Propranolol for prevention of recurrent gastrointestinal bleeding in patients with cirrhosis: A controlled study. *N Engl J Med* 1981; **305**: 1371-1374 [PMID: 7029276 DOI: 10.1056/NEJM198112033052302]

12 **Sersté T**, Melot C, Francoz C, Durand F, Rautou PE, Valla D, Moreau R, Lebrec D. Deleterious effects of beta-blockers on survival in patients with cirrhosis and refractory ascites. *Hepatology* 2010; **52**: 1017-1022 [PMID: 20583214 DOI: 10.1002/hep.23775]

13 **Sersté T**, Njimi H, Degré D, Deltenre P, Schreiber J, Lepida A, Trépo E, Gustot T, Moreno C. The use of beta-blockers is associated with the occurrence of acute kidney injury in severe alcoholic hepatitis. *Liver Int* 2015; **35**: 1974-1982 [PMID: 25611961 DOI: 10.1111/liv.12786]

14 **Mandorfer M**, Bota S, Schwabl P, Bucsics T, Pfisterer N, Kruzik M, Hagmann M, Blacky A, Ferlitsch A, Sieghart W, Trauner M, Peck-Radosavljevic M, Reiberger T. Nonselective β blockers increase risk for hepatorenal syndrome and death in patients with cirrhosis and spontaneous bacterial peritonitis. *Gastroenterology* 2014; **146**: 1680-90.e1 [PMID: 24631577 DOI: 10.1053/j.gastro.2014.03.005]

15 **Bang UC**, Benfield T, Hyldstrup L, Jensen JE, Bendtsen F. Effect of propranolol on survival in patients with decompensated cirrhosis: A nationwide study based Danish patient registers. *Liver Int* 2016; **36**: 1304-1312 [PMID: 26992041 DOI: 10.1111/liv.13119]

16 **Krag A**, Wiest R, Albillos A, Gluud LL. The window hypothesis: haemodynamic and non-haemodynamic effects of β-blockers improve survival of patients with cirrhosis during a window in the disease. *Gut* 2012; **61**: 967-969 [PMID: 22234982 DOI: 10.1136/gutjnl-2011-301348]

17 **Groszmann RJ**, Garcia-Tsao G, Bosch J, Grace ND, Burroughs AK, Planas R, Escorsell A, Garcia-Pagan JC, Patch D, Matloff DS, Gao H, Makuch R; Portal Hypertension Collaborative Group. Beta-blockers to prevent gastroesophageal varices in patients with cirrhosis. *N Engl J Med* 2005; **353**: 2254-2261 [PMID: 16306522 DOI: 10.1056/NEJMoa044456]

18 **Villanueva C**, Aracil C, Colomo A, Lopez-Balaguer JM, Piqueras M, Gonzalez B, Torras X, Guarner C, Balanzo J. Clinical trial: A randomized controlled study on prevention of variceal rebleeding comparing nadolol + ligation vs. hepatic venous pressure gradient-guided pharmacological therapy. *Aliment Pharmacol Ther* 2009; **29**: 397-408 [PMID: 19006538 DOI: 10.1111/j.1365-2036.2008.03880.x]

19 **Mandorfer M**, Reiberger T. Beta blockers and cirrhosis, 2016. *Dig Liver Dis* 2017; **49**: 3-10 [PMID: 27717792 DOI: 10.1016/j.dld.2016.09.013]

20 **Kumar A**, Sharma P, Anikhindi SA, Prajapati R, Agarwal R, Sharma B, Bansal N, Singla V, Arora A. Can Non-Selective Beta-Blockers (NSBBs) Prevent Enlargement of Small Esophageal Varices in Patients with Cirrhosis? A Meta-analysis. *J Clin Exp Hepatol* 2017; **7**: 275-283 [PMID: 29234190 DOI:

10.1016/j.jceh.2017.09.003]

21   **Engelmann C**, Jalan R. Non selective beta-blocker in cirrhosis: Not 'whether' but 'who and how'. *AME Med J* 2017; **2**: 90 [DOI: 10.21037/amj.2017.06.13]

22   **Kim SG**, Larson JJ, Lee JS, Therneau TM, Kim WR. Beneficial and harmful effects of nonselective beta blockade on acute kidney injury in liver transplant candidates. *Liver Transpl* 2017; **23**: 733-740 [PMID: 28187503 DOI: 10.1002/lt.24744]

23   **Bossen L**, Krag A, Vilstrup H, Watson H, Jepsen P. Nonselective β-blockers do not affect mortality in cirrhosis patients with ascites: Post Hoc analysis of three randomized controlled trials with 1198 patients. *Hepatology* 2016; **63**: 1968-1976 [PMID: 26599983 DOI: 10.1002/hep.28352]

24   **Leithead JA**, Rajoriya N, Tehami N, Hodson J, Gunson BK, Tripathi D, Ferguson JW. Non-selective β-blockers are associated with improved survival in patients with ascites listed for liver transplantation. *Gut* 2015; **64**: 1111-1119 [PMID: 25281417 DOI: 10.1136/gutjnl-2013-306502]

25   **Onali S**, Kalafateli M, Majumdar A, Westbrook R, O'Beirne J, Leandro G, Patch D, Tsochatzis EA. Non-selective beta-blockers are not associated with increased mortality in cirrhotic patients with ascites. *Liver Int* 2017; **37**: 1334-1344 [PMID: 28296047 DOI: 10.1111/liv.13409]

26   **Facciorusso A**, Roy S, Livadas S, Fevrier-Paul A, Wekesa C, Kilic ID, Chaurasia AK, Sadeq M, Muscatiello N. Nonselective Beta-Blockers Do Not Affect Survival in Cirrhotic Patients with Ascites. *Dig Dis Sci* 2018; **63**: 1737-1746 [PMID: 29725793 DOI: 10.1007/s10620-018-5092-6]

27   **Njei B**, McCarty TR, Garcia-Tsao G. Beta-blockers in patients with cirrhosis and ascites: type of beta-blocker matters. *Gut* 2016; **65**: 1393-1394 [PMID: 27207973 DOI: 10.1136/gutjnl-2016-312129]

28   **Sinagra E**, Perricone G, D'Amico M, Tinè F, D'Amico G. Systematic review with meta-analysis: The haemodynamic effects of carvedilol compared with propranolol for portal hypertension in cirrhosis. *Aliment Pharmacol Ther* 2014; **39**: 557-568 [PMID: 24461301 DOI: 10.1111/apt.12634]

29   **Reiberger T**, Ulbrich G, Ferlitsch A, Payer BA, Schwabl P, Pinter M, Heinisch BB, Trauner M, Kramer L, Peck-Radosavljevic M; Vienna Hepatic Hemodynamic Lab. Carvedilol for primary prophylaxis of variceal bleeding in cirrhotic patients with haemodynamic non-response to propranolol. *Gut* 2013; **62**: 1634-1641 [PMID: 23250049 DOI: 10.1136/gutjnl-2012-304038]

30   **Sinha R**, Lockman KA, Mallawaarachchi N, Robertson M, Plevris JN, Hayes PC. Carvedilol use is associated with improved survival in patients with liver cirrhosis and ascites. *J Hepatol* 2017; **67**: 40-46 [PMID: 28213164 DOI: 10.1016/j.jhep.2017.02.005]

31   **Araújo Júnior RF**, Garcia VB, Leitão RF, Brito GA, Miguel Ede C, Guedes PM, de Araújo AA. Carvedilol Improves Inflammatory Response, Oxidative Stress and Fibrosis in the Alcohol-Induced Liver Injury in Rats by Regulating Kupffer Cells and Hepatic Stellate Cells. *PLoS One* 2016; **11**: e0148868 [PMID: 26891124 DOI: 10.1371/journal.pone.0148868]

32   **Mandorfer M**, Peck-Radosavljevic M, Reiberger T. Prevention of progression from small to large varices: are we there yet? An updated meta-analysis. *Gut* 2017; **66**: 1347-1349 [PMID: 27694143 DOI: 10.1136/gutjnl-2016-312814]

33   **Bhardwaj A**, Kedarisetty CK, Vashishtha C, Bhadoria AS, Jindal A, Kumar G, Choudhary A, Shasthry SM, Maiwall R, Kumar M, Bhatia V, Sarin SK. Carvedilol delays the progression of small oesophageal varices in patients with cirrhosis: A randomised placebo-controlled trial. *Gut* 2017; **66**: 1838-1843 [PMID: 27298379 DOI: 10.1136/gutjnl-2016-311735]

34   **Zacharias AP**, Jeyaraj R, Hobolth L, Bendtsen F, Gluud LL, Morgan MY. Carvedilol versus traditional, non-selective beta-blockers for adults with cirrhosis and gastroesophageal varices. *Cochrane Database Syst Rev* 2018; **10**: CD011510 [PMID: 30372514 DOI: 10.1002/14651858.CD011510.pub2]

35   **Senzolo M**, Cholongitas E, Burra P, Leandro G, Thalheimer U, Patch D, Burroughs AK. beta-Blockers protect against spontaneous bacterial peritonitis in cirrhotic patients: A meta-analysis. *Liver Int* 2009; **29**: 1189-1193 [PMID: 19508620 DOI: 10.1111/j.1478-3231.2009.02038.x]

36   **Merli M**, Lucidi C, Di Gregorio V, Giannelli V, Giusto M, Ceccarelli G, Riggio O, Venditti M. The chronic use of beta-blockers and proton pump inhibitors may affect the rate of bacterial infections in cirrhosis. *Liver Int* 2015; **35**: 362-369 [PMID: 24836902 DOI: 10.1111/liv.12593]

37   **Mookerjee RP**, Pavesi M, Thomsen KL, Mehta G, Macnaughtan J, Bendtsen F, Coenraad M, Sperl J, Gines P, Moreau R, Arroyo V, Jalan R; CANONIC Study Investigators of the EASL-CLIF Consortium. Treatment with non-selective beta blockers is associated with reduced severity of systemic inflammation and improved survival of patients with acute-on-chronic liver failure. *J Hepatol* 2016; **64**: 574-582 [PMID: 26519600 DOI: 10.1016/j.jhep.2015.10.018]

38   **Facciorusso A**, Villani R, Bellanti F, Bruno R, Fioravanti G, Vendemiale G, Serviddio G. In-hospital mortality and length of stay in cirrhotic patients with sepsis treated with non-selective beta-blockers. *Digest Liver Dis* 2017; **49**: e15 [DOI: 10.1016/j.dld.2017.01.033]

39   **Reiberger T**, Ferlitsch A, Payer BA, Mandorfer M, Heinisch BB, Hayden H, Lammert F, Trauner M, Peck-Radosavljevic M, Vogelsang H; Vienna Hepatic Hemodynamic Lab. Non-selective betablocker therapy decreases intestinal permeability and serum levels of LBP and IL-6 in patients with cirrhosis. *J Hepatol* 2013; **58**: 911-921 [PMID: 23262249 DOI: 10.1016/j.jhep.2012.12.011]

40   **Pérez-Paramo M**, Muñoz J, Albillos A, Freile I, Portero F, Santos M, Ortiz-Berrocal J. Effect of propranolol on the factors promoting bacterial translocation in cirrhotic rats with ascites. *Hepatology* 2000; **31**: 43-48 [PMID: 10613726 DOI: 10.1002/hep.510310109]

41   **Gimenez P**, Garcia-Martinez I, Francés R, Gonzalez-Navajas JM, Mauri M, Alfayate R, Almenara S, Miralles C, Palazon JM, Carnicer F, Pascual S, Such J, Horga JF, Zapater P. Treatment with non-selective beta-blockers affects the systemic inflammatory response to bacterial DNA in patients with cirrhosis. *Liver Int* 2018; **38**: 2219-2227 [PMID: 29802788 DOI: 10.1111/liv.13890]

42   **Thiele M**, Albillos A, Abazi R, Wiest R, Gluud LL, Krag A. Non-selective beta-blockers may reduce risk of hepatocellular carcinoma: A meta-analysis of randomized trials. *Liver Int* 2015; **35**: 2009-2016 [PMID: 25581713 DOI: 10.1111/liv.12782]

43   **Herrera I**, Pascual S, Zapater P, Carnicer F, Bellot P, María Palazón J. The use of β-blockers is associated with a lower risk of developing hepatocellular carcinoma in patients with cirrhosis. *Eur J Gastroenterol Hepatol* 2016; **28**: 1194-1197 [PMID: 27294486 DOI: 10.1097/MEG.0000000000000677]

44   **Thiele M**, Wiest R, Gluud LL, Albillos A, Krag A. Can non-selective beta-blockers prevent hepatocellular carcinoma in patients with cirrhosis? *Med Hypotheses* 2013; **81**: 871-874 [PMID: 24060485 DOI: 10.1016/j.mehy.2013.08.026]

45   **Feu F**, García-Pagán JC, Bosch J, Luca A, Terés J, Escorsell A, Rodés J. Relation between portal pressure response to pharmacotherapy and risk of recurrent variceal haemorrhage in patients with cirrhosis. *Lancet* 1995; **346**: 1056-1059 [PMID: 7564785 DOI: 10.1016/S0140-6736(95)91740-3]

46   **D'Amico G**, Malizia G, Bosch J. Beta-blockers in 2016: Still the safest and most useful drugs for portal hypertension? *Hepatology* 2016; **63**: 1771-1773 [PMID: 26890689 DOI: 10.1002/hep.28502]

47   **Peeraphatdit TB**, Kamath PS, Shah VH. Beta-blockers in patients with advanced cirrhosis: Red light, green light, yellow light…. *Liver Transpl* 2017; **23**: 725-726 [PMID: 28452116 DOI: 10.1002/lt.24780]

48   **Garcia-Tsao G**, Abraldes JG, Berzigotti A, Bosch J. Portal hypertensive bleeding in cirrhosis: Risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the study of liver diseases. *Hepatology* 2017; **65**: 310-335 [PMID: 27786365 DOI: 10.1002/hep.28906]

49   **European Association for the Study of the Liver**. Electronic address: easloffice@easloffice.eu.; European Association for the Study of the Liver. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. *J Hepatol* 2018; **69**: 406-460 [PMID: 29653741 DOI: 10.1016/j.jhep.2018.03.024]

50   **Conn HO**, Leevy CM, Vlahcevic ZR, Rodgers JB, Maddrey WC, Seeff L, Levy LL. Comparison of lactulose and neomycin in the treatment of chronic portal-systemic encephalopathy. A double blind controlled trial. *Gastroenterology* 1977; **72**: 573-583 [PMID: 14049]

51   **Als-Nielsen B**, Gluud LL, Gluud C. Non-absorbable disaccharides for hepatic encephalopathy: Systematic review of randomised trials. *BMJ* 2004; **328**: 1046 [PMID: 15054035 DOI: 10.1136/bmj.38048.506134.EE]

52   **Gluud LL**, Vilstrup H, Morgan MY. Non-absorbable disaccharides versus placebo/no intervention and lactulose versus lactitol for the prevention and treatment of hepatic encephalopathy in people with cirrhosis. *Cochrane Database Syst Rev* 2016; **4**: CD003044 [PMID: 27089005 DOI: 10.1002/14651858.CD003044.pub3]

53   **Elkington SG**. Lactulose. *Gut* 1970; **11**: 1043-1048 [PMID: 4929274]

54   **Sharma BC**, Sharma P, Agrawal A, Sarin SK. Secondary prophylaxis of hepatic encephalopathy: An open-label randomized controlled trial of lactulose versus placebo. *Gastroenterology* 2009; **137**: 885-891, 891.e1 [PMID: 19501587 DOI: 10.1053/j.gastro.2009.05.056]

55   **Agrawal A**, Sharma BC, Sharma P, Sarin SK. Secondary prophylaxis of hepatic encephalopathy in cirrhosis: An open-label, randomized controlled trial of lactulose, probiotics, and no therapy. *Am J Gastroenterol* 2012; **107**: 1043-1050 [PMID: 22710579 DOI: 10.1038/ajg.2012.113]

56   **Sharma P**, Sharma BC, Agrawal A, Sarin SK. Primary prophylaxis of overt hepatic encephalopathy in patients with cirrhosis: An open labeled randomized controlled trial of lactulose versus no lactulose. *J Gastroenterol Hepatol* 2012; **27**: 1329-1335 [PMID: 22606978 DOI: 10.1111/j.1440-1746.2012.07186.x]

57   **Prasad S**, Dhiman RK, Duseja A, Chawla YK, Sharma A, Agarwal R. Lactulose improves cognitive functions and health-related quality of life in patients with cirrhosis who have minimal hepatic encephalopathy. *Hepatology* 2007; **45**: 549-559 [PMID: 17326150 DOI: 10.1002/hep.21533]

58   **Mittal VV**, Sharma BC, Sharma P, Sarin SK. A randomized controlled trial comparing lactulose, probiotics, and L-ornithine L-aspartate in treatment of minimal hepatic encephalopathy. *Eur J Gastroenterol Hepatol* 2011; **23**: 725-732 [PMID: 21646910 DOI: 10.1097/MEG.0b013e32834696f5]

59   **Vilstrup H**, Amodio P, Bajaj J, Cordoba J, Ferenci P, Mullen KD, Weissenborn K, Wong P. Hepatic encephalopathy in chronic liver disease: 2014 Practice Guideline by the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver. *Hepatology* 2014; **60**: 715-735 [PMID: 25042402 DOI: 10.1002/hep.27210]

60   **Iadevaia MD**, Prete AD, Cesaro C, Gaeta L, Zulli C, Loguercio C. Rifaximin in the treatment of hepatic encephalopathy. *Hepat Med* 2011; **3**: 109-117 [PMID: 24367227 DOI: 10.2147/HMER.S11988]

61   **Zullo A**, Hassan C, Ridola L, Lorenzetti R, Campo SM, Riggio O. Rifaximin therapy and hepatic encephalopathy: Pros and cons. *World J Gastrointest Pharmacol Ther* 2012; **3**: 62-67 [PMID: 22966484 DOI: 10.4292/wjgpt.v3.i4.62]

62   **Mullen KD**, Sanyal AJ, Bass NM, Poordad FF, Sheikh MY, Frederick RT, Bortey E, Forbes WP. Rifaximin is safe and well tolerated for long-term maintenance of remission from overt hepatic encephalopathy. *Clin Gastroenterol Hepatol* 2014; **12**: 1390-7.e2 [PMID: 24365449 DOI: 10.1016/j.cgh.2013.12.021]

63   **Mas A**, Rodés J, Sunyer L, Rodrigo L, Planas R, Vargas V, Castells L, Rodríguez-Martínez D, Fernández-Rodríguez C, Coll I, Pardo A; Spanish Association for the Study of the Liver Hepatic Encephalopathy Cooperative Group. Comparison of rifaximin and lactitol in the treatment of acute hepatic encephalopathy: Results of a randomized, double-blind, double-dummy, controlled clinical trial. *J Hepatol* 2003; **38**: 51-58 [PMID: 12480560 DOI: 10.1016/S0168-8278(02)00350-1]

64   **Bass NM**, Mullen KD, Sanyal A, Poordad F, Neff G, Leevy CB, Sigal S, Sheikh MY, Beavers K, Frederick T, Teperman L, Hillebrand D, Huang S, Merchant K, Shaw A, Bortey E, Forbes WP. Rifaximin treatment in hepatic encephalopathy. *N Engl J Med* 2010; **362**: 1071-1081 [PMID: 20335583 DOI: 10.1056/NEJMoa0907893]

65   **Kimer N**, Gluud LL, Morris RW, Morgan MY. Rifaximin for the prevention and treatment of hepatic encephalopathy: A systematic review with meta-analyses of randomised controlled trials. *J Clin Exp Hepatol* 2017; **7**: S78-S79 [DOI: 10.1016/j.jceh.2017.01.105]

66   **Wu D**, Wu SM, Lu J, Zhou YQ, Xu L, Guo CY. Rifaximin versus Nonabsorbable Disaccharides for the Treatment of Hepatic Encephalopathy: A Meta-Analysis. *Gastroenterol Res Pract* 2013; **2013**: 236963 [PMID: 23653636 DOI: 10.1155/2013/236963]

67   **Kang SH**, Lee YB, Lee JH, Nam JY, Chang Y, Cho H, Yoo JJ, Cho YY, Cho EJ, Yu SJ, Kim MY, Kim YJ, Baik SK, Yoon JH. Rifaximin treatment is associated with reduced risk of cirrhotic complications and prolonged overall survival in patients experiencing hepatic encephalopathy. *Aliment Pharmacol Ther* 2017; **46**: 845-855 [PMID: 28836723 DOI: 10.1111/apt.14275]

68   **Kabeshova A**, Ben Hariz S, Tsakeu E, Benamouzig R, Launois R. Cost-effectiveness analysis of rifaximin-α administration for the reduction of episodes of overt hepatic encephalopathy in recurrence compared with standard treatment in France. *Therap Adv Gastroenterol* 2016; **9**: 473-482 [PMID: 27366216 DOI: 10.1177/1756283X16644249]

69   **Vlachogiannakos J**, Viazis N, Vasianopoulou P, Vafiadis I, Karamanolis DG, Ladas SD. Long-term administration of rifaximin improves the prognosis of patients with decompensated alcoholic cirrhosis. *J Gastroenterol Hepatol* 2013; **28**: 450-455 [PMID: 23216382 DOI: 10.1111/jgh.12070]

70   **Hanouneh MA**, Hanouneh IA, Hashash JG, Law R, Esfeh JM, Lopez R, Hazratjee N, Smith T, Zein NN. The role of rifaximin in the primary prophylaxis of spontaneous bacterial peritonitis in patients with liver cirrhosis. *J Clin Gastroenterol* 2012; **46**: 709-715 [PMID: 22878533 DOI: 10.1097/MCG.0b013e3182506dbb]

71   **Runyon BA**; AASLD Practice Guidelines Committee. Management of adult patients with ascites due to

cirrhosis: An update. *Hepatology* 2009; **49**: 2087-2107 [PMID: 19475696 DOI: 10.1002/hep.22853]

72 **European Association for the Study of the Liver**. EASL clinical practice guidelines on the management of ascites, spontaneous bacterial peritonitis, and hepatorenal syndrome in cirrhosis. *J Hepatol* 2010; **53**: 397-417 [PMID: 20633946 DOI: 10.1016/j.jhep.2010.05.004]

73 **Praharaj D**, Taneja S, Duseja A, Chawla Y, Dhiman RK. Randomized Control Trial of Rifaximin and Norfloxacin in Primary and Secondary Prophylaxis of Spontaneous Bacterial Peritonitis (SBP) in Cirrhotic Patients. *J Clin Exp Hepatol* 2017; **7**: S71 [DOI: 10.1016/j.jceh.2017.05.133]

74 **Sidhu GS**, Go A, Attar BM, Mutneja HR, Arora S, Patel SA. Rifaximin versus norfloxacin for prevention of spontaneous bacterial peritonitis: A systematic review. *BMJ Open Gastroenterol* 2017; **4**: e000154 [PMID: 28944070 DOI: 10.1136/bmjgast-2017-000154]

75 **Kalambokis GN**, Mouzaki A, Rodi M, Pappas K, Fotopoulos A, Xourgia X, Tsianos EV. Rifaximin improves systemic hemodynamics and renal function in patients with alcohol-related cirrhosis and ascites. *Clin Gastroenterol Hepatol* 2012; **10**: 815-818 [PMID: 22391344 DOI: 10.1016/j.cgh.2012.02.025]

76 **Baik S**, Lim Y, Cho Y, Kim M, Jang Y, Suk K, Cheon G, Kim Y, Choi D. G03: Rifaximin and propranolol combination therapy is more effective than propranolol monotherapy in the hepatic venous pressure gradient response and propranolol dose reduction–A pilot study. *J Hepatol* 2015; **62**: S188 [DOI: 10.1016/S0168-8278(15)30004-0]

77 **Kimer N**, Pedersen JS, Busk TM, Gluud LL, Hobolth L, Krag A, Møller S, Bendtsen F; Copenhagen Rifaximin (CoRif) Study Group. Rifaximin has no effect on hemodynamics in decompensated cirrhosis: A randomized, double-blind, placebo-controlled trial. *Hepatology* 2017; **65**: 592-603 [PMID: 27775818 DOI: 10.1002/hep.28898]

78 **Nathwani R**. Rifaximin to reduce infection in decompensated cirrhosis. [accessed 2017 Sep 11]. In: ISRCTN registry [Internet]. Available from: https://doi.org/10.1186/ISRCTN10994757 ISRCTN number: ISRCTN10994757.

79 **Goh ET**, Stokes CS, Sidhu SS, Vilstrup H, Gluud LL, Morgan MY. L-ornithine L-aspartate for prevention and treatment of hepatic encephalopathy in people with cirrhosis. *Cochrane Database Syst Rev* 2018; **5**: CD012410 [PMID: 29762873 DOI: 10.1002/14651858.CD012410.pub2]

80 **Bai M**, Yang Z, Qi X, Fan D, Han G. l-ornithine-l-aspartate for hepatic encephalopathy in patients with cirrhosis: A meta-analysis of randomized controlled trials. *J Gastroenterol Hepatol* 2013; **28**: 783-792 [PMID: 23425108 DOI: 10.1111/jgh.12142]

81 **Sidhu SS**. L-ornithine L-aspartate in Overt Hepatic Encephalopathy (HEAL). [accessed 2017 Aug 2]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT01722578 ClinicalTrials.gov Identifier: NCT01722578. *L-ornithine L-aspartate in Overt Hepatic Encephalopathy (HEAL). [accessed*

82 **Gentile S**, Guarino G, Romano M, Alagia IA, Fierro M, Annunziata S, Magliano PL, Gravina AG, Torella R. A randomized controlled trial of acarbose in hepatic encephalopathy. *Clin Gastroenterol Hepatol* 2005; **3**: 184-191 [PMID: 15704053 DOI: 10.1016/S1542-3565(04)00667-6]

83 **Mullen KD**, Howard R. Is acarbose an effective drug for treating patients with cirrhosis and hepatic encephalopathy? *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 264-265 [PMID: 16265229 DOI: 10.1038/ncpgasthep0194]

84 **Ventura-Cots M**, Santos B, Genescà J. α1 and α2-adrenergic agonists on cirrhotic patients with refractory ascites. *Liver Int* 2016; **36**: 177-180 [PMID: 26284943 DOI: 10.1111/liv.12941]

85 **Moore KP**, Aithal GP. Guidelines on the management of ascites in cirrhosis. *Gut* 2006; **55** Suppl 6: vi1-v12 [PMID: 16966752 DOI: 10.1136/gut.2006.099580]

86 **Piano S**, Tonon M, Angeli P. Management of ascites and hepatorenal syndrome. *Hepatol Int* 2018; **12**: 122-134 [PMID: 28836115 DOI: 10.1007/s12072-017-9815-0]

87 **Ginès P**, Cárdenas A, Arroyo V, Rodés J. Management of cirrhosis and ascites. *N Engl J Med* 2004; **350**: 1646-1654 [PMID: 15084697 DOI: 10.1056/NEJMra035021]

88 **Moore KP**, Wong F, Gines P, Bernardi M, Ochs A, Salerno F, Angeli P, Porayko M, Moreau R, Garcia-Tsao G, Jimenez W, Planas R, Arroyo V. The management of ascites in cirrhosis: Report on the consensus conference of the International Ascites Club. *Hepatology* 2003; **38**: 258-266 [PMID: 12830009 DOI: 10.1053/jhep.2003.50315]

89 **Sherlock S**, Walker JG, Senewiratne B, Scott A. The complications of diuretic therapy in patients with cirrhosis. *Ann N Y Acad Sci* 1966; **139**: 497-505 [PMID: 5230290 DOI: 10.1111/j.1749-6632.1966.tb41223.x]

90 **Laffi G**, La Villa G, Carloni V, Foschi M, Bartoletti L, Quartini M, Gentilini P. Loop diuretic therapy in liver cirrhosis with ascites. *J Cardiovasc Pharmacol* 1993; **22** Suppl 3: S51-S58 [PMID: 7506337 DOI: 10.1097/00005344-199322003-00007]

91 **Tsochatzis EA**, Gerbes AL. Diagnosis and treatment of ascites. *J Hepatol* 2017; **67**: 184-185 [PMID: 28119010 DOI: 10.1016/j.jhep.2017.01.011]

92 **Senousy BE**, Draganov PV. Evaluation and management of patients with refractory ascites. *World J Gastroenterol* 2009; **15**: 67-80 [PMID: 19115470 DOI: 10.3748/wjg.15.67]

93 **Mukerji AN**, Patel V, Jain A. Improving survival in decompensated cirrhosis. *Int J Hepatol* 2012; **2012**: 318627 [PMID: 22811919 DOI: 10.1155/2012/318627]

94 **Singh V**, Singh A, Singh B, Vijayvergiya R, Sharma N, Ghai A, Bhalla A. Midodrine and clonidine in patients with cirrhosis and refractory or recurrent ascites: A randomized pilot study. *Am J Gastroenterol* 2013; **108**: 560-567 [PMID: 23419385 DOI: 10.1038/ajg.2013.9]

95 **Singh V**, Dhungana SP, Singh B, Vijayverghia R, Nain CK, Sharma N, Bhalla A, Gupta PK. Midodrine in patients with cirrhosis and refractory or recurrent ascites: A randomized pilot study. *J Hepatol* 2012; **56**: 348-354 [PMID: 21749847 DOI: 10.1016/j.jhep.2011.04.027]

96 **Guo TT**, Yang Y, Song Y, Ren Y, Liu ZX, Cheng G. Effects of midodrine in patients with ascites due to cirrhosis: Systematic review and meta-analysis. *J Dig Dis* 2016; **17**: 11-19 [PMID: 26630543 DOI: 10.1111/1751-2980.12304]

97 **Patel S**, Nguyen DS, Rastogi A, Nguyen MK, Nguyen MK. Treatment of Cirrhosis-Associated Hyponatremia with Midodrine and Octreotide. *Front Med (Lausanne)* 2017; **4**: 17 [PMID: 28352627 DOI: 10.3389/fmed.2017.00017]

98 **Lenaerts A**, Codden T, Meunier JC, Henry JP, Ligny G. Effects of clonidine on diuretic response in ascitic patients with cirrhosis and activation of sympathetic nervous system. *Hepatology* 2006; **44**: 844-849 [PMID: 17006921 DOI: 10.1002/hep.21355]

99 **Sansoè G**, Aragno M, Mastrocola R, Parola M. Dose-dependency of clonidine's effects in ascitic cirrhotic rats: Comparison with α1-adrenergic agonist midodrine. *Liver Int* 2016; **36**: 205-211 [PMID: 26121993

DOI: 10.1111/liv.12905]

100   **Yan L**, Xie F, Lu J, Ni Q, Shi C, Tang C, Yang J. The treatment of vasopressin V2-receptor antagonists in cirrhosis patients with ascites: A meta-analysis of randomized controlled trials. *BMC Gastroenterol* 2015; **15**: 65 [PMID: 26054761 DOI: 10.1186/s12876-015-0297-z]

101   **Pose E**, Cardenas A. Translating Our Current Understanding of Ascites Management into New Therapies for Patients with Cirrhosis and Fluid Retention. *Dig Dis* 2017; **35**: 402-410 [PMID: 28468013 DOI: 10.1159/000456595]

102   **Wong F**, Watson H, Gerbes A, Vilstrup H, Badalamenti S, Bernardi M, Ginès P; Satavaptan Investigators Group. Satavaptan for the management of ascites in cirrhosis: Efficacy and safety across the spectrum of ascites severity. *Gut* 2012; **61**: 108-116 [PMID: 21836029 DOI: 10.1136/gutjnl-2011-300157]

103   **Vargas JI**, Arrese M, Shah VH, Arab JP. Use of Statins in Patients with Chronic Liver Disease and Cirrhosis: Current Views and Prospects. *Curr Gastroenterol Rep* 2017; **19**: 43 [PMID: 28752475 DOI: 10.1007/s11894-017-0584-7]

104   **Cabrera L**, Abraldes JG. Statins: The Panacea of Cirrhosis? *Curr Hepatol Rep* 2016; **15**: 1-7 [DOI: 10.1007/s11901-016-0287-9]

105   **Zafra C**, Abraldes JG, Turnes J, Berzigotti A, Fernández M, Garca-Pagán JC, Rodés J, Bosch J. Simvastatin enhances hepatic nitric oxide production and decreases the hepatic vascular tone in patients with cirrhosis. *Gastroenterology* 2004; **126**: 749-755 [PMID: 14988829 DOI: 10.1053/j.gastro.2003.12.007]

106   **Abraldes JG**, Albillos A, Bañares R, Turnes J, González R, García-Pagán JC, Bosch J. Simvastatin lowers portal pressure in patients with cirrhosis and portal hypertension: A randomized controlled trial. *Gastroenterology* 2009; **136**: 1651-1658 [PMID: 19208350 DOI: 10.1053/j.gastro.2009.01.043]

107   **Kumar S**, Grace ND, Qamar AA. Statin use in patients with cirrhosis: A retrospective cohort study. *Dig Dis Sci* 2014; **59**: 1958-1965 [PMID: 24838495 DOI: 10.1007/s10620-014-3179-2]

108   **Mohanty A**, Tate JP, Garcia-Tsao G. Statins Are Associated With a Decreased Risk of Decompensation and Death in Veterans With Hepatitis C-Related Compensated Cirrhosis. *Gastroenterology* 2016; **150**: 430-40.e1 [PMID: 26484707 DOI: 10.1053/j.gastro.2015.10.007]

109   **Chang FM**, Wang YP, Lang HC, Tsai CF, Hou MC, Lee FY, Lu CL. Statins decrease the risk of decompensation in hepatitis B virus- and hepatitis C virus-related cirrhosis: A population-based study. *Hepatology* 2017; **66**: 896-907 [PMID: 28318053 DOI: 10.1002/hep.29172]

110   **Abraldes JG**, Villanueva C, Aracil C, Turnes J, Hernandez-Guerra M, Genesca J, Rodriguez M, Castellote J, García-Pagán JC, Torres F, Calleja JL, Albillos A, Bosch J; BLEPS Study Group. Addition of Simvastatin to Standard Therapy for the Prevention of Variceal Rebleeding Does Not Reduce Rebleeding but Increases Survival in Patients With Cirrhosis. *Gastroenterology* 2016; **150**: 1160-1170.e3 [PMID: 26774179 DOI: 10.1053/j.gastro.2016.01.004]

111   **Kamal S**, Khan MA, Seth A, Cholankeril G, Gupta D, Singh U, Kamal F, Howden CW, Stave C, Nair S, Satapathy SK, Ahmed A. Beneficial Effects of Statins on the Rates of Hepatic Fibrosis, Hepatic Decompensation, and Mortality in Chronic Liver Disease: A Systematic Review and Meta-Analysis. *Am J Gastroenterol* 2017; **112**: 1495-1505 [PMID: 28585556 DOI: 10.1038/ajg.2017.170]

112   **Kim RG**, Loomba R, Prokop LJ, Singh S. Statin Use and Risk of Cirrhosis and Related Complications in Patients With Chronic Liver Diseases: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2017; **15**: 1521-1530.e8 [PMID: 28479502 DOI: 10.1016/j.cgh.2017.04.039]

113   **Singh S**, Singh PP, Singh AG, Murad MH, Sanchez W. Statins are associated with a reduced risk of hepatocellular cancer: A systematic review and meta-analysis. *Gastroenterology* 2013; **144**: 323-332 [PMID: 23063971 DOI: 10.1053/j.gastro.2012.10.005]

114   **Tsochatzis EA**, Bosch J. Statins in cirrhosis-Ready for prime time. *Hepatology* 2017; **66**: 697-699 [PMID: 28543643 DOI: 10.1002/hep.29277]

115   **Chalasani N**, Aljadhey H, Kesterson J, Murray MD, Hall SD. Patients with elevated liver enzymes are not at higher risk for statin hepatotoxicity. *Gastroenterology* 2004; **126**: 1287-1292 [PMID: 15131789 DOI: 10.1053/j.gastro.2004.02.015]

116   **Lewis JH**, Mortensen ME, Zweig S, Fusco MJ, Medoff JR, Belder R; Pravastatin in Chronic Liver Disease Study Investigators. Efficacy and safety of high-dose pravastatin in hypercholesterolemic patients with well-compensated chronic liver disease: Results of a prospective, randomized, double-blind, placebo-controlled, multicenter trial. *Hepatology* 2007; **46**: 1453-1463 [PMID: 17668878 DOI: 10.1002/hep.21848]

117   **Chalasani N**, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]

118   **European Association for the Study of the Liver (EASL)**. ; European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the Management of Non-Alcoholic Fatty Liver Disease. *Obes Facts* 2016; **9**: 65-90 [PMID: 27055256 DOI: 10.1159/000443344]

119   **Moctezuma-Velázquez C**, Abraldes JG, Montano-Loza AJ. The Use of Statins in Patients With Chronic Liver Disease and Cirrhosis. *Curr Treat Options Gastroenterol* 2018; **16**: 226-240 [PMID: 29572618 DOI: 10.1007/s11938-018-0180-4]

120   **Lodato F**, Azzaroli F, Di Girolamo M, Feletti V, Cecinato P, Lisotti A, Festi D, Roda E, Mazzella G. Proton pump inhibitors in cirrhosis: Tradition or evidence based practice? *World J Gastroenterol* 2008; **14**: 2980-2985 [PMID: 18494046 DOI: 10.3748/wjg.14.2980]

121   **Dam G**, Vilstrup H, Watson H, Jepsen P. Proton pump inhibitors as a risk factor for hepatic encephalopathy and spontaneous bacterial peritonitis in patients with cirrhosis with ascites. *Hepatology* 2016; **64**: 1265-1272 [PMID: 27474889 DOI: 10.1002/hep.28737]

122   **Dultz G**, Piiper A, Zeuzem S, Kronenberger B, Waidmann O. Proton pump inhibitor treatment is associated with the severity of liver disease and increased mortality in patients with cirrhosis. *Aliment Pharmacol Ther* 2015; **41**: 459-466 [PMID: 25523381 DOI: 10.1111/apt.13061]

123   **Trikudanathan G**, Israel J, Cappa J, O'Sullivan DM. Association between proton pump inhibitors and spontaneous bacterial peritonitis in cirrhotic patients - a systematic review and meta-analysis. *Int J Clin Pract* 2011; **65**: 674-678 [PMID: 21564440 DOI: 10.1111/j.1742-1241.2011.02650.x]

124   **Khan MA**, Cholankeril G, Howden CW. Are proton pump inhibitors a threat for spontaneous bacterial peritonitis and hepatic encephalopathy in cirrhosis? Not so fast. *Hepatology* 2017; **65**: 393 [PMID: 27688049 DOI: 10.1002/hep.28858]

125   **Tsai CF**, Chen MH, Wang YP, Chu CJ, Huang YH, Lin HC, Hou MC, Lee FY, Su TP, Lu CL. Proton Pump Inhibitors Increase Risk for Hepatic Encephalopathy in Patients With Cirrhosis in A Population Study. *Gastroenterology* 2017; **152**: 134-141 [PMID: 27639806 DOI: 10.1053/j.gastro.2016.09.007]

126   **Woodhouse CA**, Patel VC, Singanayagam A, Shawcross DL. Review article: the gut microbiome as a therapeutic target in the pathogenesis and treatment of chronic liver disease. *Aliment Pharmacol Ther* 2018; **47**: 192-202 [PMID: 29083037 DOI: 10.1111/apt.14397]

127   **Bajaj JS**, Acharya C, Fagan A, White MB, Gavis E, Heuman DM, Hylemon PB, Fuchs M, Puri P, Schubert ML, Sanyal AJ, Sterling RK, Stravitz RT, Siddiqui MS, Luketic V, Lee H, Sikaroodi M, Gillevet PM. Proton Pump Inhibitor Initiation and Withdrawal affects Gut Microbiota and Readmission Risk in Cirrhosis. *Am J Gastroenterol* 2018; **113**: 1177-1186 [PMID: 29872220 DOI: 10.1038/s41395-018-0085-9]

128   **Ratuapli SK**, Ellington TG, O'Neill MT, Umar SB, Harris LA, Foxx-Orenstein AE, Burdick GE, Dibaise JK, Lacy BE, Crowell MD. Proton pump inhibitor therapy use does not predispose to small intestinal bacterial overgrowth. *Am J Gastroenterol* 2012; **107**: 730-735 [PMID: 22334250 DOI: 10.1038/ajg.2012.4]

129   **Assaraf J**, Weiss N, Thabut D. Proton Pump Inhibitor Administration Triggers Encephalopathy in Cirrhotic Patients by Modulating Blood-Brain Barrier Drug Transport. *Gastroenterology* 2017; **152**: 2077 [PMID: 28478148 DOI: 10.1053/j.gastro.2016.10.049]

130   **Khan MA**, Cholankeril G, Howden CW. Proton Pump Inhibitors and the Possible Development of Hepatic Encephalopathy in Cirrhotic Patients: True Association or Residual Confounding? *Gastroenterology* 2017; **152**: 2076 [PMID: 28478154 DOI: 10.1053/j.gastro.2016.10.050]

131   **Terg R**, Casciato P, Garbe C, Cartier M, Stieben T, Mendizabal M, Niveyro C, Benavides J, Marino M, Colombato L, Berbara D, Silva M, Salgado P, Barreyro F, Fassio E, Gadano A; Study Group of Cirrhosis Complications of the Argentine Association for the Study of Liver Disease. Proton pump inhibitor therapy does not increase the incidence of spontaneous bacterial peritonitis in cirrhosis: A multicenter prospective study. *J Hepatol* 2015; **62**: 1056-1060 [PMID: 25481567 DOI: 10.1016/j.jhep.2014.11.036]

132   **Yu T**, Tang Y, Jiang L, Zheng Y, Xiong W, Lin L. Proton pump inhibitor therapy and its association with spontaneous bacterial peritonitis incidence and mortality: A meta-analysis. *Dig Liver Dis* 2016; **48**: 353-359 [PMID: 26795544 DOI: 10.1016/j.dld.2015.12.009]

133   **Dhar A**, Mullish BH, Thursz MR. Anticoagulation in chronic liver disease. *J Hepatol* 2017; **66**: 1313-1326 [PMID: 28088580 DOI: 10.1016/j.jhep.2017.01.006]

134   **Leonardi F**, Maria N, Villa E. Anticoagulation in cirrhosis: A new paradigm? *Clin Mol Hepatol* 2017; **23**: 13-21 [PMID: 28288507 DOI: 10.3350/cmh.2016.0110]

135   **Bitto N**, Liguori E, La Mura V. Coagulation, Microenvironment and Liver Fibrosis. *Cells* 2018; **7**: pii: E85 [PMID: 30042349 DOI: 10.3390/cells7080085]

136   **Dabbagh O**, Oza A, Prakash S, Sunna R, Saettele TM. Coagulopathy does not protect against venous thromboembolism in hospitalized patients with chronic liver disease. *Chest* 2010; **137**: 1145-1149 [PMID: 20040609 DOI: 10.1378/chest.09-2177]

137   **Villa E**, Cammà C, Marietta M, Luongo M, Critelli R, Colopi S, Tata C, Zecchini R, Gitto S, Petta S, Lei B, Bernabucci V, Vukotic R, De Maria N, Schepis F, Karampatou A, Caporali C, Simoni L, Del Buono M, Zambotto B, Turola E, Fornaciari G, Schianchi S, Ferrari A, Valla D. Enoxaparin prevents portal vein thrombosis and liver decompensation in patients with advanced cirrhosis. *Gastroenterology* 2012; **143**: 1253-1260.e4 [PMID: 22819864 DOI: 10.1053/j.gastro.2012.07.018]

138   **Senzolo M**, M Sartori T, Rossetto V, Burra P, Cillo U, Boccagni P, Gasparini D, Miotto D, Simioni P, Tsochatzis E, A Burroughs K. Prospective evaluation of anticoagulation and transjugular intrahepatic portosystemic shunt for the management of portal vein thrombosis in cirrhosis. *Liver Int* 2012; **32**: 919-927 [PMID: 22435854 DOI: 10.1111/j.1478-3231.2012.02785.x]

139   **Chen H**, Liu L, Qi X, He C, Wu F, Fan D, Han G. Efficacy and safety of anticoagulation in more advanced portal vein thrombosis in patients with liver cirrhosis. *Eur J Gastroenterol Hepatol* 2016; **28**: 82-89 [PMID: 26513611 DOI: 10.1097/MEG.0000000000000482]

140   **Delgado MG**, Seijo S, Yepes I, Achécar L, Catalina MV, García-Criado A, Abraldes JG, de la Peña J, Bañares R, Albillos A, Bosch J, García-Pagán JC. Efficacy and safety of anticoagulation on patients with cirrhosis and portal vein thrombosis. *Clin Gastroenterol Hepatol* 2012; **10**: 776-783 [PMID: 22289875 DOI: 10.1016/j.cgh.2012.01.012]

141   **Amitrano L**, Guardascione MA, Menchise A, Martino R, Scaglione M, Giovine S, Romano L, Balzano A. Safety and efficacy of anticoagulation therapy with low molecular weight heparin for portal vein thrombosis in patients with liver cirrhosis. *J Clin Gastroenterol* 2010; **44**: 448-451 [PMID: 19730112 DOI: 10.1097/MCG.0b013e3181b3ab44]

142   **Bernardi M**, Maggioli C, Zaccherini G. Human albumin in the management of complications of liver cirrhosis. *Crit Care* 2012; **16**: 211 [PMID: 22429536]

143   **Arroyo V**, García-Martinez R, Salvatella X. Human serum albumin, systemic inflammation, and cirrhosis. *J Hepatol* 2014; **61**: 396-407 [PMID: 24751830 DOI: 10.1016/j.jhep.2014.04.012]

144   **Garcia-Martinez R**, Caraceni P, Bernardi M, Gines P, Arroyo V, Jalan R. Albumin: Pathophysiologic basis of its role in the treatment of cirrhosis and its complications. *Hepatology* 2013; **58**: 1836-1846 [PMID: 23423799 DOI: 10.1002/hep.26338]

145   **Salerno F**, Navickis RJ, Wilkes MM. Albumin infusion improves outcomes of patients with spontaneous bacterial peritonitis: A meta-analysis of randomized trials. *Clin Gastroenterol Hepatol* 2013; **11**: 123-30.e1 [PMID: 23178229 DOI: 10.1016/j.cgh.2012.11.007]

146   **Sanyal AJ**, Boyer T, Garcia-Tsao G, Regenstein F, Rossaro L, Appenrodt B, Blei A, Gülberg V, Sigal S, Teuber P; Terlipressin Study Group. A randomized, prospective, double-blind, placebo-controlled trial of terlipressin for type 1 hepatorenal syndrome. *Gastroenterology* 2008; **134**: 1360-1368 [PMID: 18471513 DOI: 10.1053/j.gastro.2008.02.014]

147   **Bernardi M**, Caraceni P, Navickis RJ, Wilkes MM. Albumin infusion in patients undergoing large-volume paracentesis: A meta-analysis of randomized trials. *Hepatology* 2012; **55**: 1172-1181 [PMID: 22095893 DOI: 10.1002/hep.24786]

148   **Caraceni P**, Riggio O, Angeli P, Alessandria C, Neri S, Foschi FG, Levantesi F, Airoldi A, Boccia S, Svegliati-Baroni G, Fagiuoli S, Romanelli RG, Cozzolongo R, Di Marco V, Sangiovanni V, Morisco F, Toniutto P, Tortora A, De Marco R, Angelico M, Cacciola I, Elia G, Federico A, Massironi S, Guarisco R, Galioto A, Ballardini G, Rendina M, Nardelli S, Piano S, Elia C, Prestianni L, Cappa FM, Cesarini L, Simone L, Pasquale C, Cavallin M, Andrealli A, Fidone F, Ruggeri M, Roncadori A, Baldassarre M, Tufoni M, Zaccherini G, Bernardi M; ANSWER Study Investigators. Long-term albumin administration in

decompensated cirrhosis (ANSWER): An open-label randomised trial. *Lancet* 2018; **391**: 2417-2429 [PMID: 29861076 DOI: 10.1016/S0140-6736(18)30840-7]

149   **Mullish BH**, Quraishi MN, Segal JP, McCune VL, Baxter M, Marsden GL, Moore DJ, Colville A, Bhala N, Iqbal TH, Settle C, Kontkowski G, Hart AL, Hawkey PM, Goldenberg SD, Williams HRT. The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridium difficile* infection and other potential indications: Joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut* 2018; **67**: 1920-1941 [PMID: 30154172 DOI: 10.1136/gutjnl-2018-316818]

150   **Bajaj JS**, Kassam Z, Fagan A, Gavis EA, Liu E, Cox IJ, Kheradman R, Heuman D, Wang J, Gurry T, Williams R, Sikaroodi M, Fuchs M, Alm E, John B, Thacker LR, Riva A, Smith M, Taylor-Robinson SD, Gillevet PM. Fecal microbiota transplant from a rational stool donor improves hepatic encephalopathy: A randomized clinical trial. *Hepatology* 2017; **66**: 1727-1738 [PMID: 28586116 DOI: 10.1002/hep.29306]

151   **Woodhouse C**. Trial of faecal microbiota transplantation in cirrhosis. ISRCTN registry. [accessed 2018 May 21]. In: ISRCTN registry [Internet]. Available from: https://doi.org/10.1186/ISRCTN11814003 ISRCTN number: ISRCTN11814003.

152   **Dranoff JA**. Coffee Consumption and Prevention of Cirrhosis: In Support of the Caffeine Hypothesis. *Gene Expr* 2018; **18**: 1-3 [PMID: 28893365 DOI: 10.3727/105221617X15046391179559]

153   **Salomone F**, Galvano F, Li Volti G. Molecular Bases Underlying the Hepatoprotective Effects of Coffee. *Nutrients* 2017; **9**: pii: E85 [PMID: 28124992 DOI: 10.3390/nu9010085]

154   **Petta S**, Marchesini G. Coffee and tea breaks for liver health. *J Hepatol* 2017; **67**: 221-223 [PMID: 28578838 DOI: 10.1016/j.jhep.2017.04.014]

155   **Corrao G**, Zambon A, Bagnardi V, D'Amicis A, Klatsky A; Collaborative SIDECIR Group. Coffee, caffeine, and the risk of liver cirrhosis. *Ann Epidemiol* 2001; **11**: 458-465 [PMID: 11557177 DOI: 10.1016/S1047-2797(01)00223-X]

156   **Alferink LJM**, Fittipaldi J, Kiefte-de Jong JC, Taimr P, Hansen BE, Metselaar HJ, Schoufour JD, Ikram MA, Janssen HLA, Franco OH, Darwish Murad S. Coffee and herbal tea consumption is associated with lower liver stiffness in the general population: The Rotterdam study. *J Hepatol* 2017; **67**: 339-348 [PMID: 28578837 DOI: 10.1016/j.jhep.2017.03.013]

157   **Yu C**, Cao Q, Chen P, Yang S, Deng M, Wang Y, Li L. An updated dose-response meta-analysis of coffee consumption and liver cancer risk. *Sci Rep* 2016; **6**: 37488 [PMID: 27910873 DOI: 10.1038/srep37488]

158   **Freedman ND**, Everhart JE, Lindsay KL, Ghany MG, Curto TM, Shiffman ML, Lee WM, Lok AS, Di Bisceglie AM, Bonkovsky HL, Hoefs JC, Dienstag JL, Morishima C, Abnet CC, Sinha R; HALT-C Trial Group. Coffee intake is associated with lower rates of liver disease progression in chronic hepatitis C. *Hepatology* 2009; **50**: 1360-1369 [PMID: 19676128 DOI: 10.1002/hep.23162]

MINIREVIEWS

Outcomes of per oral endoscopic pyloromyotomy in gastroparesis worldwide

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Abstract

Per oral endoscopic pyloromyotomy (POP), also known as gastric per-oral endoscopic myotomy (GPOEM), is a novel procedure with promising potential for the treatment of gastroparesis. As more data emerge and the procedure is becoming more recognized in clinical practice, its safety and efficacy need to be carefully evaluated. Appropriate patient selection for favorable clinical success prediction after GPOEM also needs additional research. This review aims to systemically summarize the existing data on clinical outcomes of POP. Symptomatologic responses to the procedure, its adverse effects, procedural techniques, and predictive factors of clinical success are also discussed.

Key words: Gastroparesis; Per oral endoscopic pyloromyotomy; Gastric per-oral endoscopic myotomy; Pyloromyotomy; Outcomes

Core tip: Per oral endoscopic pyloromyotomy (POP), or gastric per oral endoscopic myotomy is a viable therapeutic modality for patients with medically refractory gastroparesis. POP has demonstrated promising mid-term clinic outcomes in up to 18 mo follow-up period. However, most published studies were single-center and retrospective. Duration of the disease, prior response to intrapyloric botulinum injection, and increased pyloric cross-sectional area has been described as predictive factors for POP outcome. Impedance planimetry can be used to evaluate pyloric dysfunction. However, the reliability of these factors still needs clinical validation.

INTRODUCTION

Gastroparesis is a chronic disabling disease with a complex pathophysiology that is still poorly understood. Its rising prevalence and hospitalization rate are alarmingly concerning[1]. The three main etiology cited for gastroparesis include idiopathic, diabetic related and post-surgical. Gastroparesis clinically manifests as recurrent postprandial nausea and vomiting, early satiety post prandial bloating and upper abdominal pain[2]. In severe cases, it can also lead to weight loss and malnutrition[3]. The diagnosis of gastroparesis is suspected by constellation of these clinical symptoms. The diagnosis is further confirmed based on normal upper endoscopy ruling out any structural obstruction and 4 h gastric emptying study proving impaired gastric emptying[2]. Gastroparesis, due to recurrent debilitating nature of disease has significant negative impact on patients' quality of life, economic burden, and heath care utilization while only with limited therapeutic options[4]. Only few prokinetic medications are available for symptomatic control. Most of which have limited long-term usage due to their side effects or tachyphylaxis[5]. Metoclopramide and domperidone, a D2 dopamine receptor antagonist, are the most widely used but only metoclopramide is Food and Drug Administration (FDA) approved in the United States while domperidone is available in Europe, Canada, Mexico, and New Zealand[5,6]. Metoclopramide also carries a significant risk of extrapyramidal adverse effects, including tardive dyskinesia when taken longer than 12 wk. Other groups of medication, such as 5-HT3 receptor antagonists, phenothiazines, and muscarinic cholinergic receptor antagonist, have been used off-label for symptomatic relieve but they do not have effect of gastric motility. While medications and dietary modification are the first line treatment, approximately 30% of patients do not respond to conservative management[7]. These limitations of medical therapy highlights the need for an alternate therapeutic option[8].

Non-medical treatments of gastroparesis include gastric electric stimulator, surgical pyloroplasty, botulinum toxin injection, and transpyloric stenting. The concept of neurostimulation and pylorus-directed therapies stem from a physiologic knowledge of gastric emptying that involves both gastric motility and pyloric clearance[9]. Gastric motility is driven by the interstitial cells of Cajal, the pacemaker of the gut, which rhythmically generate a slow wave impulse spanning from the greater curvature toward the pylorus. Gastric electrical stimulator was developed based on the principle that the amplitude, frequency, and direction of these electrical activities help control the gastric emptying function[10-12].

Although the efficacy of gastric electrical stimulation was promising in the early open-label studies but their results were not consistently reproduced in subsequent randomized control trials, which raises concern for the true clinical impact of this modality on gastroparesis[7,13-20]. In addition, gastric electrical stimulator requires surgery for placement and has been only approved based on the humanitarian device exception rule, rather than an effective therapy by the United States FDA[21]. Implantation and device-associated adverse effects such as pocket infection, sepsis, pulmonary embolism, stroke, or even death have been reported[21].

Even though the pylorus lacks slow-wave impulses, its tone and phasic contraction determines the outlet phase of gastric emptying[11]. Abnormal physiologic characteristics of the pylorus such as narrow cross-sectional area, diameter, increased pressure, distensibility, and prolonged phasic contraction or increased tone have been shown to delay gastric emptying[9,11,22-24]. Other pathophysiology, including antroduodenal hypomotility, impaired fundic accommodation, and pylorospasm, all of which also interact with one another, are believed to play major roles in gastroparesis[25].

Therefore, mechanical disruption of the pyloric muscle is believed to have effects on global gastric emptying as well. This notion of pylorus-directed therapy was strengthened by recent studies on surgical pyloroplasty, transpyloric stenting, and intrapyloric botulinum injection that improved both symptoms score and gastric emptying time[26-33]. However, the efficacy of intrapyloric botulinum injection was not demonstrated in subsequent randomized controlled trials and is no longer recommended by American College of Gastroenterology, while transpyloric stenting is only a temporizing measure and not a long term solution[8,34,35].

With the significant advancement in the field of submucosal endoscopy in the last few years, per oral endoscopic pyloromyotomy (POP), also known as gastric per oral endoscopic myotomy (GPOEM), has emerged as a pylorus-directed therapy for gastroparesis. The procedure was conceptually originated from POEM, a similar endoscopic procedure for treatment of achalasia. They both use submucosal tunneling technique to reach and dissect the target muscles, lower esophageal sphincter in achalasia and pyloric ring in gastroparesis[36]. The submucosal space offers a safe conduit to carry out pyloromyotomy via endoscopy, rather than surgery[37-39]. Due to

its minimally-invasive nature and its promising outcomes, this novel procedure has quickly gained popularity worldwide. The first human case of POP was performed by Khashab *et al*[39] in 2013 without any adverse event and demonstrated significant clinical improvement at 12-wk follow up. Subsequently in 2014, the first human case of POP was performed successfully in Europe and later in 2015, a case series of 7 patients was published on POP outcomes[37]. Since then, many single-center and a few multicenter studies have been published reporting short term outcomes on POP in gastroparesis patients, mostly in retrospective fashion (Table 1)[39-42]. The reported symptomatic responses and clinical outcomes have been very promising[2,37,40,43-48]. This review aims to examine current evidence on clinical outcome of POP.

PROCEDURAL TECHNIQUES

The procedural steps of POP follow the established sequence of POEM (Figure 1) as: (1) Mucosotomy to create an entry to submucosal plane; (2) submucosal tunnel is created using submucosal dissection technique; (3) myotomy of the targeted muscle; and (4) mucosal defect closure. However, POP is generally considered more technical demanding than POEM for a few reasons: (1) The direction of the submucosal tunnel is curved, as opposed to a straight tube in esophagus; (2) there is more movement in the procedural field from antral contractility, compared to a aperistaltic esophagus; (3) identification of the targeted muscle, the pyloric ring in POP, is more difficult than identifying lower esophageal sphincter in POEM due to both aforementioned reasons rendering risk for tunnel deviation from the desired axis; (4) the wall of duodenal bulb is much thinner than gastric cardia, increasing risk for perforation; and (5) the maneuverability of the scope is more limited due to an inevitable loop in the stomach.

In addition, due to its novelty, there is a lack of standardized technique for POP. Extent and depth of pyloromyotomy may vary depending on endoscopist's preference and how well the pyloric ring can be identified in the submucosal tunnel. Most report suggested performing the procedure in supine position as it will be easier to orient the scope direction but left lateral decubitus position may be required when a large gastric loop is present[49]. Prolonged period of clear liquid diet for 2-3 d both before and after POP was recommended as a routine pre- and post-procedure protocol to maximize visualization and reduce the risk of procedure-related infection[2,49]. Generous irrigation should be exercised to clean the stomach content and mucosotomy site. Gentamycin rinse was advocated by some centers[47]. Other technical variations among endoscopists include site of mucosal entry (lesser *vs* greater curve), mucosotomy closure tools (clips *vs* suture), depth of pyloromyotomy, and the need for fluoroscopy[2,45,50-52]. Though general anesthesia is recommended in all studies but conscious sedation in endoscopy suite can be safely and successfully performed as well[49]. Intravenous antibiotic prophylaxis is routinely administered though there is no high-quality evidence to support the practice. Proper antibiotic, dosing, and duration are still not yet refined. How much impact on these minor variations have on clinical response is also not known.

In the early studies, mucosal entry was performed mainly on the greater curve or anterior wall of the stomach and full thickness myotomy was reported[37,53,54]. However, subsequent studies verified that submucosal tunnel can be safely and effectively performed regardless of the site of mucosal entry and selective circular myotomy can achieve clinical success without the perforation risk in full thickness myotomy[41,47,48,55,56]. While mucosotomy on the greater curve makes the scope more in a neutral position and allows greater maneuverability, performing a mucosal entry on the lesser curve has its own advantages that are: (1) Shorter scope length to mucosotomy site by minimizing the gastric loop; (2) shorter length of the submucosal tunnel, reducing the risk of tunneling in the wrong direction; and (3) the procedural field is not a dependent area when the patient is on supine position, therefore blood and food would not interfere with the endoscopic visualization[48,49,55].

