

# World Journal of *Gastroenterology*

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2014-2017

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## Hepatitis C in injection drug users: It is time to treat

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### Abstract

Injection drug users (IDUs) are at risk of hepatitis C virus (HCV) infection, due to needle and syringe sharing. Chronic HCV infection is a major cause of liver-related morbidity and mortality but can be cured with antiviral treatment leading to sustained

viral response (SVR). It is well demonstrated that, when close cooperation between specialists in drug addiction and psychiatrists is assured, patients on maintenance treatment with methadone/buprenorphine can be treated for HCV with response rate, tolerability and side effects similar to those reported in non-IDUs. Current guidelines recommend that active injection drug use should not exclude patients from HCV treatment, but many services remain reluctant to treat IDUs. No significant pharmacodynamic interactions were reported between approved direct anti-viral agents (DAAs) and buprenorphine or methadone. Dose adjustments are not recommended; therefore DAAs appear to be the "perfect" therapy for patients taking opiate substitutive therapy. These suggestions have been recently recognized by the European Association for the Study of the Liver (EASL) and included in EASL Recommendations on Treatment of Hepatitis C 2016. Guidelines confirm that HCV treatment for IDUs should be considered on an individualized basis and delivered within a multidisciplinary team setting; a history of intravenous drug use and recent drug use at treatment initiation are not associated with reduced SVR and decisions to treat must be made on a case-by-case basis.

**Key words:** Hepatitis C; Drug users; Peg-interferon; Direct antiviral agents; Hepatitis C virus treatment

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**Core tip:** It is well demonstrated that injection drug users (IDUs) on maintenance treatment with methadone/buprenorphine can be treated for hepatitis C virus (HCV) with response rate, tolerability and side effects similar to those reported in non-IDUs. European Association for the Study of the Liver Recommendations on Treatment of Hepatitis C 2016 confirm that HCV treatment for IDUs should be considered on an individualized basis and delivered within a multidisciplinary team setting; a history of intravenous drug use and recent drug use at treatment initiation are not associated with reduced sustained

viral response and decisions to treat must be made on a case-by-case basis.

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## INTRODUCTION

It is well established that injection drug users (IDUs) are at risk of hepatitis C virus (HCV) infection, due to needle and syringe sharing.

Chronic HCV infection is a major cause of liver-related morbidity and mortality but can be cured with antiviral treatment leading to sustained viral response (SVR).

Treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV) leads to SVR in 46%-52% of patients with genotype 1 (GT1) infection, and 76%-80% of those with genotype 2 or 3 (GT2/GT3) infection. It should be noted that these outcomes have been reported in large clinical trials that excluded patients with a recent history of drug addiction<sup>[1,2]</sup>.

It is well demonstrated that, when close cooperation between specialists in drug addiction and psychiatrists is assured, patients on maintenance treatment with methadone/buprenorphine can be treated for HCV with response rate, tolerability and side effects similar to those reported in non-IDUs<sup>[3]</sup>. On the contrary, IDUs are still often considered to be poor candidates for HCV treatment due to concerns about psychiatric and other medical disorders as well as ongoing drug use, eventually leading to inadequate adherence to treatment and risk for reinfection. Similar concerns were previously raised with the advent of highly active antiretroviral therapy for HIV, but clinical studies suggested that DUs with HIV could achieve similar adherence to non users<sup>[4]</sup>. Current guidelines recommend that active injection drug use should not exclude patients from HCV treatment, but many services remain reluctant to treat IDUs.

In a meta-analysis, Aspinall *et al*<sup>[5]</sup> demonstrated that acceptable treatment outcomes can be achieved in patients with active drug injection who are eligible and committed to starting HCV treatment. Nevertheless, a considerable uncertainty remains around the risk of HCV reinfection following treatment. To assess reinfection risk, cases of HCV reinfection should be clearly distinguished from cases of HCV relapse. All studies in this review excluded individuals with a positive HCV test within 6 mo from end of treatment date, but, despite this, some of these individuals might have experienced early reinfection with HCV, rather

than early relapse.

Further crucial points are the low prevalence of DUs referring to specialty clinics for evaluation of HCV related disease and the low percentage (20%) of DUs who have considered to start antiviral therapy<sup>[6]</sup>.

DUs often cite discomfort encountered in conventional medical venues as a primary obstacle limiting pursuit of an HCV evaluation; consequently, HCV therapeutic effectiveness in DUs is an issue of treatment access, acceptance and adherence rather than drug efficacy<sup>[7]</sup>.

Dimova *et al*<sup>[8]</sup> conducted a meta-analysis of studies on DUs treated with PEG-IFN/RBV to understand the role of different support services in assisting DUs to complete HCV therapy and improve treatment outcome. They observed that addiction-treated DUs have higher PEG-IFN/RBV completion rates than non addiction-treated DUs and that the availability of support services during HCV treatment significantly increased the treatment completion rates. They reported a SVR rate of 55.5% among all PEG-IFN/RBV-treated DUs and of 53% for those treated for addiction during HCV treatment; these are comparable to those obtained in PEG-IFN/RBV registration trials<sup>[1,2]</sup>. Finally, they observed that involvement of multidisciplinary team led to higher SVR rates among DUs.

In accordance with these findings, we designed a collaborative programme between our hospital's Hepatology Outpatient clinic and SERT (Drug Addiction Local Outpatient Service) to manage IDUs with chronic hepatitis C together. This plan involves scheduled interdivisional meetings (including hepatologists, psychiatrists and SERT physicians) to evaluate IDUs with chronic hepatitis C and selecting the most suitable candidate to receive antiviral therapy. This plan provided IDUs with weekly direct/reserved access to SERT and the Hepatology Outpatient clinic to examine these IDUs' hepatitis C status and to prescribe antiviral therapy. Ultimately, we treated 23 DUs for HCV related liver disease with PEG-IFN/ RBV with a 61% (14 patients) SVR rate (data not published).

Despite the possibility of reinfection, antiviral treatment to IDUs represents the most cost-effective policy option, at least in scenarios with prevalence of chronic disease less than 60%<sup>[9]</sup>. Unfortunately, despite attaining the optimal treatment outcome, it has been demonstrated that an increasing significant minority of IDUs continue to inject post-SVR at an intensity which leads to either hospitalisation or death and increased risk of reinfection<sup>[10]</sup>.

This current scenario is mainly referred to "old" PEG-IFN based therapy, used in the last twenty years; however, it will be substantially modified by the arrival of interferon-free, new direct anti viral agents (DAAs). DAAs are expected to eliminate HCV in most persons who receive treatment, without significant side effects, even in advanced disease<sup>[11]</sup>.

No significant pharmacodynamic interactions were



reported between approved DAAs and buprenorphine or methadone. Dose adjustments are not recommended; therefore DAAs appear to be the “perfect” therapy in patients taking opiate substitutive therapy<sup>[12]</sup>.

These patients often suffer from psychiatric comorbidities and thus have contraindications to interferon-based antiviral treatment. They frequently have a borderline compliance to PEG-IFN while their compliance is excellent with respect to their daily visits at the low-threshold facility or pharmacy for ingestion of their opioid substitution therapy.

Recently, Schitz *et al.*<sup>[13]</sup> used DAAs to treat fifteen consecutive IDUs with chronic hepatitis C (5 cirrhotics, 4 with METAVIR fibrosis score F3) and borderline compliance, together with opioid substitution therapy under direct observation of a physician or nurse at the “Ambulatorium Suchthilfe Wien” - a low-threshold drug treatment facility in Vienna, Austria. The results were excellent: all patients completed treatment, with 100% of SVR at 12 wk post the end of therapy. In our experience, in the last two years, we treated nine IDUs with advanced HCV related disease (89% cirrhotic; 3 GT1, 6 GT3) with DAA ± RBV obtaining an 89% SVR rate (data not published).

It should be stressed that successful treatment of these patients is beneficial not only for themselves but also for the general population because transmission of the virus is prevented.

These suggestions have been recently recognized by the European Association for the Study of the Liver (EASL) and are included in EASL Recommendations on Treatment of Hepatitis C 2016. Guidelines confirm that HCV treatment for IDUs should be considered on an individualized basis and delivered within a multidisciplinary team setting; a history of intravenous drug use and recent drug use at treatment initiation are not associated with reduced SVR and decisions to treat must be made on a case-by-case basis. The anti-HCV regimens that can be used in IDUs are the same as in non-IDUs; they do not require specific methadone and buprenorphine dose adjustment<sup>[14]</sup>.

In conclusion, considering efficacy, tolerability and the indirect beneficial effect due to prevention of HCV transmission in the general population, at present, treatment of HCV related infection in IDUs appears mandatory: it is time to treat!

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## Cyclooxygenase 2 in liver dysfunction and carcinogenesis: Facts and perspectives

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### Abstract

The biosynthesis of prostaglandins and thromboxanes has been a focus of interest in the management of many liver diseases. Cyclooxygenases are the enzymes involved in the first step of the biosynthesis of these lipid mediators and selective inhibitors for these isoenzymes as well as pharmacological analogues of prostaglandins have been developed and are currently applied therapeutically. Here we discuss the implications of these enzymes in the onset of metabolic and lipid disorders in the liver and their potential role in the progression of the diseases towards fibrosis and hepatocellular carcinogenesis.

**Key words:** Cyclooxygenase-2; NAS; Prostaglandin; Non-alcoholic steatohepatitis; Hepatocellular carcinoma

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**Core tip:** The assessment of the role of Cyclooxygenase-2 (COX-2) in hepatic diseases, ranging from non-alcoholic steatohepatitis to hepatocellular carcinoma, constitutes a field in which controversy exists probably because of the use of different experimental models. Since potent and selective inhibitors of COX-2 exist, but also stable PGE<sub>2</sub> analogues to be used in therapy, unraveling the precise contribution of this enzyme and its products to the prevention of the progress of liver dysfunctions appears to be a useful approach for managing liver diseases.

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## INTRODUCTION

Prostaglandins and thromboxanes are bioactive lipids that regulate many physiological responses but in some cases are recognized as players in inflammatory and tumor diseases, including colorectal and hepatic cancer<sup>[1,2]</sup>. Cyclooxygenase (COX or prostaglandin G/H synthase, EC 1.14.99.1) catalyzes the rate-limiting step in the synthesis of prostaglandins (PGs) and thromboxanes using arachidonic acid (AA) as substrate to generate PGH<sub>2</sub>, which is the precursor for a number of cell specific prostaglandin and thromboxane synthases that generate the biologically active products PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, PGI<sub>2</sub> and thromboxanes among other bioactive lipids<sup>[1]</sup>. Various phospholipases cleave membrane bound AA that once released, it serves as substrate for three main routes: the cyclooxygenase (producing PGs and thromboxanes), the lipoxygenase (producing lipoxins) and the cytochrome P-450 monooxygenase pathways. The COX pathway has been extensively studied in view of the important effects exerted by PGs in many physiological and pathological processes. Moreover, this pathway is clinically relevant because this enzyme is the main target of nonsteroidal anti-inflammatory drugs, and selective COX-2 inhibitors are efficient in decreasing inflammation, and mitigating pain and fever<sup>[3,4]</sup>. Prostanoids exit the cells *via* a carrier-mediated process to activate specific prostanoids-dependent G protein-coupled receptors (GPCRs). There are at least eleven known PG receptors, all of them belonging to the GPCRs superfamily of seven transmembrane spanning proteins. For PGE<sub>2</sub>, EP1 receptors are coupled to G<sub>q</sub> and activate phospholipase C and increase cytoplasmic Ca<sup>2+</sup>. EP2 and EP4 receptors are coupled to G<sub>s</sub> proteins, which activate adenylate cyclase leading to a rise in cAMP and subsequent protein kinase A activation. EP3 receptors are coupled to G<sub>i</sub> proteins and activate phosphodiesterases that decrease cAMP, contributing to the resetting of the signaling<sup>[5]</sup>. In addition to this, PGs may control gene transcription through the activation of nuclear receptors of the peroxisome proliferators-activating receptor family<sup>[6]</sup>.

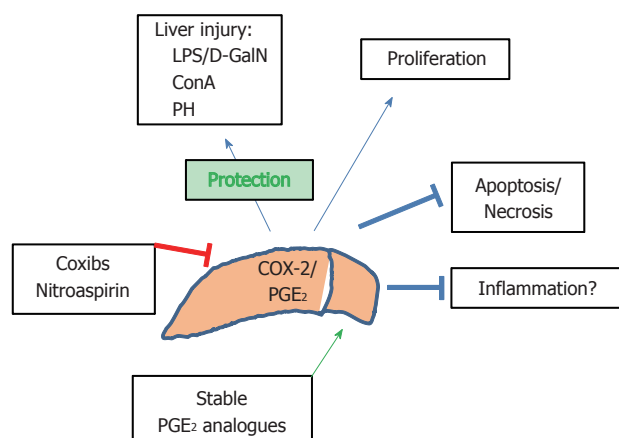
Two COX isoenzymes have been identified: COX-1, first purified from bovine vesicular glands, is ubiquitous and constitutively expressed in a wide variety of tissues where it accounts for the low and continuous PG synthesis required for tissue homeostasis; COX-2 is undetectable in tissues under normal conditions. Only few tissues have a constitutive COX-2 expression (*i.e.*, placenta, testes, kidney and some neural tissues).

However, a variety of extracellular and intracellular stimuli (inflammation, growth factors, hormones, reactive oxygen intermediates and oncogenes) can rapidly induce COX-2 expression in many cell types. Both COX isoenzymes share 61% primary sequence identity and exhibit similar kinetic properties with minimal biochemical differences. Despite the structural and kinetic similarities between COX-1 and COX-2, these close related enzymes carry out very different functions in tissues and organs due to their specific promoters, genes and mRNAs<sup>[7,8]</sup>. The second key enzyme that couples with COXs for the production of PGE<sub>2</sub> is PGE<sub>2</sub> synthase (PGES). Several isoforms of PGES have been characterized with specific enzymatic properties, cellular distribution and biochemical roles. Two main classifications for PGES have been defined: cytosolic (cPGES), and microsomal/membrane associated (mPGES). cPGES is identical to the Hsp90-associated protein 23. It is constitutively expressed in most cell types and is predominantly coupled with COX-1. However, the membrane associated mPGES is an inducible enzyme that is upregulated during inflammatory conditions. It has recently been shown that mPGES-1 is closely associated to the pathological expression of COX-2. Indeed, mPGES-1 deficiency reduces PGE<sub>2</sub> synthesis and, in turn, attenuates tumor proliferation and invasion of several tumor cells<sup>[9]</sup>.

The levels of PGE<sub>2</sub> are the result of the fine tuning between the synthesis by mPGES and cPGES, and the degradation that is mainly due to the 15-hydroxyprostaglandin dehydrogenase (15-PGDH). 15-PGDH catalyzes the NAD-dependent oxidation of PGE<sub>2</sub> (at C15) to generate the inactive PG 15-keto-PGE<sub>2</sub>. Animals lacking 15-PGDH exhibit an increase in tissue levels of PGE<sub>2</sub>. Moreover, despite a physiological action of 15-PGDH in parturition and in the uterine tract, recent data propose a prominent role for 15-PGDH in oncologic processes<sup>[10]</sup>.

## COX-2 IN LIVER PATHOLOGY

Hepatocytes express receptors for most of the stimuli that induce COX-2 transcription in other cell types, including LPS, IL-1 and TNF-α. However, adult, but not fetal or neonatal hepatocytes do not express COX-2 in response to these pro-inflammatory molecules; only Kupffer, stellate and some hepatoma liver cells (not all) exhibit the capacity to express COX-2. In the case of hepatocytes, only under long-term aggression, COX-2 is expressed due to the decrease in C/EBPα levels<sup>[11]</sup>. However, fetal hepatocytes, which contain negligible levels of C/EBPα compared to the adult counterparts, express COX-2 after pro-inflammatory challenge<sup>[12]</sup>. Interestingly, specific constitutive expression of COX-2 in liver protects against acute liver insults by combining an inhibition of apoptotic mechanisms in the hepatocytes, and promoting cell cycle progression and proliferation. To prove this, we analyzed several models of liver injury and compared the contribution



**Figure 1 Main regulation and effects of cyclooxygenase-2 in liver.** COX-2 and PGE<sub>2</sub> exert protection against many liver injuries and promote proliferation of hepatocytes and inhibition of apoptosis and necrosis of hepatic cells. The contribution to inflammation remains controversial depending on the moment of COX-2 expression.

of a COX-2 transgene (COX-2-Tg) under the control of a specific hepatocyte promoter. In this regard, in the lipopolysaccharide and D-galactosamine treated mice (LPS/D-GalN), in the concanavalin A (ConA)-induced hepatitis, and in the model of hepatocyte proliferation after partial hepatectomy (PH) the hepatic elevation of PGs due to the COX-2-Tg attenuates the injury induced by these stressors and accelerates proliferation after PH. Conversely, inhibition of COX-2 with a selective COXIB ablates these protective effects. Interestingly, constitutive COX-2 expression in the liver results in an elevation of antiapoptotic genes as well as in the activation of proteins involved in cell survival, such as phospho-Akt and phospho-AMP-kinase, after injury. Moreover, in the model of liver regeneration after PH, hepatocyte commitment to start replication is accelerated in COX-2-Tg mice due to the rapid elevation of PCNA, cyclin-D1 and E, all promoting cell cycle progression<sup>[13]</sup>. However, using a different COX-2-Tg animal model Han *et al.*<sup>[14]</sup> found that COX-2 expression notably enhanced the injury after LPS challenge. These contradictory results are of interest in order to stress the relevance of the genetic background in animal studies. In fact, the molecular mechanisms described in our case for the protection, but also the opposite observation by the Han's group can be explained in view of this circumstance (C57BL/6 vs C57BL/6XDBA)<sup>[15]</sup>. Indeed, the wild-type animals of Han's model did not showed a significant injury after LPS/D-GalN challenge as occurred in our C57BL/6XDBA animals that displayed an acute apoptotic response, in the line reported by other groups using this injury model<sup>[15]</sup>. Figure 1 summarizes these data.

## COX-2 AND NAFLD: NAS, NASH AND FIBROSIS

Non-alcoholic fatty liver disease (NAFLD) is defined as

a broad clinical pathological entity that appears in the absence of alcohol abuse, but involving fat deposition in the hepatocyte (steatosis, NAS), and worsening to non-alcoholic steatohepatitis (NASH) and fibrosis, all conditions contributing to liver failure and in some cases, to hepatic carcinogenesis. NAFLD is recognized as the hepatic manifestation of metabolic syndrome and constitutes an important health problem that affects one-third of adults and an increasing number of children in developing countries<sup>[16]</sup>. The pathological definition of metabolic syndrome includes obesity, diabetes, dyslipidemia and hypertension among other symptoms. Around 90% of NAFLD patients have at least one symptom of metabolic syndrome and about 33% have this full canonical profile. Although NAFLD is strongly associated with hyperlipidemia, diabetes mellitus, metabolic syndrome, obesity and insulin resistance (IR), its pathogenesis remains poorly understood and therapeutic options other than lifestyle modification by diet and exercise are limited.