Though the type of endoscopic knife and injectant used during POP have been heterogeneous in various reports, which included triangle-tip knife (KD-640 L, Olympus, Tokyo, Japan), hybrid knife (ERBE, Germany), a hook knife (KD-620LR; Olympus, Japan), mixed methylene blue/indigo carmine with hypertonic saline, normal saline, or hydroxy-ethyl starch, but common devices that are considered mandatory are silicone-base transparent over-the-scope cap and carbon dioxide for insufflation during the procedure. The cap facilitates submucosal entry, creates a working space in the submucosal tunnel, and also helps with hemostasis from small vessels in the tunnel. Due to submucosal nature of the procedure, pneumoperitoneum can occur. Carbon dioxide, which is absorbed 160 times faster than nitrogen gas in room air is essential to minimize this risk[57]. For hemostasis, soft coagulation mode

Table 1 Clinical outcomes of per oral endoscopic pyloromyotomy

	Type	N	Etiology of gastroparesis	Definition of refractory gastroparesis	Average Procedure duration (min)	Outcome measurement	Clinical response definition	Clinical response rate N (%)	GCSI improvement	Subscale	Adverse event	Follow-up period (mo) (Additional treatment during follow up)
Shlomovitz et al[37]	Retrospective	7	2 PSG; 4 IG; 1 patient with normal GES	Not defined	90-120	GES (3M); Gastroparesis; Symptoms	Symptomatic improvement	85.7%	NA	Nausea and epigastric burn significantly improved	One prelyloric ulcer with GI bleed	6.5 (2-11) (1 patient required Laparoscopic pyloroplasty at 7 mo, also no response after the procedure)
Chung et al[41]	Retrospective	8	4 DG; 4 PSG	Not defined		GOOSS; GES	NA	NA	NA	Nausea; Vomiting; Abdominal pain	1 bleeding pre-pyloric ulcer; 1 dumping syndome	7
Khashab et al[45]1	Retrospective	30	11 DG; 12 PSG; 7 IG	Presence of symptoms despite dietary; Modification and treatment with prokinetics and antiemetics	72 ± 42	GES (3M); Gastroparesis Symptoms (Graded self-reported symptomatic responses: Resolved, improved, unchanged or worse)	Reduction in gastroparesis symptoms with absence of recurrent hospitalization	86%	NA	97% improve nausea; 63% improve in vomiting; 73% improve in abd pain; 93% maintain or gain weight	6.7% 1 capnoperitonum; 1 prepyloric ulcer	5.5
Gonzalez et al[2] France	Retrospective	29	7 DG 5 PSG; 15 IG; 2 Other (Scleroderma)	Symptoms > 6 mo despite Rx and fail "all" prokinetic drug, GCSI > 1.5	47	GES (2M); GCSI	Improvement in GCSI and GES	79% (3M); 69% (6M)	3.3 to 1.1	All GCSI	5 pneumoperitoneum; 2 Bleeding; 1 perigastric abscess (patient ate 2 h post-procedure) 1 delayed pre-pyloric stricture	6

Dacha *et al*[43]	Retrospective	16	9 DG; 1 PSG; 5 IG; 1 PIG	Patients who failed to respond to dietary modification, prokinetic medication, or electrical stimulator	49.7 ± 22.1	GES; GCSI; SF36	A decrease in mean GCSI with an improvement of at least 2 subsets of cardinal symptoms and no gastroparesis - related hospitalization	81%	3.4 to 1.5	N/V and early satiety significantly improved but not bloating	none	12
Rodriguez *et al*[48]	Prospective observation	47	12 DG; 8 PSG; 27 IG	Patients with ongoing symptoms after at least 6 mo of medical therapy	41.2 ± 28.5	GES; GCSI	improvement in post-procedure GCSI, a decrease in the total number of gastroparesis medications used, and improved GES at 90-day	Not reported	3.6 to 3.3	All 3 subscales were significantly improved but N/V and bloating improved the most	none	3 (1 pt had lap total gastrectomy at 9 mo)
Allemang *et al*[55]	Retrospective	57	Not reported	Not clearly defined	41	GCSI	Improved GCSI	Not reported	4.6 to 3.3	Not reported	Not reported	3
Malik *et al*[47]	Case series	13	1 DG; 8 PSG; 4 IG	Not clearly defined	± 23	GES; PAGI-SYM; EndoFLIP	Improved GCSI, CPGAS, and GES	72.7%	2.1 to 1.9	Vomiting, retching, and loss of appetite improved the most by 29, 24, and 24%. None was statistically significant; Abd distension was actually worse	1 pulm embolism	3
Mekaroonkamol *et al*[62]	Retrospective	30	12 DG; 5 PSG; 12 IG; 1 PIG	Patients who failed to respond or could not tolerate to dietary modification, prokinetic medication, or electrical stimulator	48.3 ± 16.5	GES (2M); GCSI; SF36; ER visit rate; Hospitalization rate	Decrease in at least 1 averaged point of GCSI with more than a 25% decrease in at least 2 subscales > 25% increase in the mean SF-36 score with at least 50% increase in 3 categories	83.3%	3.6 to 1.4	Nausea and early satiety significantly improved; Pain only improved up to 6 mo but not thereafter	1 tension capnoperitoneum (3.3%)	18

Jacques et al[61]	Prospective	20	10 DG; 1 PSG; 4 IG; 5 Other (including 3 Sjogen, 1 Parkinson's, and 1 systemic sclerosis)	Symptoms > 6 mo despite medication, GCSI > 2.6 OR refractory vomiting, uncontrolled post-prandial hypoglycemia, need for oral medication, Fail at least 2 out of 3 prokinetic drug	56.5	GES (3M); GCSI; PAGI-QoL; GIQLI; EndoFlIP; Abdominal pain score	A decrease of more than 0.75 point of GCSI	90%	3.5 to 1.3	All 9 subscale of GCSI except for retching	4 perforation, including 1 required surgical intervention1 case of epitaxis	3
Kahaleh et al[58]2	Retrospective	33	7 DG; 12 PSG; 13 IG; 1 Other	Not clearly defined	77.6 (37-255)	GES; GCSI	Improvement in GCSI and GES	85%	3.3 to 0.8	All subscale including abd pain significantly improved	1 bleeding and 1 ulcer	11.5
Hustak et al[59]2	Prospective	7	2 DG; 4 PSG; 1 IG	Not clearly defined	70	GES; GCSI	Improvement in GCSI of > 40% and GES	100%	3.26 to 1.24	Not reported	1 bleeding ulcer	12
Mekaroonkamol et al[62]2	Retrospective	40	15 DG; 5 PSG; 18 IG; 1 PIG; 1 Other (Ehlers Danlos)	Patients who failed to respond or could not tolerate to dietary modification, prokinetic medication, or electrical stimulator	Not reported	GES (2M); GCSI; SF36	Decrease in at least 1 averaged point of GCSI with more than a 25% decrease in at least 2 subscales	Not reported	3.6 to 1.9	Only nausea/vomiting and early satiety improved, but not for bloating	1 capnoperiteum; 1 COPD exacerbation; 1 myotomy dehiscence	18

[1]Multicenter trial, two centers in the United States were involved.

[2]Abstract only publications.

DG: Diabetic gastroparesis; PSG: Post-surgical gastroparesis; IG: Idiopathic gastroparesis; PIG: Post-infectious gastroparesis; GES: Gastric emptying scintigraphy; GCSI: Gastroparesis cardinal symptoms index; GOOSS: Gastric outlet obstruction scoring system; SF36: Short form 36; PAGI-SYM: Patient Assessment of Gastrointestinal Symptoms; N/V: Nausea and vomiting; EndoFLIP: Endoscopic functional luminal imaging probe; CPGAS: Clinical Patient Grading Assessment Score; COPD: Chronic obstructive pulmonary disease.

(ERBE, Germany) for ablation of small vessels with a diameter less than 5 mm and coag-grasper (FD-411QR; Olympus, Japan) for bleeding control from a large vessel are generally used[49,55,56].

OUTCOME OF PER ORAL ENDOSCOPIC PYLOROMYOTOMY

Studied population

Since Kawai and colleagues proved that the concept of pyloromyotomy can be performed endoscopically using submucosal technique similar to that of POEM procedure in 2014[42], multiple centers have performed POP for patients with refractory gastroparesis. Most studies have been reported from The United States and France with some reports from Korea, Brazil, Australia, India, Venezuela, Mexico, and Czech Republic[2,37,40,43,45,46,48,49,54-56,58,59]. This may be in part due to high prevalence of the disease with only one approved medication for symptomatic treatment in the United States. However, as more data on its safety and efficacy emerges, it can be anticipated the procedure will become available in other centers with expertise in submucosal

Figure 1 The procedural steps of per oral endoscopic pyloromyotomy. A: Pre-pyloric area in the stomach; B: Mucosotomy site in prepyloric area; C: Submucosal tunnel creation; D: Pyloric ring; E: Myotomy; F: Closure of mucosotomy site with endoclips.

endoscopy as well.

Based on an electronic search of PubMed, Medline, Cochrane and Scopus databases for articles containing the terms "Endoscopic pyloromyotomy", "POP", or "GPOEM" between January 2013 and September 2018, there have been 13 publications on clinical outcome of POP, including 3 abstract-only publications as described in Table 1. Three case reports were excluded as the data on their clinical outcome was not available[40,54,60]. All published study, except for a study by Jacques *et al*[61] were retrospective studies.

Across all publications, 291 patients underwent POP[2,37,41,43,45,47,48,55,56,58,59,61,62]. Etiology of gastroparesis were diabetes (*n* = 69), post-surgery (*n* = 61), idiopathic (*n* = 93), post-infection (*n* = 1), and other causes (*n* = 10), which included systemic sclerosis, Sjogren syndrome, and Ehlers Danlos syndrome as described in Table 1. The inclusion criteria of all study were similar, which was patients with refractory gastroparesis, except for Two small studies where responses to medical therapy was not mentioned in the inclusion criteria and assumingly all patients with gastroparesis were included[37,41]. There were also minor differences in how each group defined "refractory". While most studies required only presence of symptoms despite dietary and prokinetics treatment, Gonzalez *et al*[2] and Jacques *et al*[61] set more strict inclusion criteria. Both studies required persistent gastroparetic symptoms of longer than 6 mo while on medical therapy. Gonzalez *et al*[2] required gastroparesis cardinal symptoms index (GCSI) of > 1.5 after all prokinetic medications, while Jacques *et al*[61] required GCSI of > 2.6 after failing at least 2 out of 3 prokinetics. Studies from Dacha *et al*[43] and Mekaroonkamol *et al*[56] were conducted by the same group from Emory University, United States. They excluded patients whose predominant symptom was abdominal pain[43,56]. Using the previous pylorus-directed therapy to predict response of POP, Rodriguez *et a*l[48] included only those who had symptomatic improvement after intrapyloric botulinum injection. This heterogeneity among inclusion criteria needs to be considered when comparing outcomes between each study.

Outcome measurements

Despite some difference on baseline characteristics of studied population, the clinical outcomes among published data have consistently suggested high clinical response rate ranging from 69%-100%. However, outcome measurements and follow-up duration are quite heterogeneous across all studies (Table 1). Gastric emptying scintigraphy (GES) was included as one of the parameters to measure clinical success in almost all of the studies even though it may not be the best tool to evaluate clinical success as it has been shown to have poor correlation with clinical symptoms[63-66]. We believe that clinical success of POP in gastroparesis cannot be solely based upon GES.

Most studies have also included objective scoring system to track improvement in clinical symptoms. Different symptomatology were evaluated. Khashab *et al*[45] used patient-report score to evaluate nausea, vomiting, and abdominal pain, while Shlomovitz *et al*[37] used a questionnaire-based scoring system to evaluate gastroparesis-related symptoms including epigastric burning and pain. Although GCSI was most commonly used scoring system, but other validated tools such as standardized short form 36 (SF36) and Patient Assessment of Gastrointestinal Symptoms (PAGI-SYM) were also used[43,47,48,56,58]. Due to significant heterogeneity in outcome measurements, not all clinical endpoints are comparable across all studies.

Technical success

Although POP is more technically demanding compared to its predecessor POEM procedure due to number of aforementioned reasons, including how well pyloric ring can be identified, complete pyloromyotomy was achieved in all patients reported in published studies (100% technical success rate) with procedural time ranging from 40-120 min[43,45,47,48,51,52]. However, it is noteworthy that only highly skilled endoscopist who had extensive experience with POEM and submucosal endoscopy performed the procedures. This number may also be subjected to reporting bias and publication bias. Trainees participated in a few studies with varying degree of hands-on involvement depending on the endoscopist's discretion[43,56]. The wide range of procedural time was possibly related to the learning curve of the procedure, difficulty in submucosal tunneling, and time needed to identify pyloric ring.

Clinical success

One of the challenges in evaluating clinical success of any gastroparesis treatment is the lack of validated objective measurements that correlate well with clinical symptoms. GCSI was the main outcome measurement in most studies with other patient-report scoring systems utilized as note above[43,47,48,56,58]. Although recall bias could not be completely eliminated, clinical response rate of POP has been very encouraging with significantly improved symptoms and quality of life ranging from 73%-100% at up to 18-mo follow-up period[37,43,45,47,56]. All studies but one showed significant drop in total GCSI after POP[43,48,61,67]. The symptoms that improve the most were nausea and vomiting, while bloating and abdominal distension were not consistently improved among existing studies[37,43,45,47,48,56,58]. Although Rodriguez *et al*[48] and Kahaleh *et al*[58] reported significant improvement in bloating in their patient population after POP, but other studies could not reproduce the results with one study even showed worsened abdominal distension after the procedure[43,45,47,56].

Abdominal pain is a common symptom in gastroparesis with some patients even have pain-predominant disease. However, pain is not one of the cardinal symptoms included in GCSI. Many studies evaluated this component by using indirect surrogate such as PAGI-DYM, SF36, or direct questioning. Improvement in abdominal pain has been reported in 56%-73% of patients after POP but the follow-up period in these studies were only up to 11.5 mo[45,48,58]. One study showed that the improvement in pain did not sustain and lasted for only 6 mo[56]. While Long term data is required to better understand the effect of POP on pain symptoms in gastroparesis.

Improvement in post procedure gastric emptying quantified by GES was included as the only objective parameter to measure clinical success in most studies. A 4-h study with retention percentage of more than 10%-20% at 4-h was generally required. GES was repeated 2-3 mo after POP in most studies. However, there was significant variability in post-procedure improvement in gastric retention. Post procedure GES was completed in as low as 34% to 100% of the studied patients[48,56,61]. Hustak *et al*[59] and Jacques *et al*[61] reported GES improvement in 100% patients, while Mekaroonkamol *et al*[56] reported GES improvement in 78% of study subjects and normalized GES in 48% patients. A few studies reported significant improvement in GES at 2-h retention but statistical significant improvement was not observed at 4-h[2,37,47]. Relatively smaller sample size in these studies may have contributed to this discrepancy. Interestingly, irrespective of the degree of improvement in GES, clinical response measured in terms of GCSI remains > 70% in all studies[2,37,43,47,48,56,61].

Despite the difference in each symptom responses to POP, overall quality of life of the patients with gastroparesis was shown to have improved after the procedure in 70%-78% of the patients[43,47,56]. Out of 8 aspects of quality of life assessed in SF36, the domains showing significant improvement included vitality, general health, social functioning, and metal health In addition, frequency in emergency room visits, gastroparesis-related hospitalization rate, and anti-emetic medication requirement have significantly reduced post-POP, when compared to the control group[43,56]. The mean length of hospital stay was 1-3.3 d[43,45,47,48]. Majority of the patients were able to tolerate oral diet and significantly gained weight[43,45,48].

The physiologic changes in gastric motility that led to these observed differences in

clinical responses of each symptom is not yet known. However, it has been hypothesized that the affected location in gastric dysmotiliy may play a role when categorizing gastroparesis into two different subtypes: (1) Fundic-predominant (proximal retention), characterized by bloating, early satiety, and abdominal pain, which are the result of visceral hypersensitivity and impaired relaxation of the fundus post-prandially[68-70]; and (2) Antral-predominant (distal retention) gastroparesis which is a result of pyloric dysfunction and impaired antral contraction causing delayed emptying from distal stomach and manifest with nausea/vomiting. It is expected that gastroparesis with distal retention from pyloric dysfunction would respond better to POP[43,71]. However, differentiating these 2 subtypes objectively remains difficult creating yet another challenge in appropriate patient selection for the procedure. Endoscopic functional luminal imaging probe (EndoFLIP) is a system which was first described in clinical practice in 2007. Impedance planimetry (IP) is a technique which allows to study the relationship of cross sectional area and pressure during volume distention in GI lumen. EndoFLIP uses multi-detector IP system to produce 3-dimentioanl images of any distensible organ within GI tract[72]. EndoFLIP recordings allow dynamic imaging of sphincter distention with a cylindrical balloon of variable diameter with instant cross sectional area measurement along with direct calculations of pyloric sphincter pressures[73]. While EndoFLIP has shown to have widely useful in esophageal disorder, its use across other gastrointestinal motility disorder has been increasing as well[72]. EndoFLIP directed therapy could have a significant role here in future but it currently remains under investigation. Considering these conflicting data on symptomatic response and the fact that most patients with gastroparesis have mixed symptoms, our practice is to advise the patient that not all symptoms will respond equally with nausea/vomiting has more likelihood of improvement than bloating and pain. The decision to proceed with the procedure should always be individualized.

Two cases were reported to undergo subsequent surgical intervention after no response to POP. One had laparoscopic total gastrectomy at 9 mo after POP[48], while the other underwent laparoscopic pyloroplasty 7 mo after the procedure, which also did not yield any significant clinical improvement[37]. The result was not surprising as both interventions offer the same therapeutic mechanism. However, one study reported a repeat POP in a patient who initially improved but had gastroparesis symptoms recurred 24 mo after the index procedure. The patient had significant clinical response even after a repeat pyloromyotomy[62].

Adverse events

Post-procedural hemorrhage, pyloric ulcer, and tension capnoperitoneum have been reported as serious adverse events of POP with complication rate ranging from 0-6.7%[43,45,47,48,58].

Bleeding: Bleeding has been reported as an adverse event by multiple studies[2,49,58,59]. All peri-procedural bleeding were controlled endoscopically and/or medically (with proton pump inhibitors) without any further interventions. Many studies have also reported pyloric ulcer after the procedure[37,45,49]. These ulcers at the incision site may be the cause of GI bleeding. Causal relationship was not established between ulcer the source of bleeding in published studies except Hustak *et al*[59] and Chung *et al*[49] where bleeding was attributed to the ulcer.

Perforation: Perforation has been reported in a recent study by Jacques *et al*[61]. While capnoperitoneum/pneumoperitoneum has been reported by a few previous studies. Despite high rate of perforation in animal studies[53,74,75], incidence of procedure-related perforation in humans was rare. This can be explained by the difference in separability of gastric muscle layers between human and porcine model. Recently, Jacques *et al*[61] reported 20% rate of perforation (4/20 patients, only 1 required surgical intervention, while others were managed conservatively). The reason for such high rate of perforation in this study remains unknown but full-thickness myotomy approach as well as extension of myotomy into duodenal side in a retrograde manner could have contributed. Allemang *et al*[55] recommends against extending myotomy into duodenal side to minimize risk of perforation. At our center, we performed selective circular pyloromyotomy as the pyloric ring without duodenal extension to minimize the risk of perforation[43,56].

Capnoperitoneum/pneumoperitoneum: In contrast to perforation, capno-peritoneum/pneumoperitoneum is encountered with reported incidence rate ranging from 0-17%[2,32,56]. Most cases are managed with either conservative treatment and resolved on its own. If severe, affecting patient ventilation or hemodynamics, it can be treated with needle decompression as described by Gonzalez *et al*[2] and Mekaroonkamol *et al*[56] At our institute, needle decompression kit is in the procedure

room and both physician and trainee staff are trained in needle decompression for tension capnoperitoneum if required during or after the procedure. There has not been any significant morbidity or mortality reported till date as a consequence of capnoperitoneum/pneumoperitoneum.

Other reported adverse events: One case of post-procedure pulmonary embolism in the setting of known hypercoagulable state and prior thrombotic event was reported[47]. There was one reported death but it was not procedural-related. Other adverse events included one case of post-procedure dysphagia and one case of pneumonia[37,48]. Regarding infection risk, there was one reported case of peri-gastric intraperitoneal abscess, which was successfully treated with antibiotics alone[2].

Surgical pyloroplasty with or without gastric pacemaker has shown successful outcomes in gastroparesis. A study comparing outcomes of POP with surgical pyloroplasty showed POP has average shorter operative time, less intraprocedural blood loss and less length of stay. Overall complication rates as well as need for post procedure intensive care unit admissions were also significantly lower for POP arm[76]. However, data in this area remains limited and till date there is no randomized controlled trial comparing surgical outcomes with endoscopic pyloromyotomy.

Predictive factors

The existing data on safety and efficacy of endoscopic pyloromyotomy in gastroparesis is limited by its small size and the retrospective nature of published studies, making it difficult to determine its validity. There is still significant lack of clarity on selecting appropriate patients for this intervention. At our center, we offer POP to the patients with refractory gastroparesis who have failed or not a candidate for medical treatment (prokinetic agents), who are not on narcotics regularly and who do not have pain predominant disease due to concern for overlapping functional pain, which is unlikely to respond to pylorus-directed therapy.

Multiple clinical parameters were evaluated as potential predictive factors of POP. Gonzalez *et al*[2] reported diabetes and female gender as predictors of poorer outcomes, but this was not shown in subsequent studies[48,61,62]. Outcomes of POP between diabetic *vs* non-diabetic gastroparesis remain conflicting. Jacques *et al*[61] showed favorable outcomes in diabetic gastroparesis post POP with the use of EndoFLIP. Pyloric physiology after POP including pyloric pressure, pyloric distensibility as well as pyloric diameter was shown to have improved more in diabetic patients as compared to non-diabetic cohort[61]. In contrast, Rodriguez *et al*[48] showed best response of POP were achieved in idiopathic and post-surgical gastroparesis while diabetic gastroparesis patients with advanced macrovascular changes such as nephropathy had worse outcomes. Mekaroonkamol *et al*[56] performed a comparative analysis between diabetic and non-diabetic cohort. Multivariate linear regression models did not show a significant association between etiology of gastroparesis and clinical improvement, rather there was a significant correlation between the duration of disease and a decrease in GCSI. Exact impact of diabetes and etiology of gastroparesis on outcomes of POP remain unclear at this point

Certain characteristics of the pylorus such as its diameter, cross-sectional area, distensibility, and compliance are known to relate to severity of gastroparesis symptoms[22,23,77]; for example, decreased pyloric diameter and cross-sectional area is associated with post-prandial fullness and early satiety[22]. Such association can explain the clinical response of POP and suggested the possible utility of pyloric measurements as a predicting tool for the procedure.

The factors predicting favorable response to POP remains unknown. Few studies have used EndoFLIP as a surrogate marker to assess pyloric sphincter indices in assessing response to POP. Malik *et al*[47] showed that while average pyloric pressure decreases, cross-sectional area as well as pyloric diameter increase significantly after POP. The only parameter associated with clinical success was increased cross-sectional area after POP, which is consistent with a prior study by the same group that found the cross sectional area to have an inverse correlation with symptoms of gastroparesis[22]. However, only a few patients (4/9) had a complete EndoFLIP measurement in this study. In contrast, the study by Jacques *et al*[61] showed with EndoFLIP use, increase in pyloric channel diameter and distensibility index was most marked in diabetic patients as well as distensibility index < 9.2 mm²/mmHg was associated with favorable outcomes after POP. Hence, the approach of using pyloric physiologic measurements to predict outcome of POP appear to be physiologically sound, further studies to validate its use are warranted.

As Malik *et al*[47] showed prior response to intrapyloric botulinum injection can be a good predictor for clinical success after POP, Rodriguez *et al*[48] took a similar approach by selecting patients for POP based on their response to intrapyloric botulinum injection, which is the least invasive pylorus directed therapy prior to

subjecting patients to either endoscopic or surgical pyloroplasty. Although the study had significant improvement in post procedure GCSI and GES, the clinical response rate was similar to other studies where clinical improvement from intrapyloric botulinum injection was not used as an inclusion criteria. There was also no direct comparison of patients who received intrapyloric botulinum injection *vs* those who didn't. Hence, it is difficult to draw any clinical conclusion on the benefit of pre-POP botulinum toxin injection, especially when 2 large randomized control trials did not demonstrate the benefit of this intervention in gastroparesis treatment and there was also a theoretical risk of submucosal fibrosis from such injection, potentially complicating a subsequent POP.

Since POP is relatively newer intervention, appropriate learning curve for POP is not yet well defined. Recently study by Suresh *et al*[78] looked into learning curve for POP and suggested about 18 procedures were required to achieve procedural efficiency (defined as < 60 min) and continued improvement in efficiency as furthermore procedures were performed. However, outcomes of procedure based on endoscopist's experience were not assessed in this study. When compared to data about learning curve for laparoscopic pyloromyotomy, about 30 cases are required to develop procedural efficiency however outcomes did not differ based on operator's experience[79].

CONCLUSION

POP has shown a promising outcome as a minimally invasive option for treatment of refractory gastroparesis. In experienced hands in high volume center, it is technically feasible with a low risk of adverse events. It significantly reduces gastroparesis symptoms at least in up to 18-mo period with nausea/vomiting being the most-responsive symptoms. In addition, it can improve quality of life and reduce hospitalization rate. Predictors of clinical outcomes and utility of pyloric physiologic measurements need to be further investigated. While initial data has shown promising results, future large multicenter trials with sham group comparison will be helpful in further assessing outcomes of POP as a standard of care approach for gastroparesis.

REFERENCES

1   **Wang YR**, Fisher RS, Parkman HP. Gastroparesis-related hospitalizations in the United States: Trends, characteristics, and outcomes, 1995-2004. *Am J Gastroenterol* 2008; **103**: 313-322 [PMID: 18047541 DOI: 10.1111/j.1572-0241.2007.01658.x]
2   **Gonzalez JM**, Benezech A, Vitton V, Barthet M. G-POEM with antro-pyloromyotomy for the treatment of refractory gastroparesis: Mid-term follow-up and factors predicting outcome. *Aliment Pharmacol Ther* 2017; **46**: 364-370 [PMID: 28504312 DOI: 10.1111/apt.14132]
3   **Parkman HP**, Camilleri M, Farrugia G, McCallum RW, Bharucha AE, Mayer EA, Tack JF, Spiller R, Horowitz M, Vinik AI, Galligan JJ, Pasricha PJ, Kuo B, Szarka LA, Marciani L, Jones K, Parrish CR, Sandroni P, Abell T, Ordog T, Hasler W, Koch KL, Sanders K, Norton NJ, Hamilton F. Gastroparesis and functional dyspepsia: Excerpts from the AGA/ANMS meeting. *Neurogastroenterol Motil* 2010; **22**: 113-133 [PMID: 20003077 DOI: 10.1111/j.1365-2982.2009.01434.x]
4   **Lacy BE**, Crowell MD, Mathis C, Bauer D, Heinberg LJ. Gastroparesis: Quality of Life and Health Care Utilization. *J Clin Gastroenterol* 2018; **52**: 20-24 [PMID: 27775961 DOI: 10.1097/MCG.0000000000000728]
5   **Parkman HP**, Hasler WL, Fisher RS; American Gastroenterological Association. American Gastroenterological Association technical review on the diagnosis and treatment of gastroparesis. *Gastroenterology* 2004; **127**: 1592-1622 [PMID: 15521026 DOI: 10.1053/j.gastro.2004.09.055]
6   **Schey R**, Saadi M, Midani D, Roberts AC, Parupalli R, Parkman HP. Domperidone to Treat Symptoms of Gastroparesis: Benefits and Side Effects from a Large Single-Center Cohort. *Dig Dis Sci* 2016; **61**: 3545-3551 [PMID: 27530760 DOI: 10.1007/s10620-016-4272-5]
7   **Williams PA**, Nikitina Y, Kedar A, Lahr CJ, Helling TS, Abell TL. Long-term effects of gastric stimulation on gastric electrical physiology. *J Gastrointest Surg* 2013; **17**: 50-5; discussion p.55-6 [PMID: 22956404 DOI: 10.1007/s11605-012-2020-5]
8   **Camilleri M**, Parkman HP, Shafi MA, Abell TL, Gerson L; American College of Gastroenterology. Clinical guideline: Management of gastroparesis. *Am J Gastroenterol* 2013; **108**: 18-37; quiz 38 [PMID: 23147521 DOI: 10.1038/ajg.2012.373]
9   **Mearin F**, Camilleri M, Malagelada JR. Pyloric dysfunction in diabetics with recurrent nausea and vomiting. *Gastroenterology* 1986; **90**: 1919-1925 [PMID: 3699409 DOI: 10.1016/0016-5085(86)90262-3]
10   **Bortolotti M**. Gastric electrical stimulation for gastroparesis: A goal greatly pursued, but not yet attained. *World J Gastroenterol* 2011; **17**: 273-282 [PMID: 21253385 DOI: 10.3748/wjg.v17.i3.273]
11   **Huizinga JD**, Lammers WJ. Gut peristalsis is governed by a multitude of cooperating mechanisms. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G1-G8 [PMID: 18988693 DOI: 10.1152/ajpgi.90380.2008]
12   **Soffer EE**. Gastric electrical stimulation for gastroparesis. *J Neurogastroenterol Motil* 2012; **18**: 131-137 [PMID: 22523722 DOI: 10.5056/jnm.2012.18.2.131]
13   **Abell T**, McCallum R, Hocking M, Koch K, Abrahamsson H, Leblanc I, Lindberg G, Konturek J, Nowak T, Quigley EM, Tougas G, Starkebaum W. Gastric electrical stimulation for medically refractory

gastroparesis. *Gastroenterology* 2003; **125**: 421-428 [PMID: 12891544 DOI: 10.1016/S0016-5085(03)00878-3]

14   **Abell TL**, Van Cutsem E, Abrahamsson H, Huizinga JD, Konturek JW, Galmiche JP, Voeller G, Filez L, Everts B, Waterfall WE, Domschke W, Bruley des Varannes S, Familoni BO, Bourgeois IM, Janssens J, Tougas G. Gastric electrical stimulation in intractable symptomatic gastroparesis. *Digestion* 2002; **66**: 204-212 [PMID: 12592096 DOI: 10.1159/000068359]

15   **Ahuja NK**, Clarke JO. Pyloric Therapies for Gastroparesis. *Curr Treat Options Gastroenterol* 2017; **15**: 230-240 [PMID: 28124202 DOI: 10.1007/s11938-017-0124-4]

16   **McCallum RW**, Snape W, Brody F, Wo J, Parkman HP, Nowak T. Gastric electrical stimulation with Enterra therapy improves symptoms from diabetic gastroparesis in a prospective study. *Clin Gastroenterol Hepatol* 2010; **8**: 947-54; quiz e116 [PMID: 20538073 DOI: 10.1016/j.cgh.2010.05.020]

17   **Gourcerol G**, Huet E, Vandaele N, Chaput U, Leblanc I, Bridoux V, Michot F, Leroi AM, Ducrotté P. Long term efficacy of gastric electrical stimulation in intractable nausea and vomiting. *Dig Liver Dis* 2012; **44**: 563-568 [PMID: 22387288 DOI: 10.1016/j.dld.2012.01.013]

18   **Levinthal DJ**, Bielefeldt K. Systematic review and meta-analysis: Gastric electrical stimulation for gastroparesis. *Auton Neurosci* 2017; **202**: 45-55 [PMID: 27085627 DOI: 10.1016/j.autneu.2016.03.004]

19   **Abell TL**, Johnson WD, Kedar A, Runnels JM, Thompson J, Weeks ES, Minocha A, Griswold ME. A double-masked, randomized, placebo-controlled trial of temporary endoscopic mucosal gastric electrical stimulation for gastroparesis. *Gastrointest Endosc* 2011; **74**: 496-503.e3 [PMID: 21872708 DOI: 10.1016/j.gie.2011.05.022]

20   **Frøkjaer JB**, Ejskjaer N, Rask P, Andersen SD, Gregersen H, Drewes AM, Funch-Jensen P. Central neuronal mechanisms of gastric electrical stimulation in diabetic gastroparesis. *Scand J Gastroenterol* 2008; **43**: 1066-1075 [PMID: 18609155 DOI: 10.1080/00365520802028221]

21   **Bielefeldt K**. Adverse events of gastric electrical stimulators recorded in the Manufacturer and User Device Experience (MAUDE) Registry. *Auton Neurosci* 2017; **202**: 40-44 [PMID: 26850819 DOI: 10.1016/j.autneu.2016.01.003]

22   **Malik Z**, Sankineni A, Parkman HP. Assessing pyloric sphincter pathophysiology using EndoFLIP in patients with gastroparesis. *Neurogastroenterol Motil* 2015; **27**: 524-531 [PMID: 25712043 DOI: 10.1111/nmo.12522]

23   **Snape WJ**, Lin MS, Agarwal N, Shaw RE. Evaluation of the pylorus with concurrent intraluminal pressure and EndoFLIP in patients with nausea and vomiting. *Neurogastroenterol Motil* 2016; **28**: 758-764 [PMID: 26813266 DOI: 10.1111/nmo.12772]

24   **Camilleri M**, Malagelada JR, Brown ML, Becker G, Zinsmeister AR. Relation between antral motility and gastric emptying of solids and liquids in humans. *Am J Physiol* 1985; **249**: G580-G585 [PMID: 4061646 DOI: 10.1152/ajpgi.1985.249.5.G580]

25   **Rao SS**, Vemuri S, Harris B, Schulze K. Fundic balloon distension stimulates antral and duodenal motility in man. *Dig Dis Sci* 2002; **47**: 1015-1019 [PMID: 12018896 DOI: 10.1023/A:1015077721427]

26   **Davis BR**, Sarosiek I, Bashashati M, Alvarado B, McCallum RW. The Long-Term Efficacy and Safety of Pyloroplasty Combined with Gastric Electrical Stimulation Therapy in Gastroparesis. *J Gastrointest Surg* 2017; **21**: 222-227 [PMID: 27896652 DOI: 10.1007/s11605-016-3327-4]

27   **Sarosiek I**, Forster J, Lin Z, Cherry S, Sarosiek J, McCallum R. The addition of pyloroplasty as a new surgical approach to enhance effectiveness of gastric electrical stimulation therapy in patients with gastroparesis. *Neurogastroenterol Motil* 2013; **25**: 134-e80 [PMID: 23113904 DOI: 10.1111/nmo.12032]

28   **Hibbard ML**, Dunst CM, Swanström LL. Laparoscopic and endoscopic pyloroplasty for gastroparesis results in sustained symptom improvement. *J Gastrointest Surg* 2011; **15**: 1513-1519 [PMID: 21720926 DOI: 10.1007/s11605-011-1607-6]

29   **Masqusi S**, Velanovich V. Pyloroplasty with fundoplication in the treatment of combined gastroesophageal reflux disease and bloating. *World J Surg* 2007; **31**: 332-336 [PMID: 17219264 DOI: 10.1007/s00268-006-0723-z]

30   **Arts J**, van Gool S, Caenepeel P, Verbeke K, Janssens J, Tack J. Influence of intrapyloric botulinum toxin injection on gastric emptying and meal-related symptoms in gastroparesis patients. *Aliment Pharmacol Ther* 2006; **24**: 661-667 [PMID: 16907899 DOI: 10.1111/j.1365-2036.2006.03019.x]

31   **Bromer MQ**, Friedenberg F, Miller LS, Fisher RS, Swartz K, Parkman HP. Endoscopic pyloric injection of botulinum toxin A for the treatment of refractory gastroparesis. *Gastrointest Endosc* 2005; **61**: 833-839 [PMID: 15933684 DOI: 10.1016/S0016-5107(05)00328-7]

32   **Khashab MA**, Besharati S, Ngamruengphong S, Kumbhari V, El Zein M, Stein EM, Tieu A, Mullin GE, Dhalla S, Nandwani MC, Singh V, Canto MI, Kalloo AN, Clarke JO. Refractory gastroparesis can be successfully managed with endoscopic transpyloric stent placement and fixation (with video). *Gastrointest Endosc* 2015; **82**: 1106-1109 [PMID: 26253017 DOI: 10.1016/j.gie.2015.06.051]

33   **Clarke JO**, Snape WJ. Pyloric sphincter therapy: Botulinum toxin, stents, and pyloromyotomy. *Gastroenterol Clin North Am* 2015; **44**: 127-136 [PMID: 25667028 DOI: 10.1016/j.gtc.2014.11.010]

34   **Arts J**, Holvoet L, Caenepeel P, Bisschops R, Sifrim D, Verbeke K, Janssens J, Tack J. Clinical trial: A randomized-controlled crossover study of intrapyloric injection of botulinum toxin in gastroparesis. *Aliment Pharmacol Ther* 2007; **26**: 1251-1258 [PMID: 17944739 DOI: 10.1111/j.1365-2036.2007.03467.x]

35   **Friedenberg FK**, Palit A, Parkman HP, Hanlon A, Nelson DB. Botulinum toxin A for the treatment of delayed gastric emptying. *Am J Gastroenterol* 2008; **103**: 416-423 [PMID: 18070232 DOI: 10.1111/j.1572-0241.2007.01676.x]

36   **Inoue H**, Minami H, Kobayashi Y, Sato Y, Kaga M, Suzuki M, Satodate H, Odaka N, Itoh H, Kudo S. Peroral endoscopic myotomy (POEM) for esophageal achalasia. *Endoscopy* 2010; **42**: 265-271 [PMID: 20354937 DOI: 10.1055/s-0029-1244080]

37   **Shlomovitz E**, Pescarus R, Cassera MA, Sharata AM, Reavis KM, Dunst CM, Swanström LL. Early human experience with per-oral endoscopic pyloromyotomy (POP). *Surg Endosc* 2015; **29**: 543-551 [PMID: 25106716 DOI: 10.1007/s00464-014-3720-6]

38   **Pasricha PJ**, Hawari R, Ahmed I, Chen J, Cotton PB, Hawes RH, Kalloo AN, Kantsevoy SV, Gostout CJ. Submucosal endoscopic esophageal myotomy: a novel experimental approach for the treatment of achalasia. *Endoscopy* 2007; **39**: 761-764 [PMID: 17703382 DOI: 10.1016/j.gie.2007.03.093]

39   **Khashab MA**, Stein E, Clarke JO, Saxena P, Kumbhari V, Chander Roland B, Kalloo AN, Stavropoulos S, Pasricha P, Inoue H. Gastric peroral endoscopic myotomy for refractory gastroparesis: First human endoscopic pyloromyotomy (with video). *Gastrointest Endosc* 2013; **78**: 764-768 [PMID: 24120337 DOI: 10.1016/j.gie.2013.07.019]

40   **Chaves DM**, de Moura EG, Mestieri LH, Artifon EL, Sakai P. Endoscopic pyloromyotomy via a gastric submucosal tunnel dissection for the treatment of gastroparesis after surgical vagal lesion. *Gastrointest Endosc* 2014; **80**: 164 [PMID: 24836745 DOI: 10.1016/j.gie.2014.03.045]

41   **Chung H**, Dallemagne B, Perretta S, Lee SK, Shin SK, Park JC, Marescaux J, Lee YC. Endoscopic pyloromyotomy for postesophagectomy gastric outlet obstruction. *Endoscopy* 2014; **46** Suppl 1 UCTN: E345-E346 [PMID: 25090473 DOI: 10.1055/s-0034-1377599]

42   **Kawai M**, Peretta S, Burckhardt O, Dallemagne B, Marescaux J, Tanigawa N. Endoscopic pyloromyotomy: A new concept of minimally invasive surgery for pyloric stenosis. *Endoscopy* 2012; **44**: 169-173 [PMID: 22271027 DOI: 10.1055/s-0031-1291475]

43   **Dacha S**, Mekaroonkamol P, Li L, Shahnavaz N, Sakaria S, Keilin S, Willingham F, Christie J, Cai Q. Outcomes and quality-of-life assessment after gastric per-oral endoscopic pyloromyotomy (with video). *Gastrointest Endosc* 2017; **86**: 282-289 [PMID: 28161449 DOI: 10.1016/j.gie.2017.01.031]

44   **Jacques J**, Legros R, Monteil J, Loustaud-Ratti V, Sautereau D. Gastric peroral endoscopic pyloromyotomy as a salvage therapy for refractory gastroparesis: A case series of different subtypes. *Neurogastroenterol Motil* 2017; 29 [PMID: 28028897 DOI: 10.1111/nmo.12932]

45   **Khashab MA**, Ngamruengphong S, Carr-Locke D, Bapaye A, Benias PC, Serouya S, Dorwat S, Chaves DM, Artifon E, de Moura EG, Kumbhari V, Chavez YH, Bukhari M, Hajiyeva G, Ismail A, Chen YI, Chung H. Gastric per-oral endoscopic myotomy for refractory gastroparesis: Results from the first multicenter study on endoscopic pyloromyotomy (with video). *Gastrointest Endosc* 2017; **85**: 123-128 [PMID: 27354102 DOI: 10.1016/j.gie.2016.06.048]

46   **Mekaroonkamol P**, Li LY, Dacha S, Xu Y, Keilin SD, Willingham FF, Cai Q. Gastric peroral endoscopic pyloromyotomy (G-POEM) as a salvage therapy for refractory gastroparesis: A case series of different subtypes. *Neurogastroenterol Motil* 2016; **28**: 1272-1277 [PMID: 27197717 DOI: 10.1111/nmo.12854]

47   **Malik Z**, Kataria R, Modayil R, Ehrlich AC, Schey R, Parkman HP, Stavropoulos SN. Gastric Per Oral Endoscopic Myotomy (G-POEM) for the Treatment of Refractory Gastroparesis: Early Experience. *Dig Dis Sci* 2018; **63**: 2405-2412 [PMID: 29468376 DOI: 10.1007/s10620-018-4976-9]

48   **Rodriguez JH**, Haskins IN, Strong AT, Plescia RL, Allemang MT, Butler RS, Cline MS, El-Hayek K, Ponsky JL, Kroh MD. Per oral endoscopic pyloromyotomy for refractory gastroparesis: Initial results from a single institution. *Surg Endosc* 2017; **31**: 5381-5388 [PMID: 28567693 DOI: 10.1007/s00464-017-5619-5]

49   **Chung H**, Khashab MA. Gastric Peroral Endoscopic Myotomy. *Clin Endosc* 2018; **51**: 28-32 [PMID: 29397648 DOI: 10.5946/ce.2018.001]

50   **Li L**, Spandorfer R, Qu C, Yang Y, Liang S, Chen H, Xue H, Cai Q. Gastric per-oral endoscopic myotomy for refractory gastroparesis: A detailed description of the procedure, our experience, and review of the literature. *Surg Endosc* 2018; **32**: 3421-3431 [PMID: 29435752 DOI: 10.1007/s00464-018-6112-5]

51   **Xue HB**, Fan HZ, Meng XM, Cristofaro S, Mekaroonkamol P, Dacha S, Li LY, Fu XL, Zhan SH, Cai Q. Fluoroscopy-guided gastric peroral endoscopic pyloromyotomy (G-POEM): A more reliable and efficient method for treatment of refractory gastroparesis. *Surg Endosc* 2017; **31**: 4617-4624 [PMID: 28409375 DOI: 10.1007/s00464-017-5524-y]

52   **Koul A**, Dacha S, Mekaroonkamol P, Li X, Li L, Shahnavaz N, Keilin S, Willingham FF, Christie J, Cai Q. Fluoroscopic gastric peroral endoscopic pyloromyotomy (G-POEM) in patients with a failed gastric electrical stimulator. *Gastroenterol Rep (Oxf)* 2018; **6**: 122-126 [PMID: 29780600 DOI: 10.1093/gastro/gox040]

53   **Chaves DM**, Gusmon CC, Mestieri LH, de Moura EG, Veras MM, Pessorrusso FC, Sakai P. A new technique for performing endoscopic pyloromyotomy by gastric submucosal tunnel dissection. *Surg Laparosc Endosc Percutan Tech* 2014; **24**: e92-e94 [PMID: 24887546 DOI: 10.1097/SLE.0b013e31829cec0e]

54   **Hernández-Mondragón OV**, Solórzano-Pineda OM, Blancas-Valencia JM, González-Martínez MA, Villanueva-Pérez RM. Gastric per-oral endoscopic pyloromyotomy in the treatment of refractory gastroparesis: Report on the first case performed in Mexico. *Rev Gastroenterol Mex* 2018; **83**: 459-461 [PMID: 28684033 DOI: 10.1016/j.rgmx.2017.03.006]

55   **Allemang MT**, Strong AT, Haskins IN, Rodriguez J, Ponsky JL, Kroh M. How I Do It: Per-Oral Pyloromyotomy (POP). *J Gastrointest Surg* 2017; **21**: 1963-1968 [PMID: 28752406 DOI: 10.1007/s11605-017-3510-2]

56   **Mekaroonkamol P**, Dacha S, Wang L, Li X, Jiang Y, Li L, Li T, Shahnavaz N, Sakaria S, LeVert F, Keilin S, Willingham F, Christie J, Cai Q. Gastric PerOral Endoscopic Pyloromyotomy Reduces Symptoms, Increases Quality of Life, and Reduces Healthcare Usage For Patients With Gastroparesis. *Clini Gastroenterol Hepatol* 2019; **17**: 82-89 [PMID: 29660525 DOI: 10.1016/j.cgh.2018.04.016]

57   **ASGE Technology Committee**. Lo SK, Fujii-Lau LL, Enestvedt BK, Hwang JH, Konda V, Manfredi MA, Maple JT, Murad FM, Pannala R, Woods KL, Banerjee S. The use of carbon dioxide in gastrointestinal endoscopy. *Gastrointest Endosc* 2016; **83**: 857-865 [PMID: 26946413 DOI: 10.1016/j.gie.2016.01.046]

58   **Kahaleh M**, Gonzalez JM, Xu MM, Andalib I, Gaidhane M, Tyberg A, Saumoy M, Baptista Marchena AJ, Barthet M. Gastric peroral endoscopic myotomy for the treatment of refractory gastroparesis: A multicenter international experience. *Endoscopy* 2018; **50**: 1053-1058 [PMID: 29649841 DOI: 10.1055/a-0596-7199]

59   **Hustak R**, Vackova Z, Rabekova Z, Spicak J, Martinek J. Su1269 per-oral endoscopic pyloromyotomy (g-poem) in the treatment of refractory gastroparesisâmid-term single centre experience. Gastrointestinal Endoscopy 2018; 87: AB304-AB305. [DOI: 10.1016/j.gie.2018.04.1656]

60   **Bapaye A**, Dubale N, Pujari R, Kulkarni A, Jajoo Naval R, Vyas V, Mahadik M. Peroral endoscopic pyloromyotomy for delayed postoperative gastroparesis. *Endoscopy* 2015; **47**: E581-E582 [PMID: 26649470 DOI: 10.1055/s-0034-1393368]

61   **Jacques J**, Pagnon L, Hure F, Legros R, Crepin S, Fauchais AL, Palat S, Ducrotté P, Marin B, Fontaine S, Boubaddi NE, Clement MP, Sautereau D, Loustaud-Ratti V, Gourcerol G, Monteil J. Peroral endoscopic pyloromyotomy is efficacious and safe for refractory gastroparesis: Prospective trial with assessment of pyloric function. *Endoscopy* 2019; **51**: 40-49 [PMID: 29895073 DOI: 10.1055/a-0628-6639]

62   **Mekaroonkamol P**, Patel V, Shah R, Li B, Tao J, Guan Q, Chen H, Shahnavaz N, Sakaria S, Dacha S. 838 duration of the disease, rather than the etiology of gastroparesis, is the key predictive factor for clinical response after gastric per oral endoscopic pyloromyotomy (GPOEM). *Gastrointest Endosc* 2018; **87**: AB119-AB120 [DOI: 10.1016/j.gie.2018.04.1311]

63   **Camilleri M**, Szarka LA. POEMs for gastroparesis. *Gastrointest Endosc* 2017; **85**: 129-131 [PMID:

27986106 DOI: 10.1016/j.gie.2016.07.012]

64   **Horowitz M**, Harding PE, Maddox AF, Wishart JM, Akkermans LM, Chatterton BE, Shearman DJ. Gastric and oesophageal emptying in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1989; **32**: 151-159 [PMID: 2753246 DOI: 10.1007/BF00265086]

65   **Horowitz M**, Harding PE, Maddox A, Maddern GJ, Collins PJ, Chatterton BE, Wishart J, Shearman DJ. Gastric and oesophageal emptying in insulindependent diabetes mellitus. *J Gastroenterol Hepatol* 1986; **1**: 97-113 [DOI: 10.1111/j.1440-1746.1986.tb00104.x]

66   **Khayyam U**, Sachdeva P, Gomez J, Ramzan Z, Smith MS, Maurer AH, Fisher RS, Parkman HP. Assessment of symptoms during gastric emptying scintigraphy to correlate symptoms to delayed gastric emptying. *Neurogastroenterol Motil* 2010; **22**: 539-545 [PMID: 20082665 DOI: 10.1111/j.1365-2982.2009.01454.x]

67   **Mekaroonkamol P**, Dacha S, Wang L, Li X, Jiang Y, Li L, Li T, Shahnavaz N, Sakaria S, LeVert F, Keilin S, Willingham FF, Christie J, Cai Q. Patient-Reported Quality of Life Assessment and Utilization of Health Care After Gastric Peroral Endoscopic Pyloromyotomy (GPOEM). *Am J Gastroenterol* 2017; **112**: S429-S490 [DOI: 10.1038/ajg.2017.304]

68   **Abell TL**, Camilleri M, Donohoe K, Hasler WL, Lin HC, Maurer AH, McCallum RW, Nowak T, Nusynowitz ML, Parkman HP, Shreve P, Szarka LA, Snape WJ, Ziessman HA; American Neurogastroenterology and Motility Society and the Society of Nuclear Medicine. Consensus recommendations for gastric emptying scintigraphy: A joint report of the American Neurogastroenterology and Motility Society and the Society of Nuclear Medicine. *Am J Gastroenterol* 2008; **103**: 753-763 [PMID: 18028513 DOI: 10.1111/j.1572-0241.2007.01636.x]

69   **Gonlachanvit S**, Maurer AH, Fisher RS, Parkman HP. Regional gastric emptying abnormalities in functional dyspepsia and gastro-oesophageal reflux disease. *Neurogastroenterol Motil* 2006; **18**: 894-904 [PMID: 16961692 DOI: 10.1111/j.1365-2982.2006.00811.x]

70   **Maurer AH**, Parkman HP. Update on gastrointestinal scintigraphy. *Semin Nucl Med* 2006; **36**: 110-118 [PMID: 16517233 DOI: 10.1053/j.semnuclmed.2005.12.003]

71   **Mekaroonkamol P**, Li L, Cai Q. The role of pyloric manometry in gastric per-oral endoscopic pyloromyotomy (G-POEM): Response to Jacques *et al*. *Neurogastroenterol Motil* 2017; **29** [PMID: 28028900 DOI: 10.1111/nmo.12949]

72   **Ata-Lawenko RM**, Lee YY. Emerging Roles of the Endolumenal Functional Lumen Imaging Probe in Gastrointestinal Motility Disorders. *J Neurogastroenterol Motil* 2017; **23**: 164-170 [PMID: 28013295 DOI: 10.5056/jnm16171]

73   **Kwiatek MA**, Pandolfino JE, Hirano I, Kahrilas PJ. Esophagogastric junction distensibility assessed with an endoscopic functional luminal imaging probe (EndoFLIP). *Gastrointest Endosc* 2010; **72**: 272-278 [PMID: 20541755 DOI: 10.1016/j.gie.2010.01.069]

74   **Geyl S**, Legros R, Charissou A, Mesturoux L, Couquet CY, Carrier P, Brayette A, El-Ouafi Z, Loustaud-Ratti V, Sautereau D, Monteil J, Jacques J. Peroral endoscopic pyloromyotomy accelerates gastric emptying in healthy pigs: Proof of concept. *Endosc Int Open* 2016; **4**: E796-E799 [PMID: 27556100 DOI: 10.1055/s-0042-108192]

75   **Jung Y**, Lee J, Gromski MA, Kato M, Rodriguez S, Chuttani R, Matthes K. Assessment of the length of myotomy in peroral endoscopic pyloromyotomy (G-POEM) using a submucosal tunnel technique (video). *Surg Endosc* 2015; **29**: 2377-2384 [PMID: 25424365 DOI: 10.1007/s00464-014-3948-1]

76   **Landreneau JP**, Strong AT, El-Hayek K, Tu C, Villamere J, Ponsky JL, Kroh MD, Rodriguez JH. Laparoscopic pyloroplasty versus endoscopic per-oral pyloromyotomy for the treatment of gastroparesis. *Surg Endosc* 2018 [PMID: 30019220 DOI: 10.1007/s00464-018-6342-6]

77   **Gourcerol G**, Tissier F, Melchior C, Touchais JY, Huet E, Prevost G, Leroi AM, Ducrotte P. Impaired fasting pyloric compliance in gastroparesis and the therapeutic response to pyloric dilatation. *Aliment Pharmacol Ther* 2015; **41**: 360-367 [PMID: 25523288 DOI: 10.1111/apt.13053]

78   **Suresh S**, Tyberg A, Martínez MG, Zamarripa F, Lambroza A, Gaidhane M, Nieto J, Kahaleh M. Su1280 gastric peroral endoscopic myotomy: a specific learning curve. *Gastrointest Endosc* 2018; **87**: AB309-AB310 [DOI: 10.1016/j.gie.2018.04.1667]

79   **Kim SS**, Lau ST, Lee SL, Waldhausen JH. The learning curve associated with laparoscopic pyloromyotomy. *J Laparoendosc Adv Surg Tech A* 2005; **15**: 474-477 [PMID: 16185119 DOI: 10.1089/lap.2005.15.474]

ORIGINAL ARTICLE

Basic Study

Dbx2 exhibits a tumor-promoting function in hepatocellular carcinoma cell lines *via* regulating Shh-Gli1 signaling

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Abstract

BACKGROUND
Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. HCC patients suffer from a high mortality-to-incidence ratio and low cure rate since we still have no specific and effective treatment. Although tremendous advances have been made in the investigation of HCC, the specific mechanisms of the progression of this disease are still only partially established. Hence, more research is needed to elucidate the underlying potential mechanisms to develop effective strategies for HCC.

AIM
To determine the role of developing brain homeobox 2 (Dbx2) gene in promoting the development of HCC.

METHODS
Dbx2 expression in clinical specimens and HCC cell lines was detected by Western blot (WB) and immunohistochemistry. Gain and loss of Dbx2 function assays were performed *in vitro* and *in vivo*. Cell viability assays were used to investigate cell growth, flow cytometry was employed to assess cell cycle and apoptosis, and trans-well assays were conducted to evaluate cell migration,

invasion, and metastasis. The expression of key molecules in the sonic hedgehog (Shh) signaling was determined by WB.

RESULTS
Compared to matched adjacent non-tumorous tissues, Dbx2 was overexpressed in 5 HCC cell lines and 76 surgically resected HCC tissues. Dbx2 overexpression was correlated with large tumor size. Both gain and loss of function assays indicated that Dbx2 promoted HCC cell proliferation by facilitating the transition from G1 to S phase, attenuating apoptosis and promoted HCC proliferation, migration, and invasion *in vitro* and *in vivo*. Mechanistically, Dbx2 modulated Shh signaling by enhancing FTCH1 and GLi1 expression in HCC cells that overexpressed Dbx2, which was reversed in HCC cells with Dbx2 knockdown.

CONCLUSION
Our results indicate that Dbx2 is significantly upregulated in HCC tissues and plays significant roles in proliferation and metastasis of HCC cells by activating the Shh pathway.

Key words: Developing brain homeobox 2; Hepatocellular carcinoma; Sonic Hedgehog pathway; Expression; Tumor tissues

Core tip: Developing brain homeobox 2 (Dbx2) plays an important role in cell differentiation and is frequently upregulated in tumor tissues, while its function in hepatocellular carcinoma (HCC) has not been reported. Our results indicate that Dbx2 is obviously upregulated in HCC tissues and strongly correlated with tumor size. Subsequently, both gain and loss of function assays indicated that Dbx2 promoted HCC migration, invasion, and proliferation by facilitating the transition from G1 to S phase and attenuating apoptosis *in vitro* and *in vivo*. Mechanistically, Dbx2 modulates sonic hedgehog signaling by enhancing FTCH1 and GLi1 expression in HCC cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide[1]. HCC patients suffer from a high mortality-to-incidence ratio and low cure rate since we still have no specific and effective treatment[2]. Although tremendous advances have been made in the investigation of HCC, the specific mechanisms of the progression of this disease are still only partially established. Hence, more research is needed to elucidate the underlying potential mechanisms to develop effective strategies for HCC.

The developing brain homeobox (Dbx) gene was first discovered in an embryonic telencephalon of mouse in 1992[3,4]. Dbx2 gene has been reported in mammals and zebrafish. Dbx1 and Dbx2 genes encode a family of homeodomain transcription factors of the H2.0 class that define an intermediate spinal progenitor domain. They are expressed within the p0, p1, and pD6 progenitor domains in vertebrate neural tube[5]. Studies on Dbx2 functions have come to focus on its important roles in neural patterning and differentiation[6-9]. More importantly, Dbx2 participates in neural tube differentiation of mouse embryonic stem cells or regulation of the sonic hedgehog (Shh) signaling pathway, while the detailed underlying mechanisms remain uncovering[10]. We performed an analysis using the cancer genome atlas (TCGA) and found that the 3-yr survival rate was significantly lower in glioma patients with high Dbx2 expression than in patients with low Dbx2 expression ($P < 0.01$). Another study has identified Dbx1, a homology of Dbx2, as a novel candidate biomarker gene in breast carcinogenesis[11]. In a previous study, we found that the methylation level of Dbx2 was significantly lower in 31 early-stage HCC patients than in 27 healthy

controls[12]. These studies indicated that Dbx2 may play an important role in tumor progression. Dbx2 was significantly upregulated in HCC in this study (Figure 1A, B), indicating that Dbx2 is involved in hepatocellular carcinogenesis. Until now, there has been no document to report the role of Dbx2 in malignant cancer. We determined to further investigate the effects of Dbx2 on HCC proliferation and metastasis *in vitro* and *in vivo*.

The Shh signaling pathway plays crucial roles in embryonic patterning and adult tissue homeostasis[13,14]. It was reported that the Shh signaling pathway was activated aberrantly in tumorigenesis and development of gastrointestinal, pancreatic, breast, lung, ovarian cancers and so on[15-18]. Some studies concluded that Shh signaling pathway was involved in invasion and metastasis in various malignant tumors[19-24]. Hyperactivation of the Shh pathway in different kinds of malignant cancers including HCC induced uncontrolled progression of cancer characteristics, such as proliferation, migration, and invasion[25,26]. An antibody and several small-molecule antagonists targeting the Shh pathway are now under development due to the important role of Shh in cancer progression[27]. Scarcely any meaningful outcome could be obtained in clinical trials, although tremendous efforts have been made. This is mainly because of an insufficient understanding of the mechanisms of tumor occurrence and progression[28]. Therefore, it is indispensable to reveal relevant molecular mechanisms of Shh pathways in hepatocarcinogenesis.

Understanding how Dbx2 plays a role in HCC may help us acquaint potential molecular mechanisms of hepatocellular carcinogenesis and progression and facilitate newly therapeutically targeted strategies for preventing or retarding HCC progression. In this study, we evaluated Dbx2 expression in matched surgically resected HCC tissues and adjacent non-tumor tissues and investigated the biological effects of Dbx2 and Shh pathway in HCC cells to explore the possible molecular mechanisms involved.

MATERIALS AND METHODS

Patients and specimen collection
Here, 76 HCC patients with tumor tissues and paired adjacent non-tumor tissues who had yet to undergo chemotherapy were recruited from the Peking University Cancer Hospital and People's Liberation Army General Hospital between August 2015 and October 2017 and included in this study. All patients had provided informed consent for their samples to be investigated in a future study. The sample collection procedure was approved by the Ethics Committee of Peking University Cancer Hospital, Beijing, China.

Hepatocellular carcinoma cell lines
Five HCC cell lines, HepG2, Li-7, Huh7, Huh7.5.1, and SMMC-7721, and one hepatic epithelial cell line, LO2, were used. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (PBS) (Gibco BRL, Carlsbad, CA, United States), and incubated in a 37 °C incubator with 5% CO_2.

Lentiviral vector transduction of hepatocellular carcinoma cells
Lentiviruses carrying shRNA targeting human Dbx2 lentiviral vectors and lentiviruses carrying overexpressing lentiviral vectors were purchased (GenePharma Co., Shanghai, China). Cells (1.5×10^5/well) transiently transduced with lentiviral vector (at a 20 nM final concentration) were seeded in 24-well plates and collected 24 h post transfection. HCC cells with stable depleted expression or overexpression of endogenous Dbx2 were selected by culturing in medium with puromycin (0.8 µg/mL). The efficiency of transduction was confirmed by Western blot assay.

Cell viability assay
HCC cells with stable overexpression or knockdown of Dbx2 and corresponding control cells (1×10^4/well) were incubated in 96-well plates. Cell viability was measured using a cell counting kit 8 assay (Dojindo, Shanghai, China) according to the manufacturer's protocols. Absorbance at 490 nm was measured using a plate reader once a day after 1-5 d of cell culture. Results were calculated by comparing OD490 to baseline.

Colony formation assay
HCC cells with stable overexpression or knockdown of Dbx2 and corresponding control cells (5×10^3/well) were incubated in a 6-well plate for 2 wk. The number of colonies stained by 5% crystal violet was counted. All experiments were performed in

F

G

H

Figure 1 Developing brain homeobox 2 expression is frequently up-regulated in tumor tissues than in non-tumor tissues from patients with hepatocellular carcinoma. A: Positive developing brain homeobox 2 (Dbx2) expression in tumor tissues and negative Dbx2 expression in non-tumor tissues as revealed by immunohistochemistry (×400); B and C: Dbx2 expression was significantly upregulated in tumor tissues compared with their matched adjacent non-tumor tissues as revealed by immunohistochemistry; D and E: Relative Dbx2 expression in tumor tissues to paired non-tumor tissues; F: There was more Dbx2 expression in hepatocellular carcinoma (HCC) cells than in normal hepatic epithelial LO2 cells; G and H: Dbx2 expression was significantly related with gender and tumor size.

triplicate.

Cell cycle assay

The paired cells were prepared as described above and fixed with 70% ethanol for 24 h at 4 °C. Cells were washed with PBS and stained with 50 μg/mL propidium iodide (PI) (BD Biosciences, NY, United States) for 30 min at room temperature in the dark. Cell cycle was assessed with an FACS scan machine (BD Biosciences, NY, United States) and data were analyzed using ModFit 3.0 software (BD Biosciences, NY, United States).

Cell apoptosis assay

For apoptotic analysis, cells were stained *via* a V-allophycocyanin (V-APC) and PI staining kit (BD Biosciences, NY, United States) according to the manufacturer's instructions, followed by flow cytometry within 1 h. Cell apoptosis was analyzed with WinMDI 2.9 software (BD Biosciences, NY, United States).

Cell migration assay

The paired cells were incubated (5×10^3/well) in a 6-well plate. Cell migration was assessed with a wound-healing assay. The confluent cell surface was scratched with a pipette tip and the width of two flanks of the wound was recorded once a day for 3 d.

Cell invasion assay

The paired cells were suspended in serum-free medium at a density of 2×10^5 cells/mL. Here, 24-well plates and Matrigel invasion assays (BD Biosciences,

Erembodegem, Belgium) were used. Cells (2×10^4) were load into the upper chamber, and 500 μL DMEM and 20% FBS were added to the lower chamber. Cells that passed through the membrane after 24-h incubation were fixed with methanol for 10 min and stained with crystal violet for 10 min. Then the stained cells were counted in five randomly selected microscopic views.

Western blot analysis

Briefly, total proteins extracted from cell pellets were lysed with CytoBuster Protein Extraction Reagent (Merck Millipore, Darmstadt, Germany) and measured using a BCA Protein Assay Kit (Beyotime Biotechnology, Jiangsu, China). About 20 to 50 μg protein of each sample was separated by 8%–15% SDS-PAGE and transferred to nitrocellulose membranes (Sartorius Stedim Biotech, Gottingen, Germany). The membranes were incubated with primary antibody at 4 °C for more than 12 h and then with secondary antibody at room temperature for 1 h. Proteins were visualized with ECL Plus Western Blot Detection Reagents (LOT16327B4, Millipore, United States). We conducted Western blot to evaluate the expression of markers with anti-Histon3 antibody (4499), anti-N-cadherin antibody (13116), anti-E-cadherin antibody (3195), anti-Vimentin antibody (5741), anti-CDK2 antibody (2546), anti-CDK4 antibody (12790), anti-CDK6 antibody (3136), anti-Cyclin D1 antibody (2978), anti-Cyclin A antibody (4656), anti-Cyclin E antibody (20808), anti-p21 antibody (2947), anti-p27 antibody (3686), anti-Bax antibody (5023), anti-bcl-2 antibody (15071), anti-Survivin antibody (2808), anti-Shh antibody (2207), anti-PTCH1 antibody (2468), anti-PTCH2 antibody (2470), anti-SUFU antibody (2522), anti-GLI1 antibody (3538), anti-cleaved caspase-9 antibody (7237), anti-cleaved caspase-8 antibody (9496), and anti-cleaved caspase-3 antibody (9664) purchased from Cell Signaling Technology.

In vivo tumorigenicity

HCC cells with stable overexpression or knockdown of Dbx2 and corresponding control cells (2×10^6/well) were injected subcutaneously into the dorsal right flanks of 6-wk-old female NOD/SCID mice (n = 5/group). Tumor size and mouse weight were measured every 3 d until animal sacrifice or experiment ending. Tumor volume was calculated using the following formula: V = (L × W^2)/2 (V, volume; L, length of tumor; W, width of tumor). All experiments were manipulated in accordance with the guidelines of Peking University Cancer Hospital Animal Care Commission.

Immunohistochemical staining for Dbx2

Four-micrometer-thick FFPE sections were deparaffinized and rehydrated, followed by antigen retrieval in EDTA (pH = 9, ZLI-9069, Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). After treatment with endogenous peroxidase, the sections were incubated with primary anti-Dbx2 monoclonal antibody (1:800, PA5-34391, Thermo, NY, United States) at 4 °C overnight, followed by incubation with relevant IgG-HRP conjugate (PV-6000, Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) and visualization using a 3,3'-diaminobenzidine kit (GK347011, GeneTech, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were calculated with SPSS 21.0 software (SPSS Inc. Chicago, IL, United States). The $\chi 2$-test was used to analyze the relationships between Dbx2 expression and clinical characteristics. The Mann-Whitney *U*-test was used to compare the difference in Dbx2 expression between tumor and non-tumor tissues. The differences in cell or tumor proliferation, and metastatic ability between two groups were compared by repeated measures analysis of variance and the $\chi 2$-test.

RESULTS

Clinicopathological characteristics of the HCC patients

A total of 76 HCC samples were collected in this study. The clinicopathological data including gender, age, tumor size, differentiation, lymph node metastasis, HBV and HCV infection, and serum alpha-fetoprotein (AFP) level are described in Table 1. Men accounted for 84.21% of this cohort (64/76) and the median age of the 76 HCC patients was 56 years (range, 26-76 years).

Dbx2 is significantly up-regulated in HCC

Dbx2 expression was detected in all 76 HCC samples and matched adjacent non-tumor tissues using immunohistochemistry. The Dbx2 positive rate in tumors (81.58%, 62/76) was higher than that in adjacent non-tumor tissues (40.79%, 31/76; P < 0.01) (Figure 1B-D). The proportion of tumor tissues with higher expression than

Table 1 Clinicopathological characteristics of hepatocellular carcinoma patients included in the study			
Characteristic		*n* = 76	Proportion (%)
Gender			
	Male	64	84.21
	Female	12	15.79
Age (yr)		Median age 56	
	<60	52	68.42
	≥60	24	31.58
Differentiation			
	Well	52	68.42
	Poor	24	31.58
Tumor size (cm)			
	< 5	53	69.74
	≥ 5	23	30.26
Lymph node metastasis			
	Yes	16	21.05
	No	60	78.95
HBV infection			
	Yes	41	53.95
	No	35	46.05
HCV infection			
	Yes	4	5.26
	No	72	94.74
Serum alpha-fetoprotein level (ng/mL)			
	< 400	56	73.68
	≥ 400	20	26.32

their respective adjacent non-tumor tissues was 61.84% (47/76), and the proportion with lower expression was 6.58% (5/76) (Figure 1E). Compared with normal hepatic epithelial LO2 cells, Dbx2 expression was up-regulated in HepG2, Li-7, Huh7, Huh7.5.1, and SMMC-7721 cells (Figure 1F), which suggested that Dbx2 may function as an oncogene in HCC.

Correlation of Dbx2 expression with clinical characteristics

To understand the potential mechanism of Dbx2 in HCC, we analyzed Dbx2 expression stratified by different characteristics. As shown in Table 2, Dbx2 expression was found to be significantly closely related to gender (P = 0.038) and tumor size (P = 0.025) (Figure 1G, H), but unrelated to age, differentiation, lymph node metastasis, HBV infection, HCV infection, or serum AFP level. Based on our data, we estimated that Dbx2 expression might be related to T stage of HCC and a poor prognosis.

Dbx2 promotes the proliferation of HCC cells in vitro and in vivo

Gain and loss of function experiments were conducted to assess the biological behavior of Dbx2 *in vitro* and *in vivo*. Cell viability assays showed that ectopic Dbx2 expression (Figure 2A) promoted the growth of SMMC-7721 and Huh7 cells. However, knockdown of Dbx2 inhibited HepG2 and Huh7 cell growth (P < 0.05; Figure 2B). These results were in accord with those obtained in colony formation assays (P < 0.01; Figure 2C).

SMMC-7721 cells with stable Dbx2 expression and control cells were injected subcutaneously into NOD/SCID mice. The results indicated that compared with the control group, tumor growth was significantly faster in the Dbx2 overexpression group (P < 0.05; Figure 2D). Meanwhile, tumor growth was inhibited in *in vivo* xenografts generated using HepG2 cells with stable knockdown of Dbx2 expression (P < 0.05; Figure 2D).

Dbx2 brings the cell cycle into the S phase

We conducted cell cycle assay to explore the mechanisms that might be responsible

Table 2 Correlation between developing brain homeobox 2 expression and characteristics of hepatocellular carcinoma *n* (%)			
Characteristic	**Dbx2 High (*n* = 29)**	**Dbx2 Low (*n* = 47)**	***P*-value**
Gender			0.038
Male	28 (93.10)	36 (76.60)	
Female	1 (6.90)	11 (23.40)	
Age (yr)	Median age 56		0.771
< 60	18 (62.07)	72.34)	
> 60	11 (37.93)	13 (27.66)	
Differentiation			0.905
Well	19 (65.52)	33 (70.21)	
Poor	10 (34.48)	14 (29.79)	
Tumor size (cm)			0.025
< 5	16 (55.17)	37 (78.72)	
≥ 5	13 (44.83)	10 (21.28)	
Lymph node metastasis			0.162
Yes	10 (34.48)	11 (23.40)	
No	19 (65.52)	36 (76.60)	
HBV infection			0.572
Yes	16 (55.17)	19 (40.43)	
No	13 (44.83)	28 (59.57)	
HCV infection			0.654
Yes	2 (6.90)	2 (4.26)	
No	27 (93.10)	45 (95.74)	
Serum alpha-fetoprotein level (ng/mL)			0.180
< 400	20 (68.97)	36 (76.60)	
≥ 400	9 (31.03)	11 (23.40)	

for the growth-promoting effect of Dbx2 in HCC cells. The percentage of S phase cells was significantly greater in SMMC-7721 and Huh7 cells that exhibited ectopic expression of Dbx2 ($P < 0.05$; Figure 3A). Consistently, the expression of related cell cycle molecules was changed by up-regulated Dbx2 expression in SMMC-7721 and Huh7 cells; the expression of cyclinA, p21, and p27 was downregulated, while cyclin-dependent kinase (CDK2, CDK4, and CDK6), cyclin B, and cyclin D were upregulated (Figure 3B). However, knockdown of Dbx2 in HepG2 and Huh7 cells induced arrest in G1 phase and the expression of cell cycle regulators was concomitantly changed (Figure 3A, B).

Dbx2 attenuates apoptosis in HCC cells by inactivating the caspase-dependent pathway

To explore the mechanisms that may underlie the ability of Dbx2 to promote growth in HCC cells, the rate of cellular apoptosis was detected using V-APC and PI staining by flow cytometry. As shown in Figure 3C, compared with control cells, the proportions of apoptotic cells in SMMC7721 and Huh7 cell lines were significantly decreased after transfection with Dbx2 ($P < 0.01$). We then conducted Western blot analysis to evaluate the expression of molecules of the caspase-dependent pathway. As shown in Figure 3D, the levels of activated caspase-9, activated caspase-8, activated caspase-3, and nuclear poly ADP-ribose polymerase were lower in the Dbx2-transfected SMMC-7721 and Huh7 cells than in the control cells, but those of the anti-apoptosis proteins Bcl2 and survivin were higher. However, the proportions of apoptotic cells in HepG2 and Huh7 cells with knockdown of Dbx2 were higher ($P < 0.05$; Figure 3C) and the expression of apoptosis-related regulators changed accordingly (Figure 3D).

Dbx2 promotes migration and invasion in HCC cells

We performed wound healing assay, transwell migration assay, and Matrigel invasion assay to assess the possible role of Dbx2 in HCC cells. Our results indicated that, relative to control cells, overexpression of Dbx2 promoted migration and invasion of SMMC-7721 and Huh7 cells ($P < 0.05$). However, knockdown of Dbx2 in

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Figure 2 Developing brain homeobox 2 promotes cell growth *in vitro* and *in vivo*. A-C: Ectopic expression of developing brain homeobox 2 (Dbx2) in SMMC-7721 and Huh7 cell lines promoted cell growth as indicated by a cell viability assay and colony formation assay. The results were reversed in HepG2 and Huh7 cell lines with knockdown of Dbx2; D: Ectopic Dbx2 expression in SMMC-7721 cells promoted tumor growth and knockdown of Dbx2 in HepG2 cells inhibited tumor growth in NOD/SCID mice. $^a P < 0.05$; $^b P < 0.01$.

HepG2 cells and Huh7 cells rendered cell migration and invasion less pronounced than in control cells ($P < 0.05$; Figure 4A, B). Consistent changes were observed in epithelial-mesenchymal transition proteins (Figure 4C).

Dbx2 activates Shh-Gli1 signaling in HCC

It was confirmed that Dbx2 plays a vital role in the development of neurons mainly through the Shh-Gli1 signaling pathway, but the role of this pathway in HCC is still not clear. Our data showed that in SMMC-7721 cells and Huh7 cells, Shh signaling was activated by up-regulation of Dbx2 and was significantly repressed in HepG2 and Huh7 cells with knockdown of Dbx2 (Figure 5A). These findings indicated that Dbx2 could activate the Shh-Gli1 signaling pathway in HCC.

DISCUSSION

The evolutionarily conserved Dbx proteins were reported to play important roles in the development of the vertebrate central nervous system. Dbx genes (Dbx1 and Dbx2) have been reported to encode a family of homeodomain transcription factors in mammals. Dbx2 is highly expressed during neuronal development and regulates differentiation of interneurons in the human brain and spinal cord. Elevated Dbx2 expression promotes age-related phenotypes in young adult neural stem/progenitor cells, including the reduced proliferation and the altered differentiation in neural stem/progenitor cell cultures. Depleting Dbx2 in aged neural stem/progenitor cells caused the reverse gene expression changes. It has been reported that Dbx1, a homology of Dbx2, could serve as a novel candidate biomarker gene in breast carcinogenesis[11]. High Dbx2 expression was associated with a shorter 3-yr survival in glioma patients by the cancer genome atlas (TCGA) analysis in another study of us. However, little was known about the role of Dbx2 in HCC pathogenesis. We have found that the methylation level of Dbx2 was significantly lower in 31 early-stage HCC patients than in 27 healthy controls in our previous study[12]. We suggested that Dbx2 may play an important role in HCC development and metastasis. In our study, we showed that Dbx2 was more often elevated in HCC tissues relative to adjacent non-tumorous tissues by immunohistochemistry. Overexpression of Dbx2 was demonstrated by Western blot analysis in HCC cell lines against normal hepatic epithelial LO2 cells. We also detected more Dbx2 overexpression in 76 HCC tissue samples than in matched adjacent non-tumor tissues by immunohistochemistry assay. What is more, high levels of Dbx2 expression were statistically significantly associated with gender and tumor size. Compared to non-tumors tissues, our data showed that aberrant upregulation of Dbx2 was found in HCC tissues.

We further investigated Dbx2 expression in several cell lines, including LO2, HepG2, Li-7, Huh7, Huh7.5.1, and SMMC-7721. Significant ectopic Dbx2 expression was found in cancer cell lines by Western blot (Figure 2A). Gain and loss of function experiments were employed to assess the effect of Dbx2 on the biological behavior of cell lines. Overexpression of Dbx2 promoted cancer cell growth in SMMC-7721 and Huh7 cells. HepG2 and Huh7 cell growth was inhibited by knockdown of Dbx2 ($P < 0.05$; Figure 2B). These results were also confirmed by colony formation assay ($P < 0.01$; Figure 2C). SMMC-7721 cells with stable Dbx2 expression were injected subcutaneously into NOD/SCID mice. Compared with the control group, tumor growth was significantly faster in the Dbx2 overexpression group ($P < 0.05$; Figure 2D). Meanwhile, the tumor growth was inhibited in *in vivo* xenografts with stable Dbx2 knockdown ($P < 0.05$; Figure 2D). We concluded that Dbx2 expression was closely correlated with HCC progression.

To explore the mechanisms of the growth-promoting effect of Dbx2 in cell lines, we further performed cell cycle analysis. The percentage of S phase cells was significantly greater in SMMC-7721 and Huh7 cells that exhibited ectopic expression of Dbx2 ($P < 0.05$; Figure 3A). G1/S transition was promoted in HCC cells after Dbx2 overexpression; conversely, knockdown of Dbx2 inhibited HCC proliferation and induced G1 phase arrest. As we know, unlimited proliferation is one of important characteristics of cancer[29,30]. Subsequently, we further found the expression levels of cell cycle-related proteins to be consistent with the progression of the cell cycle.

C

D

E

F

Figure 3 Developing brain homeobox 2 regulates cell cycle and inhibits cell apoptosis in hepatocellular carcinoma cells. A and B: Ectopic expression of developing brain homeobox 2 (Dbx2) induced cell cycle into S phase; C: Ectopic expression of Dbx2 regulated cell cycle protein expression; D and E: Ectopic expression of Dbx2 inhibited cell apoptosis; F: Ectopic expression of Dbx2 downregulated apoptotic protein expression. The results were reversed after Dbx2 knockdown.

Additionally, studies on apoptosis demonstrated that overexpression of Dbx2 could attenuate apoptosis while knockdown of Dbx2 expression could activate apoptosis through the caspase pathway. We also elucidated that Dbx2 promoted cell migration and invasion by wound healing assay and transwell migration assay. The functions were reversed after Dbx2 knockdown. In a word, our data suggested that Dbx2 plays a crucial role in liver cancer cell proliferation, migration, and invasion.

A

B

C

Figure 4 Developing brain homeobox 2 promotes cell migration and invasion in hepatocellular carcinoma cells. A: Wound healing assay; B: Transwell migration and Matrigel invasion assay demonstrated that developing brain homeobox 2 (Dbx2) promoted cell migration and invasion. The effects were reversed after Dbx2 knockdown; C: Effect of Dbx2 on expression of several epithelial-mesenchymal transition related proteins detected by Western blot analysis. $^aP < 0.05$; $^bP < 0.01$.

Shh signaling plays an important role in embryonic development and regulation of cellular functions including proliferation, survival, stemness, and differentiation. The phenotype and proliferation of cancer cells are controlled by regulating the Shh pathway *via* interaction with Shh[31-33]. Aberrant activation of Shh signaling has been demonstrated in HCC. Activation of the Shh pathway is initiated by the binding of Shh ligands to their receptor, and leads to the activation of Smo. Smo activates the Gli family of transcription factors that regulate the expression of Shh target genes including Ptch1 and Gli1. For this reason, we determined whether Dbx2 regulates the Shh pathway and cell proliferation by interacting with Shh. Our data clearly showed that Dbx2 interacted with Shh in SMMC-7721 and Huh7 cell lines. Loss of Dbx2 resulted in significant repression of the Shh pathway during short-term Shh stimulation by regulating Ptch1 and Gli1 (Figure 5B). These results indicated that Dbx2 promoted cell proliferation, presumably by activating the Shh signaling pathway. Our findings provide evidence that Dbx2 plays an important role in cell proliferation by activation of the Shh signaling pathway.

In conclusion, this study shows that Dbx2 is upregulated in HCC cell lines and tissue samples. Gain and loss of function experiments of Dbx2 demonstrated that ectopic Dbx2 expression could promote HCC cell line proliferation, migration, and invasion *in vitro* by regulating the Shh signaling pathway, and accelerate tumor growth in *in vivo* xenografts.

A

B

Figure 5 Developing brain homeobox 2 activates the Shh-Gli1 signaling pathway in hepatocellular carcinoma. A: Ectopic expression of developing brain homeobox 2 (Dbx2) upregulates the expression of Shh, PTCH1, PTCH2, SUFU, and GLI1 proteins, which are downregulated after Dbx2 knockdown; B: Proposed scheme of molecular basis for gain and loss of function of Dbx2 in HCC according to our results.

ARTICLE HIGHLIGHTS

Research background
Great efforts have been made in exploring the mechanism of hepatocellular carcinoma (HCC), but the details of the HCC pathogenesis are still only partially established. Developing brain homeobox 2 (Dbx2) is frequently upregulated in tumor tissues, while there has been no experimental evidence regarding the function of Dbx2 in HCC.

Research motivation
Investigation of Dbx2 functions may suggest potential molecular mechanisms of hepatocellular carcinogenesis and progression, and further offer the potential for developing novel therapeutic strategies for HCC treatment.

Research objectives
We measured Dbx2 expression in HCC tissues and matched non-tumor tissues and investigated biological functions and the possible molecular mechanisms of Dbx2 in HCC.

Research methods

We detected Dbx2 expression in HCC samples and adjacent non-tumor tissues by immunohistochemistry. The biological behavior of Dbx2 *in vitro* and *in vivo* was then assessed by overexpression and knockdown of the Dbx2 gene.

Research results
Dbx2 was upregulated in HCC tissues, which was related to tumor size. Dbx2 had a role of promoting proliferation and metastasis by activating sonic hedgehog (Shh) signaling in HCC.

Research conclusions
Dbx2 was overexpressed in HCC cell lines and tissues, which could promote HCC progression through the Shh signal pathway.

Research perspectives
Dbx2 might serve as a tumor promoter and to be a potential therapeutic target in the future.

REFERENCES

1 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

2 **Finn RS**, Zhu AX, Farah W, Almasri J, Zaiem F, Prokop LJ, Murad MH, Mohammed K. Therapies for advanced stage hepatocellular carcinoma with macrovascular invasion or metastatic disease: A systematic review and meta-analysis. *Hepatology* 2018; **67**: 422-435 [PMID: 28881497 DOI: 10.1002/hep.29486]

3 **Lu S**, Bogarad LD, Murtha MT, Ruddle FH. Expression pattern of a murine homeobox gene, Dbx, displays extreme spatial restriction in embryonic forebrain and spinal cord. *Proc Natl Acad Sci USA* 1992; **89**: 8053-8057 [PMID: 1355604 DOI: 10.1073/pnas.89.17.8053]

4 **Shoji H**, Ito T, Wakamatsu Y, Hayasaka N, Ohsaki K, Oyanagi M, Kominami R, Kondoh H, Takahashi N. Regionalized expression of the Dbx family homeobox genes in the embryonic CNS of the mouse. *Mech Dev* 1996; **56**: 25-39 [PMID: 8798145 DOI: 10.1016/0925-4773(96)00509-6]

5 **Ma P**, Zhao S, Zeng W, Yang Q, Li C, Lv X, Zhou Q, Mao B. Xenopus Dbx2 is involved in primary neurogenesis and early neural plate patterning. *Biochem Biophys Res Commun* 2011; **412**: 170-174 [PMID: 21806971 DOI: 10.1016/j.bbrc.2011.07.068]

6 **Seo HC**, Nilsen F, Fjose A. Three structurally and functionally conserved Hlx genes in zebrafish. *Biochim Biophys Acta* 1999; **1489**: 323-335 [PMID: 10673033 DOI: 10.1016/S0167-4781(99)00187-6]

7 **Gribble SL**, Nikolaus OB, Dorsky RI. Regulation and function of Dbx genes in the zebrafish spinal cord. *Dev Dyn* 2007; **236**: 3472-3483 [PMID: 17994542 DOI: 10.1002/dvdy.21367]

8 **Briscoe J**, Chen Y, Jessell TM, Struhl G. A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol Cell* 2001; **7**: 1279-1291 [PMID: 11430830 DOI: 10.1016/S1097-2765(01)00271-4]

9 **Pierani A**, Brenner-Morton S, Chiang C, Jessell TM. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* 1999; **97**: 903-915 [PMID: 10399918 DOI: 10.1016/S0092-8674(00)80802-8]

10 **Wetmore C**. Sonic hedgehog in normal and neoplastic proliferation: insight gained from human tumors and animal models. *Curr Opin Genet Dev* 2003; **13**: 34-42 [PMID: 12573433 DOI: 10.1016/S0959-437X(03)00002-9]

11 **Kim Y**, Yoon JW, Xiao X, Dean NM, Monia BP, Marcusson EG. Selective down-regulation of glioma-associated oncogene 2 inhibits the proliferation of hepatocellular carcinoma cells. *Cancer Res* 2007; **67**: 3583-3593 [PMID: 17440069 DOI: 10.1158/0008-5472.CAN-06-3040]

12 **Lindqvist BM**, Wingren S, Motlagh PB, Nilsson TK. Whole genome DNA methylation signature of HER2-positive breast cancer. *Epigenetics* 2014; **9**: 1149-1162 [PMID: 25089541 DOI: 10.4161/epi.29632]

13 **Penny MK**, Finco I, Hammer GD. Cell signaling pathways in the adrenal cortex: Links to stem/progenitor biology and neoplasia. *Mol Cell Endocrinol* 2017; **445**: 42-54 [PMID: 27940298 DOI: 10.1016/j.mce.2016.12.005]

14 **Cohen M**, Kicheva A, Ribeiro A, Blassberg R, Page KM, Barnes CP, Briscoe J. Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms. *Nat Commun* 2015; **6**: 6709 [PMID: 25833741 DOI: 10.1038/ncomms7709]

15 **Skoda AM**, Simovic D, Karin V, Kardum V, Vranic S, Serman L. The role of the Hedgehog signaling pathway in cancer: A comprehensive review. *Bosn J Basic Med Sci* 2018; **18**: 8-20 [PMID: 29274272 DOI: 10.17305/bjbms.2018.2756]

16 **Ma Y**, Yu W, Shrivastava A, Alemi F, Lankachandra K, Srivastava RK, Shankar S. Sanguinarine inhibits pancreatic cancer stem cell characteristics by inducing oxidative stress and suppressing sonic hedgehog-Gli-Nanog pathway. *Carcinogenesis* 2017; **38**: 1047-1056 [PMID: 28968696 DOI: 10.1093/carcin/bgx070]

17 **Feng HC**, Lin JY, Hsu SH, Lan WY, Kuo CS, Tian YF, Sun DP, Huang RS. Low folate metabolic stress reprograms DNA methylation-activated sonic hedgehog signaling to mediate cancer stem cell-like signatures and invasive tumour stage-specific malignancy of human colorectal cancers. *Int J Cancer* 2017; **141**: 2537-2550 [PMID: 28833104 DOI: 10.1002/ijc.31008]

18 **Li Z**, Jin H, Mao G, Wu L, Guo Q. Msi2 plays a carcinogenic role in esophageal squamous cell carcinoma via regulation of the Wnt/β-catenin and Hedgehog signaling pathways. *Exp Cell Res* 2017; **361**: 170-177 [PMID: 29054489 DOI: 10.1016/j.yexcr.2017.10.016]

19 **Shigemura K**, Fujisawa M. Hedgehog signaling and urological cancers. *Curr Drug Targets* 2015; **16**: 258-271 [PMID: 25429711 DOI: 10.2174/1389450115666141125122643]

20 **Konstantinou D**, Bertaux-Skeirik N, Zavros Y. Hedgehog signaling in the stomach. *Curr Opin Pharmacol* 2016; **31**: 76-82 [PMID: 27750091 DOI: 10.1016/j.coph.2016.09.003]

21 **Zhao Z**, Zhang L, Zhang J, Wang J. [Expressions of Sonic hedgehog and matrix metalloproteinase 2 in human esophageal squamous cell carcinoma and the clinicopathological implications]. *Nan Fang Yi Ke Da Xue Xue Bao* 2013; **33**: 1008-1011 [PMID: 23895842]

22 **Rucki AA**, Foley K, Zhang P, Xiao Q, Kleponis J, Wu AA, Sharma R, Mo G, Liu A, Van Eyk J, Jaffee

EM, Zheng L. Heterogeneous Stromal Signaling within the Tumor Microenvironment Controls the Metastasis of Pancreatic Cancer. *Cancer Res* 2017; **77**: 41-52 [PMID: 27821486 DOI: 10.1158/0008-5472.CAN-16-1383]

23   **Subramani R**, Gonzalez E, Nandy SB, Arumugam A, Camacho F, Medel J, Alabi D, Lakshmanaswamy R. Gedunin inhibits pancreatic cancer by altering sonic hedgehog signaling pathway. *Oncotarget* 2017; **8**: 10891-10904 [PMID: 26988754 DOI: 10.18632/oncotarget.8055]

24   **Zeng C**, Chen T, Zhang Y, Chen Q. Hedgehog signaling pathway regulates ovarian cancer invasion and migration via adhesion molecule CD24. *J Cancer* 2017; **8**: 786-792 [PMID: 28382140 DOI: 10.7150/jca.17712]

25   **Liu Z**, Tu K, Wang Y, Yao B, Li Q, Wang L, Dou C, Liu Q, Zheng X. Hypoxia Accelerates Aggressiveness of Hepatocellular Carcinoma Cells Involving Oxidative Stress, Epithelial-Mesenchymal Transition and Non-Canonical Hedgehog Signaling. *Cell Physiol Biochem* 2017; **44**: 1856-1868 [PMID: 29237157 DOI: 10.1159/000485821]

26   **Liu CH**, Lan CT, Chou JF, Tseng TJ, Liao WC. CHSY1 promotes aggressive phenotypes of hepatocellular carcinoma cells via activation of the hedgehog signaling pathway. *Cancer Lett* 2017; **403**: 280-288 [PMID: 28652022 DOI: 10.1016/j.canlet.2017.06.023]

27   **Catenacci DV**, Junttila MR, Karrison T, Bahary N, Horiba MN, Nattam SR, Marsh R, Wallace J, Kozloff M, Rajdev L, Cohen D, Wade J, Sleckman B, Lenz HJ, Stiff P, Kumar P, Xu P, Henderson L, Takebe N, Salgia R, Wang X, Stadler WM, de Sauvage FJ, Kindler HL. Randomized Phase Ib/II Study of Gemcitabine Plus Placebo or Vismodegib, a Hedgehog Pathway Inhibitor, in Patients With Metastatic Pancreatic Cancer. *J Clin Oncol* 2015; **33**: 4284-4292 [PMID: 26527777 DOI: 10.1200/JCO.2015.62.8719]

28   **Moeini A**, Cornellà H, Villanueva A. Emerging signaling pathways in hepatocellular carcinoma. *Liver Cancer* 2012; **1**: 83-93 [PMID: 24159576 DOI: 10.1159/000342405]

29   **Lee B**, Sandhu S, McArthur G. Cell cycle control as a promising target in melanoma. *Curr Opin Oncol* 2015; **27**: 141-150 [PMID: 25588041 DOI: 10.1097/CCO.0000000000000159]

30   **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

31   **Bal E**, Park HS, Belaid-Choucair Z, Kayserili H, Naville M, Madrange M, Chiticariu E, Hadj-Rabia S, Cagnard N, Kuonen F, Bachmann D, Huber M, Le Gall C, Côté F, Hanein S, Rosti RÖ, Aslanger AD, Waisfisz Q, Bodemer C, Hermine O, Morice-Picard F, Labeille B, Caux F, Mazereeuw-Hautier J, Philip N, Levy N, Taieb A, Avril MF, Headon DJ, Gyapay G, Magnaldo T, Fraitag S, Crollius HR, Vabres P, Hohl D, Munnich A, Smahi A. Mutations in ACTRT1 and its enhancer RNA elements lead to aberrant activation of Hedgehog signaling in inherited and sporadic basal cell carcinomas. *Nat Med* 2017; **23**: 1226-1233 [PMID: 28869610 DOI: 10.1038/nm.4368]

32   **Wu JB**, Yin L, Shi C, Li Q, Duan P, Huang JM, Liu C, Wang F, Lewis M, Wang Y, Lin TP, Pan CC, Posadas EM, Zhau HE, Chung LWK. MAOA-Dependent Activation of Shh-IL6-RANKL Signaling Network Promotes Prostate Cancer Metastasis by Engaging Tumor-Stromal Cell Interactions. *Cancer Cell* 2017; **31**: 368-382 [PMID: 28292438 DOI: 10.1016/j.ccell.2017.02.003]

33   **Li L**, Grausam KB, Wang J, Lun MP, Ohli J, Lidov HG, Calicchio ML, Zeng E, Salisbury JL, Wechsler-Reya RJ, Lehtinen MK, Schüller U, Zhao H. Sonic Hedgehog promotes proliferation of Notch-dependent monociliated choroid plexus tumour cells. *Nat Cell Biol* 2016; **18**: 418-430 [PMID: 26999738 DOI: 10.1038/ncb3327]

Basic Study

Dynamic changes of key metabolites during liver fibrosis in rats

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Author contributions: Li LJ and Cao HC conceived and designed the study; Yu J wrote the manuscript and performed the statistical analysis; Chen DY substantially contributed to the conception and design of the study as well as the acquisition, analysis, and interpretation of the data; Yu J and Pan QL performed the immunohistochemical detection, RT-PCR, and data collection; He JQ provided technical support and revised the manuscript; all authors reviewed and approved the final version of the manuscript.

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Abstract

BACKGROUND
Fibrosis is the single most important predictor of significant morbidity and mortality in patients with chronic liver disease. Established non-invasive tests for monitoring fibrosis are lacking, and new biomarkers of liver fibrosis and function are needed.

AIM
To depict the process of liver fibrosis and look for novel biomarkers for diagnosis and monitoring fibrosis progression.

METHODS
CCl_4 was used to establish the rat liver fibrosis model. Liver fibrosis process was measured by liver chemical tests, liver histopathology, and Masson's trichrome staining. The expression levels of two fibrotic markers including α-smooth muscle actin and transforming growth factor β1 were assessed using immunohistochemistry and real-time polymerase chain reaction. Dynamic changes in metabolic profiles and biomarker concentrations in rat serum during liver fibrosis progression were investigated using ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. The discriminatory capability of potential biomarkers was evaluated by receiver operating characteristic (ROC) curve analysis.

RESULTS
To investigate the dynamic changes of metabolites during the process of liver fibrosis, sera from control and fibrosis model rats based on pathological results were analyzed at five different time points. We investigated the association of liver fibrosis with 21 metabolites including hydroxyethyl glycine, L-threonine, indoleacrylic acid, β-muricholic acid (β-MCA), cervonoyl ethanolamide (CEA),

phosphatidylcholines, and lysophosphatidylcholines. Two metabolites, CEA and β-MCA, differed significantly in the fibrosis model rats compared to controls ($P < 0.05$) and showed prognostic value for fibrosis. ROC curve analyses performed to calculate the area under the curve (AUC) revealed that CEA and β-MCA differed significantly in the fibrosis group compared to controls with AUC values exceeding 0.8, and can clearly differentiate early stage from late stage fibrosis or cirrhosis.

CONCLUSION
This study identified two novel biomarkers of fibrosis, CEA and β-MCA, which were effective for diagnosing fibrosis in an animal model.

Key words: Ultra-performance liquid chromatography-mass spectrometry; Metabonomics; Liver fibrosis; Biomarker; Cervonoyl ethanolamide; β-muricholic acid

Core tip: Carbon tetrachloride induced model is stable and comparable with viral hepatitis. Metabolic changes occur during the progression of fibrosis. We investigated the association of liver fibrosis with 21 metabolites, and two of them, cervonoyl ethanolamide and β-muricholic acid, differed significantly in the fibrosis model rats compared to controls ($P < 0.05$) and showed prognostic value for fibrosis. The receiver operating characteristic curve analysis results showed that both metabolites had excellent diagnostic value and could be used in clinical diagnosis in the future.

INTRODUCTION

Liver fibrosis is a common pathological process of all chronic liver diseases, which can be caused by a number of factors, including long-term alcohol abuse, viral infection, fatty liver disease, metabolic disease, and cholestasis[1,2]. The mechanisms of liver fibrosis and cirrhosis are considered similar; fibrosis occurs *via* a non-specific mechanism involving excessive accumulation of extracellular matrix proteins, including collagen. This accumulation causes hepatic stellate cell activation, which persists as long as there is liver injury in most cases of chronic liver disease[3].

Liver fibrosis can be divided into four stages (0-4): Stages 0 and 1 represent normal liver; stage 2 is mild fibrosis; stages 3 and 4 indicate severe and advanced liver fibrosis that results in cirrhosis. Importantly, liver fibrosis can be reversible at any stage prior to the development of liver cirrhosis[4]. Therefore, in chronic liver disease, fibrosis level is the most important predictor of significant morbidity and mortality. Assessments of liver injury are currently based on clinical symptoms and biopsies of the liver. Alanine aminotransferase (ALT) is a simple and inexpensive surrogate marker for liver disease; however, significant fibrosis may still be present in some patients who had normal ALT levels, and there is no better index than ALT level to predict advanced fibrosis[5,6]. The gold standard for assessing liver fibrosis is still liver biopsy. Currently, magnetic resonance imaging (MRI)- and ultrasound-based elastography is widely used to assess hepatic steatosis and fibrosis. However, in the early stages of fibrosis, these techniques lack sensitivity and specificity, and cannot be used to determine inflammation and cell damage[7]. Thus, there is a need for novel liquid biomarkers, which, in combination with fibroscan and MRI, might provide significant advances in diagnosis and monitoring fibrosis progression. Metabonomics, an effective and noninvasive diagnostic method that provides quantitative measurements of metabolite changes in biofluids, is a powerful tool for biomarker discovery and helpful for understanding the pathophysiology of a disease[8-11]. In recent years, most metabolomic studies have compared only two groups, as the results are easy to interpret. However, the natural course of disease and treatment processes vary widely, and few studies have focused on the dynamic processes of

metabolic profiles and biomarkers.

In this study, we investigated biomarker concentrations and dynamic changes in metabolic profiles during liver fibrosis progression using ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS). The objective of this study was to investigate the potential utility of metabonomic biomarkers for the early diagnosis of liver fibrosis and function.

MATERIALS AND METHODS

Animal model of liver fibrosis

A total of 100 6-week-old Sprague-Dawley rats, weighing 180-200 g, were obtained from the Experimental Animal Center of Zhejiang Academy of Medical Sciences, which were housed under a 12-h daylight/darkness cycle and in an air-conditioned animal room with 50% humidity. All experimental procedures were conducted according to protocols approved by the Research Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (No. 201543). The animals were randomly divided into five groups (*n* = 20 each). To induce liver fibrosis, rats were injected subcutaneously with CCl_4 (Sigma-Aldrich, St. Louis, MO, United States) in olive oil (*v*/*v*, 50%, Sigma-Aldrich) at a dose of 0.5 mL per 100 g of body weight, twice weekly for 12 wk. The rats in the control group were administered oil using the same injection procedure. The rats were sacrificed at weeks 1, 4, 8, and 12 to collect blood samples and liver tissues.

Pathological observation

The liver tissues were rapidly isolated and immersed into 4% (*w*/*v*) paraformalde-hyde, embedded in paraffin, deparaffinized, and rehydrated with distilled water. The liver sections were stained with hematoxylin and eosin (HE) using a routine protocol, and with Masson's trichrome (MTC) using an MTC staining kit (Sigma-Aldrich) according to the manufacturer's instructions. The injury score of fibrosis was graded as described by Ishak[12]; ten representative views of each histological section from every rat were randomly selected and all rat models were scored.

Chemical analysis

Blood samples were collected *via* the caudal vein and placed into 1.5 mL centrifuge tubes. According to pathological results, serum samples were selected for MS and biochemical detection. The samples were centrifuged at 4 °C at 3000 rpm for about 15 min after incubation at room temperature for 30 min. Then, the serum samples were sent to the central clinical laboratory of the First Affiliated Hospital for total protein (TP), albumin (ALB), globulin (GLO), alkaline phosphatase (AKP), ALT, aspartate aminotransferase (AST), bile acid (BA), total bilirubin (TBIL), and creatinine (CR) concentration assays. The remaining serum was stored at - 80 °C for metabonomic analyses.

Liver immunohistochemistry

For immunohistochemistry, the paraffin sections were incubated at 4 °C overnight with primary antibodies against rat α-smooth muscle actin (α-SMA, 1:400; Abcam, United Kingdom) and rat transforming growth factor-β1 (TGF-β1, 1:500; Abcam, United Kingdom). Next day, the sections were washed with phosphate buffered saline (GenomSciences, Hangzhou, Zhejiang Province, China) three times and then incubated at 37 °C for 60 min with horseradish peroxidase-conjugated secondary antibody (1:1000; Abcam). Then, the sections were incubated with diaminobenzidine tetrahydrochloride solution (DAB kit, Abcam) for 10min, washed with distilled water, and counterstained with hematoxylin at room temperature. Finally, liver sections were sealed with neutral resin and examined microscopically.

Reverse transcription-polymerase chain reaction (PCR) and quantitative real-time PCR

Total RNA from each liver sample was extracted with Trizol reagent (Invitrogen, United States), and cDNA was synthesized using QuantiTect Reverse Transcription Kit [TAKARA Biotechnology (Dalian) Co., Ltd, Dalian, Liaoning Province, China], according to the manufacturer's instructions. The primers used were: 5'-CGA TAG AAC ACG GCA TCA TCA C-3' (forward) and 5'-GCA TAG CCC TCA TAG ATA GGC A-3' (reverse) for α-SMA; and 5'-CCT GGA AAG GGC TCA ACA C-3' (forward) and 5'-CAG TTC TTC TCT GTG GAG CTG A-3' (reverse) for TGF-β1. Quantitative real-time PCR was used to assess the mRNA levels of TGF-β1 and α-SMA [QuantiTect SYBR Green RT-PCR kit, TAKARA Biotechnology (Dalian) Co., Ltd.] on the 7500 Real

Time System (Life Technologies, Carlsbad, California, United States). All PCR products were normalized to expression levels of β-actin used as an internal standard[13].

Sample preparation and UPLC/Q-TOF-MS analysis

A total of 100 μL of each sample was mixed vigorously with 300 μL of precooled acetonitrile after the serum samples were thawed at 4 °C, followed by centrifugation at 14000 rpm at 4 °C for 10 min. Then, the supernatants were transferred to specific glass tubes for UPLC-MS analyses. To condition the column, quality control samples (10 μL of supernatants) were obtained from each sample and tested five times before the analysis, and after every eight samples throughout the procedure.

Analytical conditions of UPLC-MS

The Acquity UPLC system (Waters, Milford, MA, United States) was used for chromatographic separations, which was equipped with an Acquity UPLC BEH C18 analytical column (I.D. 2.1 mm × 100 mm, particle size 1.7 μm, pore size 130 Å). MS detection was performed with a mass spectrometer, which was equipped with an electrospray ionization source, using the negative ion electrospray mode. The nitrogen drying gas was set at a velocity of 600 L/h, and the temperatures of source and desolvation were 120 °C and 350 °C, respectively. The cone gas velocity was 50 L/h. The sampling cone voltage was set at 40.0 and the capillary voltage at 3.0 kV; the collision gas was argon, and the collision energy was set at 5.0 eV. According to the stability of the individual metabolites, tandem MS (MS/MS) analyses were performed with the mass spectrometer set at various collision energies, ranging from 30 to 80 eV.

Biomarker selection and identification

The raw UPLC–MS data files were normalized with MassLynx v4.1 software (Waters). The final peak ratio file, containing retention time, *m/z*, and signal intensity of the peaks, was analyzed with SIMCA-P+ 13.0 software (Umetrics, Umeå, Sweden). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were combined to analyze the data. The biomarkers were identified based on *m/z*, retention time, and typical MS/MS fragment and pattern. According to the variance analyses and variable importance in the projection (VIP) of the metabolites, the candidate biomarkers were selected. To identify potential biomarkers, the HMDB database (http://hmdb.ca/) and PubChem compound database (http://www.ncbi.nlm.nih.gov) were searched. The final determination of biomarkers was confirmed by comparison with corresponding standards. Metabolite set enrichment and pathway analyses were based on MetaboAnalyst (www.metaboanalyst.ca).

Statistical analyses of biomarkers

The diagnostic value of the selected biomarkers was analyzed using discriminant analyses and compared to blood biochemical parameters. The discriminant analyses relied on Fisher's functional coefficient and stepwise statistical analyses. To evaluate the discriminatory capability of potential biomarkers, we used receiver operating characteristic (ROC) curves. All statistical analyses were conducted with SPSS 19.0 software (SPSS, Inc., Chicago, IL, United States), and $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Dynamic changes in blood biochemistry

Blood biochemical parameters, including TP, ALB, GLO, AKP, ALT, AST, BA, TBIL, and CR, were measured during the fibrosis process to examine liver function (Figure 1). Serum levels of ALB, TP, and GLO showed a gradual decrease. ALT and AST levels showed a significant increase at week 1. AKP, total BA (TBA), and TBIL levels increased significantly over time ($P < 0.05$); CR levels showed no obvious trend.

Morphological changes in the liver

Liver tissues were collected from each group at five time points and subjected to histological examination using HE and MTC staining. Liver tissues from the fibrosis model groups showed a series of severe morphological changes, including inflammation, fatty metamorphosis, and necrosis compared to the normal lobes of the control group. The fibrosis model livers showed fatty metamorphoses, sinusoid congestion, and hemorrhage at week 1. At week 4, the livers showed fibroblasts between the portal area and the interlobular area, and further bubble-like degeneration and necrosis. Increased numbers of fibroblasts and hepatic lobe reconstruction were observed from weeks 8 to 12 (Figure 2A-E). The injury score of fibrosis increased gradually compared to the control group ($P < 0.05$) (Figure 2F).

Figure 1 Dynamic changes in serum biochemical parameters during the process of fibrosis. A: Albumin, total protein, and globulin; B: Alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase; C: Bile acid, total bilirubin, and creatinine. ALB: Albumin; TP: Total protein; GLO: Globulin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; BA: Bile acid; TBIL: Total bilirubin; CR: Creatinine.

MTC staining at weeks 4 to 12 revealed increasing levels of collagen deposition and fibrosis accumulation (Figure 2G-K).

Hepatic stellate cell activation markers during the fibrosis process

As shown in Figure 3, immunohistochemical staining analysis indicated that the marker of hepatic stellate cell activation α-SMA and hepatic expression of the pro-fibrogenic marker TGF-β1 showed more and more brown staining over time and reached the most brown at week 12. These results were further confirmed by the mRNA expression of α-SMA and TGF-β1, as shown in Figure 3F and L, respectively.

Metabolic profile shift during the fibrosis process

The metabolic profiles in the serum samples were acquired using UPLC/Q-TOF-MS. Trajectory analyses of the PCA score plots for liver fibrosis at various time points revealed distinct clustering of the groups. The serum parameters ($R2 Y = 0.77$, $Q2 = 0.657$) used for fibrosis classification showed good predictive ability, and several serum metabolites demonstrated time-dependent changes in the various stages of liver fibrosis (Figure 4A). OPLS-DA was used to further characterize the metabolic profiles in various stages of liver fibrosis. The control and liver fibrosis model groups showed complete separation in the OPLS-DA score plots (Figure 4B). The cumulative values of R2X, R2Y, and Q2Y in the OPLS-DA model were 0.756, 0.942, and 0.819, respectively. Hierarchical clustering and heat maps were used to investigate the metabolites detected in the five groups. The normalized intensity of each metabolite was assessed, and metabolite peaks with similar intensities were clustered together. The color distribution showed more dark red color at week 1 and week 12, with dark red representing higher intensity and dark green representing lower intensity (Figure 4C). To document the metabolite changes during 12 wk of liver fibrosis, volcano plots were constructed with thresholds of ≥ 2-fold change and $P \leq 0.05$ (red dots). The metabolites showed the greatest differences at week 1 and week 12 in the fibrosis group compared to the control group (Figure 4D-G).

Metabolite quantification and identification

Based on the VIP values (VIP > 1) using the OPLS-DA models, we identified 21 metabolites that were associated with the CCl$_4$-induced metabolic changes in the liver fibrosis model rats. These metabolites included hydroxyethyl glycine, L-threonine, indoleacrylic acid (IAA), β-muricholic acid (β-MCA), cervonoyl ethanolamide (CEA), phosphatidylcholines (PCs), and lysophosphatidylcholines (LPCs) (Table 1). To evaluate the diagnostic value, discriminant analyses were conducted in the five different groups based on selected metabolic profiles; the results showed correct classification of 96.0% of the originally grouped cases and 90.0% of the cross-validated grouped cases. Discrimination based on metabolic profiles was superior to that based on biochemical parameters, which showed correct classification of 78.0% of the originally grouped cases and 74.0% of the cross-validated grouped cases (Figure 5A and B). Correlation analyses showed a good correlation between the identified metabolites (Figure 5C). In order to further explore the impact of these selected metabolites, MetaboAnalyst 3.0 software (Metabolomics Pathway Analysis) was used to analyze the 21 positively identified metabolites to identify possible biochemical pathways during liver fibrosis. The metabolic pathways that were significantly altered by liver fibrosis included linoleic acid metabolism, glycerophospholipid metabolism, alpha-linolenic acid metabolism, glycine, serine and threonine metabolism, arachidonic acid metabolism, tryptophan metabolism, and aminoacyl-tRNA

Figure 2 Histological assessment in each group using hematoxylin and eosin and Masson's trichrome staining. A-E: Liver tissues were stained with HE in the control and fibrosis model groups at weeks 1, 4, 8, and 12; F: The injury score of fibrosis in each group; G-K: Liver tissues were stained using MTC in the control and fibrosis model groups at weeks 1, 4, 8, and 12 ([a]$P < 0.01$ *vs* control). Scale bars: 100 μm. HE: Hematoxylin and eosin; MTC: Masson's trichrome.

biosynthesis (Figure 5D). Table 2 summarizes the results of the metabolic pathway analyses.

Biomarker candidates for liver fibrosis

The MS chromatographic intensities of the 21 metabolites were analyzed by independent sample test and ROC analysis in both groups. CEA and β-MCA showed significant differences ($P < 0.05$) (Table 1). Combined box-and-whisker plots showed the key biomarker changes of liver fibrosis progression, from early and intermediate to cirrhosis stages (Figure 6A and B). These metabolites showed significant differences at the various stages. Interestingly, the metabolite alterations were most dramatic at the early stage and less pronounced in the advanced stage. To further validate the importance of these selected metabolites, ROC analyses were conducted to calculate the area under the curve (AUC); the diagnostic sensitivity and specificity of the metabolite cutoffs could be used to distinguish between individuals with cirrhosis, those with fibrosis, and normal controls. The results revealed that metabolite candidates showed significant diagnostic performance, *i.e.*, with AUC values exceeding 0.8. CEA and β-MCA levels could be used to distinguish between control and fibrosis groups with high sensitivity and specificity. In addition, these metabolite levels could be used to clearly separate the early stages of fibrosis from advanced fibrosis or cirrhosis; the AUCs of TBA and ALT were only 0.795 and 0.576 between early fibrosis and late stage fibrosis or cirrhosis (Figure 6C-G).

DISCUSSION

Liver biopsy remains the gold standard for diagnosing fibrosis in chronic liver disease; however, liver biopsy has distinct limitations, such as invasiveness, potential

Figure 3 Immunohistochemistry results and relative mRNA expression levels of α-smooth muscle actin and transforming growth factor β1. A-E: Immunohistochemical staining for α-SMA; F: qRT-PCR results of α-SMA at each time point; G-K: Immunohistochemical staining for TGF-β1; L: qRT-PCR results of TGF-β1 at each time point. The data are presented as the mean ± SD (error bars) and were statistically analyzed using a Student's *t*-test. [a]$P < 0.01$ *vs* control. Scale bars: 100 μm. α-SMA: α-smooth muscle actin; TGF-β1: Transforming growth factor-β1; qRT-PCR: Quantitative real-time polymerase chain reaction.

complications, sampling error, bleeding, and risk of injury to neighboring organs[14-16]. These limitations underscore the importance of developing reliable, non-invasive markers to evaluate the degree of hepatic fibrosis and stages of fibrosis. To date, no method has been developed to replace liver biopsy as the gold standard.

In this study, the CCl_4-induced liver fibrosis model was successfully established in rats, and liver fibrosis and its severity were determined through HE, MTC, α-SMA, and TGF-β1 staining of histological sections. Analyses of the sera from the model and control rats at five time points revealed dynamic changes in metabolites during the process of liver fibrosis. These dynamic changes, identified in a series of assessments over time, could reveal the metabolic changes that occur during the progression of liver fibrosis, particularly in the interface phase between normal status, fibrosis, and cirrhosis. The heat map directly showed more severely metabolic patterns change at week 1 and week 12. In the early stage, the injury and death of a large number of hepatocytes resulted in abnormal liver function indicators, and we believe that this is the body's stress response to CCl_4. In the late stage, changes in liver metabolic capacity occur after injury of hepatocytes, which induces secondary changes of small molecule metabolites *in vivo*, as well as strong changes in metabolic spectrum. Screening of potential biomarkers may lead to early diagnosis.

CCl_4 is used widely in liver injury animal models, and the CCl_4-induced damage is comparable to that observed with viral hepatitis. Free radicals and reactive oxygen species with oxidative stress are considered to be the main causes of the liver injury induced by CCl_4[17,18]. Oxidative stress and cell membrane damage disrupt the metabolism of hepatic cells. Pathway analyses of CCl_4-induced hepatocellular damage revealed involvement of several metabolic pathways, including alpha-linolenic acid metabolism; glycerophospholipid metabolism; linoleic acid metabolism; glycine, serine, and threonine metabolism; arachidonic acid metabolism; tryptophan metabolism; and aminoacyl-tRNA biosynthesis (Figure 5D). The various classes of
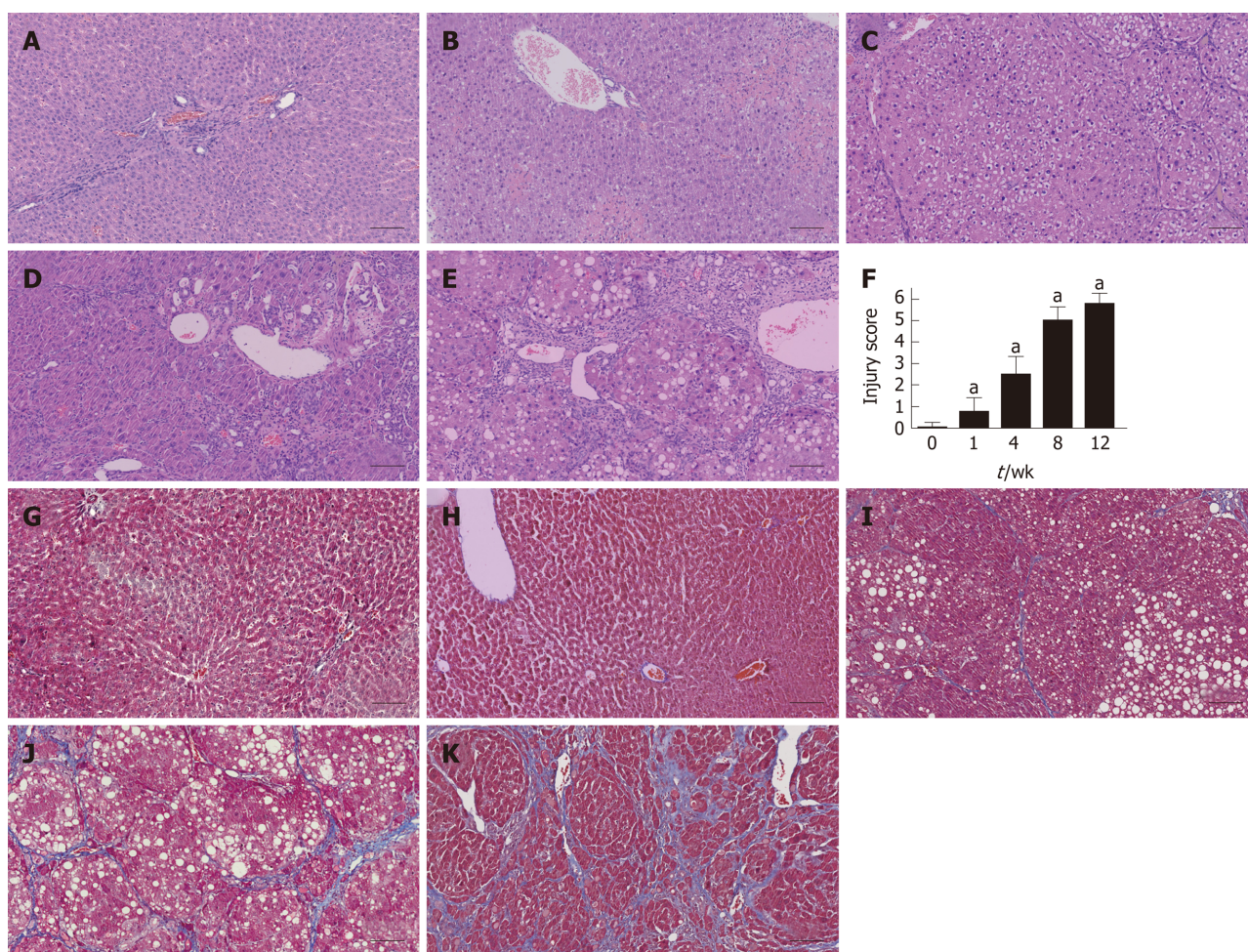
Figure 4 Metabolomic profiling and flux analyses. A: Principle component analysis for the control and fibrosis model groups at weeks 1, 4, 8, and 12; B: Orthogonal partial least squares discriminant analysis score plots for the control and fibrosis model groups at weeks 1, 4, 8, and 12; C: Heat map generated from the liquid chromatography-mass spectrometry data using the hierarchical clustering algorithm; D-G: Volcano plot analyses were used to determine the significant metabolites in the fibrosis model groups compared to controls at weeks 1, 4, 8, and 12. Data points with fold changes > 2 and $P < 0.05$ are labeled in red.

metabolites exhibited different expression patterns during the progression of liver fibrosis. From the perspective of variation amplitude, the heat maps and Volcano plots suggested that more severe metabolic disturbances occurred during the final cirrhosis stage.

Although the metabolic profile can clearly distinguish fibrosis and control groups, and liver fibrosis and cirrhosis groups, the use of complex metabolomic matrices in the clinic requires further study. Therefore, we searched for new potential biomarkers out of thousands of differential metabolites. Using the OPLS-DA model for biomarker analysis, we identified 21 markers including hydroxyethyl glycine, L-threonine, IAA, β-MCA, CEA, PCs, and LPCs. To determine whether the differential levels of these metabolites were associated with stepwise fibrosis, we compared the levels in serum samples from CCl_4-induced rats and controls at five time points. A comprehensive workflow was used to identify potential biomarkers, including visualization and metabolites of sample trajectories, multivariate screening for classification of different disease states, and stepwise univariate analyses for identification of important stages.