Steatosis is defined as the presence of cytoplasmic TG droplets in more than 5% of the hepatocytes and is the result of an imbalance between the import and/or synthesis of fatty acids by hepatocytes and the rate of usage or export, leading to the formation of the characteristic lipid droplets. Hepatic steatosis (NAS) is the first manifestation of NAFLD, and is identified by the accumulation of triglycerides (TG) as lipid droplets in the cytoplasm of hepatocytes. NAS is often limited and reversible, but it can progress to chronic hepatic inflammation, insulin resistance, liver damage and NASH. A major issue is whether the progression to NAFLD is the cause or the consequence of IR<sup>[17]</sup>. In this regard, few studies determining hepatic IR at the gene expression level have been performed in NAFLD patients. However, the data available suggest that the insulin signaling pathway, using phospho-Akt and the transcription factor phospho-FoxO1 as read-outs, shows an increase in NASH patients compared to healthy liver<sup>[18]</sup>. However, recent results from our group demonstrate that hepatic insulin signaling is decreased in NASH patients, and this process is associated with higher apoptotic rates and enhanced collagen deposition. Moreover, IR was not detected in NAS patients. Together, these data suggest that hepatic insulin signaling is preserved in NAS, at the time that point to fact that hepatic lipid overload precedes the impairment of hepatic insulin signaling<sup>[17]</sup>.

The main difference between NAS and NASH is the occurrence of hepatocyte injury, including hepatocyte ballooning and increased cell death, infiltration of circulating inflammatory cells and enhanced parenchymal collagen deposition as morphologic signature of fibrosis. Intralobular inflammation in NASH includes the presence of a small number of lymphocytes, macrophages and neutrophils<sup>[18]</sup>. Inflammation in NASH involves the contribution of both parenchymal and non-parenchymal cells through the release of bioactive soluble mediators that finally



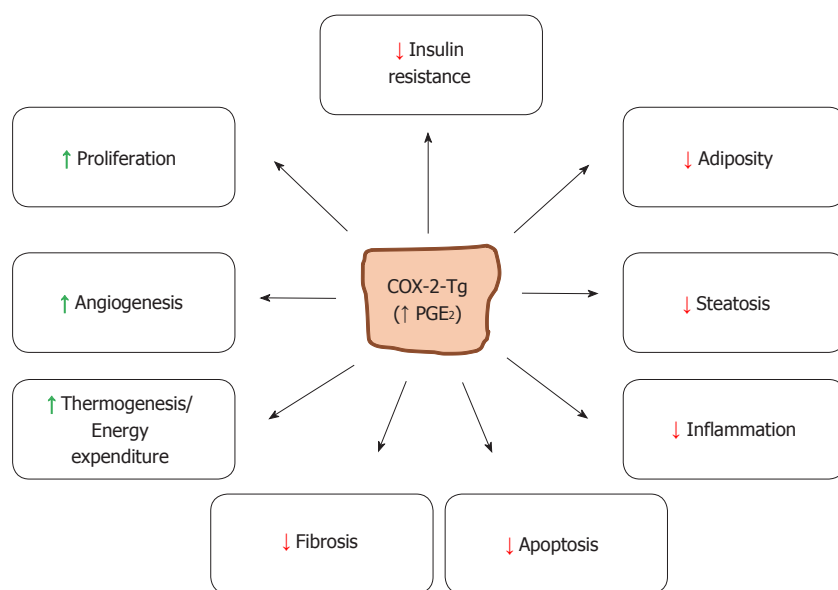
favor the recruitment of lymphoid and myeloid cells in the liver. Activated Kupffer and stellate cells contribute to cytokine expression during steatohepatitis. Among the pro-inflammatory cytokines involved in the progression to NASH, TNF- $\alpha$  and IL-6 seem to be very relevant<sup>[19]</sup>. Liver cells are also a target for adipose tissue generated factors. More specialized adipokines, such as adiponectin and leptin are also involved. Adiponectin was shown to block TNF- $\alpha$  activation of inflammatory genes, to decrease macrophage recruitment and function and to increase the synthesis of the anti-inflammatory cytokines IL-10 and IL-1Ra, displaying a beneficial effect on NAFLD in mice. Leptin appears to be a key factor in the formation of hepatic droplets due to a polarization in body fat distribution. Beside inflammation, stress signals, including oxidative stress and lipid peroxidation, lead to hepatocyte injury. Apoptotic cell death may also constitute an important component of disease progression. Oxidative stress appears to be a key factor in the pathogenesis of NASH as deduced by a significant increase of oxidative damage markers for lipids, proteins and DNA (MDA, 4-HNE, nitro-tyrosine, and 8-OH-dG), as well as a decrease in the antioxidant capacity, including a reduction in catalase and glutathione reductase and a rise the GSSG/GSH ratio<sup>[20]</sup>. Despite the high antioxidant capacity of normal liver, hepatocytes sense oxidative stress as reflected by higher rates of cell death both by necrosis and apoptosis. Our results indicated that NASH, but not NAS hepatic samples, have lesser mRNA levels of Mcl-1 and Bcl-2. As a consequence of this, the active content of caspase 3 and apoptosis were higher in NASH than in normal liver and in NAS patients<sup>[17]</sup>. NASH, in turn, can progress to fibrosis and cirrhosis. Hepatic stellate cells (HSCs) are the main contributors to extracellular matrix deposition and fibrosis as result of its activation in conditions of liver injury. Again, our data demonstrated an increase in the hepatic mRNA levels of *COL1A* in NASH and no changes in steatosis vs normal liver. In cirrhosis, HSCs are responsible of the type I collagen scar that replaces dying hepatocytes. Cirrhosis can ultimately progress to liver cancer; 4%-27% of individuals with NASH-induced cirrhosis develop hepatocellular carcinoma (HCC).

Regarding COX-2 on these lipid and metabolic disorders, whereas some studies indicate that PGs may favor fat accumulation in hepatocytes and hence the progression from NAS to NASH, others provide evidence where PGE<sub>2</sub> suppresses *de novo* lipogenesis. Therefore, the impact of PGE<sub>2</sub> on the insulin-dependent changes in hepatic metabolism is controversial: Hsieh *et al*<sup>[21]</sup> reported that rats fed a fructose or high fat diet (HFD) and treated with COX-2 inhibitors improved muscle and fat IR<sup>[22]</sup>; however, in a different context Coll *et al*<sup>[23]</sup> reported that COX-2 inhibition exacerbates palmitate-induced inflammation and IR in skeletal muscle. There are also reports using murine models of NASH induced by high fat or methionine and choline-

deficient (MCD) diet describing an increase in COX-2 expression and the beneficial effects after celecoxib or nitro-aspirin treatment<sup>[24]</sup>. The interaction between PGE<sub>2</sub>, IL-6 and the IR in hepatocytes has been studied by Henkel *et al*<sup>[25,26]</sup> reporting that PGE<sub>2</sub> enhanced fat accumulation and interrupted the intracellular signaling of insulin in hepatocytes through serine phosphorylation of IRS *via* EP3-receptor-dependent ERK1/2 activation. However, using COX-2-Tg mice we observed a protective role for COX-2 in liver injury induced by hyperglycemia in a streptozotocin diabetic mouse involving an increase in PI3K/Akt/AMPK survival pathway<sup>[27]</sup>. Recently, COX-2 was linked to cold-induced thermogenesis and this COX-2 activity in adipose tissue well correlates with the expression of specific differentiation markers of brown adipose tissue. Furthermore, COX-2 expression in white adipose tissue increased systemic energy expenditure and protected mice against HFD induced obesity<sup>[28]</sup>. Also, mice carrying a COX-2-Tg in hepatocytes are protected vs IR, adipose tissue proliferation/differentiation and low-grade inflammation when fed HFD. In these COX-2-Tg mice, PGs are associated with increased systemic energy expenditure, higher thermogenesis, and enhanced fatty acid oxidation in the liver, favoring lipid clearance in the hepatocyte<sup>[29]</sup>. In addition to this, COX-2 represses specific miRNAs. One of them is miR-183, present in liver cells and that is repressed after COX-2 expression and/or in COX-2-Tg hepatocytes. Our group has demonstrated that this decrease in miR-183 is important in the preservation, and even potentiation of the insulin signaling pathway after different hepatic challenges<sup>[30]</sup>. Accordingly, COX-2 appears as an unexpected potential therapeutic strategy against obesity-associated metabolic dysfunction. In this regard, stable analogs of PGE<sub>2</sub>, such as 16,16dmPGE<sub>2</sub>, could be considered as therapeutic alternatives to prevent steatosis progression and/or IR. In fact, PGE<sub>2</sub> and analogs are clinically used to ameliorate mild hypercholesterolemia among other liver pathologies<sup>[31,32]</sup>.

The metabolic signals governing the transition from NAS to NASH are not well defined; however, the increase of lipid content in the hepatocyte contributes to an enhancement of oxidative stress, cell death and to create a low-grade pro-inflammatory ambience that sustains the initial lipid stress<sup>[33]</sup>. Again, the impact of PGE<sub>2</sub> on this sequence from NAS to the establishment of NASH and the subsequent hepatic fibrosis remains controversial: *in vivo* studies in mice using selective COX-2 inhibitors showed a prevention in the progression to NASH, clearly pointing to COX-2-dependent PGs as mediators of the progress<sup>[34,35]</sup>. However, the opposite approach, that is administration of PGE<sub>2</sub>, has been show to prevent HSC-dependent fibrogenesis and steatohepatitis<sup>[22,36,37]</sup>. Furthermore, Cheng *et al*<sup>[38]</sup> by using a liver COX-2-Tg mice (again in a genetic background distinct from C57BL/6), failed to show any contribution of these locally produced PGs to





**Figure 2** Mice carrying a COX-2 transgene in hepatocytes have elevated PGE<sub>2</sub> and are protected against hepatic insults. PGE<sub>2</sub> produced by hepatic COX-2 has specific effects in liver against inflammation, steatosis, fibrosis and apoptosis. At the systemic level protection against insulin resistance and adiposity is observed, whereas angiogenesis and thermogenesis are enhanced.

NASH progression and steatohepatitis. In this regard, it is relevant to mention that PGE<sub>2</sub> impairs the expression of pro-fibrogenic genes in human fat explants from obese individuals as well as antagonizes the TGF- $\beta$ -dependent fibrogenic activity in adipocytes<sup>[39]</sup>. In line with this, COX-2-Tg mice fed MCD diet are protected against NASH when compared with the Wt counterparts, essentially through a mechanism that involves a lesser recruitment of circulating immune cells and, as a consequence, a minor presence of pro-inflammatory factors in the liver, resulting in a minor activation of the pro-fibrogenic cells present in the tissue, such as HSCs. Furthermore, these COX-2-Tg mice treated with a classic pro-fibrogenic insult as is CCl<sub>4</sub> exhibited a significant protection against fibrosis progression as reflected by lower synthesis of hepatic collagen and accumulation of hydroxyproline when compared with the Wt controls<sup>[40]</sup>. Figure 2 summarizes the main findings observed in the animal models carrying a COX-2 transgene.

## COX-2 AND HCC

Hepatocellular carcinoma (HCC) is one of the most common solid cancers (30% of morbidity) and is very prevalent in cirrhotic patients. The incidence of HCC is increasing worldwide associated to hepatitis C virus (HCV) infection<sup>[41,42]</sup>. Moreover, hepatitis B virus (HBV) infection, and ambient risk factors (*i.e.*, aflatoxin, alcoholic cirrhosis, *etc.*) contribute to HCC initiation and progression. Clinical diagnosis of HCC is difficult since no reliable serum markers have been clearly identified, and the therapeutic options for HCC are limited (*i.e.*, sorafenib, cis-Pt, *etc.*). Despite the recent introduction of potent preventive chemotherapeutic

protocols the study of the molecular mechanisms leading to human hepatocarcinogenesis remains an area of intense research in order to understand the progression from NASH and cirrhosis to HCC. Gene expression profiling and proteomic approaches have contributed to identify specific signatures that can be used for the identification of proteins that are differentially expressed between normal and liver tumors in an attempt to define new and more selective biomarkers for HCC. Integrative transcriptome analysis reveals three molecular HCC subclasses, each correlated with different clinical parameters and serum markers<sup>[42]</sup>. Like in other cancers, the key event driving liver carcinogenesis is the development of simultaneous deregulated proliferation and reduced cell death. Work by different groups have characterized molecular signatures implicated in tumorigenesis: (1) receptor tyrosine kinase pathways; (2) Wnt/ $\beta$ -catenin signaling pathway; (3) ubiquitin/proteasome degradation pathway; (4) epigenetic DNA methylation and histone deacetylation pathways; (5) the PI3K/Akt/mTOR pathway; (6) angiogenic pathways; and (7) telomerase activity<sup>[43]</sup>.

Regarding the involvement of COX-2-derived PGs in hepatocarcinogenesis it should be mentioned that selective COX-2 inhibitors have proved to inhibit HCC cell growth *in vitro* and in xenograft animal models. As previously mentioned, COX-2 expression in liver appears to be restricted to very special conditions. In fact, COX-2 is transiently expressed in regenerating liver after PH or after challenge with potent hepatotoxic molecules, such as thioacetamide among other<sup>[44,45]</sup>. In addition to this, COX-2 expression has been observed in animal models of cirrhosis, in human and several (but not all) mouse hepatoma cell lines, after HBV or HCV

infection and in human HCC<sup>[46-50]</sup>. However, although COX-2 expression is detected in early phases of HCC, contradictory observations have been published regarding the nature of the cells expressing the enzyme (both in normal hepatocytes and in hepatoma cells), the role of this expression pattern and the molecular mechanisms by which COX-2-dependent PGs contribute and/or induce tumorigenesis. Interestingly, work from our group<sup>[51]</sup> has shown that COX-2 expression is not sufficient to exacerbate malignant transformation after administration of chemical hepatocarcinogens. Even more, progression of liver oncogenesis in a well-established model of HCC (the c-myc and TGF- $\alpha$  double transgenic mice), is not affected by COX-2 expression<sup>[52]</sup>. However, COX-2 expression in this model facilitated the development of preneoplastic foci but failed to promote malignant transformation, probably as result of the contribution of COX-2-derived PGs to inhibit apoptosis and to provide an anti-inflammatory environment, these conditions opposing the initiation of the early phases of HCC. Using COX-2-Tg hepatocyte cell lines we also showed elevated oxidative stress and ROS accumulation after chemical hepatocarcinogenesis, together with an important decrease in the levels of GSH and higher levels of 8-OHdG. Moreover, a moderate activation of JNK, Erk and p38 was detected in COX-2-Tg cells, and COX-2 favored the growth of cell implants in nude mice, probably through the sustained activation of Akt and JNK-c-Jun survival pathways. Recently, it has been shown that PGE<sub>2</sub> is able to increase c-Myc levels through the activation of the EP4R/GS/AC/cAMP/PKA/CREB cascade that favors HCC growth and invasion of these cells, contributing PGE<sub>2</sub> in this way to hepatocellular carcinogenesis<sup>[53]</sup>.

Genetic deletion of 15-PGDH, the enzyme which degrades PGE<sub>2</sub> to an inactive 15-keto-PGE<sub>2</sub>, leads to increased tissue levels of PGE<sub>2</sub>. We have shown that 15-PGDH is repressed in human HCC cell lines, together with elevation of COX-2, in chemical and genetic murine models of HCC and in human HCC biopsies. Moreover, transfection of HCC cells with 15-PGDH induces apoptosis and attenuates the growth of these cells when implanted in nude mice; at the same time, transfection with siRNA specific for 15-PGDH promoted tumor growth indicating that the balance between COX-2 and 15-PGDH activities are relevant in the hepatocarcinogenic process<sup>[54]</sup>. Interestingly, 15-PGDH not only plays a protective role by decreasing PGE<sub>2</sub> levels, but also the 15-keto-PGE<sub>2</sub> generated has been shown to activate PPAR $\gamma$ . Indeed, activated PPAR $\gamma$  by 15-keto-PGE<sub>2</sub> favors the interaction with the p21(WAF1/Cip1) promoter, which in turn results in p21 expression and association with cyclin-dependent kinase 2 (CDK2), CDK4 and PCNA<sup>[55]</sup>. Altogether, the results of higher pulmonary metastatic incidence of HCC in COX-2-Tg mice and the promotion and migration of HCC cells induced by PGE<sub>2</sub><sup>[48]</sup> suggest that COX-2 might be involved in the expansion

and metastatic phase of HCC. COX-2 exerts pro-metastatic effects on cancer stem cells mediated partly through regulation of PDCD4 and PTEN expression<sup>[56]</sup>. Moreover, PGE<sub>2</sub> could upregulate the expression level of Snail, an inducer of epithelial-mesenchymal transition a key player in HCC invasion and metastasis, through the EP2/Src/EGFR/Akt/mTOR pathway<sup>[57]</sup>.

Besides the murine models, we have investigated whether a correlation exists between COX-2 expression and different grades of methylation at the 5' region of the COX-2 gene in hepatoma cell lines and in HCC. We also analyzed the acetylation signatures of the COX-2 promoter and the effects of inhibitors of histone deacetylase (HDAC) on COX-2 expression. Our results indicate that the low COX-2 expression in some hepatoma cell lines and HCC is associated with promoter hypermethylation of COX-2. Histone deacetylation and treatment with demethylating agents or HDAC inhibitors restored the expression of COX-2<sup>[41]</sup>. Interestingly enough, COX-2 mRNA levels were higher in the non-tumoral liver tissue than in HCC; moreover, inverse correlations between COX-2 levels and the differentiation grade of HCC were observed. Clinical studies showed a reduction of survival in patients when COX-2 expression decreased due to promoter hypermethylation and histone H3 hypoacetylation. Indeed, Giannitrapani *et al.*<sup>[47]</sup> reported a dispersed range of COX-2 expression in human HCC, from absence of expression in undifferentiated areas to a robust expression in well differentiated tissue. Moreover, COX-2 expression was significantly lower in HCC than in NASH. However, a recent meta-analysis study revealed that the presence of COX-2 in HCC is associated with stages of decreased overall and disease-free survival and represents a worse prognosis<sup>[57]</sup>. Recent studies in different cancer cells point out to a COX-dependent tumor growth through evasion of immunity and tumor-promoting inflammation. Pre-clinical data indicate that inhibition of COX-2 in breast or colorectal cancer cells favors the activation of immune mechanisms against cancer cells, reinforcing the immunosuppressive role of PGs<sup>[58]</sup>. Moreover, since HSCs are important mediators of immunosuppression and in the progress of HCC, it is suggested that they contribute to HCC through the recruitment of immunosuppressive cells, mainly myeloid-derived suppressor cells and regulatory T cells, through a mechanism involving the COX-2-PGE<sub>2</sub>-EP<sub>4</sub> pathway<sup>[59]</sup>. As a summary of these data, controversy exists in the literature regarding the precise role of COX-2 and PGE<sub>2</sub> in the development of HCC.