Table 1 The most prominent metabolites

No.	Ret. time	*m/z*	Adduct	Compound	Independent *t*-test			
					Con *vs* Week 1	Con *vs* Week 4	Con *vs* Week 8	Con *vs* Week 12
1	18.6	786.6003	[M+H]+	PC(18:0/18:2)	0.045	0.005	0.001	0.338
2	18.47	810.5999	[M+H]+	PC(18:0/20:4)	0.000	0.000	0.000	0.000
3	18.34	760.5851	[M+H]+	PC(16:0/18:1)	0.130	0.206	0.000	0.363
4	18.29	834.6003	[M+H]+	PC(18:0/22:6)	0.000	0.002	0.071	0.000
5	17.73	758.5689	[M+H]+	PC(16:0/18:2)	0.499	0.526	0.001	0.000
6	17.61	782.569	[M+H]+	PC(18:2/18:2)	0.000	0.000	0.001	0.000
7	17.49	806.5689	[M+H]+	PC(22:6/16:0)	0.000	0.037	0.808	0.439
8	17.31	782.5693	[M+H]+	PC(16:0/20:4)	0.021	0.000	0.000	0.000
9	17.2	806.5676	[M+H]+	PC(18:2/20:4)	0.000	0.041	0.000	0.000
10	17.13	756.5521	[M+H]+	PC(16:1/18:2)	0.477	0.033	0.003	0.000
11	12.94	524.3707	[M+H]+	LysoPC(18:0)	0.000	0.000	0.000	0.001
12	12.62	524.3714	[M+H]+	LysoPC(18:0)	0.000	0.001	0.003	0.335
13	11.62	496.3383	[M+H]+	LysoPC(16:0)	0.170	0.000	0.000	0.000
14	11.6	991.6714	[2M+H]+	LysoPC(16:0)	0.003	0.639	0.789	0.000
15	11.03	544.3396	[M+H]+	LysoPC(20:4)	0.000	0.000	0.000	0.003
16	10.75	544.3382	[M+H]+	LysoPC(20:4)	0.000	0.000	0.000	0.000
17	9.12	373.2716	M+H	Cervonoyl ethanolamide	0.035	0.009	0.000	0.000
18	9.11	817.5807	2M+H	β-muricholic acid	0.030	0.041	0.001	0.004
19	3.34	188.0702	M+H	Indoleacrylic acid	0.253	0.009	0.000	0.000
20	2.71	120.0802	M+H	L-threonine	0.026	0.000	0.000	0.000
21	0.97	120.0799	M+H	Hydroxyethyl glycine	0.008	0.007	0.000	0.000

Two metabolites, CEA and β-MCA, were defined as biomarker candidates.

CEA, also known as mead ethanolamide or eicosatrienoyl ethanolamide, is an N-acylethanolamine (NAE)[19,20]. NAEs are lipid mediators produced from N-acyl-phosphatidylethanolamine *via* several pathways. These endogenous bioactive lipids respond to a variety of stimuli and play critical physiological roles in a number of biological processes, including pain perception, metabolism, and inflammation, through different mechanisms[21-23]. NAEs include numerous fatty acid amides, such as palmitoylethanolamide, oleoylethanolamide, stearoylethanolamide, and CEA, and have been proposed as potential treatments for many diseases[24,25]. CEA is a novel eicosanoid; it was shown to be an agonist of central (CB1) and peripheral (CB2) cannabinoid receptors in 1995[14]. Increasing evidence indicates that the endocannabinoid system has a critical role in various liver diseases. In particular, the cannabinoid receptors CB1 and CB2 are upregulated in almost all chronic liver diseases, as well as cirrhosis and related disorders, and these receptors can be therapeutically antagonized[26-28]. Previous studies revealed that the CB2 agonists JWH-133 and 4′-O-methylhonokiol showed protective effects, such as decreased hepatocyte steatosis, inflammation, and liver regeneration[29-31]. In this study, we noted higher levels of CEA during the process of liver fibrosis, representing continuously increased concentrations of a CB2 agonist against CCl_4-induced liver damage. In addition, CEA can directly inhibit both CD8- and CD4-T cell responses by reducing their production of TNF-α, IFN-γ, and IL-17[27].

β-MCA, a natural trihydroxy hydrophilic BA, is a major BA in the rat liver and found in their BAs[32]. BAs are major endogenous metabolites of cholesterol and are involved in many metabolic processes. Under normal conditions, the liver can effectively absorb BAs through enterohepatic circulation, and bound BAs that are present at micromolar concentrations in peripheral blood. However, hepatocyte injury in hepatic diseases leads to synthesis and clearance of BA in the liver and disturbed intestinal absorption, which is characterized by elevated levels of TBAs. The resulting high concentrations of BAs can aggravate liver injury and ultimately cause cirrhosis and liver failure[33]. The serum BA (SBA) test has been suggested to use in clinical practice to screen for liver diseases[34]. In general, in the case of hepatobiliary and intestinal diseases, significant changes in individual BA concentrations and their metabolic characteristics in plasma, urine, and feces can be observed; however, there is increasing evidence that cirrhosis is closely related to significant changes in SBA

Table 2 Summary of pathway analysis results

Pathway name	Total	Expected	Hits	Raw *P*	-LOG(p)	Holm adjust	FDR	Impact
Glycerophospholipid metabolism	30	0.10699	2	0.004255	5.4597	0.34463	0.34463	0.18333
Linoleic acid metabolism	5	0.017832	1	0.01773	4.0325	1	0.71807	0
Alpha-linolenic acid metabolism	9	0.032097	1	0.031732	3.4504	1	0.85677	0
Glycine, serine, and threonine metabolism	32	0.11412	1	0.10918	2.2148	1	1	0
Arachidonic acid metabolism	36	0.12839	1	0.12213	2.1027	1	1	0
Tryptophan metabolism	41	0.14622	1	0.1381	1.9798	1	1	0
Aminoacyl-tRNA biosynthesis	67	0.23894	1	0.21745	1.5258	1	1	0

FDR: False discovery rate.

levels[10,35]. Elevated SBA levels are a more sensitive test of cirrhosis than conventional liver function detection methods[36,37]. In this study, the increased levels of β-MCA during the process of liver fibrosis were consistent with the clinical biochemical finding of increased concentrations of TBA with fibrosis progression.

The key aim of this study was to develop biomarkers for diagnosing fibrosis in the early stages. In addition, the study evaluated the diagnostic potential of these biomarkers. ROC analyses were performed for each metabolite candidate in comparison with currently available biomarkers, and these novel biomarkers achieved effective classification of both early and intermediate cirrhosis stages. Interestingly, the traditional clinical biomarkers, TBA (AUC = 0.795) and ALT (AUC = 0.576), were not good enough to distinguish between fibrosis and advanced fibrosis or cirrhosis (Figure 6), indicating the difficulty in identifying early fibrosis and advanced fibrosis[6]. This dynamic metabolomic study of the potential biomarkers of stepwise liver fibrosis might be useful for screening early metabolic characteristics related to fibrosis. In addition, the UPLC-TOF/MS-based metabolomics analysis contributes to our knowledge of liver fibrosis. This study identified two novel fibrosis biomarkers; CEA is involved in anti-inflammation and acts as an antagonist of CB2, and β-MCA is related to the processes involved in hepatocyte damage. These biomarkers correctly classified the disease stage in our fibrosis animal model. Moreover, they distinguished between fibrosis and cirrhosis more clearly than traditional ALT and TBA levels. Further mechanistic investigations are required to investigate the involvement of these metabolites in fibrosis progression and histologic changes.

Figure 5 Metabolite quantification and identification. A and B: Scatter plots of discriminant analyses in five groups based on metabolic profiles and biochemical parameters; C: Correlation analyses of the metabolic profiles. The color saturation of red and blue represents positive and negative correlation coefficients, respectively, between markers; D: Overview of pathway analyses based on selected metabolites.

Figure 6 Biomarker candidates for liver fibrosis. A and B: Dynamic changes in the identified metabolites in each group; C-H: Receiver operator characteristic curves for the diagnosis of liver fibrosis based on the potential biomarkers, TBA and ALT. TBA: Total bile acid; ALT: Alanine aminotransferase; AUC: Area under the curve; CEA: Cervonoyl ethanolamide; β-MCA: β-muricholic acid.

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis is a common chronic progressive liver disease, and alanine aminotransferase is a commonly used diagnostic indicator for liver disease. Magnetic resonance imaging- and ultrasound-based elastography has been used for further assessment of hepatic steatosis and fibrosis, but these techniques are not able to diagnose inflammation and cell damage very well. Therefore, new liver fibrosis and functional biomarkers are needed as supplements.

Research motivation

Metabolomics is an important component of systems biology which provides quantitative measurements of global changes in individual metabolic characteristics in biological fluids responding to a variety of physiological and pathological stimuli, and it can be used to discover new biomarkers for differential diagnosis of disease.

Research objectives

The main objectives were to investigate the dynamic changes in metabolic profiles during the liver fibrosis progression, and seek for potential novel biomarkers for early diagnosis of liver fibrosis.

Research methods

A liver fibrosis model was induced by subcutaneous injection with CCl$_4$. The dynamic changes in metabolic profiles during the progression of liver fibrosis were analyzed by ultra-performance liquid chromatography-mass spectrometry, and independent sample test and receiver operating characteristic analysis were used to identify potential biomarkers.

Research results

A liver fibrosis model was successfully established, which was evaluated by liver chemical tests, liver histopathology, Masson's trichrome staining, and the expression levels of α-smooth muscle actin and transforming growth factor β1. Principal component analysis and orthogonal partial least squares discriminant analysis were used to characterize the metabolic profiles, which can clearly distinguish early liver fibrosis and advanced groups. We identified 21 metabolites associated with liver fibrosis, and two of them, β-muricholic acid (β-MCA) and cervonoyl ethanolamide (CEA), had excellent diagnostic value.

Research conclusions

The dynamic metabolomics profile is useful for screening early metabolic characteristics associated with progression of fibrosis. Two new metabolic biomarkers identified in this study, β-MCA and CEA, can correctly classify the disease stage in our fibrosis animal model.

Research perspectives

According to the results of rat experiments, further mechanistic studies are needed to investigate the involvement of these metabolites in fibrotic progression. We also need to collect clinical samples for further verification, and the markers identified may be used for clinical diagnosis in the future.

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REFERENCES

1   **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218 [PMID: 15690074 DOI: 10.1172/JCI24282]

2   **Zhang S**, Wu J, Wang H, Wang T, Jin L, Shu D, Shan W, Xiong S. Liposomal oxymatrine in hepatic fibrosis treatment: Formulation, in vitro and in vivo assessment. *AAPS PharmSciTech* 2014; **15**: 620-629 [PMID: 24515270 DOI: 10.1208/s12249-014-0086-y]

3   **Eng FJ**, Friedman SL. Fibrogenesis I. New insights into hepatic stellate cell activation: The simple becomes complex. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G7-G11 [PMID: 10898741 DOI: 10.1152/ajpgi.2000.279.1.G7]

4   **Marcellin P**, Asselah T, Boyer N. Fibrosis and disease progression in hepatitis C. *Hepatology* 2002; **36**: S47-S56 [PMID: 12407576 DOI: 10.1053/jhep.2002.36993]

5   **Chao DT**, Lim JK, Ayoub WS, Nguyen LH, Nguyen MH. Systematic review with meta-analysis: The proportion of chronic hepatitis B patients with normal alanine transaminase ≤ 40 IU/L and significant hepatic fibrosis. *Aliment Pharmacol Ther* 2014; **39**: 349-358 [PMID: 24387289 DOI: 10.1111/apt.12590]

6   **Lichtinghagen R**, Pietsch D, Bantel H, Manns MP, Brand K, Bahr MJ. The Enhanced Liver Fibrosis (ELF) score: Normal values, influence factors and proposed cut-off values. *J Hepatol* 2013; **59**: 236-242 [PMID: 23523583 DOI: 10.1016/j.jhep.2013.03.016]

7   **Mann J**, Reeves HL, Feldstein AE. Liquid biopsy for liver diseases. *Gut* 2018; **67**: 2204-2212 [PMID: 30177542 DOI: 10.1136/gutjnl-2017-315846]

8   **Cao H**, Huang H, Xu W, Chen D, Yu J, Li J, Li L. Fecal metabolome profiling of liver cirrhosis and hepatocellular carcinoma patients by ultra performance liquid chromatography-mass spectrometry. *Anal Chim Acta* 2011; **691**: 68-75 [PMID: 21458633 DOI: 10.1016/j.aca.2011.02.038]

9   **Nicholson JK**, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999; **29**: 1181-1189 [PMID: 10598751 DOI: 10.1080/004982599238047]

10 **Yang L**, Xiong A, He Y, Wang Z, Wang C, Wang Z, Li W, Yang L, Hu Z. Bile acids metabonomic study on the CCl4- and alpha-naphthylisothiocyanate-induced animal models: Quantitative analysis of 22 bile acids by ultraperformance liquid chromatography-mass spectrometry. *Chem Res Toxicol* 2008; **21**: 2280-2288 [PMID: 19053324 DOI: 10.1021/tx800225q]

11 **Li M**, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L. Symbiotic gut microbes modulate human metabolic phenotypes. *Proc Natl Acad Sci U S A* 2008; **105**: 2117-2122 [PMID: 18252821 DOI: 10.1073/pnas.0712038105]

12 **Goodman ZD**. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J Hepatol* 2007; **47**: 598-607 [PMID: 17692984 DOI: 10.1016/j.jhep.2007.07.006]

13 **Yu J**, Hao G, Wang D, Liu J, Dong X, Sun Y, Pan Q, Li Y, Shi X, Li L, Cao H. Therapeutic Effect and Location of GFP-Labeled Placental Mesenchymal Stem Cells on Hepatic Fibrosis in Rats. *Stem Cells Int* 2017; **2017**: 1798260 [PMID: 28491093 DOI: 10.1155/2017/1798260]

14 **Nord HJ**. Biopsy diagnosis of cirrhosis: Blind percutaneous versus guided direct vision techniques--a review. *Gastrointest Endosc* 1982; **28**: 102-104 [PMID: 6211383 DOI: 10.1016/S0016-5107(82)73015-9]

15 **Koizumi Y**, Hirooka M, Kisaka Y, Konishi I, Abe M, Murakami H, Matsuura B, Hiasa Y, Onji M. Liver fibrosis in patients with chronic hepatitis C: Noninvasive diagnosis by means of real-time tissue

elastography--establishment of the method for measurement. *Radiology* 2011; **258**: 610-617 [PMID: 21273523 DOI: 10.1148/radiol.10100319]

16   **Kim MY**, Jeong WK, Baik SK. Invasive and non-invasive diagnosis of cirrhosis and portal hypertension. *World J Gastroenterol* 2014; **20**: 4300-4315 [PMID: 24764667 DOI: 10.3748/wjg.v20.i15.4300]

17   **Rofiee MS**, Yusof MI, Abdul Hisam EE, Bannur Z, Zakaria ZA, Somchit MN, Teh LK, Salleh MZ. Isolating the metabolic pathways involved in the hepatoprotective effect of Muntingia calabura against CCl4-induced liver injury using LC/MS Q-TOF. *J Ethnopharmacol* 2015; **166**: 109-118 [PMID: 25792013 DOI: 10.1016/j.jep.2015.03.016]

18   **Chheda TK**, Shivakumar P, Sadasivan SK, Chanderasekharan H, Moolemath Y, Oommen AM, Madanahalli JR, Marikunte VV. Fast food diet with CCl4 micro-dose induced hepatic-fibrosis--a novel animal model. *BMC Gastroenterol* 2014; **14**: 89 [PMID: 24884574 DOI: 10.1186/1471-230X-14-89]

19   **Priller J**, Briley EM, Mansouri J, Devane WA, Mackie K, Felder CC. Mead ethanolamide, a novel eicosanoid, is an agonist for the central (CB1) and peripheral (CB2) cannabinoid receptors. *Mol Pharmacol* 1995; **48**: 288-292 [PMID: 7651362 DOI: 10.1111/j.1365-2885.1995.tb00595.x]

20   **Chiurchiù V**, Leuti A, Smoum R, Mechoulam R, Maccarrone M. Bioactive lipids ALIAmides differentially modulate inflammatory responses of distinct subsets of primary human T lymphocytes. *FASEB J* 2018; **32**: 5716-5723 [PMID: 29879374 DOI: 10.1096/fj.201800107R]

21   **Inoue M**, Tsuboi K, Okamoto Y, Hidaka M, Uyama T, Tsutsumi T, Tanaka T, Ueda N, Tokumura A. Peripheral tissue levels and molecular species compositions of N-acyl-phosphatidylethanolamine and its metabolites in mice lacking N-acyl-phosphatidylethanolamine-specific phospholipase D. *J Biochem* 2017; **162**: 449-458 [PMID: 28992041 DOI: 10.1093/jb/mvx054]

22   **Hansen HS**, Diep TA. N-acylethanolamines, anandamide and food intake. *Biochem Pharmacol* 2009; **78**: 553-560 [PMID: 19413995 DOI: 10.1016/j.bcp.2009.04.024]

23   **Rahman IA**, Tsuboi K, Uyama T, Ueda N. New players in the fatty acyl ethanolamide metabolism. *Pharmacol Res* 2014; **86**: 1-10 [PMID: 24747663 DOI: 10.1016/j.phrs.2014.04.001]

24   **Gulaya NM**, Kuzmenko AI, Margitich VM, Govseeva NM, Melnichuk SD, Goridko TM, Zhukov AD. Long-chain N-acylethanolamines inhibit lipid peroxidation in rat liver mitochondria under acute hypoxic hypoxia. *Chem Phys Lipids* 1998; **97**: 49-54 [PMID: 10081148 DOI: 10.1016/S0009-3084(98)00093-0]

25   **Balvers MG**, Verhoeckx KC, Meijerink J, Bijlsma S, Rubingh CM, Wortelboer HM, Witkamp RF. Time-dependent effect of in vivo inflammation on eicosanoid and endocannabinoid levels in plasma, liver, ileum and adipose tissue in C57BL/6 mice fed a fish-oil diet. *Int Immunopharmacol* 2012; **13**: 204-214 [PMID: 22498761 DOI: 10.1016/j.intimp.2012.03.022]

26   **Tam J**, Liu J, Mukhopadhyay B, Cinar R, Godlewski G, Kunos G. Endocannabinoids in liver disease. *Hepatology* 2011; **53**: 346-355 [PMID: 21254182 DOI: 10.1002/hep.24077]

27   **Zelber-Sagi S**, Azar S, Nemirovski A, Webb M, Halpern Z, Shibolet O, Tam J. Serum levels of endocannabinoids are independently associated with nonalcoholic fatty liver disease. *Obesity (Silver Spring)* 2017; **25**: 94-101 [PMID: 27863097 DOI: 10.1002/oby.21687]

28   **Trebicka J**, Racz I, Siegmund SV, Cara E, Granzow M, Schierwagen R, Klein S, Wojtalla A, Hennenberg M, Huss S, Fischer HP, Heller J, Zimmer A, Sauerbruch T. Role of cannabinoid receptors in alcoholic hepatic injury: steatosis and fibrogenesis are increased in CB2 receptor-deficient mice and decreased in CB1 receptor knockouts. *Liver Int* 2011; **31**: 860-870 [PMID: 21645218 DOI: 10.1111/j.1478-3231.2011.02496.x]

29   **Louvet A**, Teixeira-Clerc F, Chobert MN, Deveaux V, Pavoine C, Zimmer A, Pecker F, Mallat A, Lotersztajn S. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice. *Hepatology* 2011; **54**: 1217-1226 [PMID: 21735467 DOI: 10.1002/hep.24524]

30   **Teixeira-Clerc F**, Belot MP, Manin S, Deveaux V, Cadoudal T, Chobert MN, Louvet A, Zimmer A, Tordjmann T, Mallat A, Lotersztajn S. Beneficial paracrine effects of cannabinoid receptor 2 on liver injury and regeneration. *Hepatology* 2010; **52**: 1046-1059 [PMID: 20597071 DOI: 10.1002/hep.23779]

31   **Patsenker E**, Chicca A, Petrucci V, Moghadamrad S, de Gottardi A, Hampe J, Gertsch J, Semmo N, Stickel F. 4-O'-methylhonokiol protects from alcohol/carbon tetrachloride-induced liver injury in mice. *J Mol Med (Berl)* 2017; **95**: 1077-1089 [PMID: 28689299 DOI: 10.1007/s00109-017-1556-y]

32   **Wang DQ**, Tazuma S. Effect of beta-muricholic acid on the prevention and dissolution of cholesterol gallstones in C57L/J mice. *J Lipid Res* 2002; **43**: 1960-1968 [PMID: 12401895 DOI: 10.1194/jlr.m200297-jlr200]

33   **Hofmann AF**. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med* 1999; **159**: 2647-2658 [PMID: 10597755 DOI: 10.1001/archinte.159.22.2647]

34   **Ferraris R**, Colombatti G, Fiorentini MT, Carosso R, Arossa W, De La Pierre M. Diagnostic value of serum bile acids and routine liver function tests in hepatobiliary diseases. Sensitivity, specificity, and predictive value. *Dig Dis Sci* 1983; **28**: 129-136 [PMID: 6825534 DOI: 10.1007/BF01315142]

35   **Horvatits T**, Drolz A, Roedl K, Rutter K, Ferlitsch A, Fauler G, Trauner M, Fuhrmann V. Serum bile acids as marker for acute decompensation and acute-on-chronic liver failure in patients with non-cholestatic cirrhosis. *Liver Int* 2017; **37**: 224-231 [PMID: 27416294 DOI: 10.1111/liv.13201]

36   **Chen T**, Xie G, Wang X, Fan J, Qiu Y, Zheng X, Qi X, Cao Y, Su M, Wang X, Xu LX, Yen Y, Liu P, Jia W. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. *Mol Cell Proteomics* 2011; **10**: M110.004945 [PMID: 21518826 DOI: 10.1074/mcp.M110.004945]

37   **Yin P**, Wan D, Zhao C, Chen J, Zhao X, Wang W, Lu X, Yang S, Gu J, Xu G. A metabonomic study of hepatitis B-induced liver cirrhosis and hepatocellular carcinoma by using RP-LC and HILIC coupled with mass spectrometry. *Mol Biosyst* 2009; **5**: 868-876 [PMID: 19603122 DOI: 10.1039/b820224a]

ORIGINAL ARTICLE

Basic Study

Procyanidin B2 protects against diet-induced obesity and non-alcoholic fatty liver disease *via* the modulation of the gut microbiota in rabbits

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Abstract

BACKGROUND
Procyanidins have beneficial effects on metabolic syndrome and antimicrobial activity, but the mechanisms underlying these effects are unclear.

AIM
To investigate the effects of procyanidin B2 (PB2) on non-alcoholic fatty liver disease and to explore the possible mechanism.

METHODS
Thirty male New Zealand white rabbits were randomized into three groups. All of them were fed either a high-fat-cholesterol diet (HCD) or chow diet. HCD-fed rabbits were treated with vehicle or PB2 daily for 12 wk. Body weight and food intake were evaluated once a week. Serum biomarkers, such as total cholesterols, triglycerides, and aspartate transaminase, were detected. All rabbits were sacrificed and histological parameters of liver were assessed by hematoxylin and eosin-stained sections. Moreover, several lipogenic genes and gut microbiota (by 16S rRNA sequencing) were investigated to explore the possible mechanism.

RESULTS
The HCD group had higher body weight, liver index, serum lipid profile, insulin resistance, serum glucose, and hepatic steatosis compared to the CHOW group. PB2 treatment prevented HCD-induced increases in body weight and hypertriglyceridemia in association with triglyceride accumulation in the liver. PB2 also ameliorated low-grade inflammation, which was reflected by serum

lipopolysaccharides and improved insulin resistance. In rabbit liver, PB2 prevented the upregulation of steroid response element binding protein 1c and fatty acid synthase and the downregulation of carnitine palmitoyltransferase, compared to the HCD group. Moreover, HCD led to a decrease of *Bacteroidetes* in gut microbiota. PB2 significantly improved the proportions of *Bacteroidetes* at the phylum level and *Akkermansia* at the genus level.

CONCLUSION
Our results indicate the possible mechanism of PB2 to improve HCD-induced features of metabolic syndrome and provide a new dietary supplement.

Key words: Procyanidin; Rabbit; Non-alcoholic fatty liver disease; Gut microbiota; 16S rRNA

Core tip: Procyanidins are widely recognized for their excellent antioxidant properties and fewer side effects compared to other drugs. In the past, the mechanism of procyanidin to improve insulin resistance mainly focused on the antioxidant effect. The effect of procyanidin on non-alcoholic fatty liver disease is not clear. We found that procyanidin can reduce fatty liver by remodeling intestinal flora, decreasing endotoxemia, and down-regulating fatty acid synthesis genes. Our results open a new chapter in the mechanism of action of plant compounds and suggest a safer method for the treatment of non-alcoholic fatty liver disease.

INTRODUCTION
Advances in industrialization have changed people's lifestyles and eating habits. The incidence of obesity has rapidly increased, along with an increase in obesity-related metabolic syndrome, such as non-alcoholic fatty liver disease (NAFLD). NAFLD refers to the pathological state in which lipid, carbohydrates, and other substances are metabolically disordered. It is complex and is a risk factor for cardiovascular diseases and diabetes[1]. NAFLD is characterized by insulin resistance (IR) caused by a combination of genetic and environmental factors. Common manifestations include endotoxemia, low-grade inflammation, and tissue damage[2-4]. The intestinal barrier serves as the first site of interaction between the diet and the host immune system. This interaction can affect the composition of the gut microbiota, which affects intestinal immune homeostasis and intestinal permeability[5,6]. Thus, the gut and its microflora are potential sources of pro-inflammatory molecules that can affect systemic metabolism and may be involved in early events related to IR and NAFLD.

Evidence indicates that changes in intestinal mucosal permeability caused by a dysfunctional gut microbiota and metabolites produced by bacteria initiate the development of obesity-related metabolic diseases[7,8]. Lipopolysaccharides (LPS) derived from the gut microbiota are effective inflammation-inducing agents and play important roles in the occurrence and development of inflammation and related NAFLD[7,9]. A high-fat diet is associated with elevated systemic circulating levels of LPS (endotoxemia). Moreover, it causes an imbalance in the gut microbiota, especially by changing the proportion of *Firmicutes* and *Bacteroides*, and this is thought to play a key role in the pathogenesis of NAFLD[10]. The treatment of mice with broad-spectrum antibiotics can reshape the gut microbiota and improve insulin sensitivity[11]. Therefore, the gut microbiota is a potential source of pro-inflammatory molecules that can affect systemic metabolism and appear before the development of obesity and metabolic syndrome.

Proanthocyanidins are polyphenolic plant compounds that are naturally present in the diet[12]. Owing to their anti-oxidative, anti-inflammatory, and anti-atherogenic

properties, they have been studied extensively. Procyanidin B2 (PB2), an oligomeric anthocyanin precursor, is widely distributed in grapes, cranberries, and other berries. PB2 has been reported to improve dyslipidemia, hyperglycemia, and oxidative stress in individuals with metabolic syndrome through its anti-oxidative properties[13]. However, PB2 is a natural plant compound, and its bioavailability in humans is low[12,14]. The low levels of PB2 in the body make their superior effects difficult to explain. The mechanism underlying the beneficial effects of PB2 remains largely unknown. Recent growing evidence shows that procyanidin has strong antimicrobial effects against bacteria, fungi, and viruses and can rejuvenate the gut microbiota for health benefits[15]. A diet supplemented with proanthocyanidins can regulate the microbial composition in pig intestines and reduce the infection rate of swine mites. Given the close association between metabolic syndrome, the gut microbiota, and PB2, the purpose of this study was to determine the effects of PB2 on the metabolism of a high-fat-fed New Zealand white rabbits and to determine whether these effects are related to modulation of the gut microbiota.

MATIERIALS AND METHODS

Animal model, diet, and experimental procedures
Thirty New Zealand white rabbits (male, 2-mo-old, mean weight 2.0 ± 0.2 kg) were purchased from Shanghai Silaike Experimental Animal Co. LTD (Shanghai, China). Rabbits were housed in individual cages at a controlled temperature (22 ± 2 °C), with a 12-h light/dark period. Animals were fed either a chow (standard diet: 20% corn, 30% grass powder, 20% cardamom, 25% wheat bran, 5% multivitamins, FBSH Biotechnology Inc.) or a high-fat-cholesterol diet (HCD) (10% lard, 0.5% cholesterol, 5% sucrose, 1% maltodextrin, FBSH Biotechnology Inc.). After 2 wk of a high-fat, cholesterol diet, some rabbits were excluded because of very high (> 2 mmol/L) or low (< 0.5 mmol/L) cholesterol levels[16]. Group PB2 (fed a HCD, n = 8) received daily doses (150 mg/kg, dissolved in normal saline) of PB2 by gavage[17], whereas the other two groups [groups CHOW and HCD, fed a chow or HCD, n = 8] received the vehicle (normal saline) by gavage. Body weight gain and other parameters were evaluated once a week. Feces were collected at the end of 12 wk for 16S rRNA sequencing. After 12 wk, the rabbits were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for blood and tissue collection. The livers were weighed and the liver index (the ratio of liver to body weight) was calculated. The protocol was approved by the Animal Research Committee of the Second Xiangya Hospital, Central South University, Hunan, China (permit number: 20170722). The chemicals used in this study were of analytical grade. PB2 (≥ 95% purity) was purchased from Yuanye Biological Technology Co. Ltd. (Shanghai, China).

Measurement of serum biochemical markers
Blood samples were collected from the middle artery of the ear after overnight fasting at the end of 10 wk. The concentrations of serum total cholesterols (CH), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), serum alanine transaminase (ALT), aspartic acid transferase (AST), and glucose (GLU) were detected by enzymatic methods (BioMerieux, Lyon, France). At the end of the experiment, the intravenous glucose tolerance test (IVGTT) was performed. After fasting overnight, a bolus of glucose (0.6 g/kg body weight) was injected into the ear vein, and blood samples were collected from the middle artery at 0, 5, 10, 30, 60, and 120 min. The serum insulin concentrations were analyzed according to the protocols of the Rabbit Insulin ELISA Kit (Crystal Chem Co., Elk Grove Village, IL, United States). The serum LPS concentration was determined using a kit based on a Limulus amebocyte extract (LAL Kit; Lonza, Basel, Switzerland).

Histological analysis
After the rabbits were sacrificed, a morphological analysis of liver tissues was performed on hematoxylin and eosin-stained sections. Liver tissue samples were fixed with 10% neutral buffered formalin for 24 h, dehydrated with an ethanol solution, and embedded in paraffin. Tissue sections of 5 µm-6 µm in thickness were excised, deparaffinized, rehydrated, and stained with hematoxylin and eosin.

Quantitative real-time polymerase chain reaction (PCR)
Liver was homogenized and total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). A reverse transcription reaction was performed by Taqman Reverse Transcription reagent kit (Takara, Kusatsu, Japan) according to the manufacturer's instructions. The real-time PCR reaction was carried out on Roche real-time PCR system with cDNA sample containing SYBR Green PCR master mix

(Takara) and primers. Primer sequences can be made available upon request. Relative expression of mRNA was calculated by the comparative cycle threshold method.

Bacterial DNA extraction and PCR amplification

Microbial genomic DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen). The V4 hypervariable region of 16S rRNA was amplified by PCR using specific barcoded primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 806R: 5'-GGACTACHVGGGTWTCTA AT-3'). All PCR procedures were performed using Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, United States). A mixture of PCR products was purified using the Qiagen Gel Extraction Kit. The PCR products were used to generate a sequencing library using the TruSeq DNA PCRFREE Sample Preparation Kit (Illumina, San Diego, CA, United States). Finally, the library was sequenced using the Illumina HiSeq 2500 platform.

Bioinformatics analysis of sequencing data

The 16S raw data for all samples were processed and analyzed using the QIIME pipeline (1.9.1). Operational taxonomic units (OTUs) were clustered with a 97% similarity. A representative sequence for each OTU was selected and was classified using the Greengene database gg_13_8. Based on the analysis of OTUs, the species with the highest abundance at each taxonomic level for each sample was selected to generate the relative abundances of species. An unweighted UniFrac principal coordinate analysis (PCoA) was used to analyze the similarity in the composition of fecal flora in rabbits.

Statistical analysis

The measurement data obtained in the study were expressed as means ± standard deviation, and the differences were analyzed by one-way analysis of variance. Statistical analyses were performed using SPSS 22.0 (Armonk, NY, United States). Differences were statistically significant when $P < 0.05$.

RESULTS

Effects of PB2 on body weight gain and the serum lipid profile

To investigate the metabolic effects of PB2 on HCD, body weight and serum biomarkers were detected. The treatment of HCD-fed rabbits with PB2 prevented an increase in body weight, and this effect was observed after just 3 wk (Figure 1A and B). By the end of 12-wk period, the Chow group gained the least body weight. The body weight of the PB2 group was slightly higher than that of the Chow group but was much lower than that of the HCD group ($P < 0.05$). The HCD and PB2 groups required a period of adaptation to the HCD and exhibited reduced food intake. Throughout the experiment, the PB2 group consumed the least amount of food. There was no significant difference in food intake between the three groups of rabbits after the acclimation period (Figure 1C). At the end of the experiment, the liver index of the PB2 group was significantly lower than that of the HCD group (Figure 1D).

Several serum biochemical parameters, including TG, CH, LDL-C, HDL-C, GLU, and insulin, were determined. The values of TG, CH, and LDL-C were lowest in the Chow group. PB2 administration reduced serum TG, CH, and LDL-C levels compared with levels in the HCD group, ameliorating HCD-induced hypertriglyceridemia and hypercholesterolemia in these rabbits ($P < 0.05$) (Figure 2A-C). PB2 also significantly improved HDL-C levels compared with those in the HCD group ($P < 0.05$) (Figure 2D). Although PB2-treated HCD-fed rabbits did not show improved fasting glucose compared to that of the HCD group, these animals displayed lower concentrations of fasting insulin than those in untreated HCD-fed animals ($P < 0.05$) (Figure 2E and F). The IVGTT was performed, and changes in serum glucose are presented in Figure 2G. The incremental area under the curve (AUC) for glucose in the PB2 group was lower than that in the HCD group (Figure 2H), but the difference was not statistically significant. Twelve weeks of HCD feeding produced a significant increase in circulating LPS, which was partially prevented by PB2 administration ($P < 0.05$) (Figure 2I). Thus, PB2 alleviated the endotoxemia induced by HCD.

Effects of PB2 on diet-induced hepatic steatosis in rabbits

Since PB2 could inhibit hypertriglyceridemia and hypercholesterolemia in rabbits, we next examined the effects of PB2 on HCD-induced hepatic steatosis. We performed a histological analysis of the liver by staining with hematoxylin and eosin to investigate the effect of PB2 on hepatic triglyceride accumulation. The HCD group showed obvious triglyceride accumulation in the liver. The administration of PB2 resulted in significantly less triglyceride accumulation compared with that in the HCD group.

Figure 1 Effects of procyanidin B2 on body composition. A: Weight gain curves; B: Total weight gain; C: Food intake curves; D: Liver index. The data are expressed as means ± SD. ${}^a P < 0.05$ *vs* CHOW group; ${}^b P < 0.05$ *vs* high-fat-cholesterol diet group. HCD: High-fat-cholesterol diet; PB2: Procyanidin B2; SD: Standard deviation.

The Chow group had undetectable triglycerides in the liver by hematoxylin and eosin staining (Figure 3A). At the end of the experiment, the liver weight in the HCD group was significantly higher than that of the Chow group ($P < 0.05$). PB2 treatment significantly reduced liver weight compared to the HCD group ($P < 0.05$) (Figure 3B). There was no significant difference in liver enzymes among the three groups (Figure 3C and D). Moreover, compared to the HCD group, PB2 treatment partially prevented the upregulation of the steroid response element binding protein 1c (SREBP1c) and the downregulation of carnitine palmitoyltransferase ($P < 0.05$) (Figure 3E-G). The expression of fatty acid synthase (FASN) was also decreased compared with the HCD group, but the difference was not significant.

Effects of procyanidin on the gut microbiota

The overall composition of the gut microbiota in each group was investigated. For each sample, the V4 hypervariable region of the bacterial 16S rRNA gene sequence was amplified by PCR. After filtering out sequences with a low mass and short length, 1800802 high-quality reads were obtained (average, 78295 sequences per sample). We identified 3612 OTUs based on the conventional criterion of a 97% sequence identity (equal to the species level). The coverage index (sequencing depth index) for all samples ranged from 0.984 to 0.996.

Unweighted UniFrac PCoA differentiated microbial communities based on diet and treatment were generated. As shown in Figure 4A, PCoA revealed that the HCD diet promoted major alterations in the gut microbiota. Distinct clusters were observed for PB2-treated rabbits, Chow-fed rabbits, and HCD-fed rabbits. These results demonstrated that PB2 administration has a significant effect on gut microbial

Figure 2 Effects of procyanidin B2 on serum lipid profile, insulin resistance, and endotoxemia. Serum triglycerides (A), total cholesterol (B), low-density lipoprotein cholesterol (C), high-density lipoprotein cholesterol (D) and fasting glucose (E), fasting insulin (F) and the intravenous glucose tolerance test (G), units of area under the curve (H) and lipopolysaccharides (I). The data are expressed as means ± SD. [a]$P < 0.05$ *vs* CHOW group; [b]$P < 0.05$ *vs* high-fat-cholesterol diet group. HCD: High-fat-cholesterol diet; PB2: Procyanidin B2; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; IVGTT: Intravenous glucose tolerance test; AUC: Area under the curve; LPS: Lipopolysaccharides; SD: Standard deviation.

composition in HCD-fed rabbits. A total of 10 phyla were detected, and *Firmicutes, Bacteroidetes, Proteobacteria, and Tenericutes* were the most abundant phyla in these samples (Figure 4B). At the phylum level, the proportion of *Bacteroidetes* was significantly reduced in the HCD-fed rabbits ($P < 0.05$), whereas the relative abundance of *Proteobacteria* was significantly increased compared to that of the Chow-fed rabbits ($P < 0.05$). Similar trends for *Bacteroidetes* and *Proteobacteria* were observed in Chow-fed animals and PB2-treated animals. PB2 administration led to a low ratio of *Firmicutes* to *Bacteroidetes* ($P < 0.05$).

At the genus level, there were significant alterations in *Allobaculum, Ruminococcus, Bacteroidetes*, and *Akkermansia* (Figure 4D and E). Compared with the HCD group, the relative abundance of *Allobaculum* was significantly reduced in the PB2 group and the relative abundances of *Ruminococcus* and *Bacteroidetes* were significantly increased. Of note, PB2 treatment was associated with a striking 3-fold increase in the relative abundance of *Akkermansia* compared with that of the HCD group.

Different numbers of OTUs were detected in each group, *i.e.* 2941 in the Chow group, 2940 in the HCD group, and 2712 in the PB2 group (Figure 4C). Among all OTUs, 2166 were shared by all groups. Additionally, each group had unique OTUs,

Figure 3 Effects of procyanidin B2 on hepatic steatosis. A: Hematoxylin and eosin staining of liver in CHOW group, HCD group and procyanidin B2 group; B: Liver weight; C: Alanine transaminase; D: Aspartate transaminase; E: Relative expression levels of steroid response element binding protein 1c; F: Relative expression levels of fatty acid synthase; G: Relative expression levels of carnitine palmitoyltransferase. The data are expressed as means ± SD. [a]$P < 0.05$ *vs* CHOW group; [b]$P < 0.05$ *vs* HCD group. HCD: High-fat-cholesterol diet; PB2: Procyanidin B2; ALT: Alanine transaminase; AST: Aspartate transaminase; SREBP-1c: Steroid response element binding protein 1c; FASN: Fatty acid synthase; CPT: Carnitine palmitoyltransferase; SD: Standard deviation.

including 174 in the Chow group, 210 in the HCD group, and 274 in the PB2 group.

DISCUSSION

In the current study, we used an HCD-induced model to investigate the effects of PB2 on several components of metabolic syndrome. Our results suggested that PB2 prevents HCD-induced weight gain and the development of NAFLD and improves insulin sensitivity. PB2 also prevented the upregulation of several lipogenic genes, including SREBP1c and FASN. Moreover, PB2 administration led to a dramatic alteration in the gut microbiota by increasing the proportion of *Bacteroidetes* at the phylum level and *Akkermansia* at the genus level.

Extensive evidence indicates that high polyphenol-rich fruit consumption is negatively correlated with several features of metabolic syndrome[18-20]. Although the positive health effects of fruit may be attributed to vitamins, minerals, and dietary fiber, a growing number of studies support the role of polyphenols in protection against obesity-related diseases[21]. Accordingly, we explored the impacts of PB2, a major component of berries, on several features of NAFLD[22,23]. Our results showed that PB2 reduces weight gain as well as the liver index, and this effect on weight was observed before significant change of food intake was detected. IVGTT revealed an improvement in IR by fasting and postprandial glucose in the PB2 group compared with the HCD group. PB2 treatment had an obvious lipid-lowing effect; it

A

PCoA plot

B

C

D

Figure 4 Effects of procyanidin B2 on gut microbiota. A: Principal coordinate analysis of the gut microbiota based on unweighted UniFrac distances between groups; B: Relative abundance of gut microbiota in phylum; C: Venn diagrams described the number of operational taxonomic units that are distinct and shared across the groups; D: Heatmap: Community hierarchical clustering analysis of major taxonomic groups at genus level, the red represents high relative abundance and the blue represent low relative abundance; E: Relative abundance of gut microbiota in genus. HCD: High-fat-cholesterol diet; PB2: Procyanidin B2; PCoA: Principal coordinate analysis; OTUs: Operational taxonomic units.

significantly reduced the serum levels of TG, TC, and LDL-C compared to the HCD group at the end of the experiment. Additionally, PB2 prevented the development of NAFLD to some extent by reducing liver TG deposition and weakened inflammation induced by the HCD.

SREBP-1c is a major regulator of fatty acids (FA) synthesis, which may be involved in IR and NAFLD development, with the goal of reducing lipogenesis and TG levels. Overexpressing SREBP1c increased FA synthase, leading to a higher level of liver FA and TG; whereas SREBP-1c knockout mice showed decreased FASN expression, liver FA, and serum TG levels[24]. Our results showed that PB2 significantly prevented the upregulation of SREBP-1c compared to the HCD group. The inhibition of SREBP1 upregulation may be included in the mechanisms of PB2 to alleviate the steatosis in hepatic cells.

A high ratio of *Firmicutes* to *Bacteroidetes* is often thought to be a key characteristic of obesity and NAFLD[25]. Ciubotaru *et al*[26] have reported that a high proportion of *Bacteroidetes* is associated with better glycemic control in humans. Rabot *et al*[27] found that the transplantation of a Bacteroides-rich microbiota improves glucose intolerance caused by an HCD in mice. Fernando reported that a lower *Firmicutes* to *Bacteroidetes* ratio is associated with improved glucose and insulin tolerance after cranberry extract administration[28]. Similar to previous results, we observed the partial recovery of IR and reshaping of gut microbiota induced by HCD after PB2 treatment. Future studies are warranted to determine whether the ratio of *Firmicutes* to *Bacteroidetes* influences host glucose homeostasis prior to fat mass accumulation.

The gut microbiota has a casual role in the pathogenesis of NAFLD[5]. Previous reports have suggested that polyphenolic plant compounds have poor absorption and are mainly absorbed in the intestines[13]. This prompted us to assess the impact of PB2 on gut microbiota. We demonstrated that an HCD leads to a dramatic shift in the gut microbiota of rabbits by decreasing the proportion of *Bacteroidetes* and increasing the ratio of *Firmicutes* to *Bacteroidetes*, whereas PB2 treatment changed this trend. This diet-induced remodeling of gut microbial communities in HCD-fed rabbits is a typical feature of obesity-driven dysbiosis and is in agreement with previous results[10].

According to our results, it is possible that PB2 influences metabolic phenotypes by regulating the relative abundance of *Akkermansia*. This is consistent with several previous studies that plant compounds can have a significant impact on the proportion of *Akkermansia* in the gut microbiota of an animal model[25-30]. The administration of cranberry extract to high fat-fed mice is associated with the relative proportion of *Akkermansia*, whereas a study revealed that treatment of HCD-fed mice with green tea polyphenols can modulate the gut microbial ecosystem (*in vitro*) by increasing the proportion of *Akkermansia*. In addition, the metabolic benefit of resveratrol is also associated with an increased intestinal abundance of the bacteria. Similarly, our results suggest that treatment with PB2, a plant compound, leads to an increase in the *Akkermansia* population and might be sufficient to prevent the negative metabolic phenotype caused by an HCD.

Hepatic steatosis involves the deposition of TG, which together with oxidative stress, constitutes the basis for the pathophysiology of NAFLD. In obese patients, LPS from the intestinal microbiota enters the liver through the portal vein and affects physiological metabolic processes in the host[31]. In our experiments, LPS levels in the HCD-fed rabbits were higher than those in the Chow group, and the change was partially reversed by treatment with PB2. Interestingly, the use of *Akkermansia* as a probiotic could reduce serum LPS levels in mice fed an HCD. This may be explained by the ability of *Akkermansia* to retain the thickness of the intestinal mucus layer, thus reducing intestinal permeability and LPS leakage. Therefore, the ability of PB2 to reduce serum LPS levels in our experiments could explain the reduction in liver TG accumulation and the protection against hepatic oxidative stress and inflammation in HCD-fed rabbits.

While our study provides evidence for the beneficial effects of PB2 for the treatment of NAFLD, some limitations must be acknowledged. Further studies should evaluate whether its effects on metabolic phenotype are mediated by the metabolic products of the gut microbiota, such as trimethylamine-*N*-oxide or bile acids.

In summary, we found that PB2 treatment protects against HCD-induced obesity, IR, and liver steatosis in rabbits. These effects were associated with the alleviation of intestinal inflammation and metabolic endotoxemia. These results led us to propose that PB2 may prevent obesity and NAFLD by a prebiotic effect on the gut microbiota.

ARTICLE HIGHLIGHTS

Research background

The mechanism of procyanidin to improve metabolic syndrome mainly focuses on its antioxidant effect. The latest studies show that procyanidin have commendable antibacterial properties. The evaluation of remodeling gut microbiota in non-alcoholic fatty liver disease (NAFLD) by procyanidin may provide a new therapeutic trend.

Research motivation

Procyanidin has been reported to improve dyslipidemia, hyperglycemia, and oxidative stress through its anti-oxidative properties. However, procyanidin is a natural plant compound, and its bioavailability in humans is low. The low levels of procyanidin B2 (PB2) in the body make their superior effects difficult to explain. The mechanism underlying the beneficial effects of procyanidin remains largely unknown.

Research objectives

To validate the effect of procyanidin on NAFLD and to clarify the possible mechanism of action.

Research methods

New Zealand white rabbits were fed chow or high-fat-cholesterol diet (HCD) for 12 wk. The body weight was investigated every week. The serum samples were analyzed after a 12-wk time. Hematoxylin and eosin staining of liver samples were performed, and fatty acid synthesis genes of liver were evaluated. The gut microbiota was sequenced by 16S rRNA analysis.

Research results

Our results show that procyanidin is associated with alleviated hepatic steatosis, decrease serum lipid, suppressed gut inflammation, and remolded gut microbiota.

Research conclusions

Procyanidin treatment protects against HCD-induced obesity, insulin resistance, and liver steatosis in rabbits. These effects were associated with the alleviation of intestinal inflammation and endotoxemia.

Research perspectives

Plant compounds, such as procyanidin, should be further explored for their potential therapeutic activity in NAFLD.

REFERENCES

1   **Eckel RH**, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet* 2005; **365**: 1415-1428 [PMID: 15836891 DOI: 10.1016/S0140-6736(05)66378-7]

2   **Hossain P**, Kawar B, El Nahas M. Obesity and diabetes in the developing world--a growing challenge. *N Engl J Med* 2007; **356**: 213-215 [PMID: 17229948 DOI: 10.1056/NEJMp068177]

3   **Marette A**. Mediators of cytokine-induced insulin resistance in obesity and other inflammatory settings. *Curr Opin Clin Nutr Metab Care* 2002; **5**: 377-383 [PMID: 12107372 DOI: 10.1097/00075197-200207000-00005]

4   **Xu H**, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J*

Clin Invest 2003; **112**: 1821-1830 [PMID: 14679177 DOI: 10.1172/JCI19451]

5   **Arpaia N**, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 2013; **504**: 451-455 [PMID: 24226773 DOI: 10.1038/nature12726]

6   **Ding S**, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, Jobin C, Lund PK. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. *PLoS One* 2010; **5**: e12191 [PMID: 20808947 DOI: 10.1371/journal.pone.0012191]

7   **Cani PD**, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* 2007; **50**: 2374-2383 [PMID: 17823788 DOI: 10.1007/s00125-007-0791-0]

8   **Geurts L**, Lazarevic V, Derrien M, Everard A, Van Roye M, Knauf C, Valet P, Girard M, Muccioli GG, François P, de Vos WM, Schrenzel J, Delzenne NM, Cani PD. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. *Front Microbiol* 2011; **2**: 149 [PMID: 21808634 DOI: 10.3389/fmicb.2011.00149]

9   **Cani PD**, Neyrinck AM, Maton N, Delzenne NM. Oligofructose promotes satiety in rats fed a high-fat diet: involvement of glucagon-like Peptide-1. *Obes Res* 2005; **13**: 1000-1007 [PMID: 15976142 DOI: 10.1038/oby.2005.117]

10   **Hildebrandt MA**, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD. High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* 2009; **137**: 1716-24.e1-2 [PMID: 19706296 DOI: 10.1053/j.gastro.2009.08.042]

11   **Rune I**, Rolin B, Larsen C, Nielsen DS, Kanter JE, Bornfeldt KE, Lykkesfeldt J, Buschard K, Kirk RK, Christoffersen B, Fels JJ, Josefsen K, Kihl P, Hansen AK. Modulating the Gut Microbiota Improves Glucose Tolerance, Lipoprotein Profile and Atherosclerotic Plaque Development in ApoE-Deficient Mice. *PLoS One* 2016; **11**: e0146439 [PMID: 26799618 DOI: 10.1371/journal.pone.0146439]

12   **Bladé C**, Arola L, Salvadó MJ. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol Nutr Food Res* 2010; **54**: 37-59 [PMID: 19960459 DOI: 10.1002/mnfr.200900476]

13   **Baba S**, Osakabe N, Natsume M, Terao J. Absorption and urinary excretion of procyanidin B2 [epicatechin-(4beta-8)-epicatechin] in rats. *Free Radic Biol Med* 2002; **33**: 142-148 [PMID: 12086692 DOI: 10.1016/S0891-5849(02)00871-7]

14   **Sano A**, Yamakoshi J, Tokutake S, Tobe K, Kubota Y, Kikuchi M. Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Biosci Biotechnol Biochem* 2003; **67**: 1140-1143 [PMID: 12834296 DOI: 10.1271/bbb.67.1140]

15   **Williams AR**, Krych L, Fauzan Ahmad H, Nejsum P, Skovgaard K, Nielsen DS, Thamsborg SM. A polyphenol-enriched diet and Ascaris suum infection modulate mucosal immune responses and gut microbiota composition in pigs. *PLoS One* 2017; **12**: e0186546 [PMID: 29028844 DOI: 10.1371/journal.pone.0186546]

16   **Luo F**, Guo Y, Ruan GY, Long JK, Zheng XL, Xia Q, Zhao SP, Peng DQ, Fang ZF, Li XP. Combined use of metformin and atorvastatin attenuates atherosclerosis in rabbits fed a high-cholesterol diet. *Sci Rep* 2017; **7**: 2169 [PMID: 28526884 DOI: 10.1038/s41598-017-02080-w]

17   **Wang L**, Yamashita Y, Komeda S, Saito A, Ashida H. Absorption, metabolism, distribution and faecal excretion of B-type procyanidin oligomers in mice after a single oral administration of black soybean seed coat extract. *Food Funct* 2018; **9**: 5362-5370 [PMID: 30264089 DOI: 10.1039/c8fo00852c]

18   **Cardona F**, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. *J Nutr Biochem* 2013; **24**: 1415-1422 [PMID: 23849454 DOI: 10.1016/j.jnutbio.2013.05.001]

19   **Eshak ES**, Iso H, Mizoue T, Inoue M, Noda M, Tsugane S. Soft drink, 100% fruit juice, and vegetable juice intakes and risk of diabetes mellitus. *Clin Nutr* 2013; **32**: 300-308 [PMID: 22917499 DOI: 10.1016/j.clnu.2012.08.003]

20   **Morimoto A**, Ohno Y, Tatsumi Y, Mizuno S, Watanabe S. Effects of healthy dietary pattern and other lifestyle factors on incidence of diabetes in a rural Japanese population. *Asia Pac J Clin Nutr* 2012; **21**: 601-608 [PMID: 23017319]

21   **Corder R**, Mullen W, Khan NQ, Marks SC, Wood EG, Carrier MJ, Crozier A. Oenology: red wine procyanidins and vascular health. *Nature* 2006; **444**: 566 [PMID: 17136085 DOI: 10.1038/444566a]

22   **Bellentani S**, Saccoccio G, Masutti F, Crocè LS, Brandi G, Sasso F, Cristanini G, Tiribelli C. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000; **132**: 112-117 [PMID: 10644271 DOI: 10.7326/0003-4819-132-2-200001180-00004]

23   **Choudhury J**, Sanyal AJ. Insulin resistance and the pathogenesis of nonalcoholic fatty liver disease. *Clin Liver Dis* 2004; **8**: 575-594, ix [PMID: 15331065 DOI: 10.1016/j.cld.2004.04.006]

24   **Del Bas JM**, Ricketts ML, Baiges I, Quesada H, Ardevol A, Salvadó MJ, Pujadas G, Blay M, Arola L, Bladé C, Moore DD, Fernandez-Larrea J. Dietary procyanidins lower triglyceride levels signaling through the nuclear receptor small heterodimer partner. *Mol Nutr Food Res* 2008; **52**: 1172-1181 [PMID: 18720348 DOI: 10.1002/mnfr.200800054]

25   **Ley RE**, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* 2005; **102**: 11070-11075 [PMID: 16033867 DOI: 10.1073/pnas.0504978102]

26   **Ciubotaru I**, Green SJ, Kukreja S, Barengolts E. Significant differences in fecal microbiota are associated with various stages of glucose tolerance in African American male veterans. *Transl Res* 2015; **166**: 401-411 [PMID: 26209747 DOI: 10.1016/j.trsl.2015.06.015]

27   **Rabot S**, Membrez M, Blancher F, Berger B, Moine D, Krause L, Bibiloni R, Bruneau A, Gérard P, Siddharth J, Lauber CL, Chou CJ. High fat diet drives obesity regardless the composition of gut microbiota in mice. *Sci Rep* 2016; **6**: 32484 [PMID: 27577172 DOI: 10.1038/srep32484]

28   **Anhê FF**, Nachbar RT, Varin TV, Vilela V, Dudonné S, Pilon G, Fournier M, Lecours MA, Desjardins Y, Roy D, Levy E, Marette A. A polyphenol-rich cranberry extract reverses insulin resistance and hepatic steatosis independently of body weight loss. *Mol Metab* 2017; **6**: 1563-1573 [PMID: 29107524 DOI: 10.1016/j.molmet.2017.10.003]

29   **Belzer C**, de Vos WM. Microbes inside--from diversity to function: the case of Akkermansia. *ISME J* 2012; **6**: 1449-1458 [PMID: 22437156 DOI: 10.1038/ismej.2012.6]

30   **Chen ML**, Yi L, Zhang Y, Zhou X, Ran L, Yang J, Zhu JD, Zhang QY, Mi MT. Resveratrol Attenuates Trimethylamine-N-Oxide (TMAO)-Induced Atherosclerosis by Regulating TMAO Synthesis and Bile Acid Metabolism via Remodeling of the Gut Microbiota. *MBio* 2016; **7**: e02210-e02215 [PMID: 27048804 DOI: 10.1128/mBio.02210-15]

31   **Gariani K**, Philippe J, Jornayvaz FR. Non-alcoholic fatty liver disease and insulin resistance: from bench to bedside. *Diabetes Metab* 2013; **39**: 16-26 [PMID: 23266468 DOI: 10.1016/j.diabet.2012.11.002]

ORIGINAL ARTICLE

Case Control Study

Triggers of histologically suspected drug-induced colitis

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Abstract

BACKGROUND
Drug toxicity is a common and even serious problem in the gastrointestinal tract that is thought to be caused by a broad spectrum of agents. Although withdrawal of the causative agent would cure the disease knowledge is scarce and mostly derives from case reports and series.

AIM
To investigate potential triggers of drug-induced colitis (DiC).

METHODS
We conducted a retrospective, observational case control study. Patients were assigned to DiC or one of two age- and gender-matched control groups (non-inflammatory controls and inflammatory colitis of another cause) based on histopathological findings. Histopathology was reassessed in a subset of patients (28 DiC with atherosclerosis, DiC without atherosclerosis and ischaemic colitis each) for validation purposes. Medical history was collected from the electronic database and patient records. Statistical analysis included chi-squared test, *t*-test, logistic and multivariate regression models.

RESULTS
Drug-induced colitis was detected in 211 endoscopically sampled biopsy specimens of the colon mucosa (7% of all screened colonoscopic biopsy samples); a total of 633 patients were included equally matched throughout the three groups (291 males, mean age: 62.1 ± 16.1 years). In the univariate analysis, DiC was associated with diuretics, dihydropyridines, glycosides, ASS, platelet

aggregation inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDs), statins and fibrates, and with atherosclerosis, particularly coronary heart disease, and hyperlipoproteinaemia. Echocardiographic parameters did not show substantial differences. In the multivariate analysis only fibrates [odds ratio (OR) = 9.1], NSAIDs (OR = 6.7) and atherosclerosis (OR = 2.1) proved to be associated with DiC. Both DiC reassessment groups presented milder inflammation than ischaemic colitis. The DiC patients with atherosclerosis exhibited histological features from both DiC without atherosclerosis and ischaemic colitis.

CONCLUSION
Several drugs indicated for the treatment of cardiovascular and related diseases are associated with DiC. Atherosclerosis and microcirculatory disturbances seem to play an important pathogenetic role.

Key words: Drug toxicity; Drug-induced colitis; Ischaemic colitis; Drug-associated gastrointestinal disease; Atherosclerosis; Colonic ischaemia; Nonsteroidal anti-inflammatory drugs; Fibrates

Core tip: Several drugs have been attributed to drug-induced colitis (DiC). In this systematical age- and gender-matched retrospective cohort study based on histopathological findings DiC was associated with drugs predominantly indicated for the treatment of cardiovascular and related diseases, nonsteroidal anti-inflammatory drugs, with atherosclerosis, particularly coronary heart disease, and hyperlipoproteinaemia. Histopathology was reassessed in three groups (DiC with atherosclerosis, DiC without atherosclerosis and ischaemic colitis each); both DiC groups presented milder inflammation than ischaemic colitis; DiC patients with atherosclerosis exhibited histological features from both other groups. In conclusion, atherosclerosis and microcirculatory disturbances seem to play an important pathogenetic role.

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INTRODUCTION

Drug toxicity is a common and even serious problem in the gastrointestinal tract that is thought to be caused by a broad spectrum of drugs. Related symptoms are unspecific and assumed to cover the whole set of complaints known for colitis of any other cause, *i.e.*, bloating, abdominal pain, cramping, diarrhoea, weight loss, mucosal bleeding or anaemia[1,2]. Furthermore, the continued intake of harmful medications can lead to structural bowel damage, *i.e.*, development of strictures[3], perforation[4] or severe colitis with the need for emergency colectomy[5]. Although withdrawal of the respective trigger should cure the disease, data about drug-induced colitis (DiC) are scarce. Deeper insights into the phenotype of DiC, the underlying pathomechanisms and the identification of possible triggers are mandatory particularly since intake of multiple drugs hampers the determination of a single drug as the causative agent.

Various patterns of drug-induced damage at the colonic site have been described. These include nonsteroidal anti-inflammatory drug (NSAID) colonopathy, antraquinone-induced laxative-associated damage (melanosis coli), corticosteroid-associated damage (malakoplakia), gold compound-associated damage and other drug-induced conditions, such as microscopic colitis, antibiotics associated infectious and ischaemic colitis[6]. The main pathologies of drug-induced gastrointestinal disease are ulceration, stricture formation, variable inflammatory processes and ischaemia. Within these overall major patterns, microscopic clues, such as apoptosis, cytoplasmic vacuolation, increased intraepithelial lymphocytes, melanosis coli and eosinophils, are pointers to a drug-induced pathology, though all are far from specific[2].

In 2004, Cappell comprehensively collected and critically reviewed the knowledge

about potential triggers and mechanisms of drug-induced colontoxicity and categorised them into well-established and probable associations[7]. Suspected agents having well-established associations with colonic ischaemia include cocaine, ergotamine and estrogens, and probable associations include alosetron, digitalis, dopamine, (nor)epinephrine, methysergide, NSAIDs, vasopressin, barbiturates, diuretics and tricyclic antidepressants. Gold compounds, NSAIDs and potassium chloride are thought to cause allergic, cytotoxic or inflammatory colitis; probable associations comprise alpha-methyldopa, salicylates, selective COX-2 inhibitors, carbamazepine, cimetidine, simvastatine, methodrexate, bisaodyl, penicillamine, isoretinoin[1,7]. Other drugs, such as immune checkpoint inhibitors (*i.e.*, ipilimumab) and neuroleptics, have recently been identified as causing colitis[5,8]. Multiples of the associations mentioned above, such as statin use, rely on a single case report[9], a series of case reports and case series[7]. Up to now, no study has investigated potential triggers in a larger cohort of histologically suspected DiC. Hence, we aimed to analyse DiC in comparison to two different age- and gender-matched control groups.

MATERIALS AND METHODS

Study population
We conducted a single-centre retrospective cohort study of patients undergoing colonoscopy with biopsy between 2006 and 2016, referred by the Department of Gastroenterology and Hepatology of the University Hospital Bergmannsheil gGmbH in Bochum, Germany (Supplementary Table 1 and Table 1). All patients of whom a histopathological report was available were considered eligible for inclusion. Outpatients were excluded from analysis due to insufficient information about medical history.

Patients were assigned to one of three groups based on histopathology: DiC, non-inflammatory controls (NiC) and inflammatory colitis of another cause (IC) (Figure 1). Patients of both control groups were age- and gender-matched; DiC patients without matching patients were excluded. Histology of microscopic colitis, pseudomembranous colitis and radiation-induced injury were reasons for exclusion for DiC patients. Non-inflammatory controls consisted of patients with irritable bowel syndrome, functional disorders or colorectal cancer screening. Inflammatory controls included diverticulitis, inflammatory bowel disease and ischaemic colitis.

The primary histopathological assessment was validated in a second approach. From a clinical perspective, patients were divided into three groups: DiC without atherosclerosis, DiC with atherosclerosis, and ischaemic colitis. Patients from the first two groups derived from the DiC group while the latter group was gathered from the IC group. Hereby, 28 age- and gender-matched triplets were assembled.

Objectives
The aim of the study was to reveal associations between different agents and the presence of DiC. Secondary objectives were to describe the symptomatology, to identify cofactors that support the presence of DiC and to evaluate the reliability of the histopathological criteria.

Medical history
The electronic database and patient records have been reviewed to identify all drugs prescribed up to 21 d prior to colonoscopy. The specific agents were assigned to 36 different classes, as displayed in Supplementary Table 2.

Histopathological assessment
The histopathological assessment was performed by an expert pathologist based on international standards using haematoxylin and eosin stain including low- and high-power examination. Diagnosis of DiC was based on mixed, predominantly neutrophilic or lymphocytic inflammatory infiltrates, erosions, absence of granulomas, absence of basal plasmacellular infiltration and absence of crypt architectural distortion. Laxative-, corticosteroid- or gold compound-associated damage and well-defined drug-induced conditions, such as microscopic, infectious (including clostridium-associated colitis) and neutropenic colitis, were not regarded as suitable for inclusion.

Histopathological reassessment
Representative biopsy specimens were re-evaluated using haematoxylin and eosin stain with a magnification of 100-fold and assessed regarding oedema, haemorrhage, lymphocytic, granulocytic or eosinophilic infiltration, erosions, ulcerous lesions, necrosis, fibrin plaques on erosions, and fibrosis. The same team of pathologists

Table 1 Basic demographic characteristics *n* (%)

	Drug-induced colitis, *n* = 211	Non-inflammatory controls, *n* = 211	Inflammatory controls, *n* = 211
Basic characteristics			
Age (yr)	62.3 ± 16.4	62.2 ± 16.3	61.8 ±15.7
Gender (male)	97 (46.0)	97 (46.0)	97 (46.0)
Height (cm)	168.9 ± 9.5	168.3 ± 12.4	170.0 ± 9.3
Body weight (kg)	76.9 ± 22.6	75.0 ± 20.3	72.9 ± 19.7
BMI (kg/m^2)	26.9 ± 7.2[a1b]	26.3 ± 6.8	25.0 ± 5.6[a3]
ASA[b2]			
ASA 1	0 (0)	3 (1.4)	0 (0)
ASA 2	133 (63.0)	169 (80.1)	155 (73.5)
ASA 3	70 (33.2)	37 (17.5)	52 (24.6)
ASA 4	8 (3.8)	2 (0.9)	4 (1.9)
ECOG[b2,b3]			
ECOG 0	0 (0)	1 (0.5)	2 (0.9)
ECOG 1	8 (3.8)	75 (35.5)	13 (6.2)
ECOG 2	149 (70.6)	104 (49.3)	147 (69.7)
ECOG 3	41 (19.4)	26 (12.3)	43 (20.4)
ECOG 4	13 (6.2)	5 (2.4)	6 (2.8)
Indication of colonoscopy[b1,a2]			
Diarrhoea	73 (34.6)	95 (45.0)	97 (46.0)
Constipation	6 (2.8)	5 (2.4)	1 (0.5)
Gastrointestinal bleeding	63 (29.9)	31 (14.6)	36 (17.1)
Abdominal pain	47 (22.3)	54 (25.6)	46 (21.8)
Weight loss	6 (2.8)	5 (2.4)	4 (1.9)
Scheduled survey	2 (0.9)	1 (0.5)	2 (0.9)
Miscellaneous	14 (6.6)	20 (9.5)	25 (11.8)

[1]Drug-induced colitis *vs* inflammatory controls.
[2]Drug-induced colitis *vs* non-inflammatory controls.
[3]Non-inflammatory controls *vs* inflammatory controls.
[a]$P < 0.05$.
[b]$P < 0.01$. Statistical analysis was carried out with χ^2 test, ANOVA or *t*-test as appropriate. BMI: Body mass index.

evaluated the slides unaware of the former results and the respective group.

Statistical analysis

Statistical analysis was performed with SPSS Version 24 (IBM, Armonk, United States). Arithmetic mean and standard deviation were used for the evaluation of metric variables. Categorial data were stated as absolute and relative frequencies. Nominal variables, such as the dichotomous primary and secondary objectives, were compared using the chi-squared (χ^2) test. Metric variables were analysed using the Analysis of Variance (ANOVA) and the two-tailed t-test. The binary logistic regression enter method was used for multivariate analysis. Analysis was considered statistically significant with a P-value ≤ 0.05.

Ethical concerns

The study protocol and amendment have been reviewed and approved by the institutional review board of the Ruhr-University Bochum (registration number 16-5963) based on the ethical guidelines of the Declaration of Helsinki and its later revisions. Written, informed consents were obtained from all patients before specific examinations and procedures such as colonoscopy and biopsy. For this retrospective study informed consent was neither practicable nor necessary and was exempted by the institutional review board of the Ruhr-university.

RESULTS

Basic characteristics

A total of 633 patients (291 male patients, mean age 62.1 ± 16.1 years) were included

Figure 1 Flow-chart of inclusion. DiC: Drug-induced colitis; NiC: Non-inflammatory controls; IC: Inflammatory controls.

(Figure 1). Matching referring to gender and age resulted in homogenous groups with 211 subjects in either one (Supplementary Table 3). Medium body mass index (BMI) was 25.9 ± 6.1 kg/m². Referring to American Society of Anaesthesiologist (ASA) scoring, DiC patients were characterised by higher grades, referring to the Eastern Cooperative Oncology Group (ECOG) scale, both DiC and IC patients were equally distributed, but revealed significantly higher scores than NiC patients. Indications for colonoscopy differed significantly; while diarrhoea predominated among both control groups, the major indication among DiC patients was gastrointestinal bleeding. Other indications, such as abdominal pain and scheduled survey, led to colonoscopy equally often.

Comorbidities

Most comorbidities were equally distributed, but some remarkable differences were found, as displayed in Table 2. Cardiac diseases were most common among the DiC group (52.1% *vs* 44.1% in NiC, $P = 0.098$ and 41.2% in IC, $P = 0.025$, χ^2 test). Heart failure was less frequent in the NiC group (7.6% *vs* 16.6% and 14.7%, $P = 0.014$, χ^2 test); atrial fibrillation did not differ. Coronary heart disease was most common among DiC patients (22.3% *vs* 12.3% and 16.6%, $P < 0.001$, χ^2 test). While peripheral arterial occlusive disease did not differ, the overall manifestations of atherosclerosis appeared nearly twofold more often among DiC patients (35.1% *vs* 18.5% and 19.0%, $P < 0.001$, χ^2 test). While most cardiovascular risk factors were equally distributed, hyperlipoprotenaemia was more common among DiC patients (10.4% *vs* 5.2% and 4.3%). Surgery during the same hospital stay was a relatively rare event but was overrepresented among the DiC patients (4.3% *vs* 0.5% and 1.4%, $P = 0.024$, χ^2 test).

Histological assessment

Histopathological patterns are displayed in Supplementary Table 4. Inflammatory features were rarely seen among the NiC group (between 0% and 14.7%), except for lymphoplasmacellular and granulocytic infiltration (40.3%). Oedema, erosions, regeneratory hyperplasia of the crypts and subepithelial haemorrhage occurred equally often among DiC and IC patients (24.2%, 35.5%, 12.3% and 16.4%,

Table 2 Comorbidities *n* (%)

Comorbidity	Drug-induced colitis, *n* = 211	Non-inflammatory controls, *n* = 211	Inflammatory controls, *n* = 211
Pulmonary	39 (18.5)	31 (14.7)	37 (17.5)
Cardiac	110 (52.1)	93 (44.1)	87 (41.2)
Neurological	27 (12.8)	23 (10.9)	19 (9.0)
Psychiatric	10 (4.7)	9 (4.3)	10 (4.7)
Endocrine	47 (22.3)	44 (20.9)	30 (14.2)
Renal	28 (13.2)	18 (8.5)	21 (10.0)
Hepatic	11 (5.2)	13 (6.2)	11 (5.2)
Oncological	14 (6.6)	25 (11.8)	17 (8.1)
Other	19 (9.0)	15 (7.1)	16 (7.6)
Heart failure	35 (16.6)	16 (7.6)[b2]	31 (14.7)[a3]
Renal insufficiency	24 (11.4)	14 (6.6)	18 (8.5)
Atrial fibrillation	26 (12.3)	18 (8.5)	22 (10.4)
Coronary heart disease	47 (22.3)	28 (13.3)[a2]	35 (16.6)
Peripheral arterial occlusive disease	7 (3.3)	10 (4.7)	11 (5.2)
Atherosclerosis	74 (35.1)[b1]	39 (18.5)[b2]	40 (19.0)
Arterial hypertension	98 (46.4)	97 (46.0)	87 (41.2)
Diabetes mellitus	46 (21.8)	45 (21.3)	32 (15.2)
Hypercholesterinaemia	20 (9.5)	16 (7.6)	18 (8.5)
Hyperlipoproteinaemia	22 (10.4)[a1]	11 (5.2)[a2]	9 (4.3)
Chronic obstructive lung disease	29 (13.7)	24 (11.4)	27 (12.8)
Stroke	14 (6.6)	9 (4.3)	11 (5.2)
Smoking	77 (36.7)	66 (31.3)	78 (37.0)
Surgery	9 (4.3)	1 (0.5)[a2]	3 (1.4)
Intensive care therapy	9 (4.3)	2 (0.9)[a2]	4 (3.1)

[1]Drug-induced colitis *vs* inflammatory controls.
[2]Drug-induced colitis *vs* non-inflammatory controls.
[3]Non-inflammatory controls *vs* inflammatory controls.
[a]$P < 0.05$.
[b]$P < 0.01$.
Statistical analysis was carried out with χ^2 test.

respectively). Ischaemia and ulcers were most common among the IC group (5.2% *vs* 0.5% and 14.7% *vs* 7.6%, $P < 0.001$, χ^2 test), while eosinophilia and mucosal fibrosis were reported most often in the DiC group (24.6% *vs* 21.8% *vs* 8.2%, $P < 0.001$ and 28.0% *vs* 21.8% *vs* 14.7%, $P = 0.004$, respectively).

Histological reassessment
A total of 28 age- and gender-matched triplets (84 patients altogether) were assembled for the reassessment. Among them, two-thirds were female patients (*n* = 57, 67.9%, mean age 75.6 ± 7.6 years). The inflammatory activity in both DiC groups was predominantly mild (89.3% and 82.1%, respectively), while patients with ischaemic colitis were uniformly distributed between mild, moderate and severe inflammation (Supplementary Table 5). The inflammation in both DiC groups occurred significantly more often in the ascending colon compared to ischaemic colitis (Supplementary Table 6). The same tendency, but less pronounced, was present regarding the coecum.

The histopathological reassessment revealed differences between both DiC groups and ischaemic colitis (Table 3 and Figure 2); for instance, eosinophilic infiltration was more common (42.9% and 25.0% *vs* 3.6%), while ulcers and necrosis occurred less frequently (14.3% and 10.7% *vs* 57.1% and 3.6% *vs* 57.1%, respectively). On the other hand, haemorrhage and erosions were equally present between DiC with atherosclerosis and ischaemic colitis but were more common than in the DiC group without atherosclerosis (28.6% and 21.4% *vs* 3.6% and 32.1% and 35.1% *vs* 7.1%, respectively). Oedema, lymphocytic and granulocytic infiltration, fibrin plaques and fibrosis were similar in both groups.

Drug assessment
Patients with DiC took significantly more drugs (mean 4.5 ± 2.8) than NiC (mean 3.9 ±

Figure 2 Histological appearance of drug-induced colitis with atherosclerosis, drug-induced colitis without atherosclerosis, and ischaemic colitis in hematoxylin and eosin stain. A: Drug-induced colitis (DiC) with atherosclerosis; B: DiC without atherosclerosis; C: Ischaemic colitis. Three different groups of 28 patients each were collected for histological reassessment. DiC with atherosclerosis (A) is characterised by lymphocytic, granulocytic and eosinophilic infiltration (marked with an arrow) while haemorrhage, necrosis is rarely present. In ischaemic colitis, (C) ulcers, necrosis (marked with an arrow), and erosions predominate, and haemorrhage and fibrosis also occur. Eosinophilic infiltrations are rarely seen. DiC without atherosclerosis (B) shows features of both DiC without atherosclerosis and ischaemic colitis. These include haemorrhage, eosinophilic infiltration and erosions (marked with an arrow).

3.0, $P = 0.042$) and tendentially more than IC patients (3.9 ± 3.2, $P = 0.071$). The frequencies of specific drug classes are detailed in Table 4. Betablockers, angiotensin-converting enzyme (ACE) inhibitors, benzothiazines, aldosterone antagonists, nitrates, antiarrhythmic drugs, glycosides, metamizole, potassium, vitamin K antagonists, direct thrombin inhibitors, insulin, proton pump inhibitors, thyreostatics, antibiotics, tricyclic antidepressants, neuroleptics and sedatives were equally distributed between all three study groups.

Significant differences with the highest frequencies among DiC *vs* NiC *vs* IC patients were found regarding diuretics, dihydropyridines, glycosides, ASS (31.8% *vs* 22.3% *vs* 19.0%, $P = 0.006$), platelet aggregation inhibitors, NSAIDs, statins and fibrates.

The lowest frequencies in DiC *vs* NiC *vs* IC patients were found regarding non-dihydropyridines. The lowest frequencies in IC *vs* DiC *vs* NiC patients were found regarding angiotensin II inhibitors and metformin. The highest frequencies in IC *vs* DiC *vs* NiC group were found regarding glucocorticosteroids and other unspecified drugs. The highest frequency among the NiC *vs* DiC *vs* IC group was found for levothyroxine and lowest for selective serotonin reuptake inhibitors (SSRIs).

Among the reassessment subgroups (Supplementary Table 7), DiC patients without atherosclerosis took significantly fewer drugs than both other subgroups (4.8 ± 2.5 *vs* 6.6 ± 2.3, $P < 0.01$). The use of beta blockers, dihydropyridines, ASS and aldosterone antagonists was lower, though not significantly; statin use was significantly lowest (10.7% *vs* 60.7% *vs* 46.4%, $P < 0.01$). All other drug classes, including fibrates and NSAIDs, were distributed equally; particularly, there were no remarkable differences between DiC patients with atherosclerosis and ischaemic colitis patients.

Echocardiographic parameters

Heart ultrasound was available from 111 patients (17.5%), among them were 54 DiC (25.6%), 34 NiC (16.1%) and 23 IC (11.4%) patients (Table 5). Global left ventricular function was significantly better in NiC patients than in both other groups ($P < 0.05$) in which normal function was present in 72.2% (DiC) and 73.9% (IC), respectively. There was no moderate or severe decrease among NiC, while DiC patients presented in 9.3% and 11.1% and IC patients in 8.7% and 13.0%, respectively. Additionally, dilation of the left ventricular was reported in none of the NiC patients, but in 14.8% of DiC and even 26.1% of the IC patients ($P = 0.036$, χ^2 test). Dilation of the right atrium was reported in 7.4% of DiC patients, 11.4% of NiC and 23.8% of IC patients ($P = 0.142$, χ^2 test). No differences occurred regarding hypokinesia or diastolic dysfunction. Limited sample size among the reassessment groups inhibited the subgroup analysis.

Binary logistic regression models

Different models of binary logistic regression analysis were calculated (Table 6). The model comparing DiC patients and NiC that contained the variables chronic heart failure, any manifestation of atherosclerosis, on the one hand, and dihydropyridines, diuretics, digitalis glycosides, low-dose aspirin, any other platelet aggregation inhibitor, NSAIDs, statins and fibrates, on the other hand, revealed a statistically significant model (Omnibus test $P < 0.001$; Hosmer-Lemeshow test $P = 0.598$) with low predictive power (Nagelkerke's $R2 = 0.109$). In this model, only NSAIDs ($P = 0.010$) and fibrates remained statistically significant, while atherosclerosis ($P = 0.087$)

Table 3 Histopathological reassessment *n* (%)			
Parameter (*n* = 28)	Drug-induced colitis without atherosclerosis	Drug-induced colitis with atherosclerosis	Ischaemic colitis
Oedema	2 (7.1)	2 (7.1)	4 (14.3)
Haemorrhage	1 (3.6)[a2]	8 (28.6)[a1]	6 (21.4)
Lymphocytic infiltration	27 (96.4)	27 (96.4)	27 (96.4)
Granulocytic infiltration	27 (96.4)	26 (92.9)	28 (100.0)
Eosinophilic infiltration	12 (42.9)[b2]	7 (25.0)	1 (3.6)[a3]
Erosions	2 (7.1)[b2]	9 (32.1)[a1]	10 (35.7)
Ulcerous lesions	4 (14.3)[b2]	3 (10.7)	16 (57.1)[b3]
Necrosis	1 (3.6)[b2]	1 (3.6)	16 (57.1)[b3]
Fibrin plaques on erosions	2 (7.1)	2 (7.1)	3 (10.7)
Fibrosis	3 (10.7)	3 (10.7)	7 (25.0)

[1]Drug-induced colitis without atherosclerosis *vs* drug-induced colitis with atherosclerosis.
[2]Drug-induced colitis without atherosclerosis *vs* ischaemic colitis.
[3]Drug-induced colitis with atherosclerosis *vs* ischaemic colitis.
[a]$P < 0.05$.
[b]$P < 0.01$.
Statistical analysis was carried out with χ^2 test.

and diuretics ($P = 0.070$) showed a persisting tendency. The related odds ratios were 2.2 (CI: 1.2-4.0) and 8.9 (CI: 1.1-74.2), respectively. The resembling models without dihydropyridines or additional ASA or ECOG score showed comparable results (data not shown).

The same model comparing DiC and IC patients revealed a higher but still low predictive power (Omnibus test $P < 0.001$; Hosmer-Lemeshow test $P = 0.432$, Nagelkerke's $R2 = 0.170$). Significant variables comprised atherosclerosis ($P = 0.009$), NSAIDs ($P < 0.001$) and fibrates ($P = 0.043$) while aspirin showed only a tendency ($P = 0.059$). The related ORs were 2.1 (CI: 1.2-3.7), 6.7 (CI: 3.0-15.1) and 9.1 (CI: 1.1-76.4). Skipping dihydropyridines or adding the ASA or ECOG score did not change the results substantially (data not shown).

DISCUSSION

In this age- and gender-matched retrospective cohort study, DiC was detected in about 7% of endoscopically sampled biopsy specimens of the colon mucosa. This prevalence coincides well with estimated numbers from a recently published overview[6,10]. Nevertheless, DiC (despite clostridium-associated[11,12] and microscopic colitis[13]) is a rarely reported entity in the literature and most knowledge derives from case reports or case series[7]. Therefore, to the best of our knowledge, this is the first study that systematically investigates a large set of potential triggers of DiC.

Potential triggers of DiC

In univariate analysis, DiC was associated with diuretics, dihydropyridines, glycosides, ASS, platelet aggregation inhibitors, NSAIDs, statins and fibrates. In addition to NSAIDs, that are well established to induce DiC[10,14,15], several drugs that are indicated for the treatment of heart failure, atherosclerosis and related conditions (such as fat-lowering medication) were associated with DiC. Cardiac and vascular comorbidity might, therefore, be a substantial confounding factor or the cause of the disease itself.

Several aspects were undertaken to control confounding factors. Two age- and gender-matched control groups were gathered, one of which consisted of patients with NiC while the other group was assembled from patients with IC. This matching resulted in rather equally distributed comorbidities and only a very few entities differed significantly. While overall cardiac comorbidity and atrial fibrillation were equally distributed between all three groups, atherosclerosis, particularly coronary heart disease, and hyperlipoproteinaemia were more common among DiC patients. Heart failure occurred similarly in the DiC and IC group, but more often than in NiC. Echocardiography was available for only a minority of patients (between 11% and 26%, respectively), but did not show restricted function among DiC patients. In detail, the left ventricular function did not differ between DiC and IC patients, while NiC patients preferentially showed a preserved ejection fraction. Dilation of the left

Table 4 Drug assessment *n* (%)

Group	Drug-induced colitis, *n* = 211	Non-inflammatory controls, *n* = 211	Inflammatory controls, *n* = 211
Betablocker	97 (46.0)	78 (37.0)	83 (39.3)
ACE inhibitors	70 (33.2)	55 (26.1)	62 (29.4)
Angiotensin II inhibitors	20 (9.5)[b1]	24 (11.4)	6 (2.8)[b3]
Non-Dihydropyridines	1 (0.5)[a1]	6 (2.8)	7 (3.3)
Dihydropyridines	34 (16.1)[a1]	27 (12.8)	20 (9.5)
Diuretics	55 (26.1)[b1]	29 (13.7)[b2]	33 (15.7)
Benzothiazines	28 (13.3)	33 (15.6)	24 (11.4)
Aldosterone antagonists	13 (6.2)	13 (6.2)	12 (5.7)
Nitrates	7 (3.3)	13 (6.2)	5 (2.4)
Antiarrhythmic drugs	7 (3.3)	2 (0.9)	2 (0.9)
Glycosides	10 (4.7)	2 (0.9)[a2]	8 (3.8)
ASS (100 mg to 300 mg)	67 (31.8)[b1]	47 (22.3)[a2]	40 (19.0)
Platelet aggregation inhibitors	20 (9.5)[b1]	10 (4.7)	6 (2.8)
NSAIDs	35 (16.6)[b1]	21 (10.0)[a2]	8 (3.8)[a3]
Metamizole	21 (10.0)	16 (7.6)	21 (10.0)
Potassium	4 (1.9)	3 (1.4)	6 (2.8)
Vitman K antagonists/coumarin derivates	16 (7.6)	9 (4.3)	11 (5.2)
Direct thrombin inhibitors	6 (2.8)	2 (0.9)	4 (1.9)
Glucocorticosteroids	13 (6.2)[b1]	14 (6.6)	41 (19.4)[b3]
Opioids	20 (9.5)	22 (10.4)	23 (10.9)
Metformin	8 (3.8)	16 (7.6)	4 (1.9)[b3]
Insulin	17 (8.1)	15 (7.1)	17 (8.1)
Statins	56 (26.5)[a1]	42 (19.9)	38 (18.0)
Fibrates	7 (3.3)[a1]	1 (0.5)[a2]	1 (0.5)
Levothyroxine	25 (11.8)	39 (18.5)	22 (10.4)[a3]
Thyreostatics	3 (1.4)	3 (1.4)	1 (0.5)
Proton pump inhibitors	100 (47.4)	85 (40.3)	81 (38.4)
Penicillin derivates	0 (0.0)	2 (0.9)	2 (0.9)
Macrolides	0 (0.0)	1 (0.5)	1 (0.5)
Gyrase inhibitor	0 (0.0)	0 (0.0)	0 (0.0)
Carbapenems	1 (0.5)	0 (0.0)	0 (0.0)
Imidazoles	3 (1.4)	1 (0.5)	1 (0.5)
Cephalosporins	2 (0.9)	2 (0.9)	2 (0.9)
Antbiotics	4 (1.9)	5 (2.4)	3 (1.4)
SSRIs	14 (6.6)	5 (2.4)[a2]	16 (7.6)[a3]
Tricyclic antidepressants	7 (3.3)	10 (4.7)	7 (3.3)
Neuroleptics	5 (2.4)	8 (3.8)	6 (2.8)
Sedatives	16 (7.6)	16 (7.6)	16 (7.6)
Others	127 (60.2)[a1]	125 (59.2)	147 (69.7)[a3]
Number of drugs[b]	4.5 ± 2.8	3.9 ± 3.0[a2]	3.9 ± 3.2

[1]Drug-induced colitis *vs* inflammatory controls.
[2]Drug-induced colitis *vs* non-inflammatory controls.
[3]Non-inflammatory controls *vs* inflammatory controls.
[a]$P < 0.05$.
[b]$P < 0.01$.
Statistical analysis was carried out with χ^2 test (a) or *t*-test (b). NSAIDs : Nonsteroidal anti-inflammatory drugs; SSRIs: Selective serotonin reuptake inhibitors; ACE: Angiotensin-converting enzyme.

ventricle and right atrium were most common among IC patients, while hypokinesia or diastolic dysfunction were the same among all three groups.

Only fibrates (OR = 9.1), NSAIDs (OR = 6.7), and atherosclerosis (OR = 2.1) proved to be associated with DiC in the multivariate analysis, while heart failure, dihydropyridines, glycosides, diuretics, low-dose aspirin, platelet aggregation

Table 5 Echocardiographic parameters *n* **(%)**

Parameter	Drug-induced colitis, *n* = 54 of 211 (25.6)	Non-inflammatory controls, *n* = 34 of 211 (16.1)	Inflammatory controls, *n* = 24 of 211 (11.4)
Dilated right atrium	4 (7.4)[a1]	4 (11.4)	5 (23.8)
Dilated left ventricle	8 (14.8)	0 (0.0)	6 (26.1)[b3]
Hypokinesia	10 (18.5)	5 (14.3)	3 (13.0)
Right heart failure	5 (9.3)	4 (11.4)	2 (8.7)
Diastolic dysfunction	22 (43.1)	16 (48.5)	7 (30.4)
LV Function[b2,b3]			
Normal (> 50%)	39 (72.2)	29 (85.3)	17 (73.9)
Slightly decreased (40%-50%)	4 (7.4)	5 (14.7)	1 (4.3)
Moderately decreased (30%-40%)	5 (9.3)	0 (0.0)	2 (8.7)
Severely decreased (< 30%)	6 (11.1)	0 (0.0)	3 (13.0)

[1]Drug-induced colitis *vs* inflammatory controls.
[2]Drug-induced colitis *vs* non-inflammatory controls.
[3]Non-inflammatory controls *vs* inflammatory controls.
[a]$P < 0.05$.
[b]$P < 0.01$.
Statistical analysis was carried out with χ^2 test. LV : Left ventricular.

inhibitors and statins lost their relevance. The effects of NSAIDs on the gastrointestinal tract have been extensively investigated, however, the association of fibrates and the role of atherosclerosis are novel findings.

Pathogenetic role of ischaemia

It has been proposed that medications can cause colonic ischaemia due to neuronal stimulation (*e.g.*, cocaine), by promoting thrombosis due to hormonal effects (*e.g.*, estrogen) or by extrinsic compression due to fibrosis (*e.g.*, methysergide). Other drugs may promote colonic ischaemia by shunting blood away from the mesenteric vasculature (*e.g.*, digitalis[16]) by decreasing fluid volume (*e.g.*, diuretics[17]), inducing vasculitis or vascular spasms, or by other, not yet understood mechanisms[2,7,18,19]. It was hypothesised that drugs, such as digitalis and diuretics, may predispose to ischaemic colitis in elderly patients in a state of low blood flow, often due to heart failure[6], but up to now, there has been no proof of this concept. The overall results support the idea of disturbances in microperfusion at least in a subset of patients, maybe those patients with underlying atherosclerosis.

The subgroup analysis of either 28 matched DiC patients without atherosclerosis, with atherosclerosis and patients with ischaemic colitis further strengthens this concept. The DiC patients without atherosclerosis took significantly fewer remedies than both other subgroups and used beta blockers, dihydropyridines, ASS and aldosterone antagonists less often, though not significantly. While statin intake was significantly highest among the DiC subgroup with atherosclerotic manifestations, all other drug classes, including fibrates and NSAIDs, diversified similarly; there were no specific remarkable differences between DiC patients with atherosclerosis and ischaemic colitis patients. Indeed, this distribution does not prove but at least gives a distinct clue that atherosclerosis and focal hypoxia play a substantial role in the aetiopathogenesis of DiC in a subset of patients.

Reliability of histopathological assessment

The presence of DiC in the routine histological assessment was the major inclusion criterion. The pathological changes of neither drug-induced nor ischaemic colitis are specific and significant histological overlap exists between both entities[2,18,20]. Nevertheless, acute ischaemic colitis is observed predominantly in elderly (older than 65 years) subjects[21], while the patients of our cohort were distinctly younger (62 years). None of the three different histologic variants of colitis with ischaemic features that Jessurun recently distinguished has been included in the DiC group[20]. Given the common absence of strictly specific histopathological features, the diagnosis of DiC often relies upon thorough clinicopathological correlation, but in clinical practice it is difficult to establish the correlation between a certain medication and a particular pattern of injury. The optimal establishment of a causal relationship that results from improvement with withdrawal and recurrence with rechallenge of the suspected agent is rarely available and was not a common practice in our cohort. This approach

Table 6 Binary logistic regression analysis

Parameter	Drug-induced colitis *vs* inflammatory controls OR (95%CI)	Drug-induced colitis *vs* non-inflammatory controls OR (95%CI)
Heart failure	0.6 (0.3-1.1)	1.3 (0.6-2.8)
Atherosclerosis	2.1 (1.2-3.7)[b]	1.7 (0.9-3.1)
Dihydropyridines	1.3 (0.7-2.5)	1.0 (0.5-1.8)
Diuretics	1.5 (0.8-2.6)	1.7 (1.0-3.0)
Digitalis glycosides	1.1 (0.4-3.1)	3.7 (0.8-17.9)
Low-dose ASS	1.7 (1.0-2.8)	1.1 (0.7-1.9)
Platelet aggregation inhibitors	2.0 (0.7-5.7)	1.4 (0.6-3.3)
NSAIDs	6.7 (3.0-15.1)[b]	2.2 (1.2-4.0)[a]
Statins	1.1 (0.6-1.9)	0.9 (0.6-1.6)
Fibrates	9.1 (1.1-74.3)[a]	8.9 (1.1-74.2)[a]

[a]$P < 0.05$.
[b]$P < 0.01$.
NSAIDs: Nonsteroidal anti-inflammatory drugs.

is hardly realisable when dealing with a single patient who most commonly takes a multitude of drugs; the mean number in our cohort summed up to 4.5 different agents. Thus, it is the pathologist's task to offer a strong suspicion concerning possible drug-related pathology[19].

The biopsy specimens were reassessed in a subset of patients and compared in three different groups: DiC patients with atherosclerosis, DiC patients without atherosclerosis and patients with ischaemic colitis, to gain deeper insights into the reliability of the histopathological assessment. Patients of both DiC groups presented milder inflammatory activity, suggesting common pathophysiological pathways that can be distinguished from purely ischaemic colitis. Indeed, DiC patients with atherosclerosis exhibited features from both DiC without atherosclerosis and ischaemic colitis: Haemorrhage and erosions were the same among atherosclerotic DiC and ischaemic colitis patients, while the presence of ulcers and necrosis were equal in both DiC groups. Interestingly, infiltration with eosinophils that is characteristic for DiC[2] were found most often in the DiC group without atherosclerosis, while it rarely appeared among the ischaemic colitis group (42.9% *vs* 3.6%); atherosclerotic DiC patients ranked in between (25.0%). We, therefore, conclude that focal ischaemia due to disturbances of the microcirculation, probably supported by large vessel atherosclerosis, plays a substantial role particularly in a subset of patients with DiC.

Strengths and limitations

Most patients with acute colitis are diagnosed and treated based on a combination of clinical and laboratory findings without colonoscopy and mucosal biopsy analysis. Endoscopic evaluation is reserved for patients who present with severe or atypical clinical course, do not improve within an expected timeframe or fail to respond to standard treatment[20]. Therefore, the inclusion of histologically suspected DiC bears a potential selection bias. The histopathological approach was limited to colonic manifestations with morphological changes, while functional diarrhoea, alterations on a (sub)cellular level or manifestations of the small intestine were beyond the scope of the study. Despite all potential limitations, this is the largest study that investigates potential triggers of DiC and potential confounders systematically. The fact that NSAID use has clearly been shown to induce DiC[10,14] could have been reproduced in our cohort argues seriously for the reliability of the approach chosen.

CONCLUSION

In conclusion, histopathologically suspected DiC is associated with not only NSAIDs, angiotensin II inhibitors, dihydropyridines, diuretics, ASS and other platelet aggregation inhibitors, as well as statins and fibrates, but also coronary heart disease, hyperlipoproteinaemia and, partially, heart failure. Atherosclerosis (OR = 2.1) and the intake of NSAIDs (OR = 6.7) and fibrates (OR = 9.1) were associated most strongly with DiC in the multivariate analysis. Since a subset of DiC patients with

atherosclerosis exhibited histological features of both DiC without atherosclerosis and ischaemia, we propose that focal disturbances of the microcirculation play a substantial role in the pathogenesis of a subgroup of DiC patients. The distribution of drug intake further supports this hypothesis, but the study design is not suitable to prove it. Prospective studies including larger cohorts with clearly defined cardiac function, pattern and severity of atherosclerosis and related comorbidities, such as hyperlipoprotenaemia, are warranted to unravel the underlying aetiology and pathophysiology of this under-recognised entity. Meanwhile, the histological suspicion of DiC might not necessarily reveal a drug-related mechanism but could also reflect focal ischaemia that is supported by macroangiopathy at least in a subset of patients.

ARTICLE HIGHLIGHTS

Research background
Drug-induced colitis is a common and even serious problem, but the knowledge about associated triggers is scarce.

Research motivation
Withdrawal of the respective trigger should cure the disease, so that its identification is crucial. Therefore, deeper insights into the aetiopathogenesis and knowledge about potential triggers is mandatory.

Research objectives
Consequently, the study aimed to identify potential triggers of histologically suspected drug-induced colitis.

Research methods
A retrospective case control study of 211 patients with histologically suspected drug-induced colitis and two age- and gender matched control groups was performed. The drug-induced colitis (DiC) patients showed histological changes attributable to drug-induced pathology, *i.e.*, mixed, predominantly neutrophilic or lymphocytic inflammatory infiltrates, erosions, absence of granulomas, absence of basal plasmacellular infiltration and absence of crypt architectural distortion. The control groups consisted of patients with inflammatory colitis other than DiC and inflammatory bowel disease (*i.e.*, diverticulitis, ischaemic colitis) and of patients without substantial histological changes (*i.e.*, irritable bowel syndrome, cancer screening). Clinical data including drug history was obtained from the electronic data base.

In a second approach, patients were divided into three groups from a clinical perspective: DiC without atherosclerotic comorbidity, DiC with atherosclerotic comorbidity, and ischaemic colitis. Patients from the first two groups derived from the DiC group while the latter group was gathered from the inflammatory controls.

Research results
A total of 633 patients (291 male patients, mean age 62.1 ± 16.1 years) were included. Patients with DiC took more drugs (mean 4.5 ± 2.8) than patients from both other groups (mean 3.9 ± 3.0 and 3.9 ± 3.2, respectively). In univariate analysis, DiC was associated with diuretics, dihydropyridines, glycosides, ASS, platelet aggregation inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDs), statins and fibrates. In addition to NSAIDs, that are well established to induce DiC, several drugs that are indicated for the treatment of heart failure, atherosclerosis and related conditions were associated with DiC. Cardiac and vascular comorbidity might, therefore, be a substantial confounding factor or the cause of the disease itself. In fact, atherosclerosis was more common among patients with DiC. In multivariate analysis, atherosclerosis (OR = 2.1) and the intake of NSAIDs (OR = 6.7) and fibrates (OR = 9.1) were associated most strongly with DiC. Since a subset of DiC patients with atherosclerosis exhibited histological features of both DiC without atherosclerosis and ischaemia, we propose that focal disturbances of the microcirculation play a substantial role in the pathogenesis of a subgroup of DiC patients. A total of 28 age- and gender-matched triplets were assembled for the histological reassessment. Some DiC patients with atherosclerosis exhibited histological features of both control groups, DiC without atherosclerosis and ischaemia.

Research conclusions
While most knowledge of drug-induced colitis relies on case reports and case series this is the first study that systematically investigates potential triggers of DiC. This large case control study reveals that patients with the histopathological suspicion of drug-induced colitis take more different drugs than age- and gender-matched control patients. Associated remedies include drugs that are indicated for the treatment of heart failure, atherosclerosis and related conditions. Furthermore, atherosclerosis was more common among DiC patients. We therefore hypothesise that focal disturbances of the microcirculation play a substantial role in the pathogenesis of a subgroup of DiC patients, but the study design is not suitable to prove this hypothesis.

Research perspectives
Prospective studies including larger cohorts with clearly defined cardiac function, pattern and

severity of atherosclerosis and related comorbidities, such as hyperlipoprotenaemia, are warranted to unravel the underlying aetiology and pathophysiology of this under-recognised entity. Meanwhile, the histological suspicion of DiC might not necessarily reveal a drug-related mechanism but could also reflect focal ischaemia that is supported by macroangiopathy at least in a subset of patients.

REFERENCES

1 **Sherid M**, Ehrenpreis ED. Types of colitis based on histology. *Dis Mon* 2011; **57**: 457-489 [PMID: 21944389 DOI: 10.1016/j.disamonth.2011.05.004]

2 **Price AB**. Pathology of drug-associated gastrointestinal disease. *Br J Clin Pharmacol* 2003; **56**: 477-482 [PMID: 14651719 DOI: 10.1046/j.1365-2125.2003.01980.x]

3 **Püspök A**, Kiener HP, Oberhuber G. Clinical, endoscopic, and histologic spectrum of nonsteroidal anti-inflammatory drug-induced lesions in the colon. *Dis Colon Rectum* 2000; **43**: 685-691 [PMID: 10826432 DOI: 10.1007/BF02235589]

4 **Hsu SC**, Chang SS, Lee MG, Lee SH, Tsai YW, Lin SC, Chen ST, Weng YC, Porta L, Wu JY, Lee CC. Risk of gastrointestinal perforation in patients taking oral fluoroquinolone therapy: An analysis of nationally representative cohort. *PLoS One* 2017; **12**: e0183813 [PMID: 28873440 DOI: 10.1371/journal.pone.0183813]

5 **Abdalla S**, Brouquet A, Lazure T, Costaglioli B, Penna C, Benoist S. Outcome of emergency surgery for severe neuroleptic-induced colitis: results of a prospective cohort. *Colorectal Dis* 2016; **18**: 1179-1185 [PMID: 27166739 DOI: 10.1111/codi.13376]

6 **Pusztaszeri MP**, Genta RM, Cryer BL. Drug-induced injury in the gastrointestinal tract: clinical and pathologic considerations. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 442-453 [PMID: 17667993 DOI: 10.1038/ncpgasthep0896]

7 **Cappell MS**. Colonic toxicity of administered drugs and chemicals. *Am J Gastroenterol* 2004; **99**: 1175-1190 [PMID: 15180742 DOI: 10.1111/j.1572-0241.2004.30192.x]

8 **Pernot S**, Ramtohul T, Taieb J. Checkpoint inhibitors and gastrointestinal immune-related adverse events. *Curr Opin Oncol* 2016; **28**: 264-268 [PMID: 27138569 DOI: 10.1097/CCO.0000000000000292]

9 **Rea WE**, Durrant DC, Boldy DA. Ulcerative colitis after statin treatment. *Postgrad Med J* 2002; **78**: 286-287 [PMID: 12151572 DOI: 10.1136/pmj.78.919.286]

10 **Grattagliano I**, Ubaldi E, Portincasa P. Drug-induced enterocolitis: Prevention and management in primary care. *J Dig Dis* 2018; **19**: 127-135 [PMID: 29417737 DOI: 10.1111/1751-2980.12585]

11 **McDonald LC**, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 2018; **66**: e1-e48 [PMID: 29462280 DOI: 10.1093/cid/cix1085]

12 **Crobach MJ**, Planche T, Eckert C, Barbut F, Terveer EM, Dekkers OM, Wilcox MH, Kuijper EJ. European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection. *Clin Microbiol Infect* 2016; **22** Suppl 4: S63-S81 [PMID: 27460910 DOI: 10.1016/j.cmi.2016.03.010]

13 **Cotter TG**, Pardi DS. Current Approach to the Evaluation and Management of Microscopic Colitis. *Curr Gastroenterol Rep* 2017; **19**: 8 [PMID: 28265892 DOI: 10.1007/s11894-017-0551-3]

14 **Kwak HA**, Hart J. The Many Faces of Medication-Related Injury in the Gastrointestinal Tract. *Surg Pathol Clin* 2017; **10**: 887-908 [PMID: 29103538 DOI: 10.1016/j.path.2017.07.007]

15 **Bjarnason I**, Scarpignato C, Holmgren E, Olszewski M, Rainsford KD, Lanas A. Mechanisms of Damage to the Gastrointestinal Tract From Nonsteroidal Anti-Inflammatory Drugs. *Gastroenterology* 2018; **154**: 500-514 [PMID: 29221664 DOI: 10.1053/j.gastro.2017.10.049]

16 **Keller HW**, Lorenz R, Müller JM, Pichlmaier H. [Ischemic colitis following digitalis poisoning]. *Chirurg* 1984; **55**: 830-831 [PMID: 6518857]

17 **Sharefkin JB**, Silen W. Diuretic agents: inciting factor in nonocclusive mesenteric infarction? *JAMA* 1974; **229**: 1451-1453 [PMID: 4408184 DOI: 10.1001/jama.1974.03230490039020]

18 **Yang RD**, Han MW, McCarthy JH. Ischemic colitis in a crack abuser. *Dig Dis Sci* 1991; **36**: 238-240 [PMID: 1988271 DOI: 10.1007/BF01300764]

19 **McCarthy AJ**, Lauwers GY, Sheahan K. Iatrogenic pathology of the intestines. *Histopathology* 2015; **66**: 15-28 [PMID: 25639479 DOI: 10.1111/his.12598]

20 **Jessurun J**. The Differential Diagnosis of Acute Colitis: Clues to a Specific Diagnosis. *Surg Pathol Clin* 2017; **10**: 863-885 [PMID: 29103537 DOI: 10.1016/j.path.2017.07.008]

21 **Higgins PD**, Davis KJ, Laine L. Systematic review: the epidemiology of ischaemic colitis. *Aliment Pharmacol Ther* 2004; **19**: 729-738 [PMID: 15043513 DOI: 10.1111/j.1365-2036.2004.01903.x]

Retrospective Study

Women on the liver transplantation waitlist are at increased risk of hospitalization compared to men

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Abstract

BACKGROUND
Hospital admissions are common among patients with cirrhosis, but patient factors associated with hospitalization have not been well characterized. Given recent data suggesting increased liver transplant waitlist dropout among women, we hypothesized that women on the liver transplant waitlist would have increased rates of hospitalization compared with men.

AIM
To evaluate the role of gender on risk of hospitalization for patients on the liver transplant waitlist, in order to help explain gender disparities in waitlist outcomes.

METHODS
Patients listed for liver transplant at a single center in the United States were prospectively enrolled in the Functional Assessment in Liver Transplantation Study. Patients included in this retrospective analysis included those enrolled between March 2012 and December 2014 with at least 12 mo of follow up and without hepatocellular carcinoma. The primary and secondary outcomes were

hospitalization and total inpatient days within 12 mo, respectively. Logistic and negative binomial regression associated baseline factors with outcomes.

RESULTS
Of the 392 patients, 41% were female, with median (interquartile range) age 58 years (52-63) and model for end- stage liver disease 18 (15-22). Within 12 mo, 186 (47%) patients were hospitalized $\geq$ 1 time; 48% were readmitted, with a median of 8 (4-15) inpatient days. More women than men were hospitalized (54% *vs* 43%; *P* = 0.03). In univariable analysis, female sex was associated with an increased risk of hospitalization [odds ratios (OR) 1.6, 95% confidence interval (CI) 1.0-2.4; *P* = 0.03], which remained significant on adjusted multivariable analysis (OR 1.6, 95%CI: 1.1-2.6; *P* = 0.03). Female gender was also associated with an increased number of inpatient days within 12 mo in both univariable and multivariable regression.

CONCLUSION
Women with cirrhosis on the liver transplant waitlist have more hospitalizations and inpatient days in one year compared with men, suggesting that the experience of cirrhosis differs between men and women, despite similar baseline illness severity. Future studies should explore gender-specific vulnerabilities to help explain waitlist disparities.

Key words: Gender; Cirrhosis; Liver transplantation waitlist; Hospitalization; Readmission; Women

Core tip: In this single-center study of patients on the liver transplant waitlist, women were significantly more likely to be hospitalized than men, and were hospitalized for a more days within one year. Among those who were hospitalized at least once, there was a trend toward higher rates of readmission among women compared to men. These gender differences were independent of underlying severity of illness, as measured by model for end- stage liver disease score, suggesting that perhaps traditional indicators of liver disease severity do not adequately capture all contributors to illness, such as non-hepatic comorbidities or socioeconomic factors, which may require acute inpatient care.

INTRODUCTION

Women have been shown to have worse transplant-related outcomes than men[1]. Rates of liver transplant are lower for women on the waitlist[2-6], and women are more likely to die or become too sick for transplant than men[2,7,8]. In particular, recent studies suggest that women are underserved by the model for end- stage liver disease (MELDNa) score[2], and that female sex is independently associated with a 10% higher risk of delisting[8]. The reasons for these disparities are not completely understood.

One hypothesis for the gender disparity in outcomes for patients on the liver transplant waitlist has been that women with cirrhosis have a different disease trajectory than men with cirrhosis, either because they are sicker at baseline or experience more rapid progression of cirrhosis (*i.e.*, from compensated to decompensated cirrhosis)[4,9]. In several studies, women appear to have similar or even lower MELDNa scores at listing compared with men and have fewer comorbidities, suggesting that they are not sicker at baseline[3,4,8]. However, whether their disease progresses more rapidly has not been previously explored. Hospital admissions may be a surrogate marker for disease progression that more accurately captures differences in the experience of living with cirrhosis for women and men. We therefore aimed to evaluate the role of gender on the risk of hospitalization for

patients with cirrhosis on the liver transplant waitlist.

MATERIALS AND METHODS

Patients

Our cohort included adult (≥ 18 years) patients seen as outpatients at the University of California - San Francisco (UCSF) Liver Transplant Clinic, who were listed for liver transplantation at UCSF from March 2012 to December 2014, and subsequently enrolled prospectively in the Functional Assessment in Liver Transplantation (FrAILT) Study[10,11]. Ninety-seven percent of invited participants enrolled in this study[11,12]. Patients with time to complete a Numbers Connection Test (NCT) of > 120 s were excluded because of concerns about their ability to provide informed consent[13]. For the purposes of this study, patients listed for transplant with MELD exception points for hepatocellular carcinoma were excluded, as their reasons for hospitalization (*e.g.*, complications of locoregional therapy) may differ compared to patients solely listed for decompensated cirrhosis. Patients lost-to-follow up at 12 mo were also excluded.

Baseline variables

At the time of study enrollment, patient demographics including age, gender, race, etiology of cirrhosis, as well as medical comorbidities (*e.g.*, diabetes and hypertension) and baseline laboratory values were collected from the patient's electronic health record. All patients underwent frailty assessment using the Liver Frailty Index (LFI), which is composed of hand grip, chair stands, and balance. "Frail" was defined as LFI ≥ 4.5[10]. Clinical information regarding the presence of ascites, ascertained by the patient's primary hepatologist, was recorded as absent, mild/moderate, or severe, then further classified as absent or present for the current study. Hepatic encephalopathy was classified as none/mild versus moderate/severe based on the patient's performance on the NCT Score of < or > 45 s, respectively[13].

Outcomes

The primary outcome was any hospitalization within 12 mo from study enrollment. The secondary outcome was the number of inpatient days within 12 mo. Information on number of hospitalizations and inpatient days were obtained from manual review of medical records at UCSF and review of external medical records in the case of hospital admissions elsewhere[14]. As part of their listing agreement for liver transplant at our center, all patients were required to report hospitalizations to outside institutions at time of admission. Patients who died or were transplanted within 12 mo were censored at the time of their waitlist event (*n* = 82). A 12-mo study period was selected to minimize confounding from women remaining on the waitlist longer than men due to lower rates of transplant.

Statistical analysis

Categorical data were presented as percentages; groups were compared using chi-square tests. Continuous variables were presented as medians and interquartile ranges (IQR); groups were compared using Wilcoxon Rank-Sum tests. Univariable logistic regression with odds ratios (OR) evaluated the association of all listed covariates with the primary outcome of hospitalization within 12 mo. Univariable negative binomial regression with incidence rate ratios (IRR) evaluated the association of all listed covariates with the secondary outcome of number of hospitalized days within 12 mo. Variables significant at a *P*-value < 0.2 were included in the multivariable models. Backward elimination (*P* > 0.05 for removal) was used to develop the final multivariable models. Two-sided *P*-values < 0.05 were considered statistically significant. All analyses were performed using Stata 15.1 statistical software (College Station, TA, United States). The statistical methods of this study were reviewed by multiple individuals with biomedical statistical training. This study was approved by the institutional review board at the UCSF.

RESULTS

Baseline characteristics

A total of 392 patients were enrolled between March 2012 and December 2014. Women comprised 41% of the cohort, 61% were non-Hispanic Caucasian, and median (IQR) age was 58 years (51-63). The etiology of cirrhosis was chronic hepatitis C in 43% of the cohort; median (IQR) MELDNa was 18 (15-22) and median (IQR) albumin

was 3.0 mg/dL (2.6-3.4). Thirty-four percent of patients had ascites and 42% had moderate or severe hepatic encephalopathy. Sixteen percent of the patients were characterized as frail. Baseline demographics, comorbidities and cirrhosis complications by gender are shown in Table 1. Men were more likely than women to have cirrhosis due to Hepatitis C (48% *vs* 35%, *P* < 0.01) and alcohol (23% *vs* 14%, *P* = 0.02) and more likely to have coronary artery disease (6% *vs* 2%, *P* = 0.05); women were more likely to have cirrhosis due to autoimmune liver disease (24% *vs* 9%, *P* < 0.01).

Hospitalizations

During the 12-mo study period, 186 (47%) patients were hospitalized at least once. Of these 186 patients, 89 (48%) were readmitted at least once and 47 (25%) were readmitted more than once. Among patients hospitalized at least once, median (IQR) number of hospitalizations within 12 mo was 1 (1-3), median (IQR) number of inpatient days was 8 (4-15), and median (IQR) length of stay was 5 d (3-8).

In univariable analysis, the factors associated with at least one hospitalization within 12 mo were female gender [OR 1.6, 95% confidence interval (CI) 1.0-2.4; *P* = 0.03], MELDNa (OR 1.1; 95%CI 1.1-1.2; *P* < 0.01), albumin (OR 0.4; 95%CI: 0.3-0.6; *P* < 0.01), ascites (OR 2.3; 95%CI: 1.5-3.5; *P* < 0.01), and frailty (OR 3.6; 95%CI: 2.0-6.5; *P* < 0.01).

Gender and hospitalization

More women than men were hospitalized at least once within the 12-mo study period (54% *vs* 43%, *P* = 0.03). As noted above, in univariable logistic regression, the odds of being hospitalized at least once within 12 mo were 1.6 times higher among women compared to men (*P* = 0.03). In multivariable analysis, female gender remained significantly associated with hospitalization after adjusting for MELDNa, albumin, ascites, and frailty (adjusted OR 1.6, 95%CI: 1.1-2.6; *P* = 0.03) (Table 2).

Women also had a higher median (IQR) number of total inpatient days within 12 mo compared with men [2.5 (0-10) *vs* 0 (0-6.5), *P* = 0.02]. On univariable negative binomial regression, female gender was associated with a higher number of total inpatient days within 12 mo (IRR 1.7, 95%CI 1.1-2.6, *P* = 0.02). This association persisted in multivariable analysis after adjusting for MELDNa and albumin (adjusted IRR 1.9, 95%CI: 1.2-3.0, *P* < 0.01) (Table 3). Among the 186 patients hospitalized at least once, there was a trend toward women being readmitted more often than men (54% *vs* 42%), but this did not reach statistical significance (*P* = 0.11).

DISCUSSION

Hospital admissions are common among patients with cirrhosis due to portal hypertensive complications[15]. Hospitalizations in patients with cirrhosis are also associated with high mortality, and account for a large proportion of the cost of end-stage liver disease. In 2012, liver disease accounted for nearly 250000 hospitalizations at an estimated cost of $3-12 billion each year[15-17], and rates of hospitalization as well as costs have been increasing over time[15]. A 2014 study also showed that pre-transplant spending increased exponentially with severity of illness for patients on the liver transplant waitlist, likely due in large part to increased number and complexity of hospitalizations[16]. In light of the increased mortality and high costs associated with hospitalizations in patients with cirrhosis, we wondered whether differential rates of hospitalization could help explain the gender disparity on the liver transplant waitlist.

Consistent with previous studies on hospitalizations in patients with cirrhosis, we observed that hospitalizations were quite common: Nearly half of patients on the liver transplant waitlist in our study were hospitalized at least once within one year, and approximately one half of those were readmitted at least once. But our analyses investigating gender differences in hospitalizations expand upon prior work. Specifically, in the current study, we found that in one year, women on the liver transplant waitlist were significantly more likely to be hospitalized than men. They also were hospitalized for a higher number of days within one year. Among those who were hospitalized at least once, there was a trend toward higher rates of readmission among women in comparison to men.

What might explain this gender disparity in hospitalizations? Here, we explore several possible explanations. While the most obvious hypothesis would be that the women in our cohort were sicker than the men, we found that traditional markers of illness severity for cirrhosis, including MELD and hepatic decompensation (presence of ascites or hepatic encephalopathy), did not differ at baseline by gender, confirming findings from prior studies[3,4,8]. Perhaps, then, these traditional indicators of liver disease severity do not adequately capture all contributors to illness, such as non-

Table 1 Baseline characteristics by gender[1]

Characteristics	Total (*n* = 392)	Men (*n* = 231, 59%)	Women (*n* = 161, 41%)	*P* value
Age, yr	58 (51-63)	57 (50-63)	58 (52-63)	0.51
Race				0.64
Non-Hispanic Caucasian	240 (61)	142 (62)	98 (61)	
Black	9 (2)	4 (2)	5 (3)	
Hispanic	105 (27)	59 (26)	46 (29)	
Asian	16 (4)	11 (5)	5 (3)	
Other	22 (6)	15 (7)	7 (4)	
Etiology				< 0.01
HCV	168 (43)	112 (49)	56 (35)	
Alcohol	75 (19)	53 (23)	22 (14)	
NAFLD/NASH	55 (14)	24 (10)	31 (19)	
Autoimmune[2]	59 (15)	21 (9)	38 (24)	
HBV	3 (1)	3 (1)	0 (0)	
Other	32 (8)	18 (8)	14 (9)	
BMI, kg/m^2	28.5 (25.0-33.7)	28.8 (25.3-34.1)	28.0 (24.2-33.0)	0.13
Diabetes mellitus	115 (29)	71 (31)	44 (27)	0.47
Coronary artery disease	17 (4)	14 (3)	3 (2)	0.05
Hypertension	153 (39)	98 (42)	55 (34)	0.10
Stroke	7 (2)	4 (2)	3(2)	0.92
Dialysis	16 (4)	7 (3)	9 (5)	0.21
MELDNa	18 (15-22)	18 (15-22)	19 (15-23)	0.13
Albumin, g/dL	3.0 (2.6-3.4)	3.0 (2.6-3.4)	3.1 (2.7-3.4)	0.30
Presence of ascites	133 (34)	77 (33)	56 (35)	0.77
Numbers connection test, s	41 (30-58)	42 (32-58)	40 (29-58)	0.30
Moderate/severe HE	165 (42)	98 (42)	67 (42)	0.87
Frail	62 (16)	26 (16)	26 (17)	0.88

[1]Data presented as *n* (%) or median (IQR).

[2]Combined autoimmune hepatitis, primary sclerosing cholangitis, and primary biliary cholangitis.

IQR: Interquartile range; HCV: Hepatitis C; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; HBV: Hepatitis B; BMI: Body mass index; MELDNa: Model for end-stage liver disease with serum sodium; HE: Hepatic encephalopathy.

hepatic comorbidities or socioeconomic factors, that may affect an individual's vulnerability to adverse events that necessitate acute inpatient care. Our study also raises the possibility of systematic differences in the management of women and men with cirrhosis - either there exists a lower threshold for admission in women or a gap in coordination of care from the inpatient setting to outpatient recovery. Interestingly, among those who were hospitalized at least once, women were re-admitted almost 30% more frequently than men, though this did not reach statistical significance. It is also possible that differences in etiology of cirrhosis may contribute to differences in disease progression that lead to this gender disparity. Men were more likely to have cirrhosis due to Hepatitis C and alcohol, which are often no longer active by the time of listing for liver transplant. Women, in contrast, are more likely to have autoimmune hepatitis, which often continues to cause liver injury until the time of transplant.

Furthermore, it is possible that women are hospitalized for different reasons than men. Specifically, women may be more susceptible to complications of sarcopenia, such as infection or hepatic encephalopathy, which may lead to increased risk of hospitalization. Although proportion of "frail" patients did not differ between men and women in our cohort, more subtle differences in muscle mass could lead to differences in cirrhosis complications. Unfortunately, given the complexity of ascertaining cause of hospitalization (as many patients with cirrhosis have multiple - such as acute kidney injury, hepatic encephalopathy, worsening ascites), we were not able to accurately capture indications for hospitalization, which is a limitation of this study. Other factors that could contribute to hospitalization were also not captured in this study, such as medication complexity and adherence, diuretic resistance in patients with ascites, and social support. Future studies should evaluate such predictors, though some, such as social support or adherence, may be difficult to

Table 2	Logistic regression for hospitalization within 12 mo					
Characteristics	**Univariable**			**Multivariable**		
	OR	**95%CI**	***P* value**	**aOR**	**95%CI**	***P* value**
Female gender	1.57	1.05-2.35	0.03	1.64	1.05-2.56	0.03
Non-Hispanic Caucasian	0.81	0.54-1.22	0.31			
Age per year	1.00	0.98-1.02	0.95			
Hypertension	1.26	0.84-1.89	0.26			
Diabetes mellitus	1.31	0.85-2.02	0.23			
Coronary artery disease	0.98	0.37-2.60	0.97			
Stroke	0.83	0.18-3.75	0.81			
Dialysis	1.89	0.67-5.31	0.22			
BMI per 1 kg/m^2	1.02	0.99-1.05	0.24			
Autoimmune[1]	0.61	0.35-1.08	0.09			
MELDNa per 1 point>	1.13	1.07-1.18	< 0.001	1.08	1.03-1.13	0.001
Albumin per 1 mg/dL	0.44	0.31-0.63	< 0.001	0.53	0.36-0.78	0.001
Ascites	2.28	1.49-3.50	< 0.001	1.61	1.00-2.57	0.05
> NCT per 1 s	1.01	1.01-1.02	0.006			
Moderate/severe HE	1.17	0.78-1.75	0.45			
Frail	3.59	1.97-6.55	< 0.001	2.37	1.24-4.54	0.009

[1]Combined autoimmune hepatitis, primary sclerosing cholangitis, and primary biliary cholangitis. OR: Odds ratio; CI: Confidence interval; aOR: Adjusted odds ratio; BMI: Body mass index; MELDNa: Model for end-stage liver disease with serum sodium; NCT: Numbers connection test; HE: Hepatic encephalopathy.

collect on a large scale. Another limitation is that this is a US-based single center study serving a large catchment area within an open hospital network, so it is possible that we did not capture all hospitalizations for every patient. However, all of the patients enrolled in this study were waitlisted at our center and were required to report outside hospitalizations. Therefore, we believe that our ascertainment of hospitalizations was reliable, but validation of our gender-based findings in a larger, closed health system is warranted. It is also possible that rates of and reasons for hospitalization for patients on the liver transplant waitlist differ in countries with different healthcare systems, so our findings should be replicated outside of the United States as well.