## CONCLUSION

The assessment of the role of COX-2 in hepatic diseases, ranging from NASH to HCC, constitutes a field in which controversy exists probably because of the use of different experimental models and

specific temporary guidelines of the time-dependent contribution of COX-2-derived metabolites to the onset of these hepatic dysfunctions. Since potent and selective inhibitors of COX-2 exist, but also stable PGE<sub>2</sub> analogues to be used in therapy, unraveling the precise contribution of this enzyme and its products is crucial for the prevention of the progression of liver dysfunctions and it appears to be a useful approach for managing the patients. Efforts to identify biomarkers providing indications for the correct use of these therapeutic tools are essential to underline new intervention protocols based on COX-2 and prostaglandin targeting.

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## First quarter century of laparoscopic liver resection

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### Abstract

The beginnings of laparoscopic liver resection (LLR) were at the start of the 1990s, with the initial reports being published in 1991 and 1992. These were followed

by reports of left lateral sectionectomy in 1996. In the years following, the procedures of LLR were expanded to hemi-hepatectomy, sectionectomy, segmentectomy and partial resection of posterosuperior segments, as well as the parenchymal preserving limited anatomical resection and modified anatomical (extended and/or combining limited) resection procedures. This expanded range of LLR procedures, mimicking the expansion of open liver resection in the past, was related to advances in both technology (instrumentation) and technical skill with conceptual changes. During this period of remarkable development, two international consensus conferences were held (2008 in Louisville, KY, United States, and 2014 in Morioka, Japan), providing up-to-date summarizations of the status and perspective of LLR. The advantages of LLR have become clear, and include reduced intraoperative bleeding, shorter hospital stay, and - especially for cirrhotic patients - lower incidence of complications (*e.g.*, postoperative ascites and liver failure). In this paper, we review and discuss the developments of LLR in operative procedures (extent and style of liver resections) during the first quarter century since its inception, from the aspect of relationships with technological/technical developments with conceptual changes.

**Key words:** Hepatectomy; Laparoscopic surgery; Liver cancer; History; Technology; Technique; Concept; Approach; Posture; Simulation

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**Core tip:** Laparoscopic liver resection (LLR) was introduced in early 1990s. Thereafter, LLR procedures have expanded to left lateral sectionectomy, hemi-hepatectomy, sectionectomy, segmentectomy and partial resection of posterosuperior segments, as well as parenchymal preserving limited and modified anatomical resection. This expansion is related to technological/technical developments with conceptual changes. During this period, two international consensus conferences

summarized the up-to-date status and perspective of LLR. The current advantages of LLR include reduced intraoperative bleeding, shorter hospital stay, and lower incidence of complications. Here, we review and discuss the developments of LLR in operative procedures during the first quarter century since its inception.

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## INTRODUCTION

Although laparoscopes were first introduced in the 1960s as diagnostic tools for urological and gynecological diseases, a new technology to create pneumoperitoneum and the development of the charged-coupled device (CCD) camera, which magnifies and projects laparoscopic images onto television monitors, led to the first laparoscopic cholecystectomy performed in the current style in 1987 by Philippe Mouret of Lyon, France<sup>[1]</sup>. The procedure gained immediate acceptance according to its related clinical experiences of less pain and rapid recovery, in addition to the cosmetic advantages<sup>[2,3]</sup>. Since then, the field of laparoscopic surgery has expanded rapidly to include surgery for other abdominal organs and more complex and technically demanding abdominal surgery.

The beginnings of laparoscopic liver resection (LLR) were at the start of the 1990s, with the initial reports<sup>[4-6]</sup> published in 1991 and 1992. These were followed by the reports of left lateral sectionectomy (LLS)<sup>[7,8]</sup> in 1996. In the years following, the procedures of LLR were expanded to hemi-hepatectomy, sectionectomy, segmentectomy and partial resection of posterosuperior segments, as well as parenchymal preserving limited anatomical resection and modified anatomical (extended and/or combining limited) resection. This expanded range of LLR procedures, mimicking the expansion of open liver resection (OLR) in the past, was related to advances in both technology (instrumentation) and technical skill with conceptual changes (Table 1).

During this period of remarkable development, two international consensus conferences (ICLLR) were held (2008 in Louisville, KY, United States<sup>[9]</sup> and 2014 in Morioka, Japan<sup>[10]</sup>), providing up-to-date summarizations of the status and perspective of LLR. The anxieties over LLR-specific complications, including gas-embolism, were eased by the cautious application of these procedures to and the long-term outcomes of selected patients for LLR, which were confirmed as similar to those for OLR. The advantages of LLR became clearly established, in particular, reduced

**Table 1 Development of laparoscopic liver resection over the first 25 years**

Year	Procedure [Ref]	Related developments (technological, technical, conceptual)
1991	1 <sup>st</sup> report of LLR <sup>[4-6]</sup> (partial resection in AL)	
1996	LLS <sup>[7,8]</sup>	
1997	Hemi-hepatectomy <sup>[13-15]</sup>	Energy devices (coagulating, sealing, shearing) CUSA HALS <sup>[19,20]</sup> and hybrid <sup>[21,22]</sup> , Inflow control <sup>[17,18]</sup>
2000s-2010s	Sectionectomy (right posterior, right anterior, left medial)	Glissonian approach (extra <sup>[26]</sup> , intra-hepatic <sup>[27]</sup> ) Caudal approach <sup>[10,31]</sup> Postural change <sup>[29-31]</sup>
	Segmentectomy and partial resection of segments 7, 8, 1	Postural change <sup>[29-31]</sup> Caudal approach <sup>[10,31]</sup> Lateral approach <sup>[37-39]</sup> (intercostal port) Trascopic approach <sup>[40,41]</sup>
	Limited anatomical resection and modified anatomical (extended and/or combining limited) resection <sup>[48-51]</sup>	Simulation and navigation <sup>[46,47]</sup> 3D endoscope <sup>[45]</sup>

Ref: Reference number in the References section; LLR: Laparoscopic liver resection; AL: Anterolateral segments; LLS: Left lateral sectionectomy; CUSA: Cavitron ultrasonic surgical aspirator; HALS: Hand-assisted laparoscopic surgery; Hybrid: Laparoscopic-assisted LLR; 3D: Three-dimensional.

intraoperative bleeding, shorter hospital stay, and - especially for cirrhotic patients - lower incidence of complications (e.g., postoperative ascites and liver failure).

In this review of the developments of LLR in operative procedures (extent and style of liver resections) that have occurred during the first quarter century since its inception, we discuss the relationships of these advances in technological/technical aspects of LLR with conceptual changes.

## DEVELOPMENT OF LLR

### **Partial resection of anterolateral segments and LLS: The beginnings of LLR**

The initial reports of LLR by Reich *et al.*<sup>[4]</sup>, Katkhouda *et al.*<sup>[5]</sup>, Gagner *et al.*<sup>[6]</sup> appeared in 1991 and 1992. These were followed by reports of LLS by Azagra *et al.*<sup>[7]</sup> and Kaneko *et al.*<sup>[8]</sup> in 1996. Although segment level Glissonian pedicles and thick hepatic veins should be divided in LLS, the lesions located in the anterolateral segments (segments 2, 3, 4b, 5, 6) are more accessible laparoscopically than those in the posterosuperior segments (1, 4a, 7, 8). Also, the relatively small transection plane of LLS lies in a caudal-to-cranial direction and is vertical when the patient is in supine position, making it easier to handle in the natural laparoscopic view and to access with ports below the costal-arch level. Therefore, LLS is

a big partial resection of anterolateral segments in some aspects; indeed, the first development of the LLR procedure involved anterolateral partial resection to LLS. LLS is the most straightforward sectionectomy procedure, as in OLR, and the standardization of this procedure has emerged recently as a topic of considerable discussion<sup>[11,12]</sup>.

### **Hemi-hepatectomy and feasibility studies**

The first report of hemi-hepatectomy was in 1997 by Hüscher *et al*<sup>[13]</sup>, just 1 year after the LLS reports. The transection plane of hemi-hepatectomy, like the one in LLS, lies in the caudal-to-cranial direction and is vertical in supine position, making it easier to handle *via* the laparoscopic approach. Hemi-hepatectomies are the second-most straightforward procedure, again as in OLR, after anterolateral partial resection and LLS<sup>[14,15]</sup>. However, stable transection maneuvers were required in this step of development, since the transection plane in hemi-hepatectomies is a large area. Advances in technologies and instrumentation contributed to the step<sup>[16,17]</sup>. In the early stage of LLR development, pre-transectional coagulation *via* coagulating energy devices proved important in reducing the possibility of intra-operative massive bleeding. Development of transection maneuvers that mimic open maneuvers, such as crash-clamp transection and Cavitron ultrasonic surgical aspirator (CUSA; or its equivalent) transection, was accomplished by adaptation of various energy devices (to achieve coagulation, sealing and shearing) and laparoscopic CUSA, accompanied by inflow control<sup>[17,18]</sup>. Differences exist between the right and left hemi-hepatectomy forms of the major hepatectomies, these specifically involve mobilization of the liver and handling of the caudate lobe and IVC. The mobilization procedure for the left liver is relatively straightforward, except for the dissection of the roots of the middle and left hepatic veins. Also, when it is performed without resection of the Spiegel lobe, there is no need for dissection of the IVC. On the other hand, the right hemi-hepatectomy is usually performed with resection of the para-caval caudate lobe and, therefore, necessitates dissection of the IVC and right adrenal gland. During mobilization of the right liver, handling of the heavy and large-volume right liver is also much more demanding, complicating the laparoscopic surgical procedure which occurs without the surgeon's hands being present in the operative field. As such, the procedure of laparoscopic right hemi-hepatectomy has developed more slowly than that of left<sup>[13-15]</sup>.

During this and the next step of development, the hand-assisted procedure and hybrid (laparoscopic-assisted) procedure helped to reduce the technical difficulty of LLR in pure laparoscopic setting<sup>[19-22]</sup>. Also during this step of development, an encouraging feasibility study of LLR - including left hemi-hepatectomy, LLS, segmentectomy and partial

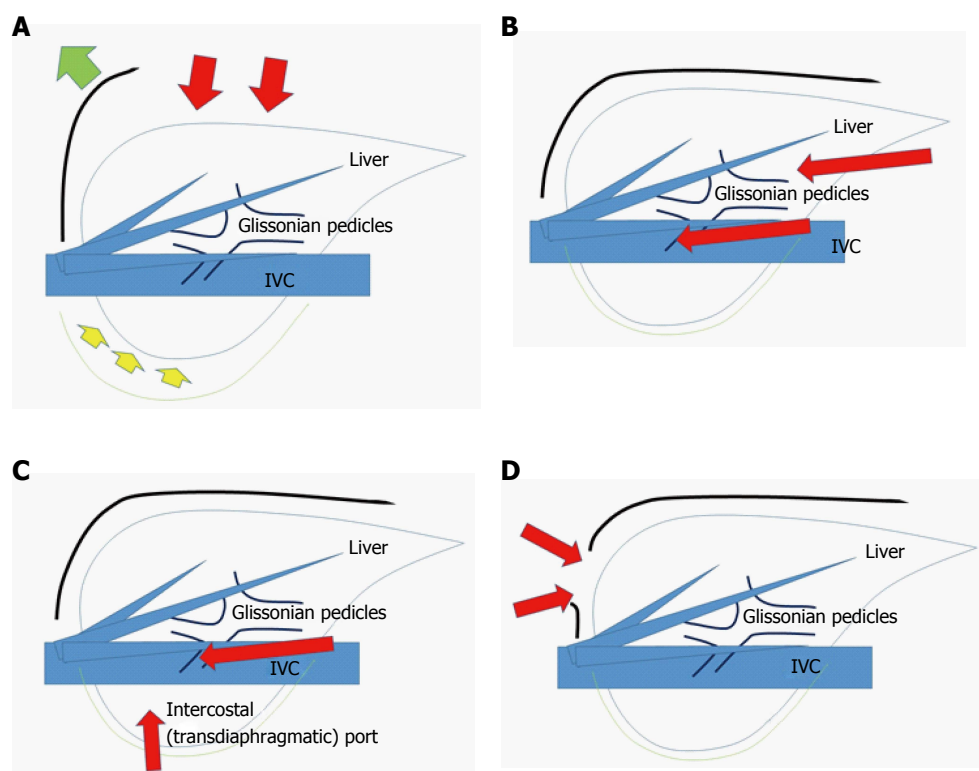
resection of segments 3, 4, 5, 6 - was reported by Cherqui *et al*<sup>[23]</sup> in 2000. This report concluded, "Laparoscopic resections are feasible and safe in selected patients with left-sided and right-peripheral lesions requiring limited resection."

### **Left medial, right anterior and posterior sectionectomies**

In the summary paper from the first ICLLR<sup>[9]</sup>, LLR was divided into the following three categories: I, small wedge resections; II, resections of the left lateral section or anterior segments (4b, 5, 6); III, hemi-hepatectomies, trisectionectomies and resections of posterior segments (4a, 7, 8). Category III was referred to as "major LLR". The section on major LLR in this summary paper concluded, "Major LLR have been performed with safety and efficacy equaling OLR in highly specialized centers." Also, in the section on hepatocellular carcinoma (HCC) treatment and LLR in this summary paper, anatomic segmental resection was recommended, instead of non-anatomical partial resection, due to the related lower rates of local recurrence. Especially for those patients with HCC and chronic liver diseases (CLDs), laparoscopic left medial, right anterior and right posterior sectionectomies were recommended as the next-step procedures after hemi-hepatectomy, in order to accomplish the preservation of residual liver function and to maintain oncological efficacy equal to that of OLR<sup>[24]</sup>.

The transection planes in sectionectomies are larger in area and more difficult to handle than those in hemi-hepatectomies. Also, hilar dissection with individual vessel preparation for processing territorial vessels cannot be performed in this level. Although the Glissonian approach has been employed for hemi-hepatectomy<sup>[25]</sup> alongside hilar dissection with individual vessel preparation, the importance of the Glissonian approach is greater in sectionectomies and in more limited anatomical resections. Both extrahepatic<sup>[26]</sup> and intrahepatic<sup>[27]</sup> laparoscopic Glissonian approaches have been reported and employed widely, as in OLR, for this step in the development of LLR.

On the other hand, handling of the transection plane-especially the border between the anterior and posterior sections - is one of the key obstacles for right anterior and posterior sectionectomies<sup>[28]</sup>. Since the liver is located in the subphrenic rib cage, in OLR, surgeons open the subphrenic cage with a large subcostal incision and lifting-up of the costal arch; after which, the surgeon dissects the retro-peritoneal attachments and physically picks-up the liver with his/her left hand in order to manipulate the intact organ (Figure 1A). However, in LLR, there are no instruments as good as the surgeon's left hand and, moreover, no anterior space available without abdominal wall incision. Therefore, laparoscopic right anterior and posterior sectionectomies are technically demanding to obtain a fine surgical field that will ensure hemostasis and an appropriate surgical margin in handling the



**Figure 1** Schema of open liver resection (A), laparoscopic liver resection (regular caudal approach, B), laparoscopic liver resection (lateral approach, C) and thoracoscopic liver resection (D). Red arrows indicate the directions of view and manipulation in each approach. A: In the open approach, the subcostal cage containing the liver is opened with a large subcostal incision and instruments are used to lift the costal arch, after which the liver is dissected and mobilized (lifted) from the retroperitoneum; B: In the regular laparoscopic caudal approach, the laparoscope and forceps are placed into the subcostal cage from the caudal direction, and the surgery is performed with minimal alteration and destruction of the associated structures; C: In the laparoscopic lateral approach, the intercostal (transdiaphragmatic) ports combined with total mobilization of the liver from the retroperitoneum can allow the direct lateral approach into the cage and to the posterosuperior tumors; D: Thoracoscopic approach is employed for lesions in segment 8, with direct exposure of the tumor into the pleural cavity upon incision on the diaphragm adjacent to the tumor, with the endoscope placed in the pleural cavity.

transection plane beneath the large and heavy right liver in the small subphrenic rib cage.

Postural changes have been employed to conquer this obstacle. Semi-prone<sup>[29,30]</sup> and left lateral<sup>[31]</sup> position LLR were reported as capable of allowing for acquirement of fine surgical view and manipulation for sectionectomies in the right liver. Also, a paper on lateral position posterior sectionectomy published by our group<sup>[31]</sup> described the new concept of “caudal approach in LLR” (Figure 1B); in this approach, the laparoscopic specific view and manipulation access is made from the caudal direction, using ports entering below the costal-arch level and going into the subphrenic rib cage. The summary paper of the second ICCLLR<sup>[10]</sup>, explains this concept as follows: “The caudal approach, which relies on visual magnification, offers improved exposure around the right adrenal gland and the vena cava and greatly facilitates identification of the Laennec’s capsule and the Glissonian pedicle at the hilar plate.”

### Segmentectomies and partial resections of segments 7, 8 and 1

Although LLS, segmentectomies for segments 5 and 6, and left medial sectionectomy (segment

4 segmentectomy) had been performed in the earlier stage of LLR development, segments 7, 8 and 1 remained unresolved challenge areas for segmentectomy and even for partial resection<sup>[32,33]</sup>.

In LLR, and distinctive from OLR, more sectionectomies or right hepatectomies have been performed than segmentectomies or partial resections as treatment of tumors involving segments 7 and 8<sup>[9,34-36]</sup>. This trend can be explained by the fact that the straightforward transection plane of the liver, from caudal edge to the diaphragm in right hepatectomy or posterior sectionectomy, is more easily handled in LLR. In the laparoscope view from the caudal direction, the transection planes of segment 7 and 8 segmentectomies or partial resections are located in the deep small subphrenic space behind the liver, with segments 5 and 6 acting as physical obstacles to the lesions. Since surgeons need to create a precisely curved or angulated transection plane in the space, the parenchymal preserving segmentectomies or partial resections of the area are technically more difficult than performance of a posterior sectionectomy or right hepatectomy.