Despite these limitations, this study describes significant gender differences in hospitalizations for patients on the liver transplant waitlist and thus, takes us one step closer to understanding the gender disparity in liver transplant waitlist mortality and dropout. Our finding that women are more likely to be hospitalized than men suggest that the experience of cirrhosis differs between women and men despite similarities in traditional measures of severity of illness. As the hepatology community moves toward developing cirrhosis-specific models of care, our data strongly suggest that these models may need to consider gender-specific vulnerabilities. Future studies are needed to evaluate gender-specific interventions in order to truly optimize the management of women and men living with cirrhosis.

Table 3 Binomial regression for number of hospitalized days within 12 mo

Characteristics	Univariable			Multivariable		
	IRR	95%CI	*P* value	aIRR	95%CI	*P* value
Female gender	1.68	1.08-2.60	0.02	1.92	1.23-2.99	0.004
Non-Hispanic Caucasian	0.86	0.55-1.35	0.52			
Age per year	1.00	0.98-1.03	0.80			
Hypertension	1.31	0.84-2.04	0.24			
Diabetes mellitus	1.05	0.65-1.69	0.85			
Coronary artery disease	0.52	0.18-1.55	0.28			
Stroke	0.20	0.03 0 1.16	0.13			
Dialysis	1.63	0.55-4.87	0.34			
BMI per 1 kg/m^2	1.01	0.98-1.05	0.42			
Autoimmune[1]	0.59	0.32-1.09	0.11			
MELDNa per 1 point	1.09	1.04-1.14	< 0.001	1.08	1.03-1.13	0.001
Albumin per 1 mg/dL	0.58	0.39-0.84	0.005	0.47	0.31-0.70	< 0.001
Ascites	1.72	1.09-2.70	0.02			
NCT per 1 s	1.00	1.00-1.01	0.13			
Moderate/severe HE	1.28	0.82-1.98	0.28			
Frail	2.12	1.17-3.81	0.007			

[1]Combined autoimmune hepatitis, primary sclerosing cholangitis, and primary biliary cholangitis. IRR: Incidence rate ratio; CI: Confidence interval; aIRR: Adjusted incidence rate ratio; BMI: Body mass index; MELDNa: Model for end-stage liver disease with serum sodium; NCT: Numbers connection test; HE: Hepatic encephalopathy.

ARTICLE HIGHLIGHTS

Research background

It is well-established in the literature that women have worse transplant-related outcomes than men, including lower rates of transplant and increased risk of waitlist mortality and dropout. The reasons for these disparities are unclear.

Research motivation

Hospital admissions are common among patients with cirrhosis, and may be a surrogate marker for disease progression that more accurately captures the differences in experience between men and women living with cirrhosis, and may help explain gender disparities in waitlist outcomes.

Research objectives

Thus, we aimed to evaluate the role of gender on risk of hospitalization for patients on the liver transplant waitlist.

Research methods

Our cohort included adults (≥ 18 years) with cirrhosis listed for liver transplant at University of California - San Francisco (UCSF) from March 2012 to December 2014 who were seen as outpatients and enrolled as a part of a prospective trial. Patients listed for transplant with model for end- stage liver disease (MELD) exception points for hepatocellular carcinoma were excluded, as were patients lost-to-follow up at 12 mo and those with severe hepatic encephalopathy. At the time of study enrollment, patient demographics and baseline laboratory values were collected. Clinical information regarding complications of patients' liver disease were assessed by enrolling clinician. The primary outcome was any hospitalization within 12 mo from study enrollment, and the secondary outcome was the number of inpatient days within 12 mo. Logistic regression and negative binomial regression evaluated the association of all listed covariates with the primary and secondary outcomes.

Research results

A total of 392 patients were enrolled during the study period; 41% were women and 61% were non-Hispanic Caucasian, with median (interquartile ranges) age of 58 years (51-63). During the 12-mo study period, 186 (47%) patients were hospitalized at least once. Of these 186 patients, 89 (48%) were readmitted at least once and 47 (25%) were readmitted more than once. More women than men were hospitalized at least once within the 12-mo study period (54% *vs* 43%, *P* = 0.03). In univariable logistic regression, the odds of being hospitalized at least once within 12 mo was 1.6 times higher among women compared to men (*P* = 0.03). In multivariable analysis, female gender remained significantly associated with hospitalization after adjusting for MELDNa, albumin, ascites, and frailty [adjusted odds ratios (OR) 1.6, 95% confidence interval (CI) 1.1-2.6; *P*

= 0.03]. Female gender was also associated with a higher number of total inpatient days within 12 mo on univariable [incidence rate ratio (IRR) 1.7, 95%CI: 1.1-2.6, P = 0.02] and multivariable analysis (adjusted IRR 1.9, 95%CI: 1.2-3.0, P < 0.01). There was a trend toward women being readmitted more often than men (54% *vs* 42%), but this did not reach statistical significance (P = 0.11).

Research conclusions

Women on the liver transplant waitlist are significantly more likely to be hospitalized than men, and are hospitalized for a higher number of days, even after adjustment for illness severity. Among those who were hospitalized at least once, there was a trend toward higher rates of readmission among women in comparison to men. These findings suggest that the clinical course of cirrhosis among women and men differs despite similarities in traditional measures of severity of illness.

Research perspectives

Our findings may help explain the gender disparities in liver transplant waitlist mortality and dropout, by highlighting differences in the experience of living with cirrhosis for women and men. Future studies are needed to evaluate gender-specific interventions in order to truly optimize the management of women and men living with cirrhosis and to eliminate waitlist disparities.

REFERENCES

1   **Sarkar M**, Watt KD, Terrault N, Berenguer M. Outcomes in liver transplantation: Does sex matter? *J Hepatol* 2015; **62**: 946-955 [PMID: 25433162 DOI: 10.1016/j.jhep.2014.11.023]
2   **Moylan CA**, Brady CW, Johnson JL, Smith AD, Tuttle-Newhall JE, Muir AJ. Disparities in liver transplantation before and after introduction of the MELD score. *JAMA* 2008; **300**: 2371-2378 [PMID: 19033587 DOI: 10.1001/jama.2008.720]
3   **Lai JC**, Terrault NA, Vittinghoff E, Biggins SW. Height contributes to the gender difference in wait-list mortality under the MELD-based liver allocation system. *Am J Transplant* 2010; **10**: 2658-2664 [PMID: 21087414 DOI: 10.1111/j.1600-6143.2010.03326.x]
4   **Mathur AK**, Schaubel DE, Gong Q, Guidinger MK, Merion RM. Sex-based disparities in liver transplant rates in the United States. *Am J Transplant* 2011; **11**: 1435-1443 [PMID: 21718440 DOI: 10.1111/j.1600-6143.2011.03498.x]
5   **Sharma P**, Schaubel DE, Messersmith EE, Guidinger MK, Merion RM. Factors that affect deceased donor liver transplantation rates in the United States in addition to the Model for End-stage Liver Disease score. *Liver Transpl* 2012; **18**: 1456-1463 [PMID: 22965903 DOI: 10.1002/lt.23548]
6   **Mindikoglu AL**, Emre SH, Magder LS. Impact of estimated liver volume and liver weight on gender disparity in liver transplantation. *Liver Transpl* 2013; **19**: 89-95 [PMID: 23008117 DOI: 10.1002/lt.23553]
7   **Myers RP**, Shaheen AA, Aspinall AI, Quinn RR, Burak KW. Gender, renal function, and outcomes on the liver transplant waiting list: Assessment of revised MELD including estimated glomerular filtration rate. *J Hepatol* 2011; **54**: 462-470 [PMID: 21109324 DOI: 10.1016/j.jhep.2010.07.015]
8   **Cullaro G**, Sarkar M, Lai JC. Sex-based disparities in delisting for being "too sick" for liver transplantation. *Am J Transplant* 2018; **18**: 1214-1219 [PMID: 29194969 DOI: 10.1111/ajt.14608]
9   **Giard JM**, Terrault NA. Women with Cirrhosis: Prevalence, Natural History, and Management. *Gastroenterol Clin North Am* 2016; **45**: 345-358 [PMID: 27261903 DOI: 10.1016/j.gtc.2016.02.010]
10   **Lai JC**, Covinsky KE, Dodge JL, Boscardin WJ, Segev DL, Roberts JP, Feng S. Development of a novel frailty index to predict mortality in patients with end-stage liver disease. *Hepatology* 2017; **66**: 564-574 [PMID: 28422306 DOI: 10.1002/hep.29219]
11   **Lai JC**, Dodge JL, Sen S, Covinsky K, Feng S. Functional decline in patients with cirrhosis awaiting liver transplantation: Results from the functional assessment in liver transplantation (FrAILT) study. *Hepatology* 2016; **63**: 574-580 [PMID: 26517301 DOI: 10.1002/hep.28316]
12   **Lai JC**, Feng S, Terrault NA, Lizaola B, Hayssen H, Covinsky K. Frailty predicts waitlist mortality in liver transplant candidates. *Am J Transplant* 2014; **14**: 1870-1879 [PMID: 24935609 DOI: 10.1111/ajt.12762]
13   **Weissenborn K**, Rückert N, Hecker H, Manns MP. The number connection tests A and B: Interindividual variability and use for the assessment of early hepatic encephalopathy. *J Hepatol* 1998; **28**: 646-653 [PMID: 9566834 DOI: 10.1016/S0168-8278(98)80289-4]
14   **Sinclair M**, Poltavskiy E, Dodge JL, Lai JC. Frailty is independently associated with increased hospitalisation days in patients on the liver transplant waitlist. *World J Gastroenterol* 2017; **23**: 899-905 [PMID: 28223735 DOI: 10.3748/wjg.v23.i5.899]
15   **Nguyen GC**, Segev DL, Thuluvath PJ. Nationwide increase in hospitalizations and hepatitis C among inpatients with cirrhosis and sequelae of portal hypertension. *Clin Gastroenterol Hepatol* 2007; **5**: 1092-1099 [PMID: 17625983 DOI: 10.1016/j.cgh.2007.04.027]
16   **Axelrod DA**, Dzebisashvili N, Lentine K, Segev DL, Dickson R, Tuttle-Newhall E, Freeman R, Schnitzler M. Assessing variation in the costs of care among patients awaiting liver transplantation. *Am J Transplant* 2014; **14**: 70-78 [PMID: 24165015 DOI: 10.1111/ajt.12494]
17   **Mathur AK**, Chakrabarti AK, Mellinger JL, Volk ML, Day R, Singer AL, Hewitt WR, Reddy KS, Moss AA. Hospital resource intensity and cirrhosis mortality in United States. *World J Gastroenterol* 2017; **23**: 1857-1865 [PMID: 28348492 DOI: 10.3748/wjg.v23.i10.1857]

Retrospective Study

Two-year delay in ulcerative colitis diagnosis is associated with anti-tumor necrosis factor alpha use

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Abstract

BACKGROUND
Ulcerative colitis (UC) is an uncommon inflammatory bowel disease (IBD). However, its incidence has recently increased in South Korea. Moreover, UC diagnoses are frequently delayed, and the relationship between diagnostic delay and UC prognosis has not been extensively studied in South Korean patients.

AIM
To identify meaningful diagnostic delay affecting UC prognosis and to evaluate risk factors associated with diagnostic delay in South Korean patients.

METHODS
Medical records of 718 patients with UC who visited the outpatient clinic of six university hospitals in South Korea were reviewed; 167 cases were excluded

because the first symptom date was unknown. We evaluated the relationship between the prognosis and a diagnostic delay of 3, 6, 12, 18, and 24 mo by comparing the prognostic factors [anti-tumor necrosis factor (TNF)-α use, admission history due to acute flare-ups, frequent admission due to flare-ups, surgery associated with UC, and the clinical remission state at the latest follow-up] at each diagnostic interval.

RESULTS
The mean diagnostic interval was 223.3 ± 483.2 d (median, 69 d; 75th percentile, 195 d). Among the prognostic factors, anti-TNFα use was significantly increased after a diagnostic delay of 24 mo. Clinical risk factors predictive of a 24-mo diagnostic delay were age < 60 years at diagnosis [odd ratio (OR) = 14.778, 95% confidence interval (CI): 1.731-126.121], smoking history (OR = 2.688, 95%CI: 1.239-5.747, $P = 0.012$), and misdiagnosis of hemorrhoids (OR = 11.066, 95%CI: 3.596-34.053). Anti-TNFα use was associated with extensive UC at diagnosis (OR = 3.768, 95%CI: 1.860-7.632) and 24-mo diagnostic delay (OR = 2.599, 95%CI: 1.006-4.916).

CONCLUSION
A diagnostic delay > 24 mo was associated with increased anti-TNFα use. Age < 60 years at diagnosis, smoking history, and misdiagnosis of hemorrhoids were risk factors for delayed diagnosis.

Key words: Ulcerative colitis; Diagnostic delay; Anti-tumor necrosis factor alpha; Smoking

Core tip: We aimed to identify the diagnostic delay that affects the prognosis in Korean patients with ulcerative colitis. We found that the group with a ≥ 24-mo diagnostic delay had used anti-tumor necrosis factor alpha drugs more frequently than the group with a < 24-mo delay. We also found that additional risk factors for a 24-mo delay in diagnosis were age < 60 years, smoking history, and a misdiagnosis of hemorrhoids by a physician.

INTRODUCTION

Ulcerative colitis (UC) is an uncommon inflammatory bowel disease (IBD). However, its incidence has recently increased in South Korea. UC is diagnosed by clinical, endoscopic, and histologic findings because there is no definite diagnosis index. Therefore, differentiating it from other diseases of the intestines, such as acute gastroenteritis or irritable bowel syndrome (IBS) is often difficult, and its diagnosis is often delayed. It is unclear whether a diagnostic delay of IBD is clinically significant. Recent studies have shown that early control of IBD affects the quality of life and the disease course, including its prognosis[1-4].

Most studies of a diagnostic delay for IBD were focused on Crohn's disease (CD), and although the duration of the delay was different, the need for surgery was closely related to the diagnostic delay of CD in both Western and Asian populations[5-9]. There have been reports of clinical factors involved in the diagnostic delay of UC, but there is a lack of information regarding whether this delay affects the prognosis and treatment of UC for Asian patients[9]. Some patients have had symptoms for a long period, but the correct diagnosis was not made because UC was mild or had an insidious onset. A few studies have focused on how various durations of diagnostic delay can affect the future treatment and prognosis of these patients. Diagnostic delay and its impact on Western and Asian populations may be significantly different due to genetic or environmental factors; therefore, it is necessary to examine the results

according to countries or regions.

Thus, we aimed to identify the period of delay in diagnosis (time from the first symptoms to UC diagnosis) that affected treatment and prognosis. We also evaluated the risk factors and clinical significance of a diagnostic delay for UC in South Korean patients.

MATERIALS AND METHODS

Patients

This retrospective study was based on patient data collected from six university-affiliated hospitals located in metropolitan areas (Incheon-si, Ansan-si, Anyang-si, Suwon-si, Sungnam-si and Dongtan-si) near Seoul, South Korea, from January 2006 to December 2016. We analyzed the medical records of 718 patients who visited the outpatient clinic in 2016, had a definite diagnosis of UC, and were followed up for more than 6 mo. The diagnosis of UC was based on clinical, radiological, endoscopic, and pathologic findings. One hundred sixty-seven patients were excluded from the study because of incomplete medical record data regarding the first day of symptoms. Patients were not required to provide informed consent for inclusion in this retrospective study. We anonymized all clinical data to protect personal information. This study was conducted with the approval of the ethics committee of Hallym University Sacred Heart Hospital in Anyang, South Korea (IRB No. 2016-I607). The study was performed in accordance with the recommendations of the Declaration of Helsinki.

Data collection, definition, and assessment of clinical outcomes

Demographic characteristics of patients at the time of the first diagnosis were collected, including sex, age, body mass index (BMI), family history of UC, history of smoking, residence at the time of diagnosis (urban or rural), and education level (university education or less). Clinical medical records regarding the first symptoms and endoscopic and laboratory findings were also investigated, such as the day when the first symptom occurred, the day of the first physician visit, the day of first diagnosis, the type of symptoms (hematochezia, chronic diarrhea, abdominal pain), cases misdiagnosed as other diseases (hemorrhoids, IBS), Mayo score including endoscopic score[10-12], disease extension (proctitis, left side, extensive)[13], and C-reactive protein level (CRP, mg/dL). During data collection, to determine the Mayo endoscopic score and the extension of the disease, endoscopy findings were reviewed again by the endoscopist from each medical center. To reduce inter-observer variation, all endoscopist reviewers were trained using the same reference material[10-13] before the review. The use of prescribed medications [oral/intravenous (IV) steroids, azathioprine/6-mercaptopurine (AZA/6MP), or anti-tumor necrosis factor alpha (anti-TNFα)] and the first day of the prescribed medication were investigated. To determine the prognostic factors, the use of anti-TNFα, the hospital admission history due to acute flare-ups[14,15], frequent admission (more than two admissions due to UC flare-ups), surgery associated with UC, and the clinical remission state at the latest follow-up were obtained from the medical records. The diagnostic interval was defined as the time from the first symptom until UC diagnosis. We divided the patients into the early and delay groups according to several diagnostic interval criteria (3 mo, 6 mo, 12 mo, 18 mo and 24 mo). Then, we compared the two groups according to the aforementioned demographic and clinical characteristics to determine the diagnostic delay having a clinical impact.

Statistical analysis

Both categorical and continuous variables of baseline characteristics were analyzed. Continuous variables are shown as mean ± standard deviation or medians with ranges. To evaluate the criteria for a diagnostic delay having a clinical impact, the chi-square test, Fisher's exact test, and Kaplan-Meier method with the log-rank test were used to compare prognostic factors between the early group and delay group. After the meaningful diagnostic delay and prognosis factors were determined, they were compared to the clinical factors (sex, age, BMI, first symptom, cases misdiagnosed as hemorrhoids or IBS, Mayo score, disease extension, and CRP level) by univariate analysis. Multivariate logistic regression analyses including significant clinical factors from the univariate analysis were performed to evaluate risk factor-related diagnostic delay and prognosis. Odd ratios (OR) and 95% confidence intervals (CI) were calculated as measures of the correlation between the clinical variables and outcomes of interest. $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Baseline characteristics of patients

Clinical characteristics of the patients were as follows: male sex, 318 (55.7%); mean age at diagnosis, 40.56 ± 16.11 years; and BMI at diagnosis, 22.28 ± 3.19 kg/m^2 (Table 1). Seventy-seven of 443 (17.4%) had a history of smoking. Presenting first symptoms were hematochezia (388/548; 70.8%), diarrhea (164/548; 29.9%), and abdominal pain (39/548; 7.1%). The days from first symptoms to UC diagnosis were 223.28 ± 483.15 (median, 69); 75% of patients were diagnosed within 195 d. Time from the first symptoms to the first hospital visit was 154.22 ± 379.140 d (median, 40 d), and the time from the first hospital visit to diagnosis was 69.06 ± 295.04 d (median, 9 d). The Mayo score at diagnosis was examined in 399 patients; 179 (44.9%) had mild disease, 204 (51.1%) had moderate disease, and 220 (4.0%) had severe disease. Disease extension at diagnosis was examined in 547 patients; 253 (46.3%) had proctitis, 160 (29.3%) had left-side colitis, and 134 (24.5%) had extensive colitis. Prescribed medications used by all patients were as follows: oral/IV steroids, 250 (45.4%); AZA/6MP, 163 (29.8%); and anti-TNFα, 50 (9.1%).

Diagnostic delay and prognosis

There were no statistically significant differences in the use of anti-TNFα, hospital admission history for acute flare-ups, frequent admission, surgery associated with UC, or clinical remission state at the latest follow-up between the early and delay groups with other diagnostic intervals (3, 6, 12 and 18 mo) (Table 2). In Figure 1, the use of anti-TNFα at the 3-mo diagnostic interval was insignificantly prevalent in the early group (34/322; 10.6%) and the delay group (16/229; 7.0%); however, the use of anti-TNFα by the early group and delay group began to decrease at the 18-mo diagnostic interval. Finally, at the 24-mo diagnostic interval, it was significantly higher in the delay group (8/42; 35.7%) than in the early group (42/509; 8.3%) (*P* = 0.019). Anti-TNFα free-survival rates between the early and delay groups according to the 24-mo diagnostic interval were significantly different (*P* = 0.034) (Figure 2). Therefore, it was determined that 24 mo was the diagnostic delay cutoff point for poor outcomes, and this delay was related to the use of anti-TNFα.

Risk factors associated with a 24-mo diagnostic delay

According to the univariate analysis (Table 3), sex, age, BMI, education level, residence at the time of diagnosis, family history of IBD, and the first symptom were not different between the early and delay groups. However, age < 60 years (*P* = 0.020), history of smoking (*P* = 0.008), and misdiagnosis of hemorrhoids (*P* = 0.000) were significantly increased in the delay group. According to the multivariate logistic regression analysis, independent risk factors predictive of 24 mo as the cutoff were age < 60 years (OR = 14.778, 95%CI: 1.731-126.121, *P* = 0.014), smoking history (OR = 2.688, 95%CI: 1.239-5.747, *P* = 0.012), and misdiagnosis of hemorrhoids (OR = 11.066, 95%CI: 3.596-34.053, *P* = 0.000) (Table 4).

Risk factors associated with the use of anti-TNFα

According to the univariate analysis (Table 5), sex, age, smoking history, BMI, CRP level at diagnosis, early steroid use within 2 mo of diagnosis, and early AZA/6MP use within 2 mo of diagnosis were not predictive factors for anti-TNFα use. However, moderate or severe Mayo score (*P* = 0.001), extensive disease (*P* = 0.000), and a diagnostic delay ≥ 24 mo (*P* = 0.019) were significantly increased in the delay group. According to the multivariate logistic regression analysis, independent risk factors predictive of anti-TNFα use were extensive disease (OR = 3.768, 95%CI: 1.860-7.632, *P* = 0.000) and a diagnostic delay of ≥ 24 mo (OR = 2.599, 95%CI: 1.006-4.916, *P* = 0.049) (Table 6).

DISCUSSION

We found that the ≥ 24-mo diagnostic delay group used anti-TNFα drugs more frequently than the < 24-mo delay group. We also found that risk factors for the 24-mo delay were age < 60 years, smoking history, and misdiagnosis of hemorrhoids by a physician.

In general, CD is known to have a longer diagnostic delay than UC[4,7,9,16,17]. This is because the main symptom of CD is abdominal pain, and it is necessary to differentiate it from IBS. Furthermore, those with UC seem to visit the hospital earlier because it is related to hematochezia. In three studies on delayed diagnosis in UC, the median delays were 1, 3.1 and 4 mo. These studies categorized patients at the 76th to 100th percentiles into the delay group, and according to this, a diagnosis of more than

Table 1 Baseline characteristics of patients (*n* = 551)	
Characteristics	**Value**
Male sex (%)	318/551 (55.7)
Age at diagnosis, yr	40.56 ± 16.11
BMI at diagnosis, kg/m^2	22.28 ± 3.19
Family history of IBD (%)	51/369 (13.8)
History of smoking (%)	77/443 (17.4)
First symptom	
Hematochezia	388/548 (70.8)
Diarrhea	164/548 (29.9)
Abdominal pain	39/548 (7.1)
Diagnostic interval, d (median)	223.28 ± 483.15 (69)
Time from first symptom to hospital visit, d (median)	154.22 ± 379.140 (40)
Time from first hospital visit to diagnosis, d (median)	69.06 ± 295.04 (9)
Mayo score at diagnosis (%)	
Mild	179/399 (44.9)
Moderate	204/399 (51.1)
Severe	220/399 (4.0)
Disease extent at diagnosis (%)	
Proctitis	253/547 (46.3)
Left side	160/547 (29.3)
Extensive	134/547 (24.5)
Steroid (oral or intravenous) use (%)	250/551 (45.4)
AZA/6MP use (%)	163/551 (29.6)
Anti-TNFα use (%)	50/551 (9.1)
Admission due to flare-up (%)	121/430 (22.0)
Frequent admission due to flare-up (%)	50/551 (9.1)
UC-related surgery (%)	7/551 (1.3)
Clinical remission at the latest follow-up (%)	333/467 (71.3)

BMI: Body mass index; IBD: Inflammatory bowel disease; AZA/6MP: Azathioprine or 6-mercaptopurine; Anti-TNFα: Anti-tumor necrosis factor alpha; UC: Ulcerative colitis.

3, 10 and 12 mo were classified as delayed diagnosis; non-steroidal anti-inflammatory drug (NSAID) use, male sex, and age < 40 years were suggested as factors related to delayed diagnosis[4,7,16]. In our study, the median delay was 3.3 mo; 75% of patients were diagnosed within 6.5 mo, and 7.6% of patients had a diagnostic delay of more than 24 mo. Therefore, the median delay and the delay for 75% of patients were not significantly different from that of other studies. In most previous studies, the definition of a diagnostic delay for IBD was defined as more than the 76th to 100th percentile of patients without a correct diagnosis[4,7,8,18]. Our initial hypothesis was that the diagnostic delay for 76th to 100th percentile of patients (> 6.5 mo in our study) would be correlated with prognostic factors; however, we did not find any significant results. Therefore, unlike previous studies, we did not predetermine the criteria for diagnostic delay; instead, we tried to determine the diagnostic delay that affects the prognostic factors. As a result, we found that a diagnostic delay of more than 24 mo was related to the use of anti-TNFα.

Age < 60 years at diagnosis, smoking history, and misdiagnosis of hemorrhoids were risk factors for the 24-mo diagnostic delay in our study. It is well known that smoking is related to UC[17]; however, a person who smokes may not be interested in health or may not be able to seek medical care because of economic reasons[19], which may have affected our results. Contrary to previous studies, we found that factors other than smoking were related to UC. Some studies showed that age older than 40 years was correlated with diagnostic delay, whereas others showed the opposite[7,16]. In our study, age of 40 years or younger and age of 40 years or older were not risk factors for diagnostic delay; however, age < 60 years was a risk factor for diagnostic delay. Additionally, misdiagnoses as hemorrhoids were risk factors, which was contradictory to the finding of a Western study that showed UC was sometimes found faster by hematochezia[4]. There were several reasons for these results. First, regarding
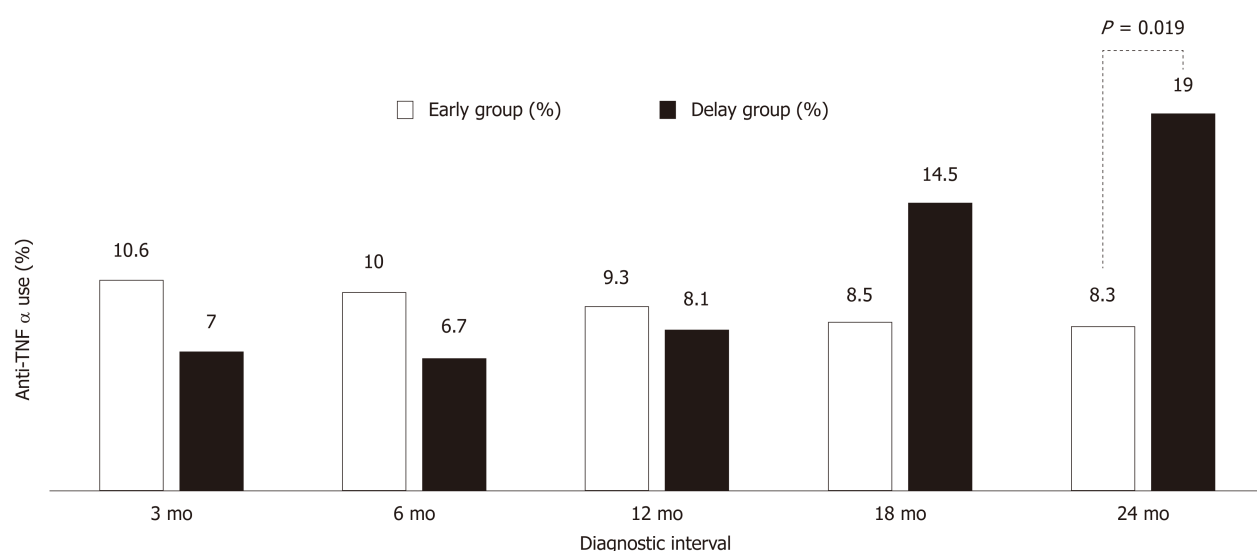
Figure 1 Difference in the anti-tumor necrosis factor alpha use rate (%) according to the diagnostic interval. Anti-TNFα: Anti-tumor necrosis factor alpha.

genetics, UC in Asian individuals, including South Koreans, may have a milder course than that in Western individuals, although the clinical features of both are similar[20-24]. Second, there are many regional differences in each country due to environmental factors such as medical accessibility and insurance status[25,26]. For example, in South Korea, colorectal cancer screening is performed after age 50 years, and hemorrhoids are common. Furthermore, recently, UC has been increasingly observed in Asian individuals compared to Western individuals. Therefore, because of environmental and genetic factors, our results may not be applicable to other populations. However, more research is necessary to determine whether our research represents the entire Asian population.

In the CD studies which examined the relation between diagnostic delay and prognosis, a delayed diagnosis of 13, 18, 25 and 34 mo was associated with CD-related surgery[5-8]. In a study of 1047 patients in South Korea, an 18-mo diagnostic delay was predictive of further development of intestinal stenosis, internal fistulas, and perianal fistulas[27]. Although the durations of the delays were different, the need for surgery was closely related to the diagnostic delay of CD in both Asian and Western populations. These studies indicated that as the duration of exposure to the disease increased, cumulative intestinal damage did not respond to medical treatment and the necessity for surgery increased. There are very few studies on the relationship between diagnostic delay and the prognosis of UC. A previous study performed in South Korea suggested that a diagnostic delay of 6.2 mo led to an increased risk for intestinal surgery[9]. Unfortunately, in our study, the number of surgeries (7/551; 1.3%) was too small, and the analysis was insufficient.

In our study, a 24-mo diagnostic delay was associated with the use of anti-TNFα drugs. In South Korea, certain factors regarding anti-TNFα use and surgery should be considered. First, a systemic analysis of IBD using population-based studies and randomized controlled trials suggested that anti-TNFα drugs had a protective effect against surgery in the biologic era, and that anti-TNFα drugs were used more frequently and earlier in South Korea[21,28]. Second, in Asia, the UC course is mild, patients resist surgery, and cases of colonic resection are rare[20-24]. In recent studies at an IBD specialized referral center in South Korea, the colectomy rate has decreased over the past 30 years for South Korean patients with UC[21]. In this study, 1119 patients were diagnosed between 2007 and 2013, the 7-year cumulative colectomy rate was 0.5% for patients diagnosed at this IBD referral hospital, and 4.7% for patients referred to this institution after diagnosis or treatment at another hospital. Therefore, because there is a possibility of a severe refractory cases in the referral population, the actual colectomy rate in South Korea is probably between 0.5% and 4.7% in biologic era, which is clearly lower than that of Western populations[29]. Third, practitioners in South Korea are unable to perform top-down treatment because of insurance problems; only patients who do not respond to steroids and AZT/6MP treatment can receive anti-TNFα treatment[30]. Thus, instead of surgery, the use of anti-TNFα drugs may be an important surrogate marker of the severity and prognosis of UC in South Korea.

The more extensive the disease, the longer the morbidity period; furthermore, primary sclerosing cholangitis and frequent admissions for flare-up were predictors

Table 2 Diagnostic delay and prognosis

Diagnostic interval	3 mo			6 mo			12 mo			18 mo			24 mo		
	Early group (%)	Delay group (%)	P value	Early group (%)	Delay group (%)	P value	Early group (%)	Delay group (%)	P value	Early group (%)	Delay group (%)	P value	Early group (%)	Delay group (%)	P value
	322	229		401	150		452	99		496	55		509	42	
	(58.4)	(41.6)		(72.8)	(27.2)		(82.0)	(18)		(90)	(10)		(92.4)	(7.6)	
Anti-TNFα use			0.150			0.249			0.847			0.137			0.019
No	288	213		361	140		410	91		454	47		467	34	
	(89.4)	(93.0)		(90.0)	(93.3)		(90.7)	(91.9)		(91.5)	(85.5)		(91.7)	(81.0)	
Yes	34	16		40	10		42	8		42	8		42	8	
	(10.6)	(7.0)		(10.0)	(6.7)		(9.3)	(8.1)		(8.5)	(14.5)		(8.3)	(19.0)	
Admission (flare-up)			0.269			0.203			1.000			0.509			0.703
No	246	184		307	123		353	77		389	41		398	32	
	(76.4)	(80.3)		(76.6)	(82.0)		(78.1)	(77.8)		(78.4)	(74.5)		(78.2)	(76.2)	
Yes	76	45		94	27		99	22		107	14		111	10	
	(23.6)	(19.7)		(23.4)	(18.0)		(21.9)	(22.2)		(21.8)	(25.5)		(21.8)	(23.8)	
Frequent admission			0.150			0.384			0.443			0.806			0.412
No	288	213		362	139		409	92		450	51		461	40	
	(89.4)	(93.0)		(90.3)	(92.7)		(90.5)	(92.9)		(90.7)	(92.7)		(90.6)	(95.2)	
Yes	34	16		39	11		43	7		46	4		48	2	
	(10.6)	(7.0)		(9.7)	(7.3)		(9.5)	(7.1)		(9.3)	(7.3)		(9.4)	(4.8)	
Surgery-related UC			0.457			0.092			0.114			0.523			0.428
No	319	225		398	146		448	96		490	54		503	41	
	(99.1)	(98.3)		(99.3)	(97.3)		(99.1)	(97.7)		(98.8)	(98.2)		(98.8)	(97.6)	
Yes	3	4		3	4		4	3		6	1		6	1	
	(0.9)	(1.7)		(0.7)	(2.7)		(0.9)	(3.0)		(1.2)	(1.8)		(1.2)	(2.4)	
Clinical remission			0.866			0.842			0.509			0.679			0.338
No	191	142		97	37		267	66		300	33		310	23	
	(71.0)	(71.7)		(29.0)	(28.0)		(70.6)	(74.2)		(71.6)	(68.8)		(71.9)	(63.9)	
Yes	78	56		238	95		111	23		119	15		121	13	
	(29.0)	(28.3)		(71.0)	(72.0)		(29.4)	(25.8)		(28.4)	(31.3)		(28.1)	(36.1)	

The early group was defined as receiving a diagnosis earlier than the diagnostic interval, and the delay group was defined as receiving a later diagnosis. Clinical remission was investigated in 467 patients. And the proportion of the delay group in 467 patients was similar to that of 551 patients: 3 mo (42.3%), 6 mo (28.2%), 12 mo (19%), 18 mo (9.6%) and 24 mo (7.7%). Anti-TNFα: Anti-tumor necrosis factor alpha; UC: Ulcerative colitis.

of colonic resection of UC[31-33]. In addition, in a study of step-up treatment for UC, risk factors for anti-TNFα use were female sex, age > 40 years, extra-intestinal manifestation, and extensive disease[34]. In another study, extensive disease was also associated with anti-TNFα and AZA/6MP for UC[35]. Therefore, if the use of anti-TNFα replaces UC-related surgery as a prognostic factor, then extensive disease is an important factor in the prognosis of UC with a diagnostic delay, as indicated by our multivariate analysis. Unfortunately, several clinical factors such as early use of steroids, use of azathioprine, and Mayo scores were not associated with anti-TNFα use in our study.

There were some limitations to our study. First, it was a retrospective study; therefore, important clinical information, the first day of UC-related symptoms, may have been inaccurate because of recall bias. In addition, nearly one-fourth of patients were excluded from the study because of incomplete medical record data regarding the first day of symptoms. Second, NSAID use, oral contraceptive use, socioeconomic status, and EIM were not investigated. Third, the use of anti-TNFα drugs was greater in the early group than in the delay group when the diagnostic interval was 3 mo. This was presumably owing to the inclusion of patients with acute flare-ups; however, in our study, the diagnostic criteria for the acute flare-up group were not clear, hence this condition could not be ruled out. Nevertheless, this was the first multicenter study of the average diagnostic delay period for UC in the South Korean

Figure 2 Anti-tumor necrosis factor alpha-free survival between the early and delay groups according to the 24-mo diagnostic interval (*P* = 0.034).

population. The prognosis was assessed according to the use of anti-TNFα in Asians avoiding surgery, and it was assumed that these patients could represent the Asian population with mild UC.

Our study suggested that a 24-mo diagnostic delay should be avoided in UC patients even those with mild UC symptoms. Care should be taken not to overlook UC in young patients with hemorrhoids who smoke.

Table 3 Comparisons of clinical characteristics between the early and delay groups according to the 24-mo diagnostic interval for patients with ulcerative colitis

		Total	Early group (< 24 mo)	Delay group (≥ 24 mo)	P value
Sex (%)					0.872
	Male	318 (57.7)	293 (57.6)	25 (59.5)	
	Female	233 (42.3)	216 (42.4)	17 (40.5)	
Age (%)		40.56 ± 16.11	40.93 ± 16.20	41.21 ± 14.47	0.903
					0.020
	≥ 60 yr	80 (14.5)	79 (15.5)	1 (2.4)	
	< 60 yr	471 (85.5)	430 (84.5)	41 (97.6)	
BMI (%)					0.306
	< 25 kg/m^2	295 (83.3)	268 (82.7)	27 (90.0)	
	≥ 25 kg/m^2	59 (16.7)	56 (17.3)	3 (10.0)	
Education (%)					0.561
	< University	170 (56.5)	152 (55.9)	18 (62.1)	
	≥ University	131 (43.5)	120 (44.1)	11 (37.9)	
Residence at diagnosis (%)					0.498
	Rural	503 (94.2)	466 (94.3)	37 (92.5)	
	Urban	31 (5.8)	28 (5.7)	3 (7.5)	
Family history of IBD (%)					0.782
	No	318 (86.2)	291 (85.8)	27 (90.0)	
	Yes	51 (13.8)	48 (14.2)	3 (10.0)	
Smoking (%)					0.008
	No	366 (82.6)	339 (84.1)	27 (67.5)	
	Yes	77 (17.4)	64 (15.9)	13 (32.5)	
First symptom (%)					
					0.540
Hematochezia	No	160 (29.2)	146 (28.9)	14 (33.3)	
	Yes	388 (70.8)	360 (71.1)	28 (66.7)	
					0.229
Chronic diarrhea	No	384 (70.1)	358 (70.8)	26 (61.9)	
	Yes	164 (29.9)	148 (29.2)	16 (38.1)	
Diagnosis before UC					
					0.000
Hemorrhoids (%)	No	487 (94.7)	453 (95.8)	34 (82.9)	
	Yes	27 (5.3)	20 (4.2)	7 (17.1)	
					0.066
IBS (%)	No	494 (96.1)	457 (96.6)	37 (90.2)	
	Yes	20 (3.9)	16 (3.4)	4 (9.8)	
Mayo score at diagnosis (%)					0.958
	Mild	179 (44.9)	163 (44.9)	16 (44.4)	
	Moderate + Severe	220 (55.1)	200 (55.1)	20 (55.6)	
Disease extent at diagnosis (%)					0.914
	Proctitis + left side	413 (75.5))	381 (75.4)	32 (76.2)	
	Extensive	134 (24.5)	124 (24.6)	10 (23.8)	

BMI: Body mass index; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; IBS: Irritable bowel syndrome.

Table 4 Risk factors predictive of 24-mo diagnostic delay according to the univariate and multivariate analyses

	Univariate		Multivariate	
	OR (95%CI)	P value	OR (95%CI)	P value
Age (≥ 60 yr *vs* < 60 yr)	7.533 (1.021-55.557)	0.020	14.778 (1.731-126.121)	0.014

Smoking history (no *vs* yes)	2.550 (1.249-5.206)	0.008	2.688 (1.239-5.747)	0.012
Misdiagnosed as hemorrhoids (no *vs* yes)	4.663 (1.842-11.803)	0.000	11.066 (3.596-34.053)	0.000

OR: Odd ratio; CI: Confidence interval.

Table 5 Risk factors related to anti-tumor necrosis factor alpha use

		Total	Anti-TNFα No	Anti-TNFα Yes	*P* value
Sex (%)					0.731
	Male	318 (57.7)	288 (57.5)	30 (60.0)	
	Female	233 (42.3)	213 (42.5)	20 (40.0)	
Age (%)		40.56 ± 16.11	41.10 ± 16.76	38.36 ± 16.05	0.268
					0.913
	< 60 yr	278 (50.5)	428 (85.4)	43 (86.0)	
	≥ 60 yr	273 (49.5)	73 (14.6)	7 (14.0)	
Smoking (%)					0.422
	No	366 (82.6)	329 (83.1)	37 (78.7)	
	Yes	77 (17.4)	67 (16.9)	10 (21.3)	
BMI (%)					0.667
	< 25 kg/m^2	295 (83.3)	260 (83.6)	35 (81.4)	
	≥ 25 kg/m^2	59 (16.7)	51 (16.4)	8 (18.6)	
Mayo score at diagnosis (%)					0.001
	Mild	179 (44.9)	170 (47.6)	9 (21.4)	
	Moderate + severe	220 (55.1)	187 (52.4)	33 (78.6)	
Disease extent at diagnosis (%)					0.000
	Proctitis + left side	413 (75.5)	394 (79.3)	19 (38.0)	
	Extensive	134 (24.5)	103 (20.7)	31 (62.0)	
CRP level at diagnosis (%)					0.536
	< 5 mg/dL	265 (79.3)	17 (73.9)	282 (79.0)	
	≥ 5 mg/dL	69 (20.7)	6 (26.1)	75 (21.0)	
Steroid use after diagnosis					0.269
	< 2 mo		116 (57.1)	31 (66.0)	
	≥ 2 mo		87 (42.9)	16 (34.0)	
AZA/6MP use after diagnosis					0.741
	< 2 mo	47 (29.2)	37 (29.8)	10 (27.0)	
	≥ 2 mo	114 (70.8)	87 (70.2)	27 (73.0)	
Diagnostic delay (%)					0.019
	< 24 mo	509 (92.4)	467 (93.2)	42 (84.0)	
	≥ 24 mo	42 (7.6)	34 (6.8)	8 (16.0)	

Anti-TNFα: Anti-tumor necrosis factor alpha; BMI: Body mass index; AZA/6MP: Azathioprine or 6-mercaptopurine.

Table 6 Risk factors predictive of anti-tumor necrosis factor alpha use according to the univariate and multivariate analyses

	Univariate		Multivariate	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Mayo score (mild *vs* moderate + severe).	3.333 (1.550-7.169)	0.002	2.168 (0.956-4.916)	0.064
Disease extent (proctitis + left side *vs* extensive)	6.241 (3.388-11.496)	0.000	3.768 (1.860-7.632)	0.000
Diagnostic delay (< 24 mo *vs* ≥ 24 mo)	2.616 (1.138-6.014)	0.019	2.599 (1.006-4.916)	0.049

OR: Odd ratio; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is diagnosed by clinical, endoscopic, and histologic findings because there is no definite diagnosis index. Therefore, differentiating it from other diseases of the intestines, such as acute gastroenteritis or irritable bowel syndrome is often difficult, and its diagnosis is often delayed. Recent studies have shown that early control of inflammatory bowel disease (IBD) affects the quality of life and the disease course, including its prognosis.

Research motivation

Most studies of a diagnostic delay for IBD were focused on Crohn's disease. There have been reports of clinical factors involved in the diagnostic delay of UC, but there is a lack of information regarding whether this delay affects the prognosis and treatment of UC. Diagnostic delay and its impact on Western and Asian populations may be significantly different owing to genetic or environmental factors; therefore, it is necessary to examine the results according to countries or regions.

Research objectives

We aimed to identify the delay in diagnosis (time from the first symptoms to UC diagnosis) that affected treatment and prognosis. We also evaluated the risk factors and clinical significance of a diagnostic delay for UC in South Korean patients.

Research methods

This retrospective study was based on patient data collected from six university-affiliated hospitals located in South Korea from January 2006 to December 2016. We analyzed the medical records of 718 patients who visited the outpatient clinic in 2016, had a definite diagnosis of UC, and were available for follow-up for more than 6 mo. One hundred sixty-seven patients were excluded from the study because of incomplete medical record data regarding the first day of symptoms. To determine the prognostic factors, the use of anti-tumor necrosis factor alpha (TNFα) drugs, the hospital admission history due to acute flare-ups, frequent admission, surgery associated with UC, and the clinical remission state at the latest follow-up were obtained from the medical records. The diagnostic interval was defined as the time from the first symptom until UC diagnosis. We divided the patients into the early and delay groups according to several diagnostic interval criteria (3 mo, 6 mo, 12 mo, 18 mo and 24 mo. Then, we compared the two groups according to the demographic and clinical characteristics to determine the diagnostic delay having a clinical impact.

Research results

The days from first symptoms to UC diagnosis were 223.28 ± 483.15 (median, 69); 75% of patients were diagnosed within 195 d. The use of anti-TNFα drugs at the 3-mo diagnostic interval was insignificantly prevalent in the early group (34/314; 10.6%) and the delay group (16/229; 7.0%); however, the use of anti-TNFα drugs by the early group and delay group started to decrease at the 18-mo diagnostic interval. Finally, at the 24-mo diagnostic interval, it was significantly higher in the delay group (8/42; 35.7%) than in the early group (42/509; 8.3%) (P = 0.019). Anti-TNFα free-survival rates between the early and delay groups according to the 24-mo diagnostic interval were significantly different (P = 0.034). Therefore, it was determined that 24 mo was the diagnostic delay cutoff point for poor outcomes. According to the multivariate logistic regression analysis, independent risk factors predictive of a diagnostic delay of 24 mo were age < 60 years [odd ratio (OR) = 14.778, 95% confidence interval (CI): 1.731-126.121, P = 0.014], smoking history (OR = 2.688, 95%CI: 1.239-5.747, P = 0.012), and misdiagnosis of hemorrhoids (OR = 11.066, 95%CI: 3.596-34.053, P=0.000).

Research conclusions

We found that the ≥ 24-mo diagnostic delay group more frequently used anti-TNFα compared to the < 24-mo delay group. We also found that risk factors for a 24-mo delay were age < 60 years, smoking history, and misdiagnosis of hemorrhoids by a physician.

Research perspectives

Prospective studies are needed to reduce recall bias for important clinical studies such as the first day of UC-related symptoms.

REFERENCES

1   **Markowitz J**. Early inflammatory bowel disease: different treatment response to specific or all medications? *Dig Dis* 2009; **27**: 358-365 [PMID: 19786765 DOI: 10.1159/000228574]

2   **Etchevers MJ**, Aceituno M, Sans M. Are we giving azathioprine too late? The case for early immunomodulation in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 5512-5518 [PMID: 18810768 DOI: 10.3748/wjg.14.5512]

3   **Ricart E**, García-Bosch O, Ordás I, Panés J. Are we giving biologics too late? The case for early versus late use. *World J Gastroenterol* 2008; **14**: 5523-5527 [PMID: 18810770 DOI: 10.3748/wjg.14.5523]

4   **Vavricka SR**, Spigaglia SM, Rogler G, Pittet V, Michetti P, Felley C, Mottet C, Braegger CP, Rogler D, Straumann A, Bauerfeind P, Fried M, Schoepfer AM; Swiss IBD Cohort Study Group. Systematic evaluation of risk factors for diagnostic delay in inflammatory bowel disease. *Inflamm Bowel Dis* 2012;

18: 496-505 [PMID: 21509908 DOI: 10.1002/ibd.21719]

5   **Schoepfer AM**, Dehlavi MA, Fournier N, Safroneeva E, Straumann A, Pittet V, Peyrin-Biroulet L, Michetti P, Rogler G, Vavricka SR; IBD Cohort Study Group. Diagnostic delay in Crohn's disease is associated with a complicated disease course and increased operation rate. *Am J Gastroenterol* 2013; **108**: 1744-1753; quiz 1754 [PMID: 23978953 DOI: 10.1038/ajg.2013.248]

6   **Nahon S**, Lahmek P, Paupard T, Lesgourgues B, Chaussade S, Peyrin-Biroulet L, Abitbol V. Diagnostic Delay Is Associated with a Greater Risk of Early Surgery in a French Cohort of Crohn's Disease Patients. *Dig Dis Sci* 2016; **61**: 3278-3284 [PMID: 27207180 DOI: 10.1007/s10620-016-4189-z]

7   **Zaharie R**, Tantau A, Zaharie F, Tantau M, Gheorghe L, Gheorghe C, Gologan S, Cijevschi C, Trifan A, Dobru D, Goldis A, Constantinescu G, Iacob R, Diculescu M; IBDPROSPECT Study Group. Diagnostic Delay in Romanian Patients with Inflammatory Bowel Disease: Risk Factors and Impact on the Disease Course and Need for Surgery. *J Crohns Colitis* 2016; **10**: 306-314 [PMID: 26589956 DOI: 10.1093/ecco-jcc/jjv215]

8   **Li Y**, Ren J, Wang G, Gu G, Wu X, Ren H, Hong Z, Hu D, Wu Q, Li G, Liu S, Anjum N, Li J. Diagnostic delay in Crohn's disease is associated with increased rate of abdominal surgery: A retrospective study in Chinese patients. *Dig Liver Dis* 2015; **47**: 544-548 [PMID: 25840874 DOI: 10.1016/j.dld.2015.03.004]

9   **Lee DW**, Koo JS, Choe JW, Suh SJ, Kim SY, Hyun JJ, Jung SW, Jung YK, Yim HJ, Lee SW. Diagnostic delay in inflammatory bowel disease increases the risk of intestinal surgery. *World J Gastroenterol* 2017; **23**: 6474-6481 [PMID: 29085197 DOI: 10.3748/wjg.v23.i35.6474]

10   **Stange EF**, Travis SP, Vermeire S, Reinisch W, Geboes K, Barakauskiene A, Feakins R, Fléjou JF, Herfarth H, Hommes DW, Kupcinskas L, Lakatos PL, Mantzaris GJ, Schreiber S, Villanacci V, Warren BF; European Crohn's and Colitis Organisation (ECCO). European evidence-based Consensus on the diagnosis and management of ulcerative colitis: Definitions and diagnosis. *J Crohns Colitis* 2008; **2**: 1-23 [PMID: 21172194 DOI: 10.1016/j.crohns.2007.11.001]

11   **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629 [PMID: 3317057 DOI: 10.1056/NEJM198712243172603]

12   **Sandborn WJ**, Feagan BG, Hanauer SB, Lochs H, Löfberg R, Modigliani R, Present DH, Rutgeerts P, Schölmerich J, Stange EF, Sutherland LR. A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. *Gastroenterology* 2002; **122**: 512-530 [PMID: 11832465 DOI: 10.1053/gast.2002.31072]

13   **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus EV, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5A-36A [PMID: 16151544]

14   **Truelove SC**, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J* 1955; **2**: 1041-1048 [PMID: 13260656 DOI: 10.1136/bmj.2.4947.1041]

15   **Choi CH**, Moon W, Kim YS, Kim ES, Lee BI, Jung Y, Yoon YS, Lee H, Park DI, Han DS; IBD Study Group of the Korean Association for the Study of Intestinal Diseases. Second Korean guidelines for the management of ulcerative colitis. *Intest Res* 2017; **15**: 7-37 [PMID: 28239313 DOI: 10.5217/ir.2017.15.1.7]

16   **Nguyen VQ**, Jiang D, Hoffman SN, Guntaka S, Mays JL, Wang A, Gomes J, Sorrentino D. Impact of Diagnostic Delay and Associated Factors on Clinical Outcomes in a U.S. Inflammatory Bowel Disease Cohort. *Inflamm Bowel Dis* 2017; **23**: 1825-1831 [PMID: 28885229 DOI: 10.1097/MIB.0000000000001257]

17   **Cantoro L**, Di Sabatino A, Papi C, Margagnoni G, Ardizzone S, Giuffrida P, Giannarelli D, Massari A, Monterubbianesi R, Lenti MV, Corazza GR, Kohn A. The Time Course of Diagnostic Delay in Inflammatory Bowel Disease Over the Last Sixty Years: An Italian Multicentre Study. *J Crohns Colitis* 2017; **11**: 975-980 [PMID: 28333328 DOI: 10.1093/ecco-jcc/jjx041]

18   **Nahon S**, Lahmek P, Lesgourgues B, Poupardin C, Chaussade S, Peyrin-Biroulet L, Abitbol V. Diagnostic delay in a French cohort of Crohn's disease patients. *J Crohns Colitis* 2014; **8**: 964-969 [PMID: 24529604 DOI: 10.1016/j.crohns.2014.01.023]

19   **Laaksonen M**, Rahkonen O, Karvonen S, Lahelma E. Socioeconomic status and smoking: analysing inequalities with multiple indicators. *Eur J Public Health* 2005; **15**: 262-269 [PMID: 15755781 DOI: 10.1093/eurpub/cki115]

20   **Kim YM**, Park SH, Yang SK, Choe JW, Kim SH, Byeon JS, Myung SJ, Cho YK, Yu CS, Choi KS, Chung JW, Kim B, Choi KD, Kim JH. Clinical characteristics and long-term course of ulcerative colitis in Korea. *Intest Res* 2006; **4**: 12-21 Accessed June 30, 2006 Available from: https://www.irjournal.org/journal/view.php?number=406

21   **Lee HS**, Park SH, Yang SK, Lee J, Soh JS, Lee S, Bae JH, Lee HJ, Yang DH, Kim KJ, Yea BD, Byeon JS, Myung SJ, Yoon YS, Yu CS, Kim JH. Long-term prognosis of ulcerative colitis and its temporal change between 1977 and 2013: a hospital-based cohort study from Korea. *J Crohns Colitis* 2015; **9**: 147-155 [PMID: 25518059 DOI: 10.1093/ecco-jcc/jju017]

22   **Lok KH**, Hung HG, Ng CH, Kwong KC, Yip WM, Lau SF, Li KK, Li KF, Szeto ML. Epidemiology and clinical characteristics of ulcerative colitis in Chinese population: experience from a single center in Hong Kong. *J Gastroenterol Hepatol* 2008; **23**: 406-410 [PMID: 17623033 DOI: 10.1111/j.1440-1746.2007.05079.x]

23   **Ling KL**, Ooi CJ, Luman W, Cheong WK, Choen FS, Ng HS. Clinical characteristics of ulcerative colitis in Singapore, a multiracial city-state. *J Clin Gastroenterol* 2002; **35**: 144-148 [PMID: 12172359 DOI: 10.1097/00004836-200208000-00005]

24   **Kim ES**, Kim WH. Inflammatory bowel disease in Korea: epidemiological, genomic, clinical, and therapeutic characteristics. *Gut Liver* 2010; **4**: 1-14 [PMID: 20479907 DOI: 10.5009/gnl.2010.4.1.1]

25   **Sewell JL**, Velayos FS. Systematic review: The role of race and socioeconomic factors on IBD healthcare delivery and effectiveness. *Inflamm Bowel Dis* 2013; **19**: 627-643 [PMID: 22623078 DOI: 10.1002/ibd.22986]

26   **Spivak W**, Sockolow R, Rigas A. The relationship between insurance class and severity of presentation of inflammatory bowel disease in children. *Am J Gastroenterol* 1995; **90**: 982-987 [PMID: 7771435]

27   **Moon CM**, Jung SA, Kim SE, Song HJ, Jung Y, Ye BD, Cheon JH, Kim YS, Kim YH, Kim JS, Han DS; CONNECT study group. Clinical Factors and Disease Course Related to Diagnostic Delay in Korean Crohn's Disease Patients: Results from the CONNECT Study. *PLoS One* 2015; **10**: e0144390 [PMID:

26647084 DOI: 10.1371/journal.pone.0144390]

28   **Olivera P**, Spinelli A, Gower-Rousseau C, Danese S, Peyrin-Biroulet L. Surgical rates in the era of biological therapy: up, down or unchanged? *Curr Opin Gastroenterol* 2017; **33**: 246-253 [PMID: 28463854 DOI: 10.1097/MOG.0000000000000361]

29   **Bernstein CN**, Ng SC, Lakatos PL, Moum B, Loftus EV; Epidemiology and Natural History Task Force of the International Organization of the Study of Inflammatory Bowel Disease. A review of mortality and surgery in ulcerative colitis: milestones of the seriousness of the disease. *Inflamm Bowel Dis* 2013; **19**: 2001-2010 [PMID: 23624887 DOI: 10.1097/MIB.0b013e318281f3bb]

30   **Yang SK**. Personalizing IBD Therapy: The Asian Perspective. *Dig Dis* 2016; **34**: 165-174 [PMID: 26982286 DOI: 10.1159/000443134]

31   **Targownik LE**, Singh H, Nugent Z, Bernstein CN. The epidemiology of colectomy in ulcerative colitis: results from a population-based cohort. *Am J Gastroenterol* 2012; **107**: 1228-1235 [PMID: 22613902 DOI: 10.1038/ajg.2012.127]

32   **Chow DK**, Leong RW, Tsoi KK, Ng SS, Leung WK, Wu JC, Wong VW, Chan FK, Sung JJ. Long-term follow-up of ulcerative colitis in the Chinese population. *Am J Gastroenterol* 2009; **104**: 647-654 [PMID: 19262521 DOI: 10.1038/ajg.2008.74]

33   **Falcone RA**, Lewis LG, Warner BW. Predicting the need for colectomy in pediatric patients with ulcerative colitis. *J Gastrointest Surg* 2000; **4**: 201-206 [PMID: 10675244 DOI: 10.1016/S1091-255X(00)80057-8]

34   **Safroneeva E**, Vavricka SR, Fournier N, Straumann A, Rogler G, Schoepfer AM. Prevalence and Risk Factors for Therapy Escalation in Ulcerative Colitis in the Swiss IBD Cohort Study. *Inflamm Bowel Dis* 2015; **21**: 1348-1358 [PMID: 25806845 DOI: 10.1097/MIB.0000000000000368]

35   **Ott C**, Takses A, Obermeier F, Schnoy E, Salzberger B, Müller M. How fast up the ladder? Factors associated with immunosuppressive or anti-TNF therapies in IBD patients at early stages: results from a population-based cohort. *Int J Colorectal Dis* 2014; **29**: 1329-1338 [PMID: 25179426 DOI: 10.1007/s00384-014-2002-z]

Retrospective Study

Big-data analysis: A clinical pathway on endoscopic retrograde cholangiopancreatography for common bile duct stones

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Abstract

BACKGROUND
A clinical pathway (CP) is a standardized approach for disease management.
However, big data-based evidence is rarely involved in CP for related common
bile duct (CBD) stones, *let al*one outcome comparisons before and after CP
implementation.

AIM
To investigate the value of CP implementation in patients with CBD stones
undergoing endoscopic retrograde cholangiopancreatography (ERCP).

METHODS
This retrospective study was conducted at Nanjing Drum Tower Hospital in
patients with CBD stones undergoing ERCP from January 2007 to December
2017. The data and outcomes were compared by using univariate and
multivariable regression/linear models between the patients who received
conventional care (non-pathway group, n = 467) and CP care (pathway group, n
= 2196).

RESULTS
At baseline, the main differences observed between the two groups were the
percentage of patients with multiple stones ($P < 0.001$) and incidence of
cholangitis complication ($P < 0.05$). The percentage of antibiotic use and
complications in the CP group were significantly less than those in the non-
pathway group [adjusted odds ratio (OR) = 0.72, 95% confidence interval (CI):
0.55-0.93, $P = 0.012$, adjusted OR = 0.44, 95%CI: 0.33-0.59, $P < 0.001$, respectively].
Patients spent lower costs on hospitalization, operation, nursing, medication, and

medical consumable materials ($P < 0.001$ for all), and even experienced shorter length of hospital stay (LOHS) ($P < 0.001$) after the CP implementation. No significant differences in clinical outcomes, readmission rate, or secondary surgery rate were presented between the patients in the non-pathway and CP groups.

CONCLUSION
Implementing a CP for patients with CBD stones is a safe mode to reduce the LOHS, hospital costs, antibiotic use, and complication rate.

Key words: Common bile duct stones; Endoscopic retrograde cholangiopancreatography; Clinical pathway; Outcomes; Costs

Core tip: We utilized a big-data process and application platform for exploring the value of clinical pathway (CP) implementation in patients with common bile duct stones undergoing endoscopic retrograde cholangiopancreatography. Univariate and multivariable regression/linear models were developed to compare the outcomes between the patients in the non-pathway and CP groups. Our findings demonstrated that a CP is a safe mode to reduce the length of hospital stay, hospital costs, antibiotic use, and complication rate. The present study provides big-data evidence for clinical standardization of CPs.

INTRODUCTION

Gallstone disease is one of the most frequent biliary diseases leading to hospitalization and imposing a significant financial burden. The worldwide prevalence of gallstones presents a rising tendency due to the change of dietary structure and routine living customs in recent years[1,2]. Of the patients who suffered from gallstones, approximately 10%-15% were found to have synchronous common bile duct (CBD) stones[3,4]. The clinical manifestations of CBD stones are varied from biliary colic to a combination of complications, such as acute pancreatitis or cholangitis; sometimes, CBD stones even may be asymptomatic[5]. Treatment and management of CBD stones have changed considerably during the last three decades. With the popularization of minimally invasive surgery in clinical practice, endoscopic retrograde cholangiopancreatography (ERCP) is currently recognized as a standard therapy for patients with CBD stones[6,7]. Despite this, a risk of complications after ERCP cannot be avoided, and it is even associated with increased morbidity and mortality[8]. In addition, there is evidence showing that in some patients with gallstones received ERCP and routine care at first admission, re-admission and longer preoperative stay were caused[9]. Due to the growing complexity of CBD stone treatments and care, it is crucial to develop a standardized multidisciplinary approach to avoid chaotic management of this disease.

A clinical pathway (CP) is an advanced medical diagnosis, treatment, and management mode, which may optimize medical treatment by facilitating clinical assessments, improving utilization efficiency of medical sources, and reducing economical expenses[10-12]. Nowadays, a CP is thought to be an effective tool to be explicit about the sequencing, timing and provision of interventions in clinical practice and can guide physicians and nursing staff in providing evidence-based results[13,14]. Moreover, analysis of evaluating indexes (including clinical outcome, efficiency indicators, financial indicators, and antibiotic use indicators) can guarantee the effectiveness of the CP implementation and optimization[15,16]. One study has demonstrated that a CP presents sustainable effects in gallstone-related care, resulting in shorter length of hospital stay (LOHS) and lower hospital expenses[9]. In addition, several studies conducted in other surgical domains also presented similar results[17-19].

However, implementation of the CP in China is in its start-up stage, especially in the field of hepatobiliary surgery. The status and value of the CP in the management of patients with CBD stones after ERCP remain to be explored. Given this concern, a retrospective study based on a big-data, intelligence database platform was launched. The aim of the present study was to analyze the impact of a CP on LOHS, readmission, treatment outcomes, hospital costs, and postoperative complication rate in patients with CBD stones undergoing ERCP.

MATERIALS AND METHODS

Study patients and data collection

This is a retrospective study of patients with CBD stones who received ERCP at Nanjing Drum Tower Hospital (Nanjing, Jiangsu Province, China) between January 2007 and December 2017. This study was approved by Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (201817001), and informed consent was obtained from all subjects.

All patients aged above 18 years old without previous ERCP history were included in this study. The exclusion criteria were: (1) Patients with previous or present hepatolithiasis; (2) patients with severe liver diseases, cardio-pulmonary or renal inadequacy; (3) patients with severe hematologic diseases and concomitant obvious coagulopathy; (4) patients with combined gallbladder, CBD, duodenal papillary neoplasm, or congenital choledochal cyst; (5) patients who underwent Billroth I and II gastrectomy or gastrojejunostomy; and (6) pregnant patients. Subjects who met criteria for this study were extracted automatically from a big-data, intelligence database platform (Yidu Cloud Technology Co. Ltd., Beijing, China) by setting the inclusion and exclusion criteria. The study population consisted of two groups which accepted conventional care (non-pathway group) and a CP (CP group), respectively.

Demographic and clinical characteristics of subjects were obtained from electronic medical records. Outcomes of pathway complementation were compared between the two groups in LOHS (total and preoperative LOHS), readmission rate (a second hospital admission within 30 d due to CBD stones and postoperative complications), treatment outcomes, hospital charges (also including medication, operation, perioperative examinations, nursing and medical consumable materials charges), antibiotic use, secondary surgery rate, and postoperative complications.

CP

A set of sophisticated CPs for patients with CBD stones was implemented at this hospital in 2012. Development and optimization of the CP involved a multidisciplinary team under the instruction of relevant guidelines, including attending surgeons and residents, an anesthesiologist, a head of pharmacy faculty, and representatives from nursing and rehabilitation department. The training of the CP was performed before implementation to relevant personnel. Pathway I was designed for patients with expected LOHS less than 5-10 d, while pathway II was used for LOHS of 7-10 d. These CPs were explicit about the sequence of strategies for diagnosis, treatment, medication, routine care, and assessment. The pathway I is shown as an example in Appendix 1.

Statistical analysis

All statistical analyses were conducted using SAS, version 9.4 (SAS Institute, Cary, NC, United States). Data following a normal distribution are presented by mean ± standard deviation (mean ± SD), and otherwise are presented as median (interquartile range). Differences between the two groups were compared using Wilcoxon signed-rank test (continuous variables) or chi-squared test (categorical variables). In addition, univariable logistic regression models were used to determine whether odds of outcomes differed between the groups. We also utilized multivariable logistic (linear) regression models for evaluating the effect of pathway complementation on each outcome by controlling age, gender, smoking and drinking habits, the number of stones, and white blood cell (WBC) count at hospital admission. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Two thousand six hundred and sixty-three eligible patients were included finally, of whom 467 were in the non-pathway group and 2196 in the clinical-pathway group (Figure 1). Table 1 shows the comparison of demographic and clinical characteristics

between the patients who received routine care and CP care. There were no differences between the two groups in terms of age, gender, insured status, health behaviors, or maximum diameter of stones. The percentage of patients with multiple stones was found to be significantly different between the two groups ($P < 0.001$). The number of patients suffering from comorbidities was similar, although the percentage of patients with cholangitis was higher in the non-pathway group ($P = 0.041$). Although WBC counts in both groups were within the normal range, there was a significantly higher WBC count among the patients in the non-pathway group ($P = 0.005$).

Table 2 presents the outcomes of efficiency, treatment, hospital costs, and antibiotic use following the CP implementation. The median total LOHS was 8 (range, 6-11) d in the non-pathway group, while it was one day shorter (7, 5-9) in the CP group (P < 0.001). The pre-operative LOHS was found to be similar between the two groups. There were no significant changes with respect to the treatment outcomes (recovered, improved, not improved, and died) in both groups, and even no patients died in our study. Thirty-four (7.28%) patients in the non-pathway group required readmission to hospital, while readmission rate (173, 7.88%) was increased after pathway complementation, although this difference was not statistically significant (P = 0.661). A considerably decreasing trend in the costs was observed among patients with the CP implementation, including hospitalization, medication, operation, nursing, materials, and preoperative examination ($P < 0.001$). In addition, implementation of the CP was associated with a reduced proportion of antibiotic use ($P < 0.001$). The median time of antibiotic use [11 (7.0-17.0) d] in the CP group was one day shorter than that before the CP complementation [12 (8.0-18.5) d] ($P = 0.004$). For patients in the CP group, secondary procedure occurred more frequently, although this difference was not statistically significant (16.94 *vs* 14.78%, *P*=0.253).

The postoperative complication rates are compared in Table 3. About 26.77% of patients with routine care had at least one complication, while the incidence of complications dropped to 14.39% after the CP complementation ($P < 0.001$). The incidence rates of acute pancreatitis and liver abscess were considerably lower in patients after CP implementation, with significant differences between the two groups ($P < 0.001$).

The effect of the CP complementation on each outcome was also assessed by univariate and multivariate logistic regression through controlling age, gender, smoking and drinking habits, the number of stones, and WBC count at hospital admission (Tables 4 and 5). After adjusting for differences between the two groups, antibiotic use and postoperative complications were less in patients with the CP complementation [odds ratio (OR) = 0.72, 95% confidence interval (CI) 0.55-0.93, $P = 0.012$; and OR = 0.44, 95%CI 0.33-0.59, $P < 0.001$, respectively]. The costs of hospitalization, operation, nursing, medication, and materials ($P < 0.001$ for all) and LOHS ($P < 0.001$) decreased significantly after implementation of the CP.

DISCUSSION

Despite wide adoption of CPs throughout different departments currently, their evaluation and optimization remain doubtful[20]. The purpose of this study was to compare the indicators of CP implementation in five domains (clinical outcome, efficiency indicators, financial indicators, and antibiotic use indicators) for patients with CBD stones undergoing ERCP. Our results confirmed that pathway implementation in CBD stones was associated with reduced total LOHS, costs, antibiotic use, antibiotic use duration, and complication rate. More importantly, the decrease has not been achieved at the expense of increased readmission rate or mortality.

With the development of endoscopic technique, ERCP is considered a preferred therapeutic method in management of CBD stones. However, it can be still challenging in some cases, such as high total hospital expenses and high risk of post-ERCP complications[21,22]. CP, one of the main modes to standardize treatment and care, is increasingly adopted by hospitals to strive to better outcomes and lower costs. However, the definition of CP has not yet been fully elucidated in clinical practice, and the impact of pathway complementation is varied by different factors and conditions[23,24]. Findings of our study are consistent with those obtained by Kristin *et al* who demonstrated a considerable reduction in terms of costs and LOHS in patients with complicated gallstone disease after the CP implementation[9]. More recent studies showed similar improvements in other specialties of diseases, such as acute pancreatitis[25], breast cancer[15], and chronic obstructive pulmonary disease[26]. However, pre-operative length of stay showed no distinct disparity in our study, and

Table 1 Demographic and clinical characteristics of patients

Characteristic	Non-pathway group (*n* = 467)	Cinical pathway group (*n* = 2196)	*P* value
Age, yr (median, range)	64 (52-75)	63 (51-75)	0.405
Female, *n* (%)	235 (50.32)	1093 (49.77)	0.829
Medical insurance, *n* (%)	217 (46.47)	945 (43.03)	0.174
Health behavior, *n* (%)			
Smoking	57 (12.21)	260 (11.84)	0.825
Alcohol use	36 (7.71)	164 (7.47)	0.858
Comorbidity, *n* (%)			
Hypertension	155 (33.19)	703 (32.01)	0.621
Diabetes	68 (14.56)	292 (13.30)	0.468
COPD	11 (2.36)	54 (2.46)	0.895
Myocardial infarction	0	6 (0.27)	0.258
Cholangitis	74 (15.85)	271 (12.34)	0.041[a]
Cholecystolithiasis	188 (40.26)	780 (35.52)	0.053
JPD	1 (0.21)	10 (0.46)	0.460
CBD stone number ≥ 5, *n* (%)	201 (43.04)	1173 (53.42)	< 0.001[a]
Maximum diameter of stones, cm (median, range)	0.8 (0.6-1.2)	0.9 (0.6-1.2)	0.949
Laboratory test (median, range)			
Temperature (°C), *n* = 359/2113	36.5 (36.3-36.8)	36.5 (36.3-36.8)	0.855
WBC count, *n* = 370/1451	6.0 (4.7-9.2)	5.7 (4.6-7.6)	0.005[a]
Direct bilirubin level, *n* = 363/1439	12.2 (5.6-44.2)	10.7 (4.8-37.2)	0.210
Total bilirubin level, *n* = 363/1439	22.6 (12.7-57.6)	21.2 (12.5-51.9)	0.628
AST, *n* = 363/1440	47.9 (22.8-104.5)	45.4 (23.5-104.8)	0.842
GGT, *n* = 363/1439	257.4 (115.3-492.4)	265.1 (109.9-519.4)	0.918
ALP, *n* = 363/1439	161.7 (99.9-283.3)	154.9 (97.4-259.3)	0.536
ALT, *n* = 364/1443	85.1 (29.6-203.2)	89.0 (32.0-206.9)	0.946
Cr, *n* = 361/1431	62.0 (52.0-74.0)	62.0 (2.0-73.0)	0.966

[a]*P* < 0.05 was considered statistically significant.

COPD: Chronic obstructive pulmonary disease; JPD: Juxtapapillary duodenal diverticulum; CBD stones: Common bile duct stones; WBC: White blood cell; AST: Aspartate transaminase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; Cr: Creatinine.

the implementation of pathway might have greater impact on postoperative hospital stay. Similar to our results in terms of antibiotic use, Dona *et al*[27] reported that there was a reduction of antibiotic prescriptions in patients with community-acquired pneumonia after introducing a CP. Our study provided further evidence that the CP implementation can also significantly reduce the duration of antibiotic use and three-line antibiotic prescriptions. Thus, it is possible to conclude that a marked reduction of costs appears to be associated with several factors, such as effective pre-operative examination and rational use of medications and materials. Moreover, CP seems to be one of key approaches to maximize cost-effectiveness, while without sacrificing good treatment outcomes[28]. The most common causes of dropout from CP were postoperative complications that needed additional treatment. The findings of the present study demonstrated that rates of complications were lower in patients operated upon admission who implemented the CP compared to patients receiving routine care. There have been multiple previous publications in various domains which have demonstrated the lower incidence of complications following critical pathways[29-31]. However, critical factors may have affected outcomes, such as patients' characteristics, living habits, disease features, and individual laboratory measurements. Thus, these factors did not affect the findings that CP use achieved a significantly shorter LOHS, lower costs, and reduced complications after adjustments.

Large-scale populations with gallstones and data process and application platform utilization are the main strengths in the present study. Furthermore, the findings were demonstrated by adjusting the potential confounders. However, this study was limited by its single-center retrospective design, indicating that further multiple-center trials with larger variable are in need to confirm the results.

In conclusion, findings of our study have demonstrated that patients with CBD stones who accepted the CP appear to be significantly lower in the LOHS, the costs,
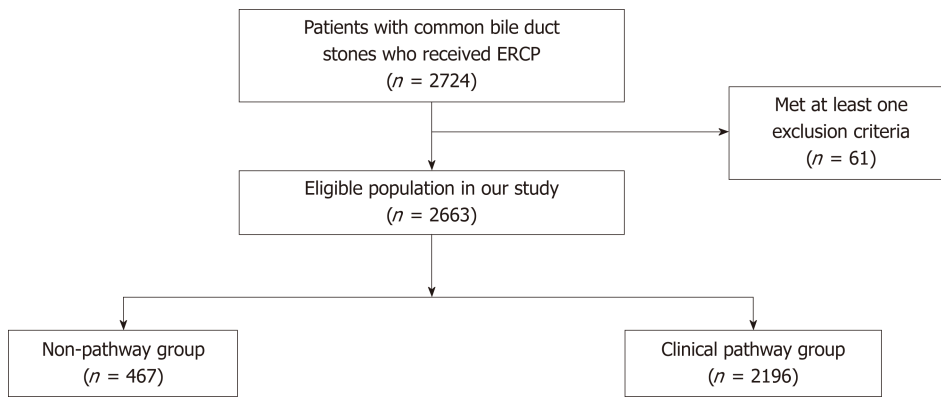
Figure 1 Flow diagram of study population.

the rate of antibiotic use, and the incidence of complications. Our study provides further evidence of CP use in Chinese patients and also standardizes gallstone management and treatment.

Table 2 Comparison of length of hospital stay, clinical outcomes, hospital charges, and drug use between the non-pathway and clinical pathway groups

Characteristic	Non-pathway group (*n* = 467)	Cinical pathway group (*n* = 2196)	*P* value
Length of total hospital stay (median, range)	8 (6-11)	7 (5-9)	< 0.001[a]
Pre-operative length of stay	2 (1-4)	2 (1-3)	0.451
Readmission, *n* (%)	34 (7.28)	173 (7.88)	0.661
Clinical outcomes, *n* (%)			0.115
Recovered	223 (47.75)	1146 (52.19)	
Improved	236 (50.54)	1028 (46.81)	
Not improved	8 (1.71)	22 (1)	
Died	0	0	
Charges of hospitalization (CNY), (median, range) *n* = 467/2183	21508.3 (17150.6-30045.8)	18362.9 (15665.8-22895.2)	< 0.001[a]
Charges of medication, *n* = 459/2153	6620.6 (3429.4-12239.7)	4900.6 (3172.4-8186.0)	< 0.001[a]
Charges of operation, *n* = 458/2151	5741.0 (4519.2-7324.0)	5455.2 (2670.0-6964.0)	< 0.001[a]
Charges of nursing, *n* = 454/2133	188.0 (115.0-288.0)	120.0 (74.0-211.0)	< 0.001[a]
Charges of materials, *n* = 409/1540	7916.4 (6696.0-10552.6)	6985.5 (6135.1-8224.7)	< 0.001[a]
Charges of examination, *n* = 459/2153	2698.0 (2224.5-3799.5)	2463.5 (2134.5-3105.5)	< 0.001[a]
Antibiotic use, *n* (%)	260/430 (60.47)	832/1737 (47.90)	< 0.001[a]
Antibiotic usage duration (d) (median, range), *n* = 260/832	12 (8.0-18.5)	11 (7.0-17.0)	0.004[a]
Three line antibiotic use, *n* (%)	51/430 (11.86)	149/1737 (8.58)	0.035[a]
Secondary surgery, *n* (%)	69 (14.78)	372 (16.94)	0.253

[a]*P* < 0.05 was considered statistically significant.

Table 3 Comparison of postoperative complication rates between the non-pathway and clinical pathway groups

Characteristic	Non-pathway group (*n* = 467)	Cinical pathway group (*n* = 2196)	*P* value
Total, *n* (%)	125 (26.77)	316 (14.39)	< 0.001[a]
Acute pancreatitis	113 (24.20)	298 (13.57)	< 0.001[a]
Gallbladder perforation	1 (0.21)	1 (0.05)	0.227
Gastrointestinal hemorrhage	0	2 (0.09)	0.514
Biliary tract infection	2 (0.43)	4 (0.18)	0.308
Liver abscess	10 (2.14)	11 (0.50)	< 0.001[a]

[a]*P* < 0.05 was considered statistically significant.

Table 4 Univariate logistic regression analysis of outcomes

Characteristic	Non-pathway group (*n* = 467)	Cinical pathway group (*n* = 2196)	OR (95%CI)	*P* value
Length of total hospital stay (median, range)	8 (6-11)	7 (5-9)		< 0.001[a]
Pre-operative length of stay	2 (1-4)	2 (1-3)		0.078
Readmission, *n* (%)	34 (7.28)	173 (7.88)	1.09 (0.74, 1.60)	0.661
Clinical outcomes, *n* (%)			1.21 (0.99, 1.47)	0.064
Charges of hospitalization (CNY) (median, range)	21508.3 (17150.6-30045.8)	18362.9 (15665.8-22895.2)		< 0.001[a]
Medication	6620.6 (3429.4-12239.7)	4900.6 (3172.4-8186.0)		< 0.001[a]
Operating	5741.0 (4519.2-7324.0)	5455.2 (2670.0-6964.0)		< 0.001[a]
Nursing	188.0 (115.0-288.0)	120.0 (74.0-211.0)		< 0.001[a]
Materials	7916.4 (6696.0-10552.6)	6985.5 (6135.1-8224.7)		< 0.001[a]
Examination	2698.0 (2224.5-3799.5)	2463.5 (2134.5-3105.5)		< 0.001[a]
Antibiotic use, *n* (%)	260/430 (60.47)	832/1737(47.90)	0.60 (0.49, 0.75)	< 0.001[a]
Antibiotic usage duration (d) (median, range)	12 (8.0-18.5)	11 (7.0-17.0)		0.310

Three line antibiotic use, *n* (%)	51/430 (11.86)	149/1437 (8.58)	0.69	0.036[a]
Secondary surgery, *n* (%)	69 (14.78)	372 (16.94)	1.18	0.254
Complications	125 (26.77)	316 (14.39)	0.46 (0.36, 0.58)	< 0.001[a]

[a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval.

Table 5 Multivariate logistic and linear regression analysis of outcomes

Characteristic	Adjusted OR (95%CI) or coefficients	*P* value
Length of total hospital stay	-1.71 (-2.30, -1.12)[1]	< 0.001[a]
Hospitalization costs	-5572.26 (-6931.48, -4213.03)[1]	< 0.001[a]
Medication costs	-2760.03 (-3738.96, -1781.11)[1]	< 0.001[a]
Operating costs	-382.08 (-635.30, -128.85)[1]	< 0.001[a]
Nursing costs	-138.25 (-195.83, -80.66)[1]	< 0.001[a]
Materials costs	-1688.35 (-2049.61, -1327.10)[1]	< 0.001[a]
Examination costs	-138.25 (-195.83, -80.66)[1]	< 0.001[a]
Three line antibiotic use	0.89 (0.60, 1.31)	0.546
Antibiotic use, *n* (%)	0.72 (0.55, 0.93)	0.012[a]
Complications	0.44 (0.33, 0.59)	< 0.001[a]

[1]Coefficients.
[a]$P < 0.05$ was considered statistically significant, adjusted for age, gender, smoking, alcohol use, the number of stones, and white blood cell count at hospital admission.
OR: Odds ratio; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Endoscopic retrograde cholangiopancreatography (ERCP) is widely recognized as a standard endoscopic technique for patients with common bile duct (CBD) stones. However, ERCP is associated with significant morbidity, mortality, and longer preoperative stay. A clinical pathway (CP) is an advanced methodology that provides a sequence of diagnosis, treatment, and management. Although CP implementation could optimize medical treatment and improve efficiency of medical sources utilization, CP implementation for CBD stones has not been fully promoted at present.

Research motivation

Current situation and value of the CP in management of CBD stones receiving ERCP still need to be explored. With the arrival of the era of big-data, we utilized a big-data process and application platform to provide a solid data base and scientific evidence for the establishment of the CP.

Research objectives

The objective of this study was to compare length of hospital stay (LOHS), costs, clinical outcomes, antibiotic use, and postoperative complication rate before and after implementing a CP for patients with CBD stones undergoing ERCP.

Research methods

Patients with CBD stones from Nanjing Drum Tower Hospital between January 2007 and December 2017 were identified from a big-data, intelligence database platform (Yidu Cloud Technology Ltd., Beijing, China). The enrolled population consisted of two groups which accepted conventional care (non-pathway group, *n* = 467) and the CP (CP group, *n* = 2196), respectively. Univariate and multivariable regression/linear models were utilized to compare the medical records and outcomes.

Research results

The percentage of antibiotic use and complications in the CP group were significantly less than those in the non-pathway group [adjusted odds ratio (OR) = 0.72, 95% confidence interval (CI) 0.55-0.93, *P* = 0.012, adjusted OR = 0.44, 95%CI 0.33-0.59, *P* < 0.001, respectively]. Patients experienced lower costs in hospitalization, operation, nursing, medication, and materials (*P* < 0.001 for all), and even shorter LOHS (*P* < 0.001) after implementation of the CP. No significant differences in clinical outcomes, readmission rate, or secondary surgery rate were presented between the patients in non-pathway and CP groups.

Research conclusion

In conclusion, implementation of the CP for patients with CBD stones undergoing ERCP significantly reduced LOHS, the costs, the rate of antibiotic use, and the incidence of complications without increasing readmission rates. A CP is confirmed to be an effective mode which is explicit about the sequencing, timing, and provision of interventions in the field of CBD stones. Meanwhile, our study provides further big-data evidence of a multidisciplinary CP in Chinese patients.

Research perspectives

Despite that this is the rare big-data evidence of a CP in Chinese patients with CBD stones, further multiple-center studies with larger variable are essential to strengthen the results.

ACKNOWLEDGEMENTS

REFERENCES

1   **Acalovschi M**. Gallstones in patients with liver cirrhosis: Incidence, etiology, clinical and therapeutical aspects. *World J Gastroenterol* 2014; **20**: 7277-7285 [PMID: 24966598 DOI: 10.3748/wjg.v20.i23.7277]

2   **Zhu Q**, Sun X, Ji X, Zhu L, Xu J, Wang C, Zhang C, Xue F, Liu Y. The association between gallstones and metabolic syndrome in urban Han Chinese: A longitudinal cohort study. *Sci Rep* 2016; **6**: 29937 [PMID: 27443986 DOI: 10.1038/srep29937]

3   **Anand G**, Patel YA, Yeh HC, Khashab MA, Lennon AM, Shin EJ, Canto MI, Okolo PI, Kalloo AN, Singh VK. Factors and Outcomes Associated with MRCP Use prior to ERCP in Patients at High Risk for Choledocholithiasis. *Can J Gastroenterol Hepatol* 2016; **2016**: 5132052 [PMID: 27446845 DOI: 10.1155/2016/5132052]

4   **Tazuma S**. Gallstone disease: Epidemiology, pathogenesis, and classification of biliary stones (common bile duct and intrahepatic). *Best Pract Res Clin Gastroenterol* 2006; **20**: 1075-1083 [PMID: 17127189 DOI: 10.1016/j.bpg.2006.05.009]

5   **Aslan F**, Arabul M, Celik M, Alper E, Unsal B. The effect of biliary stenting on difficult common bile duct stones. *Prz Gastroenterol* 2014; **9**: 109-115 [PMID: 25061492 DOI: 10.5114/pg.2014.42507]

6   **Kenny R**, Richardson J, McGlone ER, Reddy M, Khan OA. Laparoscopic common bile duct exploration versus pre or post-operative ERCP for common bile duct stones in patients undergoing cholecystectomy: Is there any difference? *Int J Surg* 2014; **12**: 989-993 [PMID: 24998206 DOI: 10.1016/j.ijsu.2014.06.013]

7   **Bray MS**, Borgert AJ, Folkers ME, Kothari SN. Outcome and management of endoscopic retrograde cholangiopancreatography perforations: A community perspective. *Am J Surg* 2017; **214**: 69-73 [PMID: 28173939 DOI: 10.1016/j.amjsurg.2017.01.034]

8   **Morris S**, Gurusamy KS, Sheringham J, Davidson BR. Cost-effectiveness analysis of endoscopic ultrasound versus magnetic resonance cholangiopancreatography in patients with suspected common bile duct stones. *PLoS One* 2015; **10**: e0121699 [PMID: 25799113 DOI: 10.1371/journal.pone.0121699]

9   **Sheffield KM**, Ramos KE, Djukom CD, Jimenez CJ, Mileski WJ, Kimbrough TD, Townsend CM, Riall TS. Implementation of a critical pathway for complicated gallstone disease: Translation of population-based data into clinical practice. *J Am Coll Surg* 2011; **212**: 835-843 [PMID: 21398156 DOI: 10.1016/j.jamcollsurg.2010.12.047]

10   **Elliott MJ**, Gil S, Hemmelgarn BR, Manns BJ, Tonelli M, Jun M, Donald M. A scoping review of adult chronic kidney disease clinical pathways for primary care. *Nephrol Dial Transplant* 2017; **32**: 838-846 [PMID: 27257274 DOI: 10.1093/ndt/gfw208]

11   **De Luca A**, Toni D, Lauria L, Sacchetti ML, Giorgi Rossi P, Ferri M, Puca E, Prencipe M, Guasticchi G; IMPLementazione Percorso Clinico Assistenziale ICtus Acuto (IMPLICA) Study Group. An emergency clinical pathway for stroke patients--results of a cluster randomised trial (isrctn41456865). *BMC Health Serv Res* 2009; **9**: 14 [PMID: 19159477 DOI: 10.1186/1472-6963-9-14]

12   **Schuur JD**, Baugh CW, Hess EP, Hilton JA, Pines JM, Asplin BR. Critical pathways for post-emergency outpatient diagnosis and treatment: Tools to improve the value of emergency care. *Acad Emerg Med* 2011; **18**: e52-e63 [PMID: 21676050 DOI: 10.1111/j.1553-2712.2011.01096.x]

13   **Bradywood A**, Farrokhi F, Williams B, Kowalczyk M, Blackmore CC. Reduction of Inpatient Hospital Length of Stay in Lumbar Fusion Patients With Implementation of an Evidence-Based Clinical Care Pathway. *Spine (Phila Pa 1976)* 2017; **42**: 169-176 [PMID: 27213939 DOI: 10.1097/BRS.0000000000001703]

14   **El Baz N**, Middel B, van Dijk JP, Oosterhof A, Boonstra PW, Reijneveld SA. Are the outcomes of clinical pathways evidence-based? A critical appraisal of clinical pathway evaluation research. *J Eval Clin Pract* 2007; **13**: 920-929 [PMID: 18070263 DOI: 10.1111/j.1365-2753.2006.00774.x]

15   **van Dam PA**, Verheyden G, Sugihara A, Trinh XB, Van Der Mussele H, Wuyts H, Verkinderen L, Hauspy J, Vermeulen P, Dirix L. A dynamic clinical pathway for the treatment of patients with early breast cancer is a tool for better cancer care: Implementation and prospective analysis between 2002-2010. *World J Surg Oncol* 2013; **11**: 70 [PMID: 23497270 DOI: 10.1186/1477-7819-11-70]

16   **de Vries M**, van Weert JC, Jansen J, Lemmens VE, Maas HA. Step by step development of clinical care pathways for older cancer patients: Necessary or desirable? *Eur J Cancer* 2007; **43**: 2170-2178 [PMID: 17870519 DOI: 10.1016/j.ejca.2007.08.004]

17   **Shin KC**, Lee HS, Park JM, Joo HC, Ko YG, Park I, Kim MJ. Outcomes before and after the Implementation of a Critical Pathway for Patients with Acute Aortic Disease. *Yonsei Med J* 2016; **57**: 626-634 [PMID: 26996561 DOI: 10.3349/ymj.2016.57.3.626]

18   **Thursky K**, Lingaratnam S, Jayarajan J, Haeusler GM, Teh B, Tew M, Venn G, Hiong A, Brown C,

Leung V, Worth LJ, Dalziel K, Slavin MA. Implementation of a whole of hospital sepsis clinical pathway in a cancer hospital: Impact on sepsis management, outcomes and costs. *BMJ Open Qual* 2018; **7**: e000355 [PMID: 30019016 DOI: 10.1136/bmjoq-2018-000355]

19   **van der Kolk M**, van den Boogaard M, Ter Brugge-Speelman C, Hol J, Noyez L, van Laarhoven K, van der Hoeven H, Pickkers P. Development and implementation of a clinical pathway for cardiac surgery in the intensive care unit: Effects on protocol adherence. *J Eval Clin Pract* 2017; **23**: 1289-1298 [PMID: 28719134 DOI: 10.1111/jep.12778]

20   **Dong W**, Huang Z. A Method to Evaluate Critical Factors for Successful Implementation of Clinical Pathways. *Appl Clin Inform* 2015; **6**: 650-668 [PMID: 26763576 DOI: 10.4338/ACI-2015-05-RA-0054]

21   **Dasari BV**, Tan CJ, Gurusamy KS, Martin DJ, Kirk G, McKie L, Diamond T, Taylor MA. Surgical versus endoscopic treatment of bile duct stones. *Cochrane Database Syst Rev* 2013; CD003327 [PMID: 23999986 DOI: 10.1002/14651858.CD003327.pub3]

22   **Platt TE**, Smith K, Sinha S, Nixon M, Srinivas G, Johnson N, Andrews S. Laparoscopic common bile duct exploration; a preferential pathway for elderly patients. *Ann Med Surg (Lond)* 2018; **30**: 13-17 [PMID: 29946453 DOI: 10.1016/j.amsu.2018.03.044]

23   **Lion KC**, Wright DR, Spencer S, Zhou C, Del Beccaro M, Mangione-Smith R. Standardized Clinical Pathways for Hospitalized Children and Outcomes. *Pediatrics* 2016; **137**: pii: e20151202 [PMID: 27002007 DOI: 10.1542/peds.2015-1202]

24   **Singh SB**, Shelton AU, Greenberg B, Starner TD. Implementation of cystic fibrosis clinical pathways improved physician adherence to care guidelines. *Pediatr Pulmonol* 2017; **52**: 175-181 [PMID: 27797455 DOI: 10.1002/ppul.23635]

25   **Vujasinovic M**, Makuc J, Tepes B, Marolt A, Kikec Z, Robac N. Impact of a clinical pathway on treatment outcome in patients with acute pancreatitis. *World J Gastroenterol* 2015; **21**: 9150-9155 [PMID: 26290642 DOI: 10.3748/wjg.v21.i30.9150]

26   **Nishimura K**, Yasui M, Nishimura T, Oga T. Clinical pathway for acute exacerbations of chronic obstructive pulmonary disease: Method development and five years of experience. *Int J Chron Obstruct Pulmon Dis* 2011; **6**: 365-372 [PMID: 21760723 DOI: 10.2147/COPD.S20423]

27   **Donà D**, Zingarella S, Gastaldi A, Lundin R, Perilongo G, Frigo AC, Hamdy RF, Zaoutis T, Da Dalt L, Giaquinto C. Effects of clinical pathway implementation on antibiotic prescriptions for pediatric community-acquired pneumonia. *PLoS One* 2018; **13**: e0193581 [PMID: 29489898 DOI: 10.1371/journal.pone.0193581]

28   **Kim HE**, Kim YH, Song KB, Chung YS, Hwang S, Lee YJ, Park KM, Kim SC. Impact of critical pathway implementation on hospital stay and costs in patients undergoing pancreaticoduodenectomy. *Korean J Hepatobiliary Pancreat Surg* 2014; **18**: 14-20 [PMID: 26155241 DOI: 10.14701/kjhbps.2014.18.1.14]

29   **Husni ME**, Losina E, Fossel AH, Solomon DH, Mahomed NN, Katz JN. Decreasing medical complications for total knee arthroplasty: Effect of critical pathways on outcomes. *BMC Musculoskelet Disord* 2010; **11**: 160 [PMID: 20630086 DOI: 10.1186/1471-2474-11-160]

30   **Burgers PT**, Van Lieshout EM, Verhelst J, Dawson I, de Rijcke PA. Implementing a clinical pathway for hip fractures; effects on hospital length of stay and complication rates in five hundred and twenty six patients. *Int Orthop* 2014; **38**: 1045-1050 [PMID: 24337751 DOI: 10.1007/s00264-013-2218-5]

31   **Walters DM**, McGarey P, LaPar DJ, Strong A, Good E, Adams RB, Bauer TW. A 6-day clinical pathway after a pancreaticoduodenectomy is feasible, safe and efficient. *HPB (Oxford)* 2013; **15**: 668-673 [PMID: 23458383 DOI: 10.1111/hpb.12016]

ORIGINAL ARTICLE

Observational Study

Lethal-7-related polymorphisms are associated with susceptibility to and prognosis of gastric cancer

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Abstract

BACKGROUND
The lethal-7 (*let-7*) family members and their targets are involved in the development and progression of tumors. *Let-7*-related polymorphisms have been reported to be associated with tumorigenesis and prognosis. In gastric cancer, however, the related studies are limited.

AIM
To investigate the role of *let-7*-related microRNA polymorphisms in the tumorigenesis and prognosis of gastric cancer in a Chinese population.

METHODS
A total of 898 gastric cancer patients and 992 tumor-free controls were recruited into this study from 2008 to 2013. Gastric cancer patients were followed periodically. Ten single nucleotide polymorphisms (SNPs) in the *let-7* gene region or their target mRNAs were genotyped using the MassARRAY system and their associations with the risk for or overall survival of gastric cancer were analyzed.

RESULTS
All the ten SNPs were in Hardy-Weinberg equilibrium. The C allele of the rs3811463 polymorphism in the 3′-untranslated region (UTR) of *LIN28A* was associated with a lower risk of gastric cancer [odds ratio (OR) = 0.74, 95% confidence interval (CI): 0.61-0.88, *P* = 0.001] after adjustment for age and *Helicobacter pylori* status. Seven hundred and thirty-five gastric cancer patients who had undergone radical tumorectomy were included in the survival analysis and their 5-year survival rate was 53.9% (95%CI: 50.1%-57.6%). The rs10889677 in

the 3'-UTR of *IL23R* was corresponded to the prognosis of gastric cancer in a dose-response manner, in which the death risk increased by 25% [hazard ratio (HR) = 1.25, 95%CI: 1.04-1.45, *P* = 0.011] with each increase in the number of C alleles after controlling for other potential clinicopathological parameters.

CONCLUSION
The *let-7*-related polymorphism rs3811463 in *LIN28A* is associated with the susceptibility to gastric cancer and the *let-7*-related polymorphism rs10889677 in *IL23R* is associated with the prognosis of gastric cancer.

Key words: Gastric cancer; Risk; Susceptibility; Prognosis; Polymorphism; Lethal-7; LIN28A; IL23R

Core tip: This study included a relatively large number of gastric cancer patients and tumor-free controls and explored the relationship of ten lethal-7 (*let-7*)-related single nucleotide polymorphisms with gastric carcinogenesis and prognosis. The results showed that the *LIN28A* rs3811463 polymorphism of the *let-7* target was associated with the development of gastric cancer and that the *IL23R* rs10889677 polymorphism was related to the overall survival of gastric cancer patients in a dose-dependent manner. This study adds evidence that polymorphisms represent a genetic factor that modifies the susceptibility to and prognosis of gastric cancer.

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide[1]. It is estimated that there were 1 million new cases in 2018, and nearly half of the new cases were in China[1]. Despite advances in the diagnosis and treatment of GC in recent decades, the prognosis for GC patients is still poor, especially in China, with the five-year survival rate of only approximately 30%[2]. GC is a heterogeneous disease with distinct clinical, epidemiological, and molecular features and is thought to be a multifactorial disease influenced by environmental factors, microbial infection, and the host's genetic background[3]. Recently, increasing numbers of studies have identified that genetic variations, of which single nucleotide polymorphisms (SNPs) are the most common type, play an important role in the development and progression of tumors including GC[4,5].

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that bind the mRNAs of the target genes to inhibit their translation and/or induce their decay[6]. It is estimated that more than 30% of human genes, involved in nearly all human physical and pathophysiological processes, are regulated by miRNAs[7]. The miRNA lethal-7 (*let-7*) is the first miRNA identified in humans, and more than ten members of the *let-7* family have been identified, including *let-7a-1*, *let-7a-2*, *let-7a-3*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f-1*, *let-7f-2*, *let-7g*, and *let-7i*[8]. *Let-7* often exhibits tumor-suppressor functions in tumorigenesis such as inhibiting proliferation, inducing apoptosis, and suppressing the invasion and metastasis of cancer cells[8]. The *let-7* family members are often downregulated in several cancers, thus derepressing the oncogenic targets such as K-ras, LIN28A, c-Myc, and HMGA2[9].

Previous studies have reported that miRNA-related SNPs could modulate the risk of tumors by altering the production of mature miRNAs or by affecting the binding affinity of miRNAs to their targets[10,11]. Xie *et al*[12] found that hepatocellular carcinoma patients carrying the C allele of rs10877887 in the promotor region of *let-7i* had a significantly increased death risk compared to patients with the TT genotype. The rs3811463 polymorphism is located in the 3'-untranslated region (UTR) of *LIN28A*, which is also near the target region of *let-7*. The C allele of rs3811463 could attenuate

the *let-7*-induced repression of *LIN28A* mRNA, resulting in the increased production of LIN28A protein, which could in turn downregulate the level of mature *let-7 via* an LIN28A/*let-7* double-negative feedback loop and alter breast cancer risk[13]. In GC, however, the related studies are limited. This study was aimed to determine the role of *let-7* related SNPs in the susceptibility to and prognosis of GC.

MATERIALS AND METHODS

Ethics statement
This study was approved by the Ethics Committee of the First Hospital of Jilin University (2013-005). All participants provided written informed consent prior to joining the study.

Subjects
GC patients who were hospitalized for potential tumor resection were invited to participate in the study from July 2008 to December 2013 at the First Hospital of Jilin University. A total of 898 patients who had not undergone chemotherapy or radiotherapy before surgery were recruited to the study, and all of them were histologically diagnosed with GC. Demographical and clinicopathological information was collected. The tumor histological type was evaluated by the World Health Organization criteria, and clinical stages were classified according to the 7th edition of the TNM staging system of the Union for International Cancer Control/American Joint Committee on Cancer (2010) based on postoperative pathologic examination.

During the same period, tumor-free controls were recruited from the Physical Examination Center of the same hospital. Controls were frequency-matched with cases by sex and age (± 5 years), and 992 controls were included in the study.

Follow-up
GC patients were followed periodically after tumorectomy. Follow-ups were performed at 3 mo, 6 mo, and 1 year after surgery and annually afterwards. Information on general status and postoperative chemotherapy was collected during each follow-up. If the patients had died, the date of death and potential cause were recorded. Survival time was defined as the duration from the date of surgery to the date of death. If the patient was alive, survival time was defined as the duration from the date of surgery to the date of the last follow-up. If the patient was lost to follow-up, survival time was defined as the duration from the date of surgery to the date of the last successful interview. Survival time was right-censored in the latter two cases. Patients were excluded from the survival analysis if they were lost to follow-up at the first interview or if they died of postoperative complications such as uncontrollable bleeding during the perioperative period.

Genotyping
All subjects donated blood samples. Genomic DNA was isolated using blood genomic DNA extraction kits (Axygen Biosciences, Union City, CA, United States). Serum immunoglobulin G antibodies to *Helicobacter pylori* (*H. pylori*) were assessed using an enzyme-linked immunosorbent assay (Biohit, Helsinki, Finland). Titers higher than 30 enzyme immunounits were defined as positive for *H. pylori* according to the manufacturer's instructions.

Let-7-related SNPs that were reportedly associated with cancer were selected and genotyped. Six SNPs are in the *let-7* gene region (rs13293512, rs562052, rs547008, rs1143770, rs629367, and rs10877887) and four SNPs are in the 3'-UTR of potential target genes of *let-7* (rs3811463 in *LIN28A*, rs10889677 in *IL23R*, rs7963551 in *RAD52*, and rs712 in *KRAS*). Genotypes were determined with the MassARRAY system (Sequenom Inc, CA, United States). The call rates were all greater than 95% (13 subjects for rs13293512, 10 for rs562052, 10 for rs547008, 26 for rs1143770, 2 for rs629367, 53 for rs10877887, 7 for rs10889677, 6 for rs7963551, and 31 for rs712, respectively, failed to genotype). Ten randomly selected samples were simultaneously genotyped twice, and the concordance rates were 100% for all loci.

Statistical analysis
Continuous variables are shown as the mean ± standard deviation (SD) and compared by Student's *t*-test. Categorical variables are presented as frequencies with percentages and were compared with the $\chi 2$ test or Fisher's exact test when appropriate. Multivariate logistic regression analysis was employed to select the independent loci associated with the development of GC, and odds ratios (ORs) with their 95% confidence intervals (CIs) were calculated. Survival curves within each

stratification of variables were plotted by the Kaplan-Meier method and compared by log-rank test. The multivariate Cox proportional hazard model was used to evaluate the prognostic role of polymorphisms, and hazard ratios (HRs) with their 95% CIs were calculated. All analyses were performed using SAS 9.2 software (SAS Institute Inc, United States). A two-tailed *P*-value < 0.05 indicated statistical significance.

RESULTS

From 2008 to 2013, 898 GC cases and 992 tumor-free controls were enrolled in the study. Gender distribution was uniform between cases and controls (*P* = 0.886). The mean age of GC patients was 2 years older than that of the controls (61.1 years *vs* 59.2 years, respectively; *P* = 0.0002). Six hundred and three GC cases were positive for *H. pylori*, and the *H. pylori* positive rate (67.5%) was much higher in GC patients than in the control group (487/992 = 49.1%, *P* < 0.001) (Table 1).

LIN28A rs3811463 is associated with gastric cancer risk

Ten SNPs were genotyped in the study. All ten loci were in agreement with the Hardy-Weinberg equilibrium in the control group (*P* > 0.05). The distribution of the genotypes is presented in Table 2. The rs3811463 polymorphism in the 3'-UTR of *LIN28A* was distributed differently between GC cases and controls (*P* = 0.024). The proportions of T/C and C/C genotypes were lower in the GC group than in the control group (T/C: 22.6% *vs* 26.7%, respectively; C/C: 2.6% *vs* 3.8%, respectively). When compared to the population with the T/T genotype, individuals with the heterozygous T/C genotype had a decreased risk of GC (OR = 0.76, 95%CI: 0.61-0.94), and the risk was even lower for individuals with the C/C genotype (OR = 0.59, 95%CI: 0.34-1.01) after adjusting for age, gender, and *H. pylori* status. When the dominant model was used, the C allele (C/T + C/C) was associated with a reduced risk of GC (OR = 0.74, 95%CI: 0.60-0.91, *P* = 0.004). In stepwise logistic regression analysis, which included all loci, age, gender, and *H. pylori* status, rs3811463 was the only SNP site in the final model, and the risk of GC was lower by 26% with each increase of the number of C alleles (OR = 0.74, 95%CI: 0.61-0.88, *P* = 0.001), in addition to *H. pylori* status (OR = 2.20, 95%CI: 1.81-2.67, *P* < 0.001) and age (> 65 years *vs* ≤ 65 years, OR = 1.31, 95%CI: 1.07-1.61, *P* = 0.010) (Table 3). The other nine SNPs, however, were not associated with GC risk.

IL23R rs10889677 variant genotype predicts overall survival of gastric cancer

Given the prognostic value of surgical tumorectomy, survival analysis was performed in patients who had undergone radical tumorectomy (Figure 1). Among the 898 GC cases included in this study, 143 were excluded for one of the following reasons: (1) distant metastasis; (2) not receiving curative tumorectomy; or (3) positive surgical margins. Of the remaining 755 patients, 5 were lost to follow-up at the first interview, and 15 died of postoperative complications within one month of surgery. These 20 patients were also excluded from the survival analysis. Thus, 735 patients were included in the final survival analysis (Figure 1). The median follow-up time until September 2017 for these 735 patients was 59.8 mo. During the follow-up period, 334 patients (45.4%) died, 383 (52.1%) survived, and 18 (2.4%) were lost to follow-up. The 5-year survival rate was 53.9% (95% CI 50.1%-57.6%) and the median survival time was estimated to be 77.7 mo.

We tested the association between SNP genotypes and the overall survival of GC patients with radical resection. We observed that patients with different rs10889677 genotypes had different survival curves (log-rank *P* = 0.049, Figure 2). More precisely, patients had worse survival with each increase in the number of C alleles of rs10889677. By the end of the study, 41.7% of patients with no rs10889677 C allele (A/A genotype) had died, while 48.5% of patients with one C allele (A/C genotype) and 56.6% of patients with two C alleles (C/C genotype) had died (Table 4). The five-year survival rate for A/A genotype patients was 57.7%, which was higher than that of patients with the A/C genotype (50.3%) or C/C genotype (46.0%). We further transformed the rs10889677 genotypes to a quantifiable trait (the number of C alleles) and observed that the death HR increased by 23% with each additional C allele (HR = 1.23, 95%CI: 1.04-1.45, *P* = 0.015). The other nine SNP loci were not significantly associated with the overall survival of GC patients (Table 4).

Age (*P* < 0.001), tumor differentiation level (*P* = 0.010), depth of tumor invasion (T stage, *P* < 0.001), local lymph node metastasis (N stage, *P* < 0.001), TNM stage (*P* < 0.001), lymphovascular invasion (*P* < 0.001), and neural invasion (*P* < 0.001) were associated with the long-term survival of GC patients in univariate survival analysis (Table 5). Postoperative chemotherapy was marginally related to the outcome (*P* = 0.054). *H. pylori* status, which was previously reported to be related to GC

Table 1 General characteristics of subjects included in this study			
	Cases	**Controls**	***P* value**
n	898	992	
Gender			
Male	649 (72.3)	714 (72.0)	0.886
Female	249 (27.7)	278 (28.0)	
Age (yr)	61.1 ± 11.4	59.2 ± 10.0	< 0.001
≤ 65	582 (64.8)	703 (70.9)	0.005
> 65	316 (35.2)	289 (29.1)	
Helicobacter pylori			
Negative	290 (32.5)	505 (50.9)	< 0.001
Positive	603 (67.5)	487 (49.1)	

prognosis[14], was not associated with the long-term OS in our study (P = 0.364, Table 5).

Further multivariate analysis showed that the rs10889677 polymorphism was an independent prognostic predictor and that the death HR increased by 23% with each increase in the number of C alleles (HR = 1.25, 95%CI: 1.04-1.45, P = 0.012) (Table 6). In addition, old age (> 65 years, HR = 1.39, 95%CI: 1.10-1.75), high TNM stage [stage II (HR = 3.49, 95%CI: 1.88-6.48) or III (HR = 11.12, 95%CI: 6.0-20.61)], and positive lymphovascular invasion (HR = 1.89, 95%CI: 1.32-2.72) were also independently associated with decreased long-term survival. Additionally, chemotherapy after radical tumorectomy reduced the death hazard (HR = 0.75, 95%CI: 0.59-0.96, P = 0.021) (Table 6).

DISCUSSION

The *let-7* family members play an important role in several hallmarks of cancer, including repressing cellular proliferation, inducing cell apoptosis, suppressing aerobic glycolysis, inhibiting invasion and metastasis, and regulating tumor innate immune reactions by interacting with their targets[8]. *Let-7*-related SNPs, which are located in the *let-7* gene region or in the target gene region, were reported to be associated with the development or prognosis of tumors. In this study, we explored the association of *let-7*-related SNPs with the susceptibility to and long-term overall survival of GC in a Chinese population. We observed that the rs3811463 in the 3'-UTR of *LIN28A* was associated with the susceptibility of GC and that rs10889677 in the 3'-UTR of *IL23R* was associated with the OS of resectable GC.

The *let-7* family and LIN28A compose a double-negative feedback loop. LIN28A represses *let-7* by inhibiting the biogenesis and inducing the degradation of the *let-7* family; meanwhile, *let-7* inhibits the expression and function of LIN28A *via* binding to complementary sites at the 3'-UTR of *LIN28A* mRNA[8]. In contrast to the role of *let-7* in tumors, LIN28A promotes the proliferation of cancer cells and is related to the poor prognosis[15]. Rs3811463 is located in the 3'-UTR of *LIN28A*, near the binding region of *let-7*. Chen *et al*[13] reported that the C allele of rs3811463 corresponded to an increased risk of breast cancer by attenuating the *let-7*-induced LIN28A repression, which in turn downregulates the mature *let-7 via* the LIN28A/*let-7* double-negative feedback loop. A subsequent study on oral cancer did not report any association between oral cancer risk and rs3811463 polymorphism in 384 cases and 731 controls[16]. In our study, however, the C allele of rs3811463 was found to be associated with a reduced risk of GC. Moreover, this association demonstrated a dose-response manner in which the risk was lower with each increase in the number of C alleles compared to that of the patients without the C allele (*i.e.*, genotype T/T). The online bioinformatics tool SNPinfo (http://snpinfo.niehs.nih.gov/) predicts that the change from T to C might make *LIN28A* gain the target of miR-490, which would reduce the level of *LIN28A*, thus associating with a lower risk of GC. However, further studies are needed to verify this hypothesis.

Interleukin-23 receptor (IL23R), a crucial subunit of the IL-23 receptor complex, is involved in innate and adaptive immune processes by interacting with IL-23, which plays a crucial role in many human diseases including autoimmune diseases and tumors[17,18]. Because IL23R presents tumor-promoting effects, knockdown of *IL23R* restricts tumor growth and decreases cancer metastasis[17,18]. The rs10889677

Table 2 The genotype distribution between gastric cancer cases and controls

Gene	SNP	Genotypes	Cases	Controls	*P* value[a]	OR (95% CI)[b]
let-7a-1	rs13293512	T/T	270 (30.3)	310 (31.5)	0.593	1.00
		T/C	442 (49.6)	494 (50.2)		1.03 (0.83-1.28)
		C/C	180 (20.2)	181 (18.4)		1.13 (0.86-1.48)
let-7a-2	rs562052	G/G	382 (43.0)	439 (44.3)	0.814	1.00
		G/A	413 (46.5)	446 (45.0)		1.06 (0.87-1.29)
		A/A	94 (10.6)	106 (10.7)		0.96 (0.70-1.32)
let-7a-2	rs547008	C/C	529 (59.2)	575 (58.3)	0.875	1.00
		C/T	321 (35.9)	358 (36.3)		0.96 (0.79-1.17)
		T/T	44 (4.9)	53 (5.4)		0.85 (0.56-1.30)
let-7a-2	rs1143770	T/T	255 (28.9)	282 (28.7)	0.262	1.00
		T/C	456 (51.8)	482 (49.0)		1.04 (0.83-1.29)
		C/C	170 (19.3)	219 (22.3)		0.83 (0.64-1.09)
let-7a-2	rs629367	A/A	532 (59.2)	597 (60.3)	0.583	1.00
		A/C	323 (36.0)	355 (35.9)		1.01 (0.83-1.22)
		C/C	43 (4.8)	38 (3.8)		1.21 (0.76-1.92)
let-7i	rs10877887	T/T	370 (42.3)	406 (42.2)	0.777	1.00
		T/C	399 (45.6)	449 (46.7)		0.96 (0.78-1.17)
		C/C	106 (12.1)	107 (11.1)		1.04 (0.76-1.42)
LIN28A	rs3811463	T/T	672 (74.8)	689 (69.5)	0.024	1.00
		T/C	203 (22.6)	265 (26.7)		0.76 (0.61-0.94)
		C/C	23 (2.6)	38 (3.8)		0.59 (0.34-1.01)
	Dominant	T/T	672 (74.8)	689 (69.5)	0.009	1.00
		T/C+C/C	226 (25.2)	303 (30.5)		0.74 (0.60-0.91)
IL23R	rs10889677	A/A	476 (53.2)	545 (55.2)	0.685	1.00
		A/C	356 (39.8)	375 (38.0)		1.10 (0.91-1.34)
		C/C	63 (7.0)	68 (6.9)		1.07 (0.74-1.55)
RAD52	rs7963551	T/T	597 (66.6)	672 (68.0)	0.666	1.00
		T/G	267 (29.8)	287 (29.0)		1.06 (0.87-1.31)
		G/G	32 (3.6)	29 (2.9)		1.18 (0.69-2.01)
KRAS	rs712	G/G	540 (61.0)	589 (60.5)	0.646	1.00
		G/T	296 (33.4)	339 (34.8)		0.94 (0.77-1.15)
		T/T	49 (5.5)	46 (4.7)		1.14 (0.74-1.75)

[a]*P* was computed using the $\chi 2$ test.
[b]ORs with 95%CIs were computed using a logistic model after adjusting for age, gender, and *Helicobacter pylori* status.
SNP: Single nucleotide polymorphism; ORs: Odds ratios; CIs: Confidence intervals.

polymorphism, located in the 3'-UTR of the *IL23R* gene, has been reported to be associated with cancer risk, including the risk of breast cancer[19], esophageal cancer[20], bladder cancer[21], ovarian cancer[22], oral cancer[23], and GC[24,25]. The results of these studies, however, are controversial[26]. In GC, Chen *et al* showed that the presence of the C allele of rs10889677 predicted lower GC risk (A/C: OR = 0.81, 95%CI: 0.66–0.99; C/C: OR = 0.47, 95%CI: 0.31–0.71)[24], while another study by Dong *et al*[25] observed an opposite relationship; the presence of the C allele increased the GC risk by 24% (C_{allele} *vs* A_{allele}: OR = 1.24, 95%CI: 1.02–1.52). In our study, however, we did not find any significant association between rs10889677 and GC risk ($P > 0.05$). Nonetheless, we observed that the C allele was related to decreased survival of GC patients after curative tumorectomy in a dose-dependent manner, and the death HR increased by 25% (HR = 1.25, 95%CI: 1.05-1.49) with each increment in the number of the C alleles. This is the first study reporting that the rs10889677 of *IL23R* is related to the prognosis of GC, as most studies only focused on cancer susceptibility. Zwiers *et al*[27] showed that the C allele of rs10889677 was corresponded to reduced *IL23R* levels possibly through the target gain of *let-7*. However, this could not explain what we had observed, and another unknown mechanism might be involved.

Studies have reported a significant correlation between SNPs in the *let-7* gene

Table 3 Multivariate stepwise logistic regression analysis of gastric cancer risk

Factor	OR	95%CI	*P* value
rs3811463 (each increase in C allele)	0.74	0.61-0.88	0.001
Helicobacter pylori (positive *vs* negative)	2.20	1.81-2.67	< 0.001
Age (> 65 *vs* ≤ 65 yr)	1.31	1.07-1.61	0.010

OR: Odds ratio; CI: Confidence interval.

region and the susceptibility to or prognosis of malignant tumors, for example, rs629367 in *let-7a* and the risk of GC[10], rs10877887 in *let-7i* and the risk of papillary thyroid carcinoma[28] and survival of hepatocellular carcinoma[12], and rs1143770 in *let-7a* and the overall survival and disease-free survival of surgically resected non-small cell lung cancer[29]. Other studies, however, observed no such associations[30]. In our study of a relatively large number of GC patients, we did not find that the six polymorphisms in the *let-7* gene region (rs13293512, rs562052, rs547008, rs1143770, rs629367, and rs10877887) were associated with the risk of development and the overall survival of GC. Several factors might contribute to the discrepancies among studies, such as different origins of the tumors studied and study populations from different ethnic groups. Therefore, more studies with a large sample size, including populations from different ethnic groups and tumors from multiple origins, are warranted in the future.

Two limitations should be noted in our study. First, although we observed that two SNPs were associated with GC, we could not verify the underlying mechanism for these associations because of our study design. Second, GC patients and controls from only one hospital were included in the study. The generalization of these results to other populations should be cautious because there has been no external validation. Therefore, more studies are needed to verify the results.

In summary, this study provides evidence that polymorphisms represent a genetic factor that modifies the susceptibility to and prognosis of GC. Individuals carrying the C allele of rs3811463 in the 3'-UTR of *LIN28A* have a lower risk of GC, and GC patients with the C allele of rs10889677 in the 3'-UTR of *IL23R* have a shorter lifespan than patients without it.

Table 4 Genotypes of single nucleotide polymorphisms and overall survival of gastric cancer patients

SNP	Genotype	Total	Death (%)	5-yr survival rate	Median survival time	P value[a]
rs13293512	T/T	213	100 (46.9)	51.6%	85.2	0.508
	T/C	370	172 (46.5)	53.3%	75.8	
	C/C	148	60 (40.5)	59.0%	-[b]	
rs562052	G/G	313	150 (47.9)	51.5%	76.2	0.253
	G/A	337	141 (41.8)	56.9%	-[b]	
	A/A	77	40 (51.9)	50.7%	65.4	
rs547008	C/C	434	200 (46.1)	53.0%	85.2	0.148
	C/T	264	112 (42.4)	56.2%	-[b]	
	T/T	34	21 (61.8)	46.7%	33.8	
rs1143770	T/T	215	98 (45.6)	54.3%	-[b]	0.535
	T/C	370	164 (44.3)	54.4%	-[b]	
	C/C	138	68 (49.3)	51.3%	65.4	
rs629367	A/A	435	205 (47.1)	52.8%	75.8	0.834
	A/C	264	113 (42.8)	55.6%	-[b]	
	C/C	36	16 (44.4)	55.8%	85.2	
rs10877887	T/T	301	147 (48.8)	50.4%	64.7	0.235
	T/C	330	141 (42.7)	57.2%	-[b]	
	C/C	88	39 (44.3)	53.7%	-[b]	
rs3811463	T/T	555	251 (45.2)	53.7%	85.2	0.971
	T/C	162	75 (46.3)	54.9%	77.7	
	C/C	18	8 (44.4)	52.0%	-[b]	
rs10889677	A/A	379	158 (41.7)	57.7%	-[b]	0.049
	A/C	301	146 (48.5)	50.3%	65.4	
	C/C	53	30 (56.6)	46.0%	41.0	
rs7963551	T/T	508	236 (46.5)	52.56%	85.2	0.144
	T/G	199	82 (41.2)	59.35%	-[b]	
	G/G	27	16 (59.3)	38.22%	41.3	
rs712	G/G	452	215 (47.6)	51.51%	73.7	0.227
	G/T	235	95 (40.4)	58.64%	-[b]	
	T/T	36	17 (47.2)	55.01%	77.7	

[a]*P* was computed by log-rank test.
[b]Median survival time could not be estimated as fewer than 50% of patients died.