Adequate functional reserve of the liver after resection is as important as oncological efficacy,



especially in impaired livers, as encountered in CLD patients with HCC<sup>[24]</sup>. An important consideration for LLR of this area, therefore, is how to obtain good and stable access that allow for sufficient and safe handling of the liver and tumors, so that a well-visualized transection plane can be acquired. To this end, intercostal (transdiaphragmatic) ports with total mobilization of the liver from the retroperitoneum have been applied to facilitate the direct lateral approach into the rib cage (in the abdominal cavity) and to segment 7 (Figure 1C)<sup>[37-39]</sup>. In addition, the thoracoscopic approach was employed for lesions in segment 8 (Figure 1D), with direct exposure of the tumor into the pleural cavity being achieved by incision on the diaphragm adjacent to the tumor<sup>[40,41]</sup>. Endoscopes have been placed in the abdominal cavity for the lateral approach using intercostal ports (Figure 1C), and in the pleural cavity for the thoracoscopic approach (Figure 1D).

On the other hand, postural changes, such as semi-prone positioning for tumors located in segment 7<sup>[28-30]</sup>, have also been applied to solve the same problem. Although segment 7 is located in the bottom of the abdominal cavity when the patient is in supine position, that same area is located almost on the top of the abdominal cavity when the patient is in semi-prone position. Adapting those postural changes allows for the weight of the liver itself to facilitate its own mobilization, ultimately providing a good and stable surgical space above the liver. Ikeda *et al.*<sup>[42]</sup> applied semi-prone position LLR with the use of intercostal ports to treat tumors in the anterosuperior and posterior segments.

There are still only a few reports, all with small numbers of cases, for laparoscopic isolated resection of the caudate lobe<sup>[33,43,44]</sup>. Although further experiences are needed for segment 1 LLR, especially for the total isolated resection of caudate lobe (Spiegel lobe, caudate process and paracaval portion), the fine laparoscopic caudal view to the vena cava area and behind the hilar plate, particularly from the left side with the incision on the gastro-hepatic ligament, could facilitate LLR for this area<sup>[44]</sup>.

## FUTURE PERSPECTIVES OF LLR

During the development of the LLR procedures, disadvantages of LLR have also been recognized. The lack of three-dimensional (3D) view was overcome by the development of the 3D laparoscope<sup>[45]</sup>. However, the lack of overview in the operative field (despite the local fine magnified view) combined with the lack of tactile sensation easily leads to disorientation on the perspective of the organs, tumors and the intrahepatic structures during LLR. Therefore, intraoperative laparoscopic ultrasonography and preoperative simulation/intraoperative navigation using reconstruction of preoperative imaging scans and the intraoperative implementation of near-

infrared fluorescence scans with indocyanine green have become more important and are continued to be developed<sup>[46,47]</sup>. Based on the development of the imaging techniques, parenchymal preserving limited anatomical resection and modified (extended and/or combining limited) anatomical resection are advocated<sup>[48-51]</sup>. Robotic-assisted LLR holds the promise of facilitating a more precise surgery in certain situations, such as bile duct reconstruction<sup>[47,52,53]</sup>.

On the other hand, there are specific advantages in LLR, besides those advantages common to all laparoscopic surgeries. For one, improved direct exposure with magnification could be obtained under the laparoscopic specific view to the liver inside the rib cage. This allows clearer access to the surgical field without the destruction of the surrounding environments, such as collateral vessels in patients with HCC and liver cirrhosis, and without inducing compression damage on the liver parenchyma<sup>[54,55]</sup>. Pneumoperitoneum pressure during laparoscopic surgery could reduce the amount of bleeding from the hepatic vein concomitantly with inflow control<sup>[54]</sup>. This creates a very dry surgical field, with clear visualization of the detailed internal structures of the liver. After the second ICCLR in Morioka (2014), two important studies using propensity score analysis on about 5,000 patients' data were published, and both showed the short-term benefits of LLR without deteriorating long-term results, compared with open procedure<sup>[56,57]</sup>.

In the context of these advantages, several endeavors have now been attempted with the aim of increasing the adoption rate of LLR to clinical practice. To help ensure the safe and consistent extended application of the procedure, studies aimed at determining the learning curve of LLR were published<sup>[58,59]</sup> and a difficulty scoring system<sup>[60]</sup> (*i.e.*, calculated according to tumor condition, resection style and liver condition) for the appropriate selection of the patient according to the surgeon's skill set was proposed in the second ICCLR<sup>[10]</sup>. Randomized clinical trials are underway and two have been completed<sup>[61,62]</sup>, and registries have been started in several nations and areas<sup>[63,64]</sup>. It is likely that LLR will become a more standardized procedure with wider application in the second quarter century based upon the experiences in the first quarter century.

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## Hepatitis A virus infection and hepatitis A vaccination in human immunodeficiency virus-positive patients: A review

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### Abstract

Hepatitis A virus (HAV) is one of the most common infectious etiologies of acute hepatitis worldwide. The virus is known to be transmitted fecal-orally, resulting in symptoms ranging from asymptomatic infection to fulminant hepatitis. HAV can also be transmitted through oral-anal sex. Residents from regions of low endemicity for HAV infection often remain susceptible in their adulthood. Therefore, clustered HAV infections or outbreaks of acute hepatitis A among men who have sex with men and injecting drug users have been reported in countries of low endemicity for HAV infection. The



duration of HAV viremia and stool shedding of HAV may be longer in human immunodeficiency virus (HIV)-positive individuals compared to HIV-negative individuals with acute hepatitis A. Current guidelines recommend HAV vaccination for individuals with increased risks of exposure to HAV (such as from injecting drug use, oral-anal sex, travel to or residence in endemic areas, frequent clotting factor or blood transfusions) or with increased risks of fulminant disease (such as those with chronic hepatitis). The seroconversion rates following the recommended standard adult dosing schedule (2 doses of HAVRIX 1440 U or VAQTA 50 U administered 6-12 mo apart) are lower among HIV-positive individuals compared to HIV-negative individuals. While the response rates may be augmented by adding a booster dose at week 4 sandwiched between the first dose and the 6-mo dose, the need of booster vaccination remain less clear among HIV-positive individuals who have lost anti-HAV antibodies.

**Key words:** Epidemiology; Viral hepatitis; Acute hepatitis; Fecal-oral transmission; Oral-anal sex; Men who have sex with men; Injecting drug use; Immunosuppression; Immunization

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**Core tip:** We provide an updated review of hepatitis A virus (HAV) coinfection among human immunodeficiency virus (HIV)-positive individuals, focusing on the epidemiology, clinical manifestations, and prevention for HAV infection. The reported outbreaks of acute hepatitis A among men who have sex with men and injecting drug users are summarized. Updated vaccination guidelines for prevention of HIV-positive individuals against HAV infection are presented. We also review the published data of effectiveness or efficacy of HAV vaccination studies and the different approaches to improvement of the serological responses to conventional HAV vaccines among HIV-positive individuals.

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## INTRODUCTION

Hepatitis A virus (HAV) is one of the most common infectious etiologies of acute hepatitis worldwide. According to the WHO estimates, HAV resulted in 13.7 million illnesses and 28000 deaths in 2010<sup>[1]</sup>. HAV is primarily transmitted fecal-orally *via* contaminated food or water, or through close contact with an infected

person. With improved sanitation and provision of HAV vaccination, areas or populations with high HAV endemicity show patterns of declining endemicity, according to their socioeconomic backgrounds<sup>[2]</sup>. Based on the different age-specific HAV seroprevalence profiles, the world can be divided into countries of high, intermediate, low, and very low HAV endemicity<sup>[3]</sup>. In countries of high endemicity, most people acquire HAV in their early childhood and are immune to the virus. On the contrary, adults from low endemic areas are first exposed to HAV during travel to or residence in endemic areas, or being engaged in risky behaviors, such as contact with infected persons, being men who have sex with men (MSM), or using illicit drugs<sup>[2,4]</sup>.

Several outbreaks of acute HAV infection among the MSM and injecting drug users' (IDUs') communities have been reported in several developed countries of low endemicity for HAV infection. The duration of HAV viremia and stool shedding of HAV may be longer in HIV-positive individuals, increasing the window of opportunity for wider transmission of HAV to those engaged in risk behaviors. HAV vaccination is the most efficient approach to prevention of acquiring HAV infection. However, the seroconversion rates following the recommended standard 2-dose HAV vaccination schedule are lower among HIV-positive individuals compared to HIV-negative individuals, and the vaccination effectiveness among HIV-positive individuals is rarely investigated in the outbreak setting<sup>[5]</sup>. In this article, we review the epidemiology and clinical manifestations of acute HAV infection and HAV vaccination among HIV-positive individuals in the era of combination antiretroviral therapy (cART).

## HAV VIROLOGY

HAV, first identified by Feinstone *et al.*<sup>[6]</sup> in 1973, belongs to the *Hepatovirus* genus of the family *Picornaviridae*. The genome of HAV is a positive-strand RNA (range, 7470 to 7478 nucleotides) and encodes only a single open reading frame, which is translated into a polyprotein. The polyprotein is then cleaved by the virus-encoded protease (3C<sup>pro</sup>) to yield 8 viral proteins, including VP0, VP3, VP1-2A, 2B, 2C, 3AB, 3C<sup>pro</sup>, and RNA-dependent RNA polymerase (RDRP, 3D<sup>pol</sup>). The virus particle is composed of 3 proteins, VP0, VP1-2A, and VP3. During the assembly of the virus capsid, 2A will be removed from the VP1-2A by cellular protease or 3C<sup>pro</sup>, and at the final stage of maturation, VP0 will be cleaved into VP2 and VP4. Five copies of each protein will be assembled to form a pentamer, and 12 copies of the pentamer will form a virus capsid. Despite that there are some amino acid variations between different HAV strains, the detection of anti-HAV antibody is not as complicated as other RNA viruses due to the fact that HAV exists as a single serotype. Due to the advances of molecular technology, 7 unique genotypes (I to VII) of HAV are defined by analysis of a 168-base region, located

between the C terminus of VP1 and N terminus of P2A<sup>[7]</sup>. These 7 genotypes exhibit less than 85% of sequence identity between genotypes and no more than 15% of divergence within a genotype, a criterion used for polioviruses, another member of the family *Picornaviridae*. However, further detailed analyses of other viral regions reveal that the genotypes II and VII should be reclassified as subtypes A and B of genotype II<sup>[8]</sup>, and genotypes I and III could also be divided into subgenotypes A and B<sup>[9]</sup>. Four genotypes (I, II, III, and VII) are of human origin, and 3 (IV, V, VI) are of simian origin. Genotypes I and III are the most prevalent genotypes identified in humans. Subgenotypes IA and IB are often found in North and South Americas, Europe, China, and Japan<sup>[7]</sup>. Clusters within genotypes predominant in certain geographic regions have been reported, such as a group of subgenotype IA strains from the United States<sup>[10]</sup>, and genotype II in the Netherlands, France, and Sierra Leone<sup>[7,11]</sup>. However, in other regions, the presence of variant genotypes was reported in Europe and Japan, likely representing international spread from the endemic regions.

## EPIDEMIOLOGY OF HAV INFECTION AMONG HIV-POSITIVE PATIENTS

### *HAV seroprevalence among HIV-positive patients*

Previous studies have shown higher seroprevalence and incidence of HAV infection among MSM compared to the general population<sup>[12-14]</sup>, which were associated with oral-anal sex and the number of sexual contacts and partners<sup>[12,15-20]</sup>. The HAV seroprevalence also increases with age, indicating the cohort effect<sup>[2,12,19,21]</sup>. Unlike MSM, heterosexual men with risky sexual behaviors has been inconsistently associated with higher HAV seroprevalence. While a few studies reported a lower seroprevalence and incidence among heterosexual men with sexually transmitted diseases (STDs) compared to MSM<sup>[15,16]</sup>, others indicated that the risks for HAV infection among heterosexual men with STDs and MSM were similar<sup>[12,19,21]</sup>. IDUs also had a higher HAV seroprevalence than the general population<sup>[13,14,22,23]</sup>. However, the high seroprevalence might not be solely attributable to needle contamination, since some reported similar elevation of the HAV seroprevalence between IDUs and non-injecting illicit drug users<sup>[22,23]</sup>.

Although the direct evidence on the correlation between contracting HIV and HAV was scarce, observational data suggested that HIV-positive individuals, especially MSM and IDUs, are at increased risk of acquiring HAV<sup>[24]</sup>. In addition, one small study including 15 HIV-positive individuals demonstrated that the duration of HAV viremia in HIV-positive individuals with acute hepatitis A was prolonged compared to that in HIV-negative individuals with acute hepatitis A, which may increase the probability of HAV transmission

to others<sup>[25]</sup>. Several studies have reported the HAV seroprevalence among HIV-positive individuals and at-risk persons in areas of different HAV endemicities and vaccine coverage (Table 1)<sup>[12-23,26-42]</sup>. In these studies, the HAV seroprevalence among HIV-positive individuals ranged from 15.1% in Taiwan to 96.3% in Iran<sup>[31,35]</sup>. While studies conducted in countries of high HAV endemicity showed no differences in the HAV seroprevalence between HIV-positive and HIV-negative individuals<sup>[27]</sup>, the seroprevalence in countries of low endemicity was higher among HIV-positive individuals compared to HIV-negative individuals<sup>[26,30]</sup>. Among HIV-positive individuals, older age and injecting drug use were identified as the independent factors associated with seropositivity for HAV; the HAV seroprevalence was lower in HIV-positive MSM despite the at-risk sexual behaviors<sup>[29,30,33-36]</sup>.

### *Hepatitis A outbreaks in the MSM population*

In countries of low HAV endemicity, the majority of HAV-seronegative adults remain susceptible to acute HAV infection. Outbreaks of acute hepatitis A are often caused by introduction of HAV through contaminated foods and person-to-person transmission<sup>[2]</sup>. Numerous outbreaks of acute hepatitis A have been reported in the MSM population through sexual contacts, which are summarized in Table 2<sup>[43-70]</sup>. Since the early 1980s, outbreaks of acute hepatitis A among MSM have been described in Denmark<sup>[43]</sup>, Sweden<sup>[44]</sup>, the United Kingdom<sup>[45]</sup>, and the United States<sup>[61,62]</sup>. The incidence of acute HAV infection among MSM peaked in the 1990s, and the affected countries included the United Kingdom<sup>[46,47,49,51]</sup>, the Netherlands<sup>[48]</sup>, Norway<sup>[50]</sup>, the United States<sup>[63,65,66]</sup>, Canada<sup>[64]</sup> and Australia<sup>[67-70]</sup>. One of the largest epidemics of acute hepatitis A occurred in Sydney, Australia, where 2 outbreaks affected 323 and 186 MSM during 1991-1992 and 1995-1996, respectively<sup>[69]</sup>. Since 2015, Taiwan reported a large outbreak involving more than 1000 indigenous cases, with more than 70% of the affected individuals being MSM<sup>[71]</sup>. While the HAV vaccine was licensed and recommended for MSM since the mid-1990s<sup>[47]</sup>, the emergence of HAV infection continued to pose a health threat to MSM in several developed European countries during the 2000s, including Italy<sup>[52,54,55,60]</sup>, Denmark<sup>[53]</sup>, Spain<sup>[56,58]</sup>, Poland<sup>[57]</sup>, and the United Kingdom<sup>[59]</sup>.

The duration of outbreaks of acute hepatitis A among MSM were mostly curtailed at 2 years; however, the outbreak in Canada extended from December 1994 to February 1998<sup>[64]</sup>. The cyclical outbreaks were noted in Australia during 1991-1996<sup>[69]</sup> and in Spain during 1989-2010<sup>[56]</sup>, which might be facilitated by the continuous circulation of particular HAV strains in the MSM population<sup>[50,55,60]</sup>. The predominant circulating HAV strains among MSM belonged to genotype IA<sup>[50,55,59,60,72]</sup>. The patients contracting HAV during the outbreaks were mostly young adults with a mean or median age of 28-36 years<sup>[55,57]</sup>. HAV was recognized

**Table 1 Seroprevalence of hepatitis A virus infection among human immunodeficiency virus-positive patients and at-risk populations**

Ref.	Location	Study period	Study population	Age (yr)	HIV-positive population	Other populations	Associated factors <sup>1</sup> and comments
HIV-positive population Nandwani <i>et al</i> <sup>[26]</sup>	London, United Kingdom	1993	255 men attending genitourinary clinics	32	41.3%	MSM, 32.4% Heterosexuals, 30.0% Unknown HIV status, 26.4%	No difference between homosexual and heterosexual men
Fainboim <i>et al</i> <sup>[27]</sup>	Buenos Aires, Argentina	1994-1995	484 HIV-positive patients	29	84.0%	HIV-positive MSM, 83.3% HIV-positive heterosexuals, 86.3% HIV-positive IDUs, 85.7% Blood donors, 82.4%	High seroprevalence without difference between HIV-positive and HIV-negative individuals
Aloise <i>et al</i> <sup>[28]</sup>	Rio de Janeiro, Brazil	1988-2004	581 HIV-positive patients	35	79.8%	NA	Older age and lower educational level
Lee <i>et al</i> <sup>[29]</sup>	Tainan, Taiwan	2000-2005	484 patients with recent diagnosed HIV infection	36	65.8%	HIV-positive MSM, 40.0%; HIV-positive heterosexuals, 85.2% HIV-positive IDUs, 70.1%	Seroprevalence increased with age and among heterosexuals
Sun <i>et al</i> <sup>[30]</sup>	Taiwan	2004-2007	1580 HIV-positive patients	39	60.9%	HIV-positive MSM, 50.5% HIV-positive heterosexuals, 79.3% HIV-positive IDUs, 62.0% HIV-negative individuals, 48.0%	Older age and injecting drug use Higher seroprevalence in HIV-positive individuals
Davoudi <i>et al</i> <sup>[31]</sup>	Tehran, Iran	2005-2006	247 HIV-positive patients	36	96.3%	NA	
Hoover <i>et al</i> <sup>[32]</sup>	6 major cities <sup>2</sup> , United States	2004-2007	627 HIV-positive MSM	41	16.1% <sup>3</sup>	NA	Low HAV screening and vaccination rates (28.5%)
Linkins <i>et al</i> <sup>[33]</sup>	Bangkok, Thailand	2006-2008	1291 MSM	27	32.4% <sup>3</sup>	HIV-negative MSM, 25.5%	Older age and lower education level
Baek <i>et al</i> <sup>[34]</sup>	Seoul, South Korea	2008-2010	188 HIV-positive patients	39	62.8%	HIV-positive MSM, 57.1% HIV-positive heterosexuals, 65.8%	Older age
Tseng <i>et al</i> <sup>[35]</sup>	Taipei, Taiwan	2009-2010	1128 MSM	18-40	15.1% <sup>3</sup>	HIV-negative MSM, 7.4%	Older age No difference between HIV-positive and HIV-negative individuals
Kourkounti <i>et al</i> <sup>[36]</sup>	Athens, Greece	2007-2011	897 HIV-positive MSM	41	35.7% <sup>3</sup>	NA	Older age and being foreigners
At-risk populations (MSM and IDUs) Corey <i>et al</i> <sup>[15]</sup>	Seattle, United States	1977-1979	159 patients from STD clinics	31	NA	MSM, 30.4% (annual incidence, 22%) Heterosexuals, 12.3% (annual incidence, 0%)	Oral-anal sexual contact Higher seroprevalence and incidence in MSM
McFarlane <i>et al</i> <sup>[12]</sup>	Nova Scotia, Canada	1977-1978	421 patients from STD clinics	25	NA	MSM, 42.4% Heterosexuals, 39.2% Blood donors, 12.6% Student nurses, 13.2%	Higher number of sex partners and older age
Kryger <i>et al</i> <sup>[16]</sup>	Copenhagen, Denmark	1979	269 men with previous syphilis	33	NA	MSM, 36.0%; Heterosexual, 20.0%	More episodes of syphilis in younger MSM
Coutinho <i>et al</i> <sup>[17]</sup>	Amsterdam, the Netherlands	1980-1982	689 MSM	31	NA	MSM, 42.0% (incidence, 14.0%)	Longer duration of homosexual activity
Crofts <i>et al</i> <sup>[22]</sup>	Victoria, Australia	1990-1992	2175 prison entrants 293 IDUs	30	NA	IDU, 43.7% Prison entrants, 60.1% Blood donors, 30.0%	History of incarceration
Katz <i>et al</i> <sup>[18]</sup>	San Francisco and Berkeley, United States	1992-1993	411 MSM	21	NA	MSM, 28.0%	Sexual and drug-using behaviors

Villano <i>et al</i> <sup>[13]</sup>	Baltimore, United States	1993-1994	294 MSM 292 IDUs	NA	NA	MSM, 32.3% IDU, 66.4% Blood donors, 13.7%	Increased risk for HAV infection in MSM and IDUs
Corona <i>et al</i> <sup>[19]</sup>	Rome, Italy	1997	432 male patients from STD clinics	NA	NA	MSM, 60.3% Heterosexual, 62.2%	Older age and more sexual partner
Ochnio <i>et al</i> <sup>[14]</sup>	Vancouver, Canada	1998	494 individuals from street outreach clinics	32	NA	MSM, 25.5% IDU, 42.6% Street youth, 6.3%	Increased risk for HAV infection in MSM and IDUs
Ross <i>et al</i> <sup>[21]</sup>	Birmingham, United Kingdom	2000	210 men attending genitourinary clinics	NA	NA	MSM, 23.0%; Heterosexual men, 32.0%	Ethnicity, older age, and history of sex in a sauna
Diamond <i>et al</i> <sup>[37]</sup>	Washington, United States	1997-2000	833 MSM	15-29	NA	MSM, 21.0%	Ethnicity, IDU, HBV and HIV infection Vaccination rate, 21%
Bialek <i>et al</i> <sup>[20]</sup>	7 major cities <sup>4</sup> , United States	1994-2000	2708 MSM	15-29	NA	MSM, 18.4%	More male sex partners and unprotected anal sex
O'Riordan <i>et al</i> <sup>[38]</sup>	London, United Kingdom	2004	395 MSM attending genitourinary clinics	NA	NA	MSM, 49.9%	
Van Rijckevorsel <i>et al</i> <sup>[39]</sup>	Amsterdam, the Netherlands	1992-2006	1697 hepatitis A patients	NA	NA	Incidence, 0.97/1000 MSM	Clustered transmission in social MSM networks
Removille <i>et al</i> <sup>[23]</sup>	Luxembourg	2005	368 problem drug users	NA	NA	IDUs, 57.1% nIDUs, 65.9%	
Bozicevic <i>et al</i> <sup>[40]</sup>	Zagreb, Croatia	2006	360 MSM	27	NA	MSM, 14.2%	
Weerakoon <i>et al</i> <sup>[41]</sup>	Melbourne, Australia	2002-2011	3055 MSM	33	NA	MSM, 39.0%	Vaccination levels over 40%-50% to prevent outbreaks
Ali <i>et al</i> <sup>[42]</sup>	Sydney, Australia	1996-2012	14799 MSM	30	NA	MSM, 31.9% in 1996 to 63.8% in 2012	Vaccination rate, 9.8% in 1996 to 45.2% in 2012

<sup>1</sup>Factors associated with HAV seropositivity were identified by bivariate or multivariable logistic regression analysis; <sup>2</sup>The 6 major cities included Atlanta, Chicago, Los Angeles, Miami, New York City, and San Francisco; <sup>3</sup>Only HIV-positive MSM were included; <sup>4</sup>The 7 major cities included Baltimore, Dallas, Los Angeles, Miami, New York City, San Francisco, and Seattle. HAV: Hepatitis A virus; IDUs: Injecting drug users; MSM: Men who have sex with men; NA: Not available; nIDUs: Non-injecting drug users; STD: Sexually transmitted disease.

as being transmitted among MSM through sexual contacts<sup>[73]</sup>, and case-control studies have identified several associated factors such as having anonymous sex partners, group sex, oral-anal and digital-rectal intercourse<sup>[63]</sup>, contact with patients with acute hepatitis A<sup>[66]</sup>, having sex in gay saunas<sup>[51,53]</sup>, and visiting saunas and darkrooms<sup>[48]</sup>. In light of the risky sexual behavior, the largest HAV vaccination campaign for MSM was launched in Montréal, in which 9500-15000 first doses of HAV vaccine were administered to achieve a coverage rate between 20% and 41%. However, the decrease in the incidence of acute hepatitis A shortly after the vaccination campaign might indicate the relatively late implementation of HAV vaccination and the natural decline after herd immunity was established at the end of the outbreak<sup>[64]</sup>. The vaccination campaigns targeting MSM in Atlanta and Barcelona recruited 3,000 persons, which resulted in a 16% decrease of reported acute hepatitis A cases<sup>[56,65]</sup>.

Coinfections with HAV and HIV were identified during the 2000s in Italy<sup>[52,54,55]</sup>, Spain<sup>[56]</sup>, and Poland<sup>[57]</sup>. Most HAV/HIV-coinfected individuals were males with known HIV status, while others were found to be HIV-positive concomitantly with acute HAV infections<sup>[52,54-57]</sup>. Among all male patients who received a diagnosis of acute hepatitis A during 2002-2008 in Italy, 15.2% (56/368) were HIV-positive<sup>[54]</sup>. After excluding those without available HIV serology, the HIV seroprevalence among was 27.6%<sup>[54]</sup>. The high proportion of HAV/HIV coinfection in the areas of low

HAV endemicity highlights the importance of routine HIV testing in patients with acute hepatitis A<sup>[54]</sup>.

### **Hepatitis A outbreak in the IDU population**

Outbreaks of acute hepatitis A in the IDU population have been reported since 1970s as the numbers of IDUs increased<sup>[74]</sup>. The studies of outbreaks of acute hepatitis A among IDUs are summarized in Table 3<sup>[74-88]</sup>. During 1970-1979, the cyclic occurrence of outbreaks of acute hepatitis A in Sweden suggested a continuously increasing pool of susceptible young IDUs in the closed communities<sup>[74]</sup>. The outbreaks were mostly described in Europe<sup>[75-78]</sup> and the United States<sup>[82,83,85]</sup> in the 1980s and 1990s, but were seldom described after the early 2000s<sup>[79-81,86]</sup>. Up to 492 IDUs were infected with HAV in Norway between 1995 and 1996<sup>[77]</sup>. In Terni, Italy; 47 cases of acute hepatitis A were reported during 2002-2003, among which included 35 IDUs and 2 HIV-positive individuals. The most recent outbreak of acute HAV infection among IDUs was described in Israel during 2012-2013, which occurred in IDUs and homeless adults with subsequent spread to the general population in Tel Aviv, despite the nation-wide implementation of universal toddler's vaccination in 1999<sup>[88]</sup>.

The outbreaks of acute hepatitis A among IDUs mainly lasted between 1 and 2 years, and young patients with a mean or median age of 20-34 years were predominantly affected<sup>[74,81]</sup>. HAV could be transmitted fecal-orally through poor personal hygiene



**Table 2 Outbreaks of acute hepatitis A in the men who have sex with men population**

Ref.	Location	Study period	Case number	Male	MSM	HIV-positive patients	Age (yr)	Risk factors <sup>1</sup> and comments
Europe								
Høybye <i>et al</i> <sup>[43]</sup>	Copenhagen, Denmark	1977-1978	45	45	21	NA	29	Multiple partners and oral-anal sexual contact
Christenson <i>et al</i> <sup>[44]</sup>	Stockholm, Sweden	1979-1980	145	145	145	NA	NA	
Mindel <i>et al</i> <sup>[45]</sup>	London, United Kingdom	1980	24	NA	23	NA	NA	
Kani <i>et al</i> <sup>[46]</sup>	London, United Kingdom	1989-1990	7000	NA	41	NA	NA	Oral-anal sexual contact
Atkins <i>et al</i> <sup>[47]</sup>	London, United Kingdom	1989-1992	206	121	65	NA	NA	Oral-anal sexual contact and sexual promiscuity
Leentvaar-Kuijpers <i>et al</i> <sup>[48]</sup>	Amsterdam, the Netherlands	1992-1993	293	NA	39	NA	NA	Visiting saunas and darkrooms
Walsh <i>et al</i> <sup>[49]</sup>	Thames region, United Kingdom	1995	481	NA	58	NA	NA	Oral-anal and digital-rectal intercourse
Stene-Johansen <i>et al</i> <sup>[50]</sup>	Oslo, Norway	1995-1998	26	26	26	NA	NA	Eating shellfish and sex in gay saunas
Bell <i>et al</i> <sup>[51]</sup>	London and East Sussex, United Kingdom	1997	48	NA	41	NA	NA	
Manfredi <i>et al</i> <sup>[52]</sup>	Bologna, Italy	1999-2004	122	104	81	11	28	
Mazick <i>et al</i> <sup>[53]</sup>	Copenhagen, Denmark	2004	18	18	18	NA	NA	Casual sex and sex in gay saunas
Girardi <i>et al</i> <sup>[54]</sup>	Rome, Italy	2002-2008	473	368	115	57	25-64	Same gender sex
Bordi <i>et al</i> <sup>[55]</sup>	Rome, Italy	2008-2010	162	143	34	14	36	Routine HIV test in HAV-infected patients should be considered
Tortajada <i>et al</i> <sup>[56]</sup>	Barcelona, Spain	2002	48	47	NA	28%	31	Monophyletic HAV strain sustained the outbreak
		2003-2004	60	60	NA	24%	32	
		2008-2009	189	185	NA	21%	33	
Dabrowska <i>et al</i> <sup>[57]</sup>	Warsaw, Poland	2007-2008	860	NA	50	6	28	No difference in disease severity between HIV-positive and HIV-negative individuals
Tortajada <i>et al</i> <sup>[58]</sup>	Barcelona, Spain	2008-2009	150	126	87	NA	33	The outbreak strain was indistinguishable from that in Czech Republic
Sfetcu <i>et al</i> <sup>[59]</sup>	Northern Ireland, United Kingdom	2008-2009	38	36	26	NA	29	
Taffon <i>et al</i> <sup>[60]</sup>	Tuscany, Italy	2008	240	NA	32%	NA	NA	A unique circulating HAV strain
Kosatsky <i>et al</i> <sup>[61]</sup>	Anchorage, Alaska	1982-1983	17	17	17	NA	19-31	Anonymous sex partner, group sex, oral-anal and digital-rectal intercourse
Desenclos <i>et al</i> <sup>[62]</sup>	Florida, United States	1988-1989	311	69	26	NA	NA	
Henning <i>et al</i> <sup>[63]</sup>	New York, United States	1991	180	180	62	NA	20-49	
Allard <i>et al</i> <sup>[64]</sup>	Montréal, Canada	1996-1997	376	376	376	NA	33	Vaccination campaign achieving 20%-41% coverage in MSM decreased incidence rapidly
Finton <i>et al</i> <sup>[65]</sup>	Atlanta, United States	1996	222	NA	75%	NA	NA	Vaccination campaign in MSM decreased reported cases
Cotter <i>et al</i> <sup>[66]</sup>	Ohio, United States	1998-1999	136	118	47	NA	33	Contact with hepatitis A cases
Stewart <i>et al</i> <sup>[67]</sup>	Melbourne, Australia	1991	495	407	210	NA	NA	Sexual and social contact
Stokes <i>et al</i> <sup>[68]</sup>	Sydney, Australia	1991-1992	570	515	330	NA	31	Sexual contact was the most reported contact type
Ferson <i>et al</i> <sup>[69]</sup>	Sydney, Australia	1991-1996	1138	991	587	NA	30	Household or sexual contact
Delpech <i>et al</i> <sup>[70]</sup>	Sydney, Australia	1997-1999	354	265	139	NA	32	A total of 1296 cases reported as of February, 2017
Chen <i>et al</i> <sup>[71]</sup>	Taiwan	2015-2016	> 1000	NA	> 70%	> 60%	NA	

<sup>1</sup>Risk factors of acquiring HAV infection were identified by case-control studies. HAV: Hepatitis A virus; MSM: Men who have sex with men; NA: Not available.

and living conditions, or percutaneously through contamination of illicit drugs or injecting equipment by fecal materials or blood<sup>[81]</sup>. Three case-control studies identified not washing hands after using the toilet or before preparing food, not washing hands prior to

preparing drugs, sharing of needles or syringes, use of contaminated illicit drugs, and contact with jaundiced persons to be factors associated with acute hepatitis A in IDUs<sup>[80,81,85]</sup>. To curb the epidemic of acute hepatitis A, HAV vaccination programs were implemented in

**Table 3 Outbreaks of acute hepatitis A in the injecting drug user population**

Ref.	Location	Study period	Total patients	IDU	HIV-positive individuals	Age (yr)	Risk factors <sup>1</sup> and comments
Widell <i>et al.</i> <sup>[74]</sup>	Europe Malmo, Sweden	1970-1979	323	188	NA	NA	
Sundkvist <i>et al.</i> <sup>[75]</sup>	Helsingborg, Sweden	1983-1984	36	32	NA	18-35	The outbreak was associated with intrarectal transportation of illicit drugs
Leino <i>et al.</i> <sup>[76]</sup>	Helsinki, Finland	1994-1995	238	131	NA	31	The outbreak was associated with intrarectal transportation of illicit drugs
Stene-Johansen <i>et al.</i> <sup>[77]</sup>	Oslo, Norway	1995-1996	621	492	NA	NA	The outbreak was associated with needle sharing
O'Donovan <i>et al.</i> <sup>[78]</sup>	United Kingdom	1998-1999	27	14	NA	25	
Syed <i>et al.</i> <sup>[79]</sup>	Bristol, United Kingdom	2000	123	69	NA	25	The outbreak was associated with parenteral transmission from contaminated illicit drugs; HAV vaccination of IDUs decreased the reported cases
Roy <i>et al.</i> <sup>[80]</sup>	Aberdeen, Scotland	2000-2002	106	74	NA	NA	Not washing hands after using the toilet, or before preparing food or drugs, sharing needles/syringes, and injecting contact with jaundiced persons
Spada <i>et al.</i> <sup>[81]</sup>	Terni, Italy	2002-2003	47	35	2	34	Contact with jaundiced persons, but not related to injecting practices; HAV vaccination of IDUs decreased the reported cases
Harkess <i>et al.</i> <sup>[82]</sup>	North America Oklahoma, United States	1984-1987	79	42	NA	23-27	
Jenkerson <i>et al.</i> <sup>[83]</sup>	New York, United States	1986-1987	256	70	NA	NA	
Jin <i>et al.</i> <sup>[84]</sup>	Canada	1987-1989	65	59	NA	NA	
Hutin <i>et al.</i> <sup>[85]</sup>	Iowa, United States	1996-1997	158	9.7%	NA	NA	Methamphetamine injection, sharing methamphetamine use, using brown methamphetamine, and needle sharing
Vong <i>et al.</i> <sup>[86]</sup>	Florida, United States	2001-2002	403	11%	NA	32	HAV vaccination in jail decreased the reported cases
Shaw <i>et al.</i> <sup>[87]</sup>	Queensland, Australia	1997	875	118	NA	NA	Sharing of instruments for smoking marijuana
Manor <i>et al.</i> <sup>[88]</sup>	Tel-Aviv, Israel	2012-2013	75	9	NA	33	

<sup>1</sup>Risk factors of acquiring HAV infection were identified by case-control studies. HAV: Hepatitis A virus; HIV: Human immunodeficiency virus; IDU: Injecting drug user; NA: Not available.

**Table 4 Clinical symptoms and signs of patients with acute hepatitis A infection<sup>[92-96]</sup>**

Symptoms	Frequency
Asymptomatic	14%
Fever	48%-87%
Nausea/vomiting	56%-88%
Anorexia	66%-96%
Fatigue/malaise	49%-80%
Upper abdominal pain	42.5%-82%
Diarrhea	8%-23%
Signs	
Jaundice	24%-99%
Hepatomegaly	7%-78%
Splenomegaly	18%-30%

the United Kingdom<sup>[79]</sup>, Norway<sup>[89]</sup> and Italy<sup>[81]</sup>, and harm reduction program by providing clean injecting equipment was implemented in Switzerland<sup>[90]</sup>.

## CLINICAL MANIFESTATIONS OF ACUTE HAV INFECTION

The incubation period of acute HAV infection is 2.5 to 5 wk<sup>[91]</sup>. The typical symptoms of acute hepatitis A include fatigue, malaise, nausea, vomiting, anorexia, fever, and right upper quadrant pain. The frequencies of symptoms or signs of acute hepatitis A are listed in Table 4<sup>[92-96]</sup>. While most of acute HAV infections are self-limited, the severity of the symptoms may vary with age and concurrent comorbidities, particularly chronic viral hepatitis. Acute HAV infection is usually silent or subclinical in children, but approximately 30% of the infected patients older than 6 years have symptoms including hepatitis, jaundice, and abdominal pain<sup>[97]</sup>. Less than 25% of the patients have diarrhea though HAV is transmitted through fecal-oral route<sup>[98]</sup>. The data on the symptoms of acute hepatitis A

**Table 5** Comparison of clinical manifestations of hepatitis A virus between human immunodeficiency virus-positive patients or human immunodeficiency virus-negative patients with acute hepatitis A

	HIV-positive patients	HIV-negative patients
Natural course of acute HAV infection		
Incubation period (wk)	NA	2.5-5 <sup>[91]</sup>
Duration of stool shedding (d)	NA	25 (HAV antigen) <sup>[105]</sup> 81 (HAV RNA) <sup>[106]</sup>
Duration of viremia (d)	53 (10-89) <sup>[25]</sup>	22-95 <sup>[25,106-108]</sup>
Laboratory findings		
Peak T-bilirubin (mg/dL)	5.1-5.9 <sup>[25]</sup>	5.7-8.7 <sup>[25,92,93,95,98,99]</sup>
Peak AST (IU/L)	929-1339 <sup>[25,57]</sup>	1231-2271 <sup>[25,92,93,99]</sup>
Peak ALT (IU/L)	1995-2368 <sup>[25,57]</sup>	1079-3442 <sup>[25,92,93,99,100]</sup>
Duration of elevated AST/ALT (d)	63 ± 38 <sup>[109]</sup>	51 <sup>[92]</sup>
Peak ALP (IU/L)	807 <sup>[25,57]</sup>	228-396 <sup>[25,92]</sup>

HIV: Human immunodeficiency virus; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HAV: Hepatitis A virus; NA: Not available.

among HIV-positive individuals are limited, and the study by Ida *et al.*<sup>[25]</sup> of 15 HIV-positive and 15 HIV-negative individuals with acute hepatitis A suggested no differences in the frequency and severity of clinical symptoms of acute hepatitis A between the two groups.

Patients with acute hepatitis A usually have significantly elevated levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin. In previous studies, the average peak levels of total bilirubin were 7-8 mg/dL and the levels of AST and ALT were higher than 1000 IU/L<sup>[25,92,93,98-100]</sup>. Alkaline phosphatase (ALP) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) are also elevated in patients with acute hepatitis A. Resolution of the abnormal biochemical tests generally occurs within 1 to 6 wk after the onset of the illness<sup>[99]</sup>. Approximately 85% of the patients who are infected with HAV have full clinical and biochemical recovery within 3 mo and nearly all have a complete recovery by 6 mo<sup>[92]</sup>. The study by Ida *et al.*<sup>[25]</sup> reported lower elevations in total bilirubin, AST, and ALT in HIV-positive individuals during acute hepatitis A than HIV-negative individuals, which were considered to be related to the weaker immune responses in HIV-positive patients or clonal spreading of a specific HAV strain that was able to escape from immunity in the study. Regulatory T cells (Tregs) normally suppress the T-cell responses directed against hepatitis viruses and down-regulate the immune reaction that is responsible for liver damage in viral hepatitis<sup>[101]</sup>. The study by Choi *et al.*<sup>[102]</sup> suggested a decrease in Tregs leading to a severe liver injury during acute hepatitis A. HIV-positive individuals however are known to have high Tregs, compared to their HIV-negative counterparts, hence they may experience less severe injury during acute hepatitis A<sup>[103]</sup>. On the other hand, Ida *et al.*<sup>[25]</sup> reported higher levels of ALP and  $\gamma$ -GT during acute

hepatitis A in HIV-positive individuals than HIV-negative patients. Biliary tract is not the primary target of HAV infection. Lymphocytic cholangitis is rarely seen with acute HAV infection<sup>[104]</sup>. However, HIV-related cholangitis or cholangiopathy is a well-recognized late complication of acquired immunodeficiency syndrome (AIDS). Opportunistic infections such as cytomegalovirus infection or cryptosporidiosis may also cause cholangitis. HIV is also able to cause direct cytopathic effects on the biliary tract mucosa. Hence, the higher levels of ALP and  $\gamma$ -GT observed in HIV-positive patients with acute hepatitis A may be explained by multiple factors other than the liver injury caused by HAV itself.

In the general population, stool shedding of HAV antigen can be detected 19 d before the peak elevation of ALT levels and continue for at least 25 d<sup>[105]</sup> and even up to 80 d<sup>[106]</sup>. The duration of viremia is estimated to last around 20 to 40 d<sup>[25,106,107]</sup> and even longer than 3 mo<sup>[108]</sup>. In the study by Ida *et al.*<sup>[25]</sup>, the median duration of HAV viremia in HIV-positive individuals with acute hepatitis A was 53 d, which was longer than that of HIV-negative individuals. A longer duration of HAV viremia may be related to impaired host immunity<sup>[100]</sup>. Besides, the relationship between duration of viremia and specific HAV genotypes is still inconclusive<sup>[106,107]</sup>. The comparisons of clinical manifestations of acute hepatitis A between HIV-positive and HIV-negative individuals are summarized in Table 5<sup>[25,57,91-93,95,98-100,105-109]</sup>.

Other atypical presentations of acute hepatitis A include renal insufficiency and relapsing hepatitis<sup>[93]</sup>, which are usually present in children. Some individuals experienced a prolonged hepatitis (5.8%)<sup>[93]</sup> or cholestasis (6.8%), especially in the presence of hepatitis B virus<sup>[94]</sup>. Severe hepatic failure is rare and occurs more commonly in patients with underlying diseases or advanced age. Reported case fatality rates were 0.1% in infants and children, 0.45% in those aged 15 to 39 years, and 1.1% in those aged > 40 years. Patients with chronic hepatitis C virus (HCV) infection have a substantial risk of fulminant hepatitis and death associated with HAV superinfection<sup>[110]</sup>. HIV-positive individuals acquire HAV infection mostly in their adulthood and often have other underlying liver disease<sup>[25,57]</sup>, which may increase the risk of hepatic failure and fatality caused by HAV. Therefore, prevention by HAV vaccination is important, especially for the HIV/HCV-coinfected individuals.

## HAV VACCINATION AND FACTORS ASSOCIATED WITH IMMUNOGENICITY AND PERSISTENT PROTECTION

### *Vaccine immunogenicity and factors associated with immunogenicity*

HAV vaccination is not universally recommended for HIV-positive individuals but specifically for those with

**Table 6** Hepatitis A virus vaccination recommendations by the British human immunodeficiency virus Association, the European AIDS Clinical Society, the US Advisory Committee for Immunization Practices and the World Health Organization

Health Authority	Target candidates	Dosing Schedule	Comments
BHIVA <sup>[111]</sup>	Household and sexual contacts of infected persons Travellers MSM Injecting and non-injecting drug users Individuals at risk of infection during outbreaks Those with occupational exposure to HAV (e.g., laboratory workers, sewage workers) Hemophiliacs Residents of care institutions, and their care givers	Monovalent HAV vaccine recommended Patients with CD4 counts > 350 cells/mm <sup>3</sup> should be offered 2 vaccine doses at 0 and 6 mo Patients with CD4 counts < 350 cells/mm <sup>3</sup> should receive 3 vaccine doses at 0, 1, and 6 mo Patients at continued risk of exposure receive a boosting vaccine dose every 10 yr Following a significant exposure, HIV-positive contacts who are HAV-seronegative receive post-exposure prophylaxis with the HAV vaccine, with the first dose given as soon as possible and within 14 d of exposure; if the CD4 count is < 200 cells/mm <sup>3</sup> , they should also receive human normal immunoglobulin	We support the BHIVA's recommendations of targeted vaccination during outbreaks and of stratifying dosing schedule by CD4 counts, particularly administering a 3-dose schedule for those with lower CD4 counts. Despite waning antibody levels, we could not find evidence to justify routine boosters every 10 yr for those at risk. It may be preferable to follow antibody titers and revaccinate seroreverters
EACS <sup>[112]</sup>	Travellers MSM IDUs Active hepatitis B or C infection	Vaccinate if seronegative. Did not specify how	Shorter list of at risk candidates for vaccination. Our review supports their recommendation to check antibody titers in individuals with risk profile to guide the need for primary or booster vaccinations
ACIP <sup>[113]</sup>	MSM Injection or non-injection illicit drugs users Persons working with HAV-infected primates or with HAV in a research laboratory setting Persons with chronic liver disease Persons who receive clotting factor concentrates Travellers Close personal contact (e.g., household or regular babysitting) with an international adoptee during the first 60 d after arrival in the United States from a country with high or intermediate endemicity	Monovalent vaccine formulations should be administered in a 2-dose schedule at either 0 and 6-12 mo (Havrix), or 0 and 6-18 mo (Vaqta)  If the combined hepatitis A and hepatitis B vaccine (Twinrix) is used, administer 3 doses at 0, 1, and 6 mo; alternatively, a 4-dose schedule may be used, administered on days 0, 7, and 21-30 followed by a booster dose at 12 mo	Unlike BHIVA, in addition to the monovalent vaccine formulations, ACIP also recommends the combined hepatitis A and B vaccine No mention of the need to follow antibody titers or booster vaccines or the application of immunization during outbreaks
WHO <sup>[114]</sup>	Travellers Immunosuppressed patients Patients with chronic liver disease	Inactivated vaccine: 2 doses, the second dose normally 6 mo after the first. If needed, this interval may be extended to 18-36 mo	Does not specify whether all HIV-positive persons should be considered as immunosuppressed patients although evidence from Table 5 suggests that except for the duration of viremia acute HAV is not more severe in HIV-positive compared to HIV-negative patients

HAV: Hepatitis A virus; HIV: Human immunodeficiency virus; IDUs: Injecting drug users.

increased risks of exposure (such as from injecting drug use, oral-anal sex, travel to or residence in endemic areas, frequent clotting factor or blood transfusions) or with increased risks of fulminant disease (such as those with chronic hepatitis) (Table 6)<sup>[111-114]</sup>. Of the two types of HAV vaccines that are currently available internationally, the live attenuated vaccine (based on H2 or LA-1 HAV strains and manufactured as well as mainly used in China or India) and the inactivated HAV vaccine (based on clinical trials since 1991 and licensed in the United States since 1995), only the latter is recommended for HIV-positive individuals. There are 3 formulations of inactivated HAV vaccines that have been assessed in HIV-positive individuals with varying degrees of immunodeficiency as shown in Table 7<sup>[115-129]</sup>. Although different specific anti-HAV IgG titers have been used to define seroconversion (10, 18, 20, or 33 MIU/mL), the

majority of these studies have adopted 20 mIU/mL as the surrogate titer for seroprotection.

The earliest studies of HAV vaccination in moderately to severely immunodeficient HIV-positive individuals preceded the licensure of the adult formulation of HAVRIX 1440 U wherein a triple-mini dosing scheme (3 pediatric doses of HAVRIX 720 U administered at 0, 1, and 6 mo) was applied to hemophiliac patients and MSM with or without HIV<sup>[127-129]</sup>. The seroconversion rates among such HIV-positive hemophiliacs and MSM at month 7 were consistently between 76.0%-76.9% and lower than their HIV-negative counterparts at 100%<sup>[127-129]</sup>. Later studies of HIV-positive individuals without hemophilia but with other risk factors such as MSM confirmed that the seroconversion rates following the recommended standard adult dosing schedule (2 doses of HAVRIX 1440 U or VAQTA 50 U administered 6-12 mo apart) were lower among HIV-positive adults



**Table 7 Primary response rates and predictors of seroconversion after hepatitis A virus vaccination in human immunodeficiency virus-positive patients**

Ref.	Dates	Design/ Country	No. of patient <sup>1</sup>	HAV/ dosing schedules (mo)	CD4, cells/ mm <sup>3</sup>	PVL, log <sub>10</sub> , copies/ mL	ART	Timing of response <sup>2</sup> , mo/cut-off <sup>3</sup> , mIU/mL/assay	Response rate (%): ITT/PP	Predictors and comments <sup>4</sup>
Tseng <i>et al</i> <sup>[115]</sup>	2009-2010	Prospective, Taiwan	Standard 2-dose	HAVRIX 1440 U/ 2 doses (0, 6)	Mean, 538	Mean, 2.5	67.1%	12, 18/20, a. CIA (ARCHITECT HAVAb-IgG)	12 m (CIA): 75.7/81.7 12 m (ELISA): NA/88.6	MSM only study; Higher baseline CD4 and suppressed PVL; 3 doses over 2 doses
			All 126; CD4 matched, 114					b. ELISA (ETIAB- HAVK PLUS)	18 m (ELISA): NA/86.6	
			3-dose	HAVRIX 1440/ 3 doses (0, 1, 6)	Mean, 452	Mean, 3	58.2%		12 m (CIA): 77.8/81.8 12 m (ELISA): NA/89.2	
			All, 213; CD4 matched, 114						18 m (ELISA): NA/86.9	
			Standard 2-dose	HAVRIX 1440/ 2 doses (0, 6)	NA	NA	NA		12 m (CIA): 88.5/97.9 12 m (ELISA): NA/100	
			HIV-negative, 193						18 m (ELISA): NA/100	
Mena <i>et al</i> <sup>[116]</sup>	1997-2009	Retrospective, Spain	Standard 2-dose, 241	HAVRIX 1440/ (0, 6-12)	Median, 531	55.3% <sup>5</sup>	61.4%	10-16/20, CIA (Advia Centaur)	NA/80.7	Higher CD4/CD8 ratio; 2 or more doses compared to 1 dose only; female; no HCV infection
			Accelerated, 41	TWINRIX 720/ (0, 7, 21 d, 6-12)	Median, 543	73.2%	80.5%	5/20, CIA (Advia Centaur)	NA/70.7	
Jimenez <i>et al</i> <sup>[117]</sup>	2002-2008	Retrospective, United States	Standard 2-dose, 125	HAVRIX 1440/ (0, 6-12)	Median, 410	Median, 3.1	70.0%	Variable/< 0.8 signal relative to cut-off, CIA (Vitros ECi)	NA/54	Higher baseline CD4 count and suppressed PVL
			101	TWINRIX 720/ (0, 1, 6-12)					NA/53	
Kourkounti <i>et al</i> <sup>[118]</sup>		Retrospective, Greece	cART- experienced, 63	HAVRIX 1440 or	628	< 1.7	100.0%	7-13/20, ELFA	NA/78	Higher baseline CD4 count
			cART-naïve, 50	Vaqta 50/ (0, 6-12)	472	3.9	0.0%	(VIDAS)	NA/76	
Weinberg <i>et al</i> <sup>[119]</sup>	1994-2010	Prospective observational, United States	Hormone oral contraceptive, 13 No contraceptive, 149	2 doses (0, 6) or 3 doses (0, 2, 6)	478	47% <sup>5</sup>	78.0%	NA/20, ELISA (Mediagnost)	NA/62 NA/51	Women only study; Higher baseline CD4 count and suppressed PVL
Launay <i>et al</i> <sup>[120]</sup>	2003-2005	Randomized controlled trial, France	Standard 2-dose, 49	HAVRIX 1440/ (0, 6)	Median, 355	Median, < 1.7	78.0%	6-18/20, ELISA (ETIAB- HAVK PLUS)	6 m: 44.9/46.8 7 m: 69.4/72.3 18 m: 61.2/69.8	Absence of tobacco smoking
			3-dose, 46	HAVRIX 1440/ (0, 1, 6)	Median, 351	Median, < 1.7	83.0%		6 m: 69.6/74.4 7 m: 82.6/88.4 18 m: 78.3/85.7	
Overton <i>et al</i> <sup>[121]</sup>	1997-2004	Retrospective, United States	1 or 2-dose, 268	HAVRIX 1440/ NA (1 or 2 doses)	Mean, 447	Mean, 2.9	67.5%	NA/NA ELISA (Not specified)	NA/49.6	Male; PVL < 1000 copies/mL
Weissman <i>et al</i> <sup>[122]</sup>	2001-2003	Retrospective, United States	Standard 2-dose, 138	HAVRIX 1440/ (0, 6-12)	Mean, 424	NA	81.9%	6-13/18, EIA (Abbot IMx HAV Ab)	48.6 (67/138)	Female; CD4 count at vaccination > 200 cells/mm <sup>3</sup>
Wallace <i>et al</i> <sup>[123]</sup>	1997-1998	Randomized controlled trial, United States	Standard 2-dose, HIV-positive, 55	Vaqta 50/ (0, 6)	Mean, 457.5	4.52	76.0%	1, 6, 7, 12/10, Quantitative modified HAVAb assay (NA)	1 m: NA/61, CD4 < 300/ 300+, 48/74 7 m: NA/94, CD4 < 300/ 300+, 87/100 12 m: NA/90, CD4 < 300/ 300+, 80/100	100% of subjects with CD4 counts ≥ 300 cells/mm <sup>3</sup> seroconverted
			Standard 2-dose, HIV-negative, 72	Vaqta 50/ (0, 6)	NA	NA	NA		1 m: NA/90 7 m: NA/100 13 m: NA/90	

Kemper <i>et al</i> <sup>[124]</sup>	1995-1997	Double-blind, placebo-controlled trial, United States	Standard 2-dose, HIV-positive, 48	HAVRIX 1440/ (0, 6)	376	3.29	91.0%	1, 6, 7, 9/33, ELISA (Enzymun; Boehringer Mannheim)	1 m: NA/11 CD4 < 200/200+, 0/16 6 m: NA/9 CD4 < 200/200+, 0/13 7 m: NA/49, CD4 < 200/200+, 11/62 9 m: NA/52, CD4 < 200/200+, 9/67	Subjects with higher baseline CD4 counts were more likely to seroconvert and to have higher antibody titers
Neilsen <i>et al</i> <sup>[125]</sup>	Pre-1996	Randomized controlled trial, Australia	Accelerated 2-dose, HIV-positive, 48	HAVRIX 1440/ (0, 1)	Mean 569	NA	NA	1, 3/20, ELISA (Enzymun; Boehringer Mannheim)	1 m: NA/80.0 7 m: NA/93.2 CD4 ≤ 200, 64	MSM only study; subjects with higher baseline CD4 counts were more likely to seroconvert and to have higher antibody titers; Vaccine schedule did not affect response; HIV-negative subjects had higher seroconversion rates and GMTs
			Standard 2-dose, HIV-positive, 42	HAVRIX 1440/ (0, 6)	Mean 454	NA	NA	1, 7/20, ELISA (Enzymun; Boehringer Mannheim)	1 m: NA/75.6 7 m: NA/81.3 CD4 ≤ 200, 64	
			Standard 2-dose, HIV-negative, 46	HAVRIX 1440/ (0, 6)	NA	NA	NA	1, 7/20, ELISA (Enzymun; Boehringer Mannheim)	1 m: NA/90.2 7 m: NA/100	
Wilde <i>et al</i> <sup>[126]</sup>	Pre-1995	Prospective, United Kingdom	Three mini-dose, HIV-positive hemophiliacs, 31	HAVRIX 720/ (0, 1, 6)	Median 450 (IgG positive after 2 doses) Median 335 (IgG positive after 3 doses)	NA	0	1, 2, 7/20, EIA (SORIN Biomedica INCstar, Italy)	2 m: NA/29 7 m: NA/55	Hemophiliacs only (all anti-HCV positive); no patients with CD4 counts < 170 cells/mm <sup>3</sup> seroconverted
Tilzey <i>et al</i> <sup>[127]</sup>	Pre-1995	Prospective, United Kingdom	Three mini-dose, HIV-positive hemophiliacs, 25	HAVRIX 720/ (0, 1, 6)	NA	NA	NA	1, 2, 6, 7/20, ELISA (Boehringer-Mannheim)	1 m: NA/26 2 m: NA/50 6 m: NA/47 7 m: NA/76	Men only study; After 3 doses, all HIV-positive hemophiliacs with anti-HAV titers of < 50 mIU/mL had CD4 counts < 100 cells/mm <sup>3</sup> . HAVRIX 1440 was given as a 4 <sup>th</sup> booster dose to the 4 HIV vaccinees with anti-HAV < 50 mIU/mL after 3 doses; only 1 subsequently developed anti-HAV > 50 mIU/mL
			Three mini-dose, HIV-negative hemophiliacs, 8	HAVRIX 720/ (0, 1, 6)	NA	NA	NA		1 m: NA/57 2 m: NA/86 6 m: NA/100 7 m: NA/100	
			Three mini-dose, HIV-negative healthy controls, 25	HAVRIX 720/ (0, 1, 6)	NA	NA	NA		1 m: NA/100 2 m: NA/100 6 m: NA/100 7 m: NA/100	
Hess <i>et al</i> <sup>[128]</sup>	Pre-1994	Prospective, controlled, Germany	Three mini-dose, HIV-positive MSM, 26	HAVRIX 720/ (0, 1, 6)	495	NA	NA	1, 2, 6, 7/20, ELISA (SB Biologicals)	2 m: NA/78.6 7 m: NA/76.9	MSM only study; Seroconversion rates were independent of CD4 counts
			Three mini-dose, HIV-negative MSM, 20	HAVRIX 720/ (0, 1, 6)	NA	NA	NA		2 m: NA/100 7 m: NA/100	
Santagostino <i>et al</i> <sup>[129]</sup>	Pre-1994	NA, Italy	Three mini-dose, HIV-positive hemophiliacs, 47	HAVRIX 720/ (0, 1, 6)	NA	NA	NA	1, 2, 7, 12/20	12 m: NA/76.6	Hemophiliacs; Seroconversion rates were dependent on stage of HIV disease
			Three mini-dose, HIV-negative hemophiliacs, 66	HAVRIX 720/ (0, 1, 6)	NA	NA	NA	NA	12 m: NA/100	

<sup>1</sup>Number of HIV-positive individuals with baseline negative anti-HAV and data available; <sup>2</sup>Duration specified after the first dose when primary serological response was assayed; <sup>3</sup>Cut-off value of specific anti-HAV IgG used to define serological response; <sup>4</sup>Factors identified by multivariate analysis in HIV-positive individuals unless specified; <sup>5</sup>Percentage of patients with undetectable plasma HIV RNA load. cART: Combination of antiretroviral therapy; CIA: Chemiluminescence immunoassay; EIA: Enzyme immunoassay; ELISA: Enzyme linked immunosorbent assay; HAV: Hepatitis A virus; HCV: Hepatitis C virus; ITT: Intention-to-treat; NA: Not available; PVL: Plasma HIV RNA load; PP: Per-protocol.

compared to HIV-negative healthy adults, ranging from 48.6%-94.0%<sup>[122-125]</sup>. In a meta-analysis including 8 studies, combining a total of 458 HIV-positive patients, the overall rate of serological response to HAV vaccination was 64%<sup>[130]</sup>. In addition, the geometric mean titers (GMTs) of specific antibodies were also lower among HIV-positive individuals compared to the healthy population<sup>[115,123,127]</sup>.

Overall, factors that correlated best with the poor response to HAV vaccination among HIV-positive individuals were surrogates of immune status such as low CD4 cell counts and high plasma HIV RNA loads at the time of vaccination as shown in Table 7<sup>[115-129]</sup>. Other factors identified with low rates of seroconversion were HCV coinfection and tobacco smoking<sup>[116,120]</sup>. Both male and female genders have been associated with seroconversion<sup>[121,122]</sup>.

While the vaccination effectiveness among HIV-positive individuals was mostly evaluated by seroconversion rates in the countries of low endemicities, the serological and clinical responses to HAV vaccination were rarely investigated in the outbreak setting. In a recent prospective observational study during the outbreak of acute hepatitis A among MSM in Taiwan, the overall seroconversion rate among HIV-positive MSM was 39.7% and 93.4% after receiving 1 dose and completing 2-dose series of HAV vaccination, respectively. Despite the delayed serological response, HAV vaccination had led to a 93% reduction in the risk of acute HAV infection among HIV-positive MSM during the outbreak setting. Higher CD4 cell counts were consistently correlated with higher seroconversion rates<sup>[131]</sup>.

Studies published after the meta-analysis in 2006 made various attempts to augment the immune response to the inactivated HAV vaccine despite the aforementioned non-modifiable adverse factors. One attempt was by using a virosome-formulated HAV vaccine (Epaxal1, Berna Biotech Ltd.) to enhance the immune responses of 14 HIV-positive individuals compared to 64 healthy adults<sup>[132]</sup>. After a primary dose at day 1 and a booster dose 12 mo later, the seroconversion rates (anti-HAV IgG > 20 mIU/mL) at month 13 were 91.7% and 100% in HIV-positive adults and in healthy adults, respectively. The GMTs of anti-HAV increased from 25.5 mIU/mL after the primary immunization to 659.2 mIU/mL after the booster dose in HIV-positive adults<sup>[132]</sup>.

Other attempts were by increasing the number of doses of vaccine administered<sup>[115,120,121]</sup>. Two doses over 1 dose of HIV vaccine increased seroconversion rates in HIV-positive individuals<sup>[121,123,124]</sup>. There is less convincing evidence to show that 3 doses over 2 doses further increased seroconversion rates, possibly due to the smaller margin of benefit and the relatively larger sample size of adequate power needed to demonstrate the benefit. However, 2 studies showed trends of augmented responses in terms of

seroconversion rates and GMTs by adding a booster dose at week 4 sandwiched between the first dose and the second dose at week 24<sup>[115,120]</sup>. In the intention-to-treat (ITT) analysis, seroconversion at week 28 was observed in 82.6% vs 69.4% ( $P = 0.13$ ) and at week 48 in 84.2% vs 78.1% ( $P = 0.23$ ) in the 3-dose vs the 2-dose group for the French and Taiwanese studies, respectively.

When multiple doses have been used, the timing of the second and third dose did not affect immunogenicity in persons with limited immunodeficiency<sup>[125]</sup>. Hence, in the outbreak settings, an accelerated schedule, *i.e.*, delivering the second or third booster dose at an interval of less than 3 mo from the first dose may be preferable although more studies are needed<sup>[131]</sup>. However, in HIV-positive individuals with more advanced immunodeficiency (CD4 < 300 cells/mm<sup>3</sup> or AIDS status), it may be preferable to wait for the CD4 count to recover before delivering the booster doses<sup>[123,127]</sup>. In the most primitive example, of the 2 HIV-positive hemophiliacs with CD4 counts below 100 cells/mm<sup>3</sup> who, after the third dose of HAVRIX 720 U, went on to receive a fourth booster dose of HAVRIX 1440 U, neither seroconverted<sup>[127]</sup>.

To our knowledge, there is limited experience with using HAV vaccination as post-exposure prophylaxis in HIV-positive individuals. Although in healthy individuals, HAV vaccine has been demonstrated to be capable of protecting susceptible contacts with benefits of long-term protection when compared to passive immunization by immunoglobulins<sup>[133]</sup>.

### ***Durability of seroprotection and factors associated with persistent seroprotection***

In healthy adults following a primary 2-dose schedule, mathematical models indicate that anti-HAV antibodies may persist in > 90% of vaccinees for 40 years or more<sup>[134]</sup>. In HIV-positive individuals, a slight decrease was observed over time; 88.6%-100% of responders were still seroprotected after 1 year<sup>[115,120]</sup>, 86.8%-90% after 3 years<sup>[135,136]</sup>, 85%-85.4% after 4 years<sup>[136,137]</sup>, and 75.5%-88.4% after 5 years<sup>[135,136,138]</sup>. Percentages of seroprotection at the end of 5 years of follow-up were 78.9% vs 76.4% by ITT analysis ( $P = 0.61$ ) (Table 8)<sup>[135-138]</sup>. GMTs were significantly higher throughout each consecutive year with the 3-dose schedule as compared to the standard 2-dose schedule<sup>[136]</sup>. Factors associated with persistent seroprotection include virologic suppression at vaccination and maintained lower levels of HIV viremia as denoted by time-updated plasma HIV RNA load<sup>[135,137]</sup>, 3-dose compared to 2-dose schedule (adjusted odds ratio 3.36; 95%CI: 1.14-9.93), acute syphilis and absence of acute hepatitis C<sup>[136,138]</sup>.

Given the lower initial antibody levels, the apparent waning of antibody levels and the increasing life expectancy of HIV-positive individuals, post-vaccination booster doses may be necessary to maintain anti-

**Table 8** Long-term response rates and predictors of sustained seroprotection after hepatitis A virus vaccination in human immunodeficiency virus-positive patients

Ref.	Dates	Design/ Country	No. of patient <sup>1</sup>	HAV/ dosing schedules (mo)	CD4, cells/ mm <sup>3</sup>	PVL, log <sub>10</sub> , copies/mL	ART (%)	Timing of assay <sup>2</sup> , yr/cut- off <sup>3</sup> , mIU/ mL/Assay	Response rate (%): ITT/PP	Predictors of persistent response and comments <sup>4</sup>
Cheng <i>et al</i> <sup>[136]</sup>	2010-2015	Prospective, Taiwan	Primary responders: 2 doses, 110  3 doses, 185  Non- responders:  2 doses, 16  3 doses, 23	HAVRIX 1440 U/ 2 doses (0, 6) 3 doses (0, 1, 6)	560/415  470/315	2.5/2.8  2.9/3.3	70/56  59/63	2, 3, 4, 5/20 ELISA (ETIAB- HAVK PLUS)	At 1.5 yr: 2 doses: 90.0/93.4 3 doses: 87.0/94.7 At 5 yr: 2 doses: 76.4/88.4 3 doses: 78.9/94.2	MSM only study; 3-doses over 2-dose, syphilis, lack of acute HCV
Kernéis <i>et al</i> <sup>[137]</sup>	2006-2009	Prospective, France	Primary responders: 71 (52)	HAVRIX 1440/ 2 doses (0, 6) 3 doses (0, 1, 6)	362	62% <sup>5</sup>	NA	7, 43/20 ELISA (ETIAB- HAVK PLUS)	At 3.7 yr: Overall: 61.9/84.6	PVL < 50 copies/mL at time of last vaccine dose and a short duration of HIV infection
Jablonowska <i>et al</i> <sup>[138]</sup>	2004	Prospective, Poland	Primary responders: 66	HAVRIX 1440 (0, 6)	450	NA	37	1.5, 5/20 CIA (Cobas, Roche)	At 1.5 yr: 75.8/81.9  At 5 yr: 56.1/75.5	Lack of co-infection with HCV
Crum- Cianflone <i>et al</i> <sup>[135]</sup>	1996-2003	Retrospective, United States	116	Vaqta 50 or HAVRIX 1440 (0, 6-18)	Median, 467	50% <sup>5</sup>	62	3, 6-10/10	At 3 yr: 90 At 6-10 yr: 85	Lower PVL; PVL < 400 copies/mL

<sup>1</sup>Number of vaccinees with primary seroconversion after the last dose of vaccine; (figure in parentheses is the number of vaccinees with primary conversion and subsequent sera for follow-up of antibody persistence); <sup>2</sup>Duration specified after the first dose when primary serological response was assayed; <sup>3</sup>Cut-off value of specific anti-HAV IgG used to define serological response; <sup>4</sup>Factors identified by multivariate analysis in HIV-positive individuals unless specified; <sup>5</sup>Percentage of patients with undetectable plasma HIV RNA load. ART: Antiretroviral therapy; CIA: Chemiluminescence immunoassay; ELISA: Enzyme linked immunosorbent assay; HAV: Hepatitis A virus; HCV: Hepatitis C virus; ITT: Intention-to-treat; MSM: Men who have sex with men; NA: Not available; PVL: Plasma HIV RNA load; PP: Per-protocol.

HAV levels after 10 years in HIV-positive individuals in the absence of virologic suppression<sup>[111]</sup>. Currently, only the British HIV Association (BHIVA) recommends delivering booster vaccination every 10 years whilst other health authorities recommend regular monitoring of anti-HAV IgG and booster vaccinations only if at continued risk after seroconversion (Table 6)<sup>[111-114]</sup>. However, among immunocompetent hosts, memory responses to HAV may exist even in the absence of detectable antibodies<sup>[139]</sup>, and in the era of cART, the same may apply to HIV-positive patients with immune reconstitution<sup>[131]</sup>. Nevertheless, the strategies of booster HAV vaccination to those with waning immunity or non-responders need more studies to confirm the effectiveness.

### Vaccine safety

Serious adverse events following HAV vaccination in HIV-positive individuals are rare and not more common among HIV-positive individuals compared to HIV-negative vaccinees. HAV vaccination does not

have a significant impact on plasma HIV RNA load, progression to AIDS, or CD4 cell count<sup>[123,124,130]</sup>.

## CONCLUSION

In this review, we have found that, in developed countries of low HAV endemicity, HIV-positive individuals remain susceptible to HAV infection because of low adherence to recommended HAV vaccination, at-risk sexual behaviors, and injecting drug use, as demonstrated by the recent outbreaks of acute HAV infections among MSM and IDUs in Taiwan and Israel, respectively<sup>[71,88]</sup>, despite the implementation of HAV vaccination programs in children. Serological response rates to the recommended 2-dose HAV vaccination are lower in HIV-positive individuals than HIV-negative individuals; an additional dose of HAV vaccine may improve serological responses and durability of seroprotection in HIV-positive individuals with initial low CD4 cell counts. While clinical trials are warranted to confirm the HAV vaccine efficacy in the outbreak



setting of acute HAV infection, the recent observational study suggested that implementation of the 2-dose HAV vaccination was effective in preventing acute HAV infection among MSM. With ongoing improvements in survival and quality of life with modern cART, the importance of awareness of and adherence to HAV vaccination recommendations cannot be overemphasized among health care providers as well as at-risk populations.

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## Brain changes detected by functional magnetic resonance imaging and spectroscopy in patients with Crohn's disease

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### Abstract

Crohn's disease (CD) is a chronic, non-specific granulomatous inflammatory disorder that commonly affects the small intestine and is a phenotype of inflammatory bowel disease (IBD). CD is prone to relapse, and its incidence displays a persistent increase in developing countries. However, the pathogenesis of CD is poorly understood, with some studies emphasizing the link between CD and the intestinal microbiota. Specifically, studies point to the brain-gut-enteric microbiota axis as a key player in the occurrence and development of CD. Furthermore, investigations have shown white-matter lesions and neurologic deficits in patients with IBD. Based on these findings, brain activity changes in CD patients have been detected by blood oxygenation level dependent functional magnetic resonance imaging (BOLD-fMRI). BOLD-fMRI functions by detecting a local increase in relative blood oxygenation that results from neurotransmitter activity and thus reflects local neuronal firing rates. Therefore, biochemical concentrations of neurotransmitters or metabolites may change in corresponding brain regions of CD patients. To further study this phenomenon, brain changes of CD patients can be detected non-invasively, effectively and accurately by BOLD-fMRI combined with magnetic resonance spectroscopy (MRS). This approach can further shed light on the mechanisms of the occurrence and development of neurological CD. Overall, this

paper reviews the current status and prospects on fMRI and MRS for evaluation of patients with CD based on the brain-gut-enteric microbiota axis.

**Key words:** Brain-gut-enteric microbiota axis; Crohn's disease; Functional magnetic resonance spectroscopy; Functional magnetic resonance imaging; Gut microbiota; Inflammatory bowel disease; Metabolite; Spectroscopy

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**Core tip:** The occurrence and development of Crohn's disease (CD) have strong links to the brain-gut-enteric microbiota axis and are associated with psychological factors such as stress, anxiety and depression. In patients with inflammatory bowel disease, studies have revealed white-matter lesions and neurologic disorders. Brain activity and biochemical changes in brain regions can be detected accurately by blood oxygenation level dependent functional magnetic resonance imaging combined with magnetic resonance spectroscopy in patients with CD. This approach can further shed light on the mechanism of occurrence of neurologic CD.

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## INTRODUCTION

Crohn's disease (CD) is a chronic, non-specific granulomatous inflammatory disorder which can affect any part of the digestive tract. Most commonly, CD affects the small intestine. CD is a phenotype of inflammatory bowel disease (IBD). Geographically, CD is most prevalent in developed Western countries. However, recent epidemiologic studies definitely support a rapid increase in the incidence of CD in developing countries. Factors such as urbanization, improved sanitation, increased use of antibiotics and modern Western diets have all contributed to the rise of CD<sup>[1,2]</sup>. Therefore; it should be highly valued that the prevalence of CD will increase in the near future.

To date, the pathogenesis of CD is not fully understood. The current dogma attributes CD to biological factors such as immune response, genetic susceptibility, intestinal dysbiosis, and external environmental factors<sup>[3]</sup>. Many studies have demonstrated that the onset and development of CD are closely related to the intestinal microbiome, which has a strong relationship with the brain-gut-enteric microbiota axis in particular. In recent years, the mechanism of the brain-gut-enteric microbiota axis in CD patients has gained more

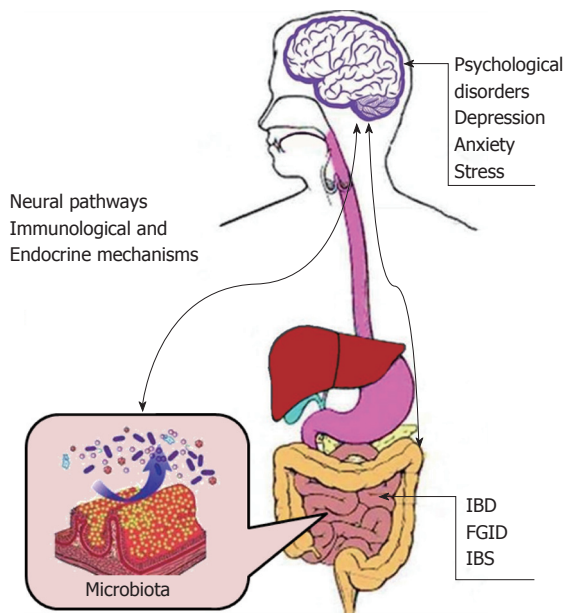
attention from researchers. In particular, the advent of blood oxygenation level dependent functional magnetic resonance imaging (BOLD-fMRI) and hydrogen proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS) has allowed non-invasive detection of brain activity and biochemical changes. Using fMRI, abnormal functional activity has been detected in the cerebral cortex of patients diagnosed with CD. This paper therefore serves as a comprehensive review of the current status and prospects on MRS and fMRI for evaluation of patients with CD based on the brain-gut-enteric microbiota axis.

## ROLE OF MICROBIOTA IN ONSET AND DEVELOPMENT OF CD

The microbiome refers to the microorganisms, their genomes and the local environment in the human intestine<sup>[4]</sup>. A healthy gut microbiome is traditionally comprised of around 100 trillion species of microbes, most of which are bacteria<sup>[5]</sup>. Interruption of the symbiotic relationship between microbiota and the gastrointestinal tract is referred to as dysbiosis, which perturbs host functions and is a precursor to disorders, such as IBD<sup>[6,7]</sup>. As Prosberg *et al.*<sup>[8]</sup> demonstrated through a systematic review, patients with active IBD had lower abundance of intestinal flora compared to patients in remission. Furthermore, the phenotypes of CD and ulcerative colitis (UC) are distinct. Therefore, the pathogenesis of IBD involves complex interactions between the immune system, the microbiome and environmental factors in genetically susceptible individuals. Specifically, an imbalance in intestinal microflora for genetically susceptible individuals can lead to abnormal immune responses within the gut and damage in the intestinal mucosal barrier<sup>[9]</sup>. This imbalance plays a key role in the progression of CD inflammation. In a study conducted by Erickson *et al.*<sup>[10]</sup>, the ileum of CD patients was found to exhibit altered carbohydrate metabolism, bacterial-host interactions and the presence of human host-secreted enzymes. It is hypothesized that the aforementioned changes in intestinal function are directly induced by an imbalanced microbiota. This result further highlights potential targets for the treatment of IBD patients<sup>[11]</sup>.

## MICROBIOTA AND ITS LINK TO THE BRAIN-GUT AXIS

The bidirectional signaling between the gastrointestinal tract and the brain is vital for sustaining homeostasis and is regulated at the neural level by both the central and enteric nervous systems. Hormonal and immunological regulations are also known to play a part. Studies<sup>[12,13]</sup> have indicated that bacteria such as commensal, probiotic, and pathogenic bacteria in the gastrointestinal tract can activate peripheral



**Figure 1 Brain-gut-enteric microbiota axis.** The bidirectional brain-gut-enteric microbiota axis between the brain and gut involves neural pathways, immunological and endocrine mechanisms, and it is closely associated with microbiota and psychological disorders such as depression, anxiety and stress. These disorders may result in FGID, IBD and IBS. FGID: Functional gastrointestinal disorders; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome.

neural pathways and central nervous system (CNS) signaling pathways. This result is hardly surprising as the gut microbiome plays an important role in basic neuroregenerative processes such as the formation of the blood-brain barrier, myelination, neurogenesis, and microglia maturation. Therefore, neural pathways in the enteric, autonomic and limbic systems along with the intestinal microbiota, immunological and endocrine systems are all regulated by the enteric microbiome. Meanwhile, the enteric nervous system is composed of small nerve cells, enteric ganglia, the nerve connectors between these ganglia, and nerve fibers that supply effector tissues. Effector tissues include intestinal smooth muscle, mucosal epithelium, intrinsic vascular and gastroenteropancreatic endocrine cells<sup>[14]</sup>. This relationship between the brain and enteric system involving the nervous system and microbiome is known as the brain-gut-enteric microbiota axis (Figure 1). A malfunction in just one of these pathways can influence the progression of CD<sup>[15-17]</sup>.

Evidently, the enteric microbiome strongly impacts brain-gut communication in the brain-gut-enteric microbiota axis. Not surprisingly, intestinal bacterial colonization plays a major role in the development and maturation of immune and endocrine systems, which are the key factors underlying CNS signaling<sup>[18]</sup>. Under control of the CNS, cells from the intrinsic layer of the lumen release chemokines into the intestinal lumen, which can lead to gastrointestinal motility, secretions, and changes in the intestinal permeability. All these factors perturb the gastrointestinal bacterial

environment<sup>[19,20]</sup>.

Animal studies<sup>[21,22]</sup> have confirmed that behavioral disorders (such as stress) can change the composition of the intestinal flora. One proposed mechanism regards mucus and norepinephrine secretion by epithelial cells under stress, which results in gastrointestinal motility changes and specific strain growth. A large number of studies<sup>[23-27]</sup> have confirmed that factors such as stress, anxiety and depression can affect the activity and recurrence of CD. Recent data suggest that gastrointestinal inflammation caused by stress may be induced upon the dysfunction of the hypothalamic-pituitary-adrenal axis. This inflammation is known to alter the interaction between bacterial and mucosal mast cells through corticotrophin releasing factor.

Current research<sup>[28]</sup> has also confirmed that intestinal microbiota can directly alter neural biochemistry and that dysbiosis may directly contribute to mental illnesses in patients with intestinal disorders. The study compared parameters of anxiety-like behavior and motor activity between specific pathogen free mice with a normal gut microbiota and germ free (GF) mice. The GF mice exhibited decreased anxiety and increased motor activity. Further studies<sup>[29-31]</sup> also revealed that animals with non-invasive infections with pathogens in the cecum showed rapid activation of brainstem nuclei and exhibited anxiety like behavior. This reaction is believed to be mediated by signals from the vagal afferents to the nuclei of the solitary tract and the lateral parabrachial nuclei.

These studies have proved that the occurrence and development of CD have strong links to the brain-gut-enteric microbiota axis and involve psychological factors such as stress, anxiety and depression.

## BOLD-fMRI FOR DETECTION OF BRAIN CHANGES IN CD

BOLD-fMRI<sup>[32,33]</sup> was first reported by Ogawa *et al.*<sup>[33]</sup> in 1990, and since then, it has become a powerful method for detecting brain activity. BOLD-fMRI functions by detecting a local increase in relative blood oxygenation that results from neurotransmitter activity and thus reflects local neuronal firing rates (Figure 2). The activity of nervous system activity is therefore detected indirectly by assaying the proportion between deoxyhemoglobin and oxyhemoglobin in blood. Inspection methods are divided into the task state and resting state (rs-fMRI). The task state is further subdivided into block design and event related methods.

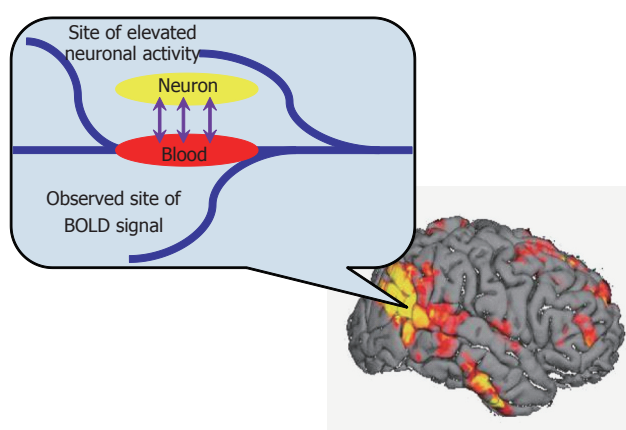
The resting state is characterized by behaviors such as slow breathing and minimal physical or mental activity. In the absence of design tasks, subjects are known to cooperate willingly with high consistency. Replicative measures are therefore more easily obtained in large numbers in the resting state. During the resting state, Rs-fMRI detects low frequency



**Table 1 Studies of blood oxygenation level dependent functional magnetic resonance imaging and magnetic resonance spectroscopy in Crohn's disease**

Ref.	Destination	Inspection	Location	Main metabolites	Results
Agostini <i>et al</i> <sup>[37]</sup> , 2013	Habituation to stress in CD	BOLD-fMRI (Task-state)	Brain	NA	Different neural activities in the amygdala, hippocampus, insula, putamen and cerebellar between CD patients and controls
Bao <i>et al</i> <sup>[38]</sup> , 2016	Brain activity in paracastic CD patients	BOLD-fMRI (Resting-state)	Brain	NA	ReHo values: Abdominal pain: insula, MCC, SMA↑, temporal pole↓; Without abdominal pain: hippocampal/parahippocampal cortex↑, dorsomedial prefrontal cortex↓
Bezabeh <i>et al</i> <sup>[41]</sup> , 2001	Diagnosis in CD and UC	MRS	Colonic mucosal	Taurine, lysine, lipid, choline, creatine	The diagnostic spectral regions include taurine, lysine, and lipids
Varma <i>et al</i> <sup>[42]</sup> , 2007	Early screening of IBD	MRS	Colonic mucosal	Creatinine and phosphatidylcholine	Triglycerides, creatine, phosphocholine and glycerol backbone of lipids are the most discriminatory metabolites
Fathi <i>et al</i> <sup>[43]</sup> , 2014	Biomarkers of CD	MRS	Serum	Alanine, glutamine, leucine/isoleucine, lysine and valine	Two chemical shifts of isoleucine (0.99 ppm) and valine (1.03 ppm) have considerable impact for discriminating patient and normal samples

BOLD-fMRI: Blood oxygenation level dependent functional magnetic resonance imaging; CD: Crohn's disease; IBD: Inflammatory bowel disease; MCC: Middle cingulate cortex; MRS: Magnetic resonance spectroscopy; NA: Not available; ReHo: Regional homogeneity; SMA: Supplementary motor area; UC: Ulcerative colitis.



**Figure 2 Mechanism of blood oxygenation level dependent functional magnetic resonance imaging.** BOLD-fMRI functions by detecting a local increase in relative blood oxygenation resulting from neurotransmitter activity and reflecting local neuronal firing rates. The nervous system activity is detected indirectly by assaying the proportion between deoxyhemoglobin and oxyhemoglobin in blood. BOLD-fMRI: Blood oxygenation level dependent functional magnetic resonance imaging.

fluctuations in functional brain regions based on blood oxygen level. Rs-fMRI analytical methods include regional homogeneity (ReHo)<sup>[34]</sup>, functional connectivity (FC)<sup>[35]</sup> and amplitude of low-frequency fluctuation (ALFF)<sup>[36]</sup>. Using fMRI, Agostini *et al*<sup>[37]</sup> and Bao *et al*<sup>[38]</sup> found abnormal functional activity in the cerebral cortex of patients with CD (Table 1).

Agostini *et al*<sup>[37]</sup> hypothesized inadequate habituation to stress as a characteristic for CD patients, and their study sought to compare neural habituation between CD patients and healthy subjects. During a high-stress task, different neural regions were activated between the two groups. Particular differences arose in the activation of the amygdala, hippocampus, insula, putamen and cerebellar regions. These contrasts revealed a stark difference in the habituation to stress between CD affected individuals and controls. Particularly, CD patients demonstrated inadequate

habituation to stress as previously hypothesized, which contribute a link between stress and exacerbated inflammation. These results suggested that the self-regulation of stress levels in CD patients is decreased, which can be an important factor in exacerbating intestinal inflammation.

Bao *et al*<sup>[38]</sup> investigated changes in resting-state brain activity in paracastic CD patients with and without abdominal pain. Regional homogeneity (ReHo) was used to assess resting-state brain activity. They found that patients with abdominal pain exhibited lower ReHo values in the insula, middle cingulate cortex (MCC) and supplementary motor area (SMA), with higher ReHo values in the temporal pole. In contrast, patients without abdominal pain exhibited lower ReHo values in the hippocampal/parahippocampal cortex and higher ReHo values in the dorsomedial prefrontal cortex. These results showed a significant negative correlation between the ReHo values of the insular and MCC activities with the daily pain scores of patients with abdominal pain. The results of this study confirmed a difference in resting state brain activity between CD patients with and without abdominal pain. Furthermore, the abnormal activity of insular and MCC regions was closely related to the severity of abdominal pain.

## MRS AND METABOLOMICS IN CD

MRS<sup>[39]</sup> utilizes nuclear magnetic resonance phenomena and chemical shifts to quantitatively analyze specific atomic nuclei and their compounds. As a non-invasive, quantitative measurement for physiological and biochemical changes of internal organs and tissue metabolism, MRS offers unparalleled versatility and safety. Not only does MRS characterize functional groups, but describes the relationships between appropriate nuclei different constitutional isomers and stereoisomers. To date, *in vivo* MRS of the brain in CD

**Table 2** Metabolites for magnetic resonance spectroscopy and their functions in normal adult human brain<sup>[66]</sup>

Metabolite	Chemical shift (ppm)	Concentration range (mmol/kgww)	Functions
NAA	2.02	7.9-16.6 (average 10.3)	An osmolyte, a storage form of aspartate, a precursor of NAAG, a marker of neuronal density
GABA	3.01	1.3-1.9	A primary inhibitory neurotransmitter
tCho	3.20	0.9-2.5	An essential nutrient that is required for synthesis of the neurotransmitter acetylcholine, and of phosphatidylcholine, a major constituent of membranes
Cr	3.05	5.1-10.6	A concentration reference
Glu	2.04-2.35	6.0-12.5	An excitatory neurotransmitter
Gln	2.12-2.46	3.0-5.8	A precursor and storage form of glutamate
mI	3.56	3.8-8.1	An essential requirement for cell growth, and a storage form for glucose
Lac	1.33-1.35	0.4	The end product of anaerobic glycolysis

Cr: Creatine; GABA:  $\gamma$ -aminobutyric acid; Glu: Glutamate; Gln: Glutamine; Lac: Lactate; mI: Myo-inositol; mmol/kgww: mmol/kg of wet weight; NAA: N-acetylaspartate; ppm: Parts per million; tCho: Choline (total), free choline, glycerophosphorylcholine, and phosphorylcholine.

patients has yet to be implemented. Studies of MRS frequently use *ex vivo* samples of CD patients, such as serum/plasma, urine, stool and colonic mucosal samples (Table 1).

MRS proves useful in the study of metabolomics, which involves the high throughput analysis, characterization and quantification of small molecular metabolites. By assaying different fluids, such as serum/plasma, urine and stool samples, the presence or absence of different metabolites can be used to distinguish between IBD and healthy volunteers. MRS can even be used to discern the different subtypes (CD and UC) of IBD as metabolite changes are directly associated with changes in intestinal bacteria<sup>[40]</sup>. These findings demonstrate that IBD is a disorder of the intestinal flora.

The differential diagnosis between CD and UC is often difficult. However, Bezabeh<sup>[41]</sup> utilized <sup>1</sup>H-MRS combined with spectral data to delineate UC and CD. Tissue samples from the colon of affected patients were assayed by spectral analysis, with a 98.6% accuracy rate for discerning UC and CD. The diagnostic spectral regions include taurine, lysine, and lipids.

In another study using MRS for metabolomics, Varma *et al.*<sup>[42]</sup> performed <sup>1</sup>H-MRS on *ex vivo* colonic mucosal samples for the early screening of IBD. Their results revealed differing levels of creatinine and phosphatidylcholine over time between IBD affected and healthy groups, and suggest the existence of biochemical changes in IBD.

Separately, Fathi *et al.*<sup>[43]</sup> explored the biomarkers of metabolism in patients with CD. Using <sup>1</sup>H-MRS metabolic profiling of the serum samples, it was shown that valine and isoleucine levels are also useful in the differential diagnosis of CD metabolites, and these metabolites can be used for high risk screening for early diagnosis of CD patients.

## CLINICAL APPLICATIONS OF MRS AND FUTURE DIRECTIONS

MRS has been most widely used in the assessment of neurologic disorders. Previous studies<sup>[44,45]</sup> have shown

that *in vivo* quantitative or semi-quantitative detection of brain tissue metabolites including glutamine (Glu), glutamate (Gln),  $\gamma$ -aminobutyric acid (GABA), N-acetylaspartate, myo-inositol, choline, creatine, glycerophosphorylcholine and phosphorylcholine can indicate neurological cell density, metabolism, permeability and other factors. The functions of clinically detectable neurochemical metabolites are listed in Table 2. Therefore, *in vivo* MRS is instrumental in the diagnosis of brain diseases such as tumors, ischemia, infection, epilepsy, metabolic disorders, dementia, mental diseases and so on<sup>[46,47]</sup>. MRS combined with clinical evaluations and conventional MRI is essential for diagnosing certain entities. Further studies<sup>[48,49]</sup> have shown that white-matter lesions and neurologic deficits in IBD patients may be an additional extra-intestinal manifestation of this disease. Despite the versatility of MRS, however, current studies more frequently employed CD spectrum analysis of urine and fecal samples of CD patients. Meanwhile, *in vivo* MRS in the brain of CD patients has yet to be implemented.

Neurotransmitter mediated signal transduction plays an important role in the regulation of cerebral blood flow, which is mainly controlled by astrocytes<sup>[50]</sup>. BOLD-fMRI studies have confirmed changes in local cerebral blood oxygen concentrations in patients with CD. Based on previous observations<sup>[37,38]</sup>, it is hypothesized that metabolites of functional brain areas will be changed accordingly in patients with CD. Specifically, the excitatory and inhibitory neurotransmitters associated with mental and psychological factors such as Glu and GABA<sup>[51,52]</sup>, respectively, are thought to vary. The coordination between excitatory and inhibitory neurotransmitters is the basis of regulated neuronal activity, and is correlated with the amplitude of the BOLD-fMRI signal. GABA baseline levels were negatively correlated with BOLD-fMRI signals of brain activity, and this result indicates the correlation between the BOLD-fMRI signals and GABA levels<sup>[53]</sup>.

In addition, Glu and Gln serve as major excitatory neurotransmitters. Due to their similar molecular structures, the pair is often referred to as Glx. Studies

of MRS in chronic pain<sup>[54-56]</sup> suggested that altered levels of Glx and GABA are present in patients with chronic pain, suggesting the role of neurotransmitters in pain management. Mullins *et al.*<sup>[57]</sup> further used <sup>1</sup>H-MRS to investigate changes of brain metabolites in patients scoring high on the self-assessed pain scale. Results indicated that the onset of pain can induce a dynamic increase in Glu concentration in the anterior cingulate cortex. It was also found that an increased Glu concentration was significantly related to the pain level of participants' subjective experience.

While increases in Glx are corresponded to increased pain perception, GABA<sup>[44,58,59]</sup> is an inhibitory neurotransmitter which plays an important role with Glx in neurotransmission and pain. GABA concentration in brain tissue *in vivo* is relatively