Table 5 Associations between clinical factors and overall survival of gastric cancer patients

Variable		Classification	*n*[a]	Death (%)	5-yr survival (%)	P value[b]
Age (yr)		≤ 65	494	197 (39.9)	59.7 (55.0-64.0)	< 0.001
		> 65	241	137 (56.8)	42.3 (35.6-48.8)	
Gender		Female	195	82 (42.1)	56.8 (49.2-63.7)	0.261
		Male	540	252 (46.7)	52.9 (48.4-57.2)	
Differentiation		Poor	508	241 (47.4)	50.9 (46.2-55.3)	0.010
		Moderate and high	209	85 (40.7)	61.6 (54.4-68.0)	
WHO type		Tubular adenocarcinoma	614	275 (44.8)	54.8 (50.6-58.8)	0.205
		Signet ring cell	72	32 (44.4)	52.8 (39.8-64.2)	
		Other	45	25 (55.6)	44.5 (29.2-58.6)	
T stage		T1	100	13 (13.0)	85.8 (76.5-91.6)	< 0.001
		T2	102	16 (15.7)	84.4 (74.8-90.6)	
		T3	423	220 (52.0)	48.1 (43.1-52.9)	
		T4	103	78 (75.7)	17.4 (9.2-27.8)	
N stage		N0	219	38 (17.4)	84.2 (78.4-88.5)	< 0.001
		N1	184	63 (34.2)	66.0 (58.2-72.6)	

	N2	150	87 (58.0)	38.7 (30.3-47.1)	
	N3	175	139 (79.4)	17.3 (11.7-23.9)	
TNM	I	137	12 (8.8)	90.6 (83.9-94.6)	< 0.001
	II	278	92 (33.1)	68.3 (62.1-73.6)	
	III	313	223 (71.2)	25.8 (20.7-31.2)	
Lymphovascular invasion	Negative	213	38 (17.8)	83.0 (77.0-87.6)	< 0.001
	Positive	511	287 (56.2)	42.6 (38.1-47.0)	
Neural invasion	Negative	328	100 (30.5)	70.2 (64.6-75.0)	< 0.001
	Positive	396	225 (56.8)	41.1 (36.0-46.2)	
Chemotherapy	No	488	218 (44.7)	54.8 (50.0-59.3)	0.054
	FOLFOX-4	136	63 (46.3)	52.1 (43.2-60.3)	
	XELOX	73	28 (38.4)	61.7 (49.1-72.0)	
	Other	38	25 (65.8)	34.0 (19.1-49.4)	
Helicobacter pylori	Negative	228	109 (47.8)	49.8 (42.7-56.4)	0.364
	Positive	503	222 (44.1)	56.1 (51.4-60.4)	

[a]Some of the variables have missing values (The missing number was 4 for the variable of WHO type, 18 for differentiation, 7 for T stage, 7 for N stage, 7 for TNM stage, 11 for lymphovascular invasion, 11 for neural invasion, and 4 for *Helicobacter pylori*, respectively).
[b]*P* was computed by log-rank test.

Table 6 Multivariate Cox regression analysis of gastric cancer survival

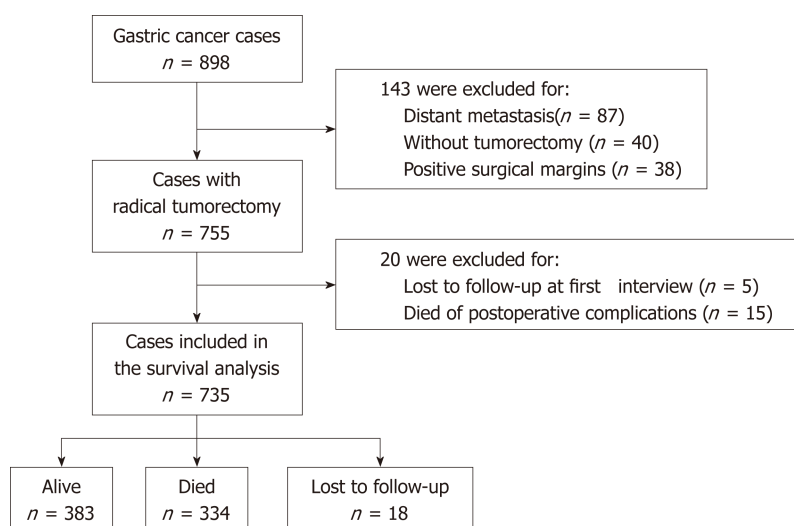| Variable	Comparison	HR	95%CI	*P* value
rs10889677	each increase in C allele	1.25	1.05-1.49	0.012
Age (yr)	> 65 *vs* ≤ 65	1.39	1.10-1.75	0.005
TNM stage	II *vs* I	3.49	1.88-6.48	< 0.001
	III *vs* I	11.12	6.00-20.61	< 0.001
Lymphovascular invasion	Positive *vs* Negative	1.89	1.32-2.72	0.001
Chemotherapy	Yes *vs* No	0.74	0.59-0.95	0.016

HR: Hazard ratio; CI: Confidence interval.

Figure 1 The flow chart of patients included in the survival analysis.
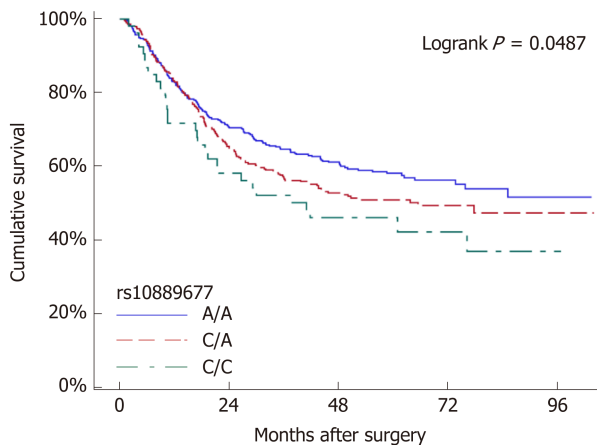
Figure 2 Survival plot of gastric cancer patients stratified by rs10889677 genotypes. Compared with the survival of patients with no C allele of rs10889677 (genotype A/A, blue line), patients with one C allele (genotype C/A, red line) exhibited a decreased survival rate and patients with two C alleles (genotype C/C, cyan line) exhibited the lowest survival rate.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most common malignancies worldwide. Despite the advances in diagnosis and treatment of GC in recent decades, prognosis of GC patients is still poor. It is of great importance to identify biomarkers that could be helpful in the improvement of screening of high-risk individuals, early diagnosis, and predicting outcome for the individualized therapy.

Research motivation

The microRNA lethal-7 (*let-7*) often exhibits tumor-suppressor functions in tumorigenesis. Single nucleotide polymorphisms (SNPs) in the *let-7* gene region or *let-7* target genes have been reported to modulate the risk of several cancers including breast cancer and lung cancer. In GC, the related studies are limited

Research objectives

By including a relatively large number of GC patients, this study aimed to determine the role of *let-7*-related polymorphisms in GC in a Chinese population.

Research methods

From 2008 to 2013, 898 consecutive GC patients and 992 tumor-free controls were recruited into the study. GC patients were followed periodically to determine their prognosis. Ten SNPs in the *let-7* gene region or their target mRNAs were genotyped using MassArray system and the associations with the risk or overall survival of GC were analyzed.

Research results

Two SNPs in *let-7* target genes were associated with GC in a dose-dependent manner. Rs3811463 in the 3'-UTR of *LIN28A* was associated with lower risk of GC and the risk was reduced by 26% with each increase of the C allele of rs3811463. The rs10889677 in the 3'-UTR of *IL23R* was corresponded to the prognosis of GC, and the death risk increased by 25% with each increment of the C allele of rs10889677, after controlling for clinicopathological parameters.

Research conclusions

Let-7-related SNPs were related to GC. The rs3811463 in *LIN28A* is associated with the susceptibility to and rs10889677 in *IL23R* is associated with the prognosis of GC.

Research perspectives

Our research adds evidence that polymorphisms represent a genetic factor to modify the susceptibility to and prognosis of GC. The underlying mechanisms of the associations should be elucidated in future studies.

REFERENCES

1   **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]
2   **Zeng H**, Chen W, Zheng R, Zhang S, Ji JS, Zou X, Xia C, Sun K, Yang Z, Li H, Wang N, Han R, Liu S,

Li H, Mu H, He Y, Xu Y, Fu Z, Zhou Y, Jiang J, Yang Y, Chen J, Wei K, Fan D, Wang J, Fu F, Zhao D, Song G, Chen J, Jiang C, Zhou X, Gu X, Jin F, Li Q, Li Y, Wu T, Yan C, Dong J, Hua Z, Baade P, Bray F, Jemal A, Yu XQ, He J. Changing cancer survival in China during 2003-15: A pooled analysis of 17 population-based cancer registries. *Lancet Glob Health* 2018; **6**: e555-e567 [PMID: 29653628 DOI: 10.1016/S2214-109X(18)30127-X]

3   **Karimi P**, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: Descriptive epidemiology, risk factors, screening, and prevention. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 700-713 [PMID: 24618998 DOI: 10.1158/1055-9965.EPI-13-1057]

4   **Stenholm L**, Stoehlmacher-Williams J, Al-Batran SE, Heussen N, Akin S, Pauligk C, Lehmann S, Senff T, Hofheinz RD, Ehninger G, Kramer M, Goekkurt E. Prognostic role of microRNA polymorphisms in advanced gastric cancer: A translational study of the Arbeitsgemeinschaft Internistische Onkologie (AIO). *Ann Oncol* 2013; **24**: 2581-2588 [PMID: 23975664 DOI: 10.1093/annonc/mdt330]

5   **Espinosa-Parrilla Y**, Muñoz X, Bonet C, Garcia N, Venceslá A, Yiannakouris N, Naccarati A, Sieri S, Panico S, Huerta JM, Barricarte A, Menéndez V, Sánchez-Cantalejo E, Dorronsoro M, Brennan P, Duarte-Salles T, B As Bueno-de-Mesquita H, Weiderpass E, Lund E, Clavel-Chapelon F, Boutron-Ruault MC, Racine A, Numans ME, Tumino R, Canzian F, Campa D, Sund M, Johansson M, Ohlsson B, Lindkvist B, Overvad K, Tjønneland A, Palli D, Travis RC, Khaw KT, Wareham N, Boeing H, Nesi G, Riboli E, Gonzalez CA, Sala N. Genetic association of gastric cancer with miRNA clusters including the cancer-related genes MIR29, MIR25, MIR93 and MIR106: Results from the EPIC-EURGAST study. *Int J Cancer* 2014; **135**: 2065-2076 [PMID: 24643999 DOI: 10.1002/ijc.28850]

6   **Engels BM**, Hutvagner G. Principles and effects of microRNA-mediated post-transcriptional gene regulation. *Oncogene* 2006; **25**: 6163-6169 [PMID: 17028595 DOI: 10.1038/sj.onc.1209909]

7   **Dalmay T**, Edwards DR. MicroRNAs and the hallmarks of cancer. *Oncogene* 2006; **25**: 6170-6175 [PMID: 17028596 DOI: 10.1038/sj.onc.1209911]

8   **Wang T**, Wang G, Hao D, Liu X, Wang D, Ning N, Li X. Aberrant regulation of the LIN28A/LIN28B and let-7 loop in human malignant tumors and its effects on the hallmarks of cancer. *Mol Cancer* 2015; **14**: 125 [PMID: 26123544 DOI: 10.1186/s12943-015-0402-5]

9   **Sun X**, Liu J, Xu C, Tang SC, Ren H. The insights of Let-7 miRNAs in oncogenesis and stem cell potency. *J Cell Mol Med* 2016; **20**: 1779-1788 [PMID: 27097729 DOI: 10.1111/jcmm.12861]

10   **Xu Q**, Dong Q, He C, Liu W, Sun L, Liu J, Xing C, Li X, Wang B, Yuan Y. A new polymorphism biomarker rs629367 associated with increased risk and poor survival of gastric cancer in chinese by up-regulated miRNA-let-7a expression. *PLoS One* 2014; **9**: e95249 [PMID: 24760009 DOI: 10.1371/journal.pone.0095249]

11   **Jiang Y**, Qin Z, Hu Z, Guan X, Wang Y, He Y, Xue J, Liu X, Chen J, Dai J, Jin G, Ma H, Wang S, Shen H. Genetic variation in a hsa-let-7 binding site in RAD52 is associated with breast cancer susceptibility. *Carcinogenesis* 2013; **34**: 689-693 [PMID: 23188672 DOI: 10.1093/carcin/bgs373]

12   **Xie K**, Liu J, Zhu L, Liu Y, Pan Y, Wen J, Ma H, Zhai X, Hu Z. A potentially functional polymorphism in the promoter region of let-7 family is associated with survival of hepatocellular carcinoma. *Cancer Epidemiol* 2013; **37**: 998-1002 [PMID: 24103425 DOI: 10.1016/j.canep.2013.09.005]

13   **Chen AX**, Yu KD, Fan L, Li JY, Yang C, Huang AJ, Shao ZM. Germline genetic variants disturbing the Let-7/LIN28 double-negative feedback loop alter breast cancer susceptibility. *PLoS Genet* 2011; **7**: e1002259 [PMID: 21912531 DOI: 10.1371/journal.pgen.1002259]

14   **Tsai KF**, Liou JM, Chen MJ, Chen CC, Kuo SH, Lai IR, Yeh KH, Lin MT, Wang HP, Cheng AL, Lin JT, Shun CT, Wu MS; Taiwan Gastrointestinal Disease and Helicobacter Consortium. Distinct Clinicopathological Features and Prognosis of Helicobacter pylori Negative Gastric Cancer. *PLoS One* 2017; **12**: e0170942 [PMID: 28152027 DOI: 10.1371/journal.pone.0170942]

15   **Xu C**, Shen J, Xie S, Jiang Z, Huang L, Wang L. Positive expression of Lin28 is correlated with poor survival in gastric carcinoma. *Med Oncol* 2013; **30**: 382 [PMID: 23292830 DOI: 10.1007/s12032-012-0382-x]

16   **Zhang Y**, Zhu L, Wang R, Miao L, Jiang H, Yuan H, Ma H, Chen N. Genetic variants in let-7/Lin28 modulate the risk of oral cavity cancer in a Chinese Han population. *Sci Rep* 2014; **4**: 7434 [PMID: 25503985 DOI: 10.1038/srep07434]

17   **Yan J**, Allen S, Vijayan D, Li XY, Harjunpää H, Takeda K, Liu J, Cua DJ, Smyth MJ, Teng MWL. Experimental Lung Metastases in Mice Are More Effectively Inhibited by Blockade of IL23R than IL23. *Cancer Immunol Res* 2018; **6**: 978-987 [PMID: 29921599 DOI: 10.1158/2326-6066.CIR-18-0011]

18   **Langowski JL**, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Basham B, McClanahan T, Kastelein RA, Oft M. IL-23 promotes tumour incidence and growth. *Nature* 2006; **442**: 461-465 [PMID: 16688182 DOI: 10.1038/nature04808]

19   **Zheng J**, Jiang L, Zhang L, Yang L, Deng J, You Y, Li N, Wu H, Li W, Lu J, Zhou Y. Functional genetic variations in the IL-23 receptor gene are associated with risk of breast, lung and nasopharyngeal cancer in Chinese populations. *Carcinogenesis* 2012; **33**: 2409-2416 [PMID: 23042301 DOI: 10.1093/carcin/bgs307]

20   **Ni B**, Chen S, Xie H, Ma H. Functional polymorphisms in interleukin-23 receptor and susceptibility to esophageal squamous cell carcinoma in Chinese population. *PLoS One* 2014; **9**: e89111 [PMID: 24586528 DOI: 10.1371/journal.pone.0089111]

21   **Tang T**, Xue H, Cui S, Gong Z, Fei Z, Cheng S, Gui C. Association of interleukin-23 receptor gene polymorphisms with risk of bladder cancer in Chinese. *Fam Cancer* 2014; **13**: 619-623 [PMID: 24861772 DOI: 10.1007/s10689-014-9731-6]

22   **Zhang Z**, Zhou B, Zhang J, Chen Y, Lai T, Yan L, Liang A, Li Y, Wang Y, Chen Y, Zhang L, Xi MR. Association of interleukin-23 receptor gene polymorphisms with risk of ovarian cancer. *Cancer Genet Cytogenet* 2010; **196**: 146-152 [PMID: 20082850 DOI: 10.1016/j.cancergencyto.2009.09.006]

23   **Chien MH**, Hsin CH, Chou LS, Chung TT, Lin CH, Weng MS, Chou MY, Chen MK, Yang SF. Interleukin-23 receptor polymorphism as a risk factor for oral cancer susceptibility. *Head Neck* 2012; **34**: 551-556 [PMID: 21717520 DOI: 10.1002/hed.21779]

24   **Chen B**, Zeng Z, Xu L, Wu X, Yu J, Xue L, Hao Y, Wang Y, Sung JJ, Chen M, Hu P. IL23R +2199A/C polymorphism is associated with decreased risk of certain subtypes of gastric cancer in Chinese: a case-control study. *Cancer Epidemiol* 2011; **35**: 165-169 [PMID: 20863779 DOI: 10.1016/j.canep.2010.08.006]

25   **Dong K**, Xu Y, Yang Q, Shi J, Jiang J, Chen Y, Song C, Wang K. Associations of Functional MicroRNA Binding Site Polymorphisms in IL23/Th17 Inflammatory Pathway Genes with Gastric Cancer Risk. *Mediators Inflamm* 2017; **2017**: 6974696 [PMID: 29118466 DOI: 10.1155/2017/6974696]

26   **Liu XH**, Dai ZM, Kang HF, Lin S, Ma XB, Wang M, Liu K, Dai C, Wang XJ, Dai ZJ. Association of IL-

23R Polymorphisms (rs6682925, rs10889677, rs1884444) With Cancer Risk: A PRISMA-Compliant Meta-Analysis. *Medicine (Baltimore)* 2015; **94**: e2361 [PMID: 26717375 DOI: 10.1097/MD.0000000000002361]

27   **Zwiers A**, Kraal L, van de Pouw Kraan TC, Wurdinger T, Bouma G, Kraal G. Cutting edge: A variant of the IL-23R gene associated with inflammatory bowel disease induces loss of microRNA regulation and enhanced protein production. *J Immunol* 2012; **188**: 1573-1577 [PMID: 22262659 DOI: 10.4049/jimmunol.1101494]

28   **Wang Y**, Wei T, Xiong J, Chen P, Wang X, Zhang L, Gao L, Zhu J. Association Between Genetic Polymorphisms in the Promoter Regions of Let-7 and Risk of Papillary Thyroid Carcinoma: A Case-Control Study. *Medicine (Baltimore)* 2015; **94**: e1879 [PMID: 26512603 DOI: 10.1097/MD.0000000000001879]

29   **Shin KM**, Jung DK, Hong MJ, Kang HJ, Lee WK, Yoo SS, Lee SY, Cha SI, Lee J, Kim CH, Seok Y, Cho S, Son JW, Lee EB, Jheon S, Kim YT, Park JY. The pri-let-7a-2 rs1143770C>T is associated with prognosis of surgically resected non-small cell lung cancer. *Gene* 2016; **577**: 148-152 [PMID: 26625972 DOI: 10.1016/j.gene.2015.11.036]

30   **Huang F**, Hu LM, Liu JB, Zhang YX, Hu ZB. [Relationship between genetic polymorphism of promoter region let-7 and genetic susceptibility to hepatocellular carcinoma]. *Zhonghua Yu Fang Yi Xue Za Zhi* 2011; **45**: 1093-1098 [PMID: 22336344]

EVIDENCE-BASED MEDICINE

Establishing a model to measure and predict the quality of gastrointestinal endoscopy

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Abstract

BACKGROUND
Tens of millions of gastrointestinal endoscopic procedures are performed every year in China, but the quality varies significantly and related factors are complex. Individual endoscopist- and endoscopy division-related factors may be useful to establish a model to measure and predict the quality of endoscopy.

AIM
To establish a model to measure and predict the quality of gastrointestinal endoscopic procedures in mainland China.

METHODS
Selected data on endoscopy experience, equipment, facility, qualification of endoscopists, and other relevant variables were collected from the National Database of Digestive Endoscopy of China. The multivariable logistic regression analysis was used to identify the potential predictive variables for occurrence of medical malpractice and patient disturbance. Linear and nonlinear regressions were used to establish models to predict incidence of endoscopic complications.

RESULTS
In 2012, gastroscopy/colonoscopy-related complications in mainland China included bleeding in 4,359 cases (0.02%) and perforation in 914 (0.003%).

Endoscopic-retrograde-cholangiopancreatography-related complications included severe acute pancreatitis in 593 cases (0.3%), bleeding in 2,151 (1.10%), perforation in 257 (0.13%) and biliary infection in 4,125 (2.11%). Moreover, 1,313 (5.0%) endoscopists encountered with medical malpractice, and 5,243 (20.0%) encountered with the disturbance from patients. The length of endoscopy experience, weekly working hours, weekly night shifts, annual vacation days and job satisfaction were predictors for the occurrence of medical malpractice and patient disturbance. However, the length of endoscopy experience and the ratio of endoscopists to nurses were not adequate to establish an effective predictive model for endoscopy complications.

CONCLUSION
The workload and job satisfaction of endoscopists are valuable predictors for medical malpractice or patient disturbance. More comprehensive data are needed to establish quality-predictive models for endoscopic complications.

Key words: Endoscopy; Gastroscopy; Colonoscopy; Endoscopic retrograde cholangiopancreatography; Quality control; Predictive model; Performance predictor

Core tip: Tens of millions of gastrointestinal endoscopic procedures are performed each year in China, but there is significant variation in the quality of endoscopy, and the method of measuring quality remains unknown. We collected data from the National Database of Digestive Endoscopy of China to establish a model to measure and predict the quality of gastrointestinal endoscopy in mainland China. The length of endoscopy experience, weekly working hours, weekly night shifts, annual vacation days and job satisfaction were predictors of medical malpractice and patient disturbance. The length of experience and endoscopist/nurse ratio were not adequate to establish a model for the prediction of endoscopic complications.

INTRODUCTION

Tens of millions of endoscopic procedures are performed every year in China, but there is significant variation in the quality of endoscopy[1-8]. Factors affecting the quality of endoscopy are complex and include personnel who perform the procedure, as well as procedures and equipment, which all may result in outcome variation.

Various organizations have developed structured procedures for the management of underperforming endoscopists. There have been studies about performance measures generated by evidence-based consensus that can be used for upper gastrointestinal endoscopy[9-11]. Performance measures can be used to measure the quality of organizational structure, healthcare procedures or clinical outcomes, providing feedbacks to endoscopists with suboptimal performance to improve their quality of procedures. Hence, it might be beneficial to predict the overall quality of endoscopy in a system consisting of human resources, procedures and equipment. This particularly matters for large developing countries, which urgently need to improve the overall quality of endoscopy.

MATERIALS AND METHODS

Conduct of the survey

As part of its endoscopy quality initiative, the Society of Digestive Endoscopy of the Chinese Medical Association, with support from the National Health and Family

Planning Commission, conducted a nationwide survey in hospitals in all 31 provinces, autonomous regions and municipalities of mainland China in 2013, to investigate the digestive endoscopy infrastructure and overall performance of endoscopic procedures.

Two sets of questionnaires were used in the survey: one was completed by the endoscopic division of each hospital, collecting data that included hospital characteristics, composition and number of endoscopic staff, type and volume of endoscopic procedures, and endoscopic facilities and equipment. The other was completed by individual endoscopists, collecting clinical practice data that included staff work status, weekly working hours, night shifts, vacation days, revenue, work volume, work satisfaction, medical malpractice and patient disturbance. The endoscopic divisions of 6,127 hospitals and 26,203 endoscopists completed and returned the respective questionnaires. All data were imported into the National Database of Digestive Endoscopy.

Data synthesis

The endoscopic divisions of each hospitals were required to follow the Digestive Endoscopy Diagnosis and Treatment Technological Management Regulations by the National Health and Family Planning Commission of China. The survey showed that all of the hospitals had equivalent equipment that had been acquired in accordance with government standards. In the analysis for this study, it was therefore assumed that procedures and equipment were equivalent and there was no difference among the hospitals that participated in the nationwide survey. This assumption meant that the analysis focused on the characteristics of human resources as potential predictive variables at the level of an endoscopic division or endoscopist.

At the level of an endoscopic division, the nationwide survey collected data about gastroscopy, colonoscopy, duodenoscopy, endoscopic ultrasonography, endoscopic retrograde cholangiopancreatography (ERCP), and other endoscopic procedures. We selected the three most common procedures to be analyzed, which included gastroscopy, colonoscopy and ERCP. In the analysis, we only considered the overall complications and did not analyze individual complications. Major complications in the records included gastroscopy/colonoscopy/ERCP-related bleeding and perforation, severe acute pancreatitis and biliary infection.

Statistical analysis

Multivariable logistic regression analyses were used to examine and identify the potential predictive variables for the occurrence of medical malpractice and patient disturbance. Covariates with $P < 0.20$ were selected to be included in the full multivariable model for prediction. Linear and nonlinear (root, square, log) regressions were used to establish models for predicting the incidence of endoscopic complications. The outcome variables (dependent) and predictor variables (independent) are listed in Table 1.

RESULTS

Overview of gastrointestinal endoscopy in mainland China in 2012

In 2012, gastrointestinal endoscopic procedures were performed in 6,128 hospitals in mainland China, invloving 26,203 endoscopists and 14,532 endoscopic nurses. The total number of gastrointestinal endoscopy procedures was 28.77 million. The numbers of gastroscopy, colonoscopy and ERCP procedures were 22.25 million, 5.83 million and 200,000, respectively.

Gastroscopy- and colonoscopy-related complications included bleeding in 4,359 cases (0.02%) and perforation in 914 cases (0.003%). ERCP-related complications included severe acute pancreatitis in 593 cases (0.3%), bleeding in 2,151 cases (1.10%), perforation in 257 cases (0.13%) and biliary infection in 4,125 cases (2.11%).

According to reports from endoscopists, 1,313 (5.0%) of them encountered with medical malpractice and 5243 (20.0%) encountered with the disturbance from patients or their relatives.

Establishment of a model to measure and predict quality of gastrointestinal endoscopy

Five workload-related factors (length of endoscopy experience, weekly working hours, weekly night shifts, annual vacation days and job satisfaction) were included in the final multivariable model as impact factors for the occurrence of medical malpractice and patient disturbance (Table 2).

Linear and nonlinear (root, square, log) regression models were used to test the

Table 1 Description of outcome variables and predictor variables

	Individual endoscopist-related	Endoscopy division-related
Outcome variable	Occurrence of medical malpractice and patient disturbance	Incidence of endoscopic complications
Predictor variable	Length of experience in endoscopy	Length of experience in endoscopy
	Workload (weekly working hours)	Ratio of endoscopists to nurses
	Workload (weekly night shifts)	
	Workload (annual vacation days)	
	Job satisfaction	
	Endoscopist status (part- or full-time)	

correlation between the incidence of endoscopic complications and two selected impact factors, which included the length of endoscopy experience and the ratio of endoscopists to nurses. However, these two factors alone were not adequate to establish an effective predictive model for gastroscopy/colonoscopy- or ERCP-related complications (Tables 3 and 4).

DISCUSSION

Quality control is an important issue in the practice of gastrointestinal endoscopy[12-21]. The primary objective of this study was to explore the feasibility of establishing a model to measure and predict the overall quality of endoscopic procedures in a system consisting of human resources, procedures and equipment, rather than to measure the performance of specific endoscopic procedures[22-28].

With the exception of endoscopist status (part- or full-time), the other five impact factors were included in the final multivariable logistic model for predicting the occurrence of medical malpractice and patient disturbance. Even the secondary data were not optimal. They did help examine the relationship between clinical outcomes (*e.g.*, complications) and predictors based on a large sample size, which decreased the effect of selection bias. The clinical outcomes, including the incidence of endoscopic complications, occurrence of medical malpractice and disturbance from patients and their families, were regarded as valuable performance measures or quality indicators in other studies[12,13,29]. The predictive variables were deemed to be risk factors that might affect the performance of endoscopic procedures in clinical practice, although there was a lack of strong evidence.

Medical professionals, procedures and equipment were included in the system. One assumption of the study was that the processes and equipment were equivalent among all of the hospitals in the survey. The assumption was made on the basis that all of the hospitals were required by government to follow the same regulations and all of the equipment was accredited by a government agency. It was assumed that these hospitals did not violate the rules and breach the standards, however we did not know the true status of each participant. To overcome this limitation in the future, information about process compliance and equipment accreditation status should be collected and updated regularly in the database.

We used linear and nonlinear (root, square, log) regression models to test the correlation between the incidence of endoscopic complications with the two corresponding impact factors. However, the results were not optimal and the two selected impact factors were inadequate for an effective predictive model. In the current analysis of human resource-related factors, there were only endoscopist data. However, it was clear in clinical practice that patient data are important. For example, the severity of the primary disease could affect the outcome of any endoscopic procedure. The suboptimal results indicated that there could be more impact factors and that their interactions are complex. Nevertheless, this study helped create a framework to establish a model that can help predict the quality of endoscopic procedures to some extent.

The major limitation of this study was that the analysis was based on suboptimal data. The data relevant to the quality of endoscopy from the Chinese National Database of Digestive Endoscopy could not cover all aspects of personnel, procedures and equipment. However, considering the large numbers of included hospitals and endoscopists, these data are still deemed valuable.

In conclusion, endoscopist workload-related factors might be valuable predictors for medical malpractice or patient disturbance. More comprehensive data are needed to establish a quality predictive model for endoscopic complications.

Table 2 Results of multivariable logistic regression analysis for occurrence of medical malpractice and patient disturbance

Impact factors	OR (95%CI)	*P* value
Length of experience in endoscopy	0.77 (0.75-0.78)	< 0.0001
Workload as weekly working hours	0.90 (0.86-0.94)	< 0.0001
Workload as weekly night shifts	0.96 (0.92-0.99)	0.0132
Workload as annual vacation days	1.02 (1.0-1.03)	0.0178
Job satisfaction	2.24 (2.12-2.36)	< 0.0001

Table 3 Results of regression analysis for incidence of endoscopic retrograde cholangiopancreatography-related complications

Impact factors	Linear regression analysis		Nonlinear (root) regression analysis		Nonlinear (square) regression analysis		Nonlinear (log) regression analysis	
	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
Intercept	0.0326	0.0000	0.0343	0.0022	0.0312	0.0000	0.0222	0.0888
Length of experience in endoscopy	0.0000	0.9895	0.0007	0.7124	-0.0000	0.5579	0.0029	0.4635
Ratio of endoscopists to nurses	-0.0018	0.1499	-0.0065	0.1307	-0.0001	0.3407	-0.0048	0.1389

Table 4 Results of regression analysis for incidence of gastroscopy/colonoscopy-related complications

Impact factor	Linear regression analysis		Nonlinear (root) regression analysis		Nonlinear (square) regression analysis		Nonlinear (log) regression analysis	
	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
Intercept	0.0064	0.0000	0.0075	0.0057	0.0059	0.0000	0.0070	0.0173
Length of experience in endoscopy	-0.0000	0.4461	-0.0003	0.5274	-0.0000	0.4250	-0.0006	0.5821
Ratio of endoscopists to nurses	-0.0000	0.8001	-0.0005	0.6422	-0.0000	0.8793	-0.0002	0.8611

ARTICLE HIGHLIGHTS

Research background

There are increasingly more gastrointestinal endoscopic procedures performed every year. However, there is significant variation in quality, and the causative factors are complex. Well-accepted predictive models have not been developed.

Research motivation

No related research has focused on this field in China before. However, the quality control of gastrointestinal endoscopy is an important issue. According to our national survey, we found that a number of adverse effects were related to gastrointestinal endoscopy. Therefore, we collected data from the National Database of Digestive Endoscopy of China, aiming to establish a model to measure and predict the quality of gastrointestinal endoscopy in mainland China.

Research objectives

Quality control is an important issue in gastrointestinal endoscopy. The primary objective of this study was to explore the feasibility of establishing a model to measure and predict the overall quality of endoscopic procedures in a system consisting of human resources as well as processes and equipment, rather than to measure the performance of specific endoscopic procedures.

Research methods

Related data were obtained from the nationwide survey in hospitals in all 31 provinces, autonomous regions and municipalities of mainland China in 2013. Multivariable logistic regression analyses were used to examine and identify the potential predictive variables for the occurrence of medical malpractice and patient disturbance.

Research results

In 2012, gastroscopy and colonoscopy-related complications included bleeding (0.02%) and perforation (0.003%). Endoscopic retrograde cholangiopancreatography (ERCP)-related complications included severe acute pancreatitis (0.3%), bleeding (1.10%), perforation (0.13%) and biliary infection (2.11%). Moreover, 5.0% of endoscopists encountered with medical

malpractice and 20.0% encountered with the disturbance from patients or their relatives. Multivariable logistic regression analyses showed that five workload-related factors, including length of endoscopy experience, weekly working hours, weekly night shifts, annual vacation days and job satisfaction, were predictors for medical malpractice and patient disturbance. However, the length of endoscopy experience and the ratio of endoscopists to nurses were not adequate to establish an effective predictive model for gastroscopy/colonoscopy or ERCP.

Research conclusions

In this study, we found for the first time that the workload and job satisfaction of endoscopists are valuable predictors for medical malpractice or patient disturbance. These findings suggest that in the clinical practice, decreasing the workload and increasing the welfare of endoscopists may improve the quality of gastrointestinal endoscopy.

Research perspectives

This study cannot build an ideal model for predicting the quality of gastrointestinal endoscopy. In the future, more comprehensive data are needed to establish quality-predictive models for endoscopic complications. The optimal method would be a multicenter prospective structured study.

REFERENCES

1 **Raftopoulos SC**, Segarajasingam DS, Burke V, Ee HC, Yusoff IF. A cohort study of missed and new cancers after esophagogastroduodenoscopy. *Am J Gastroenterol* 2010; **105**: 1292-1297 [PMID: 20068557 DOI: 10.1038/ajg.2009.736]

2 **Cohen J**, Safdi MA, Deal SE, Baron TH, Chak A, Hoffman B, Jacobson BC, Mergener K, Petersen BT, Petrini JL, Rex DK, Faigel DO, Pike IM; ASGE/ACG Taskforce on Quality in Endoscopy. Quality indicators for esophagogastroduodenoscopy. *Am J Gastroenterol* 2006; **101**: 886-891 [PMID: 16635232 DOI: 10.1111/j.1572-0241.2006.00676.x]

3 **Faigel DO**, Pike IM, Baron TH, Chak A, Cohen J, Deal SE, Hoffman B, Jacobson BC, Mergener K, Petersen BT, Petrini JL, Rex DK, Safdi MA; ASGE/ACG Taskforce on Quality in Endoscopy. Quality indicators for gastrointestinal endoscopic procedures: an introduction. *Am J Gastroenterol* 2006; **101**: 866-872 [PMID: 16635230 DOI: 10.1111/j.1572-0241.2006.00677.x]

4 **Hu LH**, Xin L, Liao Z, Pan J, Qian W, Wang LW, Li ZS; Endoscopy Audit of the Chinese Society of Digestive Endoscopy. ERCP development in the largest developing country: a national survey from China in 2013. *Gastrointest Endosc* 2016; **84**: 659-666 [PMID: 26996289 DOI: 10.1016/j.gie.2016.03.1328]

5 **Gavin DR**, Valori RM, Anderson JT, Donnelly MT, Williams JG, Swarbrick ET. The national colonoscopy audit: a nationwide assessment of the quality and safety of colonoscopy in the UK. *Gut* 2013; **62**: 242-249 [PMID: 22661458 DOI: 10.1136/gutjnl-2011-301848]

6 **Enochsson L**, Swahn F, Arnelo U, Nilsson M, Löhr M, Persson G. Nationwide, population-based data from 11,074 ERCP procedures from the Swedish Registry for Gallstone Surgery and ERCP. *Gastrointest Endosc* 2010; **72**: 1175-1184, 1184.e1-1184.e3 [PMID: 20970787 DOI: 10.1016/j.gie.2010.07.047]

7 **Baron TH**, Petersen BT, Mergener K, Chak A, Cohen J, Deal SE, Hoffinan B, Jacobson BC, Petrini JL, Safdi MA, Faigel DO, Pike IM; ASGE/ACG Taskforce on Quality in Endoscopy. Quality indicators for endoscopic retrograde cholangiopancreatography. *Am J Gastroenterol* 2006; **101**: 892-897 [PMID: 16635233 DOI: 10.1111/j.1572-0241.2006.00675.x]

8 **Cotton PB**, Garrow DA, Gallagher J, Romagnuolo J. Risk factors for complications after ERCP: a multivariate analysis of 11,497 procedures over 12 years. *Gastrointest Endosc* 2009; **70**: 80-88 [PMID: 19286178 DOI: 10.1016/j.gie.2008.10.039]

9 **Rutter MD**, Senore C, Bisschops R, Domagk D, Valori R, Kaminski MF, Spada C, Bretthauer M, Bennett C, Bellisario C, Minozzi S, Hassan C, Rees C, Dinis-Ribeiro M, Hucl T, Ponchon T, Aabakken L, Fockens P. The European Society of Gastrointestinal Endoscopy Quality Improvement Initiative: developing performance measures. *United European Gastroenterol J* 2016; **4**: 30-41 [PMID: 26966520 DOI: 10.1177/2050640615624631]

10 **Bisschops R**, Areia M, Coron E, Dobru D, Kaskas B, Kuvaev R, Pech O, Ragunath K, Weusten B, Familiari P, Domagk D, Valori R, Kaminski MF, Spada C, Bretthauer M, Bennett C, Senore C, Dinis-Ribeiro M, Rutter MD. Performance measures for upper gastrointestinal endoscopy: A European Society of Gastrointestinal Endoscopy quality improvement initiative. *United European Gastroenterol J* 2016; **4**: 629-656 [PMID: 27733906 DOI: 10.1177/2050640616664843]

11 **Chang JJ**, Xu J, Fan D. A comparative method of evaluating quality of international clinical studies in China: Analysis of site visit reports of the Clinical Research Operations and Monitoring Center. *Contemp Clin Trials* 2008; **29**: 654-662 [PMID: 18450522 DOI: 10.1016/j.cct.2008.03.002]

12 **Feurer ME**, Draganov PV. Training for advanced endoscopic procedures. *Best Pract Res Clin Gastroenterol* 2016; **30**: 397-408 [PMID: 27345648 DOI: 10.1016/j.bpg.2016.04.005]

13 **Williams EJ**, Taylor S, Fairclough P, Hamlyn A, Logan RF, Martin D, Riley SA, Veitch P, Wilkinson ML, Williamson PR, Lombard M. Risk factors for complication following ERCP; results of a large-scale, prospective multicenter study. *Endoscopy* 2007; **39**: 793-801 [PMID: 17703388 DOI: 10.1055/s-2007-966723]

14 **Hu LH**, Xin L, Wang LW, Qian W, Liao Z, Li ZS. ERCP practitioners in China: results from national surveys in 2007 and 2013. *Endoscopy* 2016; **48**: 358-363 [PMID: 26760603 DOI: 10.1055/s-0035-1563805]

15 **ASGE Quality Assurance in Endoscopy Committee**; Calderwood AH, Day LW, Muthusamy VR, Collins J, Hambrick RD 3rd, Brock AS, Guda NM, Buscaglia JM, Petersen BT, Buttar NS, Khanna LG, Kushnir VM, Repaka A, Villa NA, Eisen GM. ASGE guideline for infection control during GI endoscopy. *Gastrointest Endosc* 2018; **87**: 1167-1179 [PMID: 29573782 DOI: 10.1016/j.gie.2017.12.009]

16 **Pullens HJ**, Siersema PD. Quality indicators for colonoscopy: Current insights and caveats. *World J Gastrointest Endosc* 2014; **6**: 571-583 [PMID: 25512766 DOI: 10.4253/wjge.v6.i12.571]

17 **Lachter J**, Bluen B, Waxman I, Bellan W. Establishing a quality indicator format for endoscopic

ultrasound. *World J Gastrointest Endosc* 2013; **5**: 574-580 [PMID: 24255750 DOI: 10.4253/wjge.v5.i11.574]

18   **Denis B**, Sauleau EA, Gendre I, Piette C, Bretagne JF, Perrin P. Measurement of adenoma detection and discrimination during colonoscopy in routine practice: an exploratory study. *Gastrointest Endosc* 2011; **74**: 1325-1336 [PMID: 21958899 DOI: 10.1016/j.gie.2011.07.038]

19   **Cha JM**, Moon JS, Chung IK, Kim JO, Im JP, Cho YK, Kim HG, Lee SK, Lee HL, Jang JY, Kim ES, Jung Y, Moon CM, Kim Y, Park BY. National Endoscopy Quality Improvement Program Remains Suboptimal in Korea. *Gut Liver* 2016; **10**: 699-705 [PMID: 27282270 DOI: 10.5009/gnl15623]

20   **Kastenberg D**, Bertiger G, Brogadir S. Bowel preparation quality scales for colonoscopy. *World J Gastroenterol* 2018; **24**: 2833-2843 [PMID: 30018478 DOI: 10.3748/wjg.v24.i26.2833]

21   **Gkolfakis P**, Tziatzios G, Dimitriadis GD, Triantafyllou K. New endoscopes and add-on devices to improve colonoscopy performance. *World J Gastroenterol* 2017; **23**: 3784-3796 [PMID: 28638218 DOI: 10.3748/wjg.v23.i21.3784]

22   **Rutter MD**, Rees CJ. Quality in gastrointestinal endoscopy. *Endoscopy* 2014; **46**: 526-528 [PMID: 24788539 DOI: 10.1055/s-0034-1365738]

23   **Graham DG**, Banks MR. Advances in upper gastrointestinal endoscopy. *F1000Res* 2015; **4** [PMID: 26918137 DOI: 10.12688/f1000research.6961.1]

24   **Bretthauer M**, Aabakken L, Dekker E, Kaminski MF, Rösch T, Hultcrantz R, Suchanek S, Jover R, Kuipers EJ, Bisschops R, Spada C, Valori R, Domagk D, Rees C, Rutter MD; ESGE Quality Improvement Committee. Reporting systems in gastrointestinal endoscopy: Requirements and standards facilitating quality improvement: European Society of Gastrointestinal Endoscopy position statement. *United European Gastroenterol J* 2016; **4**: 172-176 [PMID: 27087943 DOI: 10.1177/2050640616629079]

25   **Bretthauer M**, Aabakken L, Dekker E, Kaminski MF, Rösch T, Hultcrantz R, Suchanek S, Jover R, Kuipers EJ, Bisschops R, Spada C, Valori R, Domagk D, Rees C, Rutter MD; ESGE Quality Improvement Committee. Requirements and standards facilitating quality improvement for reporting systems in gastrointestinal endoscopy: European Society of Gastrointestinal Endoscopy (ESGE) Position Statement. *Endoscopy* 2016; **48**: 291-294 [PMID: 26841269 DOI: 10.1055/s-0042-100186]

26   **Denzer UW**. Quality Assurance in Endoscopy: Which Parameters? *Visc Med* 2016; **32**: 42-51 [PMID: 27588295 DOI: 10.1159/000443653]

27   **Cho YK**. How to Improve the Quality of Screening Endoscopy in Korea: National Endoscopy Quality Improvement Program. *Clin Endosc* 2016; **49**: 312-317 [PMID: 27484810 DOI: 10.5946/ce.2016.084]

28   **Baillie J**, Testoni PA. Are we meeting the standards set for ERCP? *Gut* 2007; **56**: 744-746 [PMID: 17519478 DOI: 10.1136/gut.2006.113456]

29   **Zhao L**, Zhang XY, Bai GY, Wang YG. Violence against doctors in China. *Lancet* 2014; **384**: 744 [PMID: 25176545 DOI: 10.1016/S0140-6736(14)61436-7]

CASE REPORT

Crohn's-like acute severe colitis associated with Hermansky-Pudlak syndrome: A case report

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Abstract

BACKGROUND
Hermansky-Pudlak syndrome (HPS) is a rare autosomal recessive disorder characterized by oculocutaneous albinism, platelet storage pool deficiency and systemic complications associated with ceroid deposition in the reticuloendothelial system. HPS types 1 and 4 are associated with Crohn's disease (CD)-like gastrointestinal disorders, such as granulomatous enterocolitis or perianal disease. Cases of colitis can be particularly severe and, before the use of anti-tumor necrosis factor alpha (TNFα) therapy had become common, were reported as showing poor responsiveness to medical treatment.

CASE SUMMARY
We present the case of a 51-year-old albino woman who presented with acute severe colitis that led to the diagnosis of HPS. Histologic findings of biopsy samples showed chronic inflammation with deep ulcerations, and granulomas without caseous necrosis. Molecular genetic analysis confirmed HPS type 1, with a homozygous 27 base-pair deletion in exon 20 of the *HPS1* gene. Once the patient's bleeding diathesis was corrected by platelet transfusion, the granulomatous colitis responded dramatically to a medical treatment regimen that included corticosteroids, azathioprine and infliximab; this regimen is similar to that used in CD treatment. Although it remains unclear if the granulomatous enterocolitis in HPS is due to ceroid deposition or reflects the co-existence of CD and HPS, the fact that this case of HPS-related granulomatous colitis responded to the same therapeutic approach used in CD suggests that this type of colitis may result from HPS patients' genetic susceptibility to CD.

CONCLUSION
We report a case of severe colitis that led to the diagnosis of HPS, which was

responsive to azathioprine and infliximab.

Key words: Hermansky-Pudlak syndrome; Acute severe colitis; Infliximab; Azathioprine; Inflammatory bowel disease; Case report

Core tip: Hermansky-Pudlak syndrome (HPS) is a rare autosomal recessive disorder characterized by oculocutaneous albinism, platelet storage pool deficiency and systemic complications. HPS can be associated with Crohn's disease (CD)-like gastrointestinal disorders. We present a case of acute severe colitis in an albino woman that led to the diagnosis of HPS. Following bleeding diathesis correction by platelet transfusion, the granulomatous colitis responded dramatically to medical treatment with corticosteroids, azathioprine and infliximab. The fact that this HPS-related granulomatous colitis responded to the same therapeutic approach used in CD suggests that this type of colitis may result from HPS patients' genetic susceptibility to CD.

INTRODUCTION

Hermansky-Pudlak syndrome (HPS) is a rare hereditary autosomal recessive disorder of lysosome-related organelles, characterized by oculocutaneous albinism and storage pool deficiency of platelets with increased bleeding tendency[1]. To date, there are eight subtypes, identified based on the particular genetic mutation responsible for the disorder[2]. Visceral disorders such as pulmonary fibrosis, colitis, cardiomyopathy or renal failure are associated with some but not all subtypes of HPS, with varying degrees of severity. Indeed, HPS types 1 and 4 have been reported as associated with inflammatory bowel disease (IBD)-like disorders of the gastrointestinal tract, such as granulomatous colitis, ileitis, enterocolitis, intestinal fistulization or perianal disease[3-5]. Their clinical, endoscopic and histologic features remain indistinguishable from Crohn's disease (CD) and ulcerative colitis, suggesting that their physio-pathology might be related[3]. We report here a case of HPS type 1 that was diagnosed as a result of acute severe colitis.

CASE PRESENTATION

Chief complaints
A 51-year-old woman was admitted to the Gastroenterology Intensive Care Unit for hemorrhagic shock secondary to massive rectal bleeding.

History of present illness
The patient had lost about 15 kg over the past 5 mo, with recurrent episodes of diffuse abdominal pain that worsened following meals. Bloody diarrhea had started a few days before hospitalization, being intermittently mixed with stool first and progressing to massive rectal bleeding.

History of past illness, personal and family history
The patient had very limited medical history, comprised of very low visual acuity only. She reported having been abandoned at birth, and thus not knowing her family.

Physical examination upon admission
Initial physical examination demonstrated oculocutaneous albinism with whitish hair, pale skin, horizontal nystagmus and strabismus. Abdominal palpation elicited pain, and perineal examination did not show any anal fissure or fistula. The patient had a slight dyspnea, requiring low flow oxygen therapy, and pulmonary auscultation

revealed bilateral crackles. She also had a severe undernutrition status, with a weight of 32 kg and a body mass index of 13.6 kg/m^2.

Laboratory examinations

Laboratory findings were: hemoglobin of 5.4 g/dL (normal range: 13-18 g/dL), C-reactive protein (commonly known as CRP) of 73 mg/L (normal: < 5 mg/L), and hypoalbuminemia (1.6 g/dL; normal range: 3.4-5.4 g/dL). The white blood cell count (8.66 × 10^3/μL) and platelet count (268 × 10^3/μL) were normal. Bleeding time, activated partial thromboplastin time, and prothrombin time were normal. Stool samples cultures were negative, including for *Clostridium difficile*.

Imaging examinations

A computed tomography scan revealed diffuse bilateral interstitial infiltrates consistent with a severe pulmonary fibrosis (Figure 1A), and thickening of the descending/sigmoid colon associated with a transverse and ascending colon mild dilatation. Sigmoidoscopy showed active bleeding colitis, with continuous deep ulcerations (Figure 1B).

FINAL DIAGNOSIS

Histologic findings of biopsy samples showed chronic inflammation with deep ulcerations, extending into the muscularis mucosae, and granulomas without caseous necrosis. Molecular genetic analysis confirmed HPS type 1 with a homozygous 27 base-pair deletion in exon 20 of the *HPS1* gene (c.2037_2064del).

TREATMENT

Intravenous methylprednisolone at a dose of 60 mg daily along with repeated transfusions of red blood cells concentrates was started upon admission. After 48 h, the patient's CRP had decreased but the substantial rectal bleeding remained, necessitating additional transfusions. Thus, a colon rescue therapy was attempted with an infusion of infliximab at a dose of 5 mg/kg. Because of the HPS suspicion, platelet transfusions were also initiated, despite the normal findings for both platelet count and bleeding time; ultimately, this led to the bleeding ending.

Two weeks later, while the patient was getting better, the second infusion of infliximab was complicated by a severe anaphylactic reaction with bronchospasm that precluded continuance of this treatment. Azathioprine (50 mg daily) was started.

OUTCOME AND FOLLOW-UP

Two months later, deep remission was obtained, characterized by the absence of symptoms, normalization of inflammatory biologic markers, and mucosal healing (Figure 2). Unfortunately, the patient was not eligible for lung transplantation due to severe undernutrition and severity of pulmonary fibrosis, and she died of respiratory failure 3 mo later.

DISCUSSION

HPS was originally documented in 1959 by two Czechoslovakian physicians, who described two adults with a triad of albinism, hemorrhagic diathesis, and pigmented reticuloendothelial cells[1]. Except in the north-western quarter of the island of Puerto Rico, where HPS affects approximately 1/1800 persons and where approximately 1/22 persons are carriers of the gene, HPS remains extremely rare in the general population, with an estimated incidence between 1/500000 and 1/1000000[6]. HPS type 1 is the most common subtype and is associated with Puerto Rican heritage due to a founder mutation in this population. Diagnosis of HPS can be clinically suspected and is confirmed by molecular genetic analysis that allows classification into a particular HPS subtype (HPS 1-8).

Rarity of this syndrome can lead to delayed diagnosis and underlies the general lack of knowledge about its pathology, as was the case with our patient. HPS includes a platelet storage pool deficiency characterized by abnormally low contents of platelet α granules and/or δ granules[7] that results in a bleeding diathesis; this can be accompanied by normal findings in the usual blood tests, such as platelet count and
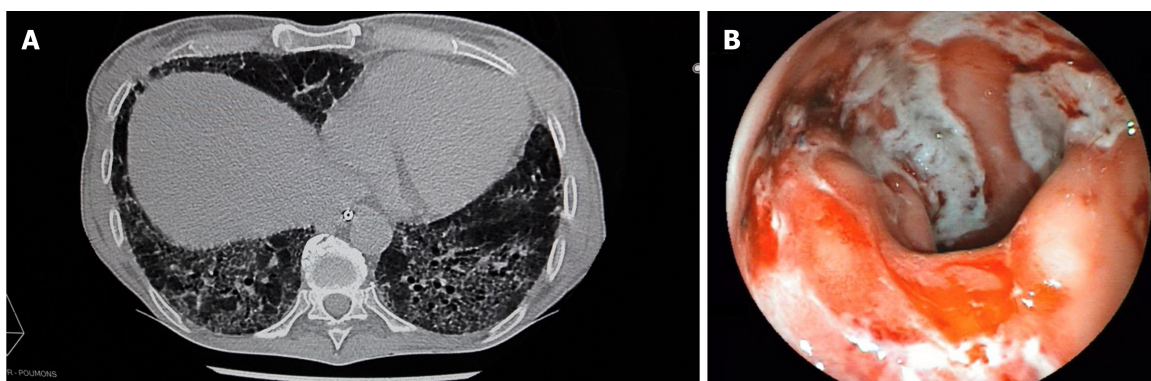
Figure 1 Computed tomography scan and sigmoidoscopy performed just after the patient's admission to the Gastroenterology Intensive Care Unit. A: Chest computed tomography scan demonstrating an old and severe bilateral pulmonary fibrosis; B: Sigmoidoscopy showing active bleeding colitis with edema, erythema and linear deep ulcerations.

bleeding time. There is no specific treatment, but transfusion of even limited numbers of normal platelets have been reported to alleviate the platelet dysfunction seen in HPS[7].

Granulomatous colitis was described as a complication of HPS for the first time in 1980[8]. Since then, many cases of inflammatory bowel disorders have been described, including those of colitis, enterocolitis or perianal disease[3,4,9-12]. These gastrointestinal complications are associated with HPS 1 and HPS 4 subtypes, occur in 20%-30% of the cases[3,4], and have been the cause of death in 9% of the deceased HPS patients in Puerto Rico[6]. The granulomatous colitis associated with HPS shares features with both ulcerative colitis and CD. In fact, clinical presentation and friable erythematous mucosa with ulcerations from rectum to cecum are suggestive of ulcerative colitis[10-12], but they present pathologic findings consistent with CD and can be associated with perianal and perirectal disease resembling the typical anorectal findings often seen in patients with CD[4,9,12].

Although HPS is caused by a single-gene defect that alters endosome trafficking and in spite of the fact that the proteins encoded by *HPS1* and *HPS4* genes are identified, the pathogenesis of the granulomatous colitis found in patients with HPS remains unclear. Moreover, it is also unclear if colitis associated with HPS is part of the syndrome, or if it represents an independent but associated process, such as CD[11]. Initially, it was suggested that granulomatous colitis of HPS results from the accumulation of ceroid lipofuscin in intestinal macrophages because of the defects in synthesis, processing and trafficking of lysosome-related organelles. As there is no degradative pathway for ceroid, this accumulation was supposed to induce macrophages' burst and a release of enzymes and cytokines, resulting in an inflammatory response[3,4,8,11]. But, some patients have had granulomatous colitis without ceroid pigments[10] and it has been noted that granulomas are not formed in relation to deposits of the ceroid-like pigments[8].

Thus, an alternative hypothesis has been proposed which is based on recent advances in the understanding of the pathophysiology of both HPS and CD[12]. HPS1 and HPS4 form a protein complex called the Biogenesis of Lysosome-related Organelles Complex 3 (BLOC-3), that triggers local activation of Rab GTP-ase proteins, called Rab32 and Rab38[13]. These small GTP-ases interact and co-locate with the protein LRRK2 to transport vesicles and recycling endosomes, and play an important role in the biogenesis and traffic of melanosomes and lysosomes. This system is disordered in HPS, accounting for the characteristic albinism[14], and LRRK2 has been recently identified by genome-wide association studies as a susceptibility gene for CD[15]. Therefore, this hypothesis suggests that granulomatous colitis in HPS results from genetic susceptibility to classical IBD.

In this case, medical treatment based on corticosteroids, azathioprine and one infusion of anti-tumor necrosis factor alpha (TNFα) therapy resulted in rapid improvement of clinical symptoms and endoscopic lesions. Among the nine published cases of HPS patients who received anti-TNFα therapy for a digestive complication[9-12], five had a good response, three necessitated surgical treatment, and the last one did not have any response to the anti-TNFα therapy but was improved by tacrolimus then vedolizumab[12]. The fact that therapeutic approaches similar to those for CD can also be effective in patients with HPS digestive complications reinforces the idea that HPS may be linked to CD. Further research is needed to determine the cause of the granulomatous enterocolitis of HPS, but all information gathered
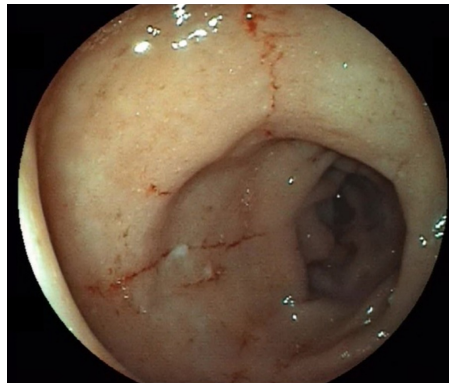
Figure 2 Sigmoidoscopy performed 2 mo after beginning the treatment. This image shows the improvement of edema and healing of linear ulcers.

concerning the genetics of HPS enterocolitis might also shed light on CD pathogenesis.

CONCLUSION

We report herein the case of acute severe colitis in an albino woman, that led to the diagnosis of HPS. This granulomatous colitis responded dramatically to a therapeutic approach based on corticosteroids, azathioprine and infliximab, which is similar to the results seen with this regimen in IBD treatment.

REFERENCES

1   **Hermansky F**, Pudlak P. Albinism associated with hemorrhagic diathesis and unusual pigmented reticular cells in the bone marrow: report of two cases with histochemical studies. *Blood* 1959; **14**: 162-169 [PMID: 13618373]

2   **Wei ML**. Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. *Pigment Cell Res* 2006; **19**: 19-42 [PMID: 16420244 DOI: 10.1111/j.1600-0749.2005.00289.x]

3   **Hussain N**, Quezado M, Huizing M, Geho D, White JG, Gahl W, Mannon P. Intestinal disease in Hermansky-Pudlak syndrome: occurrence of colitis and relation to genotype. *Clin Gastroenterol Hepatol* 2006; **4**: 73-80 [PMID: 16431308 DOI: 10.1016/S1542-3565(05)00858-X]

4   **Hazzan D**, Seward S, Stock H, Zisman S, Gabriel K, Harpaz N, Bauer JJ. Crohn's-like colitis, enterocolitis and perianal disease in Hermansky-Pudlak syndrome. *Colorectal Dis* 2006; **8**: 539-543 [PMID: 16919103 DOI: 10.1111/j.1463-1318.2006.01046.x]

5   **Mora AJ**, Wolfsohn DM. The management of gastrointestinal disease in Hermansky-Pudlak syndrome. *J Clin Gastroenterol* 2011; **45**: 700-702 [PMID: 21085008 DOI: 10.1097/MCG.0b013e3181fd2742]

6   **Witkop CJ**, Nuñez Babcock M, Rao GH, Gaudier F, Summers CG, Shanahan F, Harmon KR, Townsend D, Sedano HO, King RA. Albinism and Hermansky-Pudlak syndrome in Puerto Rico. *Bol Asoc Med P R* 1990; **82**: 333-339 [PMID: 2261023]

7   **Hurford MT**, Sebastiano C. Hermansky-pudlak syndrome: report of a case and review of the literature. *Int J Clin Exp Pathol* 2008; **1**: 550-554 [PMID: 18787629]

8   **Schinella RA**, Greco MA, Cobert BL, Denmark LW, Cox RP. Hermansky-Pudlak syndrome with granulomatous colitis. *Ann Intern Med* 1980; **92**: 20-23 [PMID: 7350869 DOI: 10.7326/0003-4819-92-1-20]

9   **Erzin Y**, Cosgun S, Dobrucali A, Tasyurekli M, Erdamar S, Tuncer M. Complicated granulomatous colitis in a patient with Hermansky-Pudlak syndrome, successfully treated with infliximab. *Acta Gastroenterol Belg* 2006; **69**: 213-216 [PMID: 16929618]

10   **Kouklakis G**, Efremidou EI, Papageorgiou MS, Pavlidou E, Manolas KJ, Liratzopoulos N. Complicated Crohn's-like colitis, associated with Hermansky-Pudlak syndrome, treated with Infliximab: a case report and brief review of the literature. *J Med Case Rep* 2007; **1**: 176 [PMID: 18067668 DOI: 10.1186/1752-1947-1-176]

11   **Grucela AL**, Patel P, Goldstein E, Palmon R, Sachar DB, Steinhagen RM. Granulomatous enterocolitis associated with Hermansky-Pudlak syndrome. *Am J Gastroenterol* 2006; **101**: 2090-2095 [PMID: 16848805 DOI: 10.1111/j.1572-0241.2006.00733.x]

12   **Sofia MA**, Sakuraba A, Rubin DT. Two Complex Cases of Hermansky-Pudlak Syndrome Highlight a Potential Biologic Explanation for an Associated Crohn's Disease Phenotype. *ACG Case Rep J* 2017; **4**: e14 [PMID: 28144619 DOI: 10.14309/crj.2017.14]

13   **Gerondopoulos A**, Langemeyer L, Liang JR, Linford A, Barr FA. BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. *Curr Biol* 2012; **22**: 2135-2139 [PMID: 23084991 DOI: 10.1016/j.cub.2012.09.020]

14   **Segal AW**. Making sense of the cause of Crohn's - a new look at an old disease. *F1000Res* 2016; **5**: 2510 [PMID: 28105308 DOI: 10.12688/f1000research.9699.2]

15   **Derkinderen P**, Neunlist M. Crohn's and Parkinson disease: is LRRK2 lurking around the corner? *Nat Rev Gastroenterol Hepatol* 2018; **15**: 330-331 [PMID: 29666431 DOI: 10.1038/s41575-018-0006-9]

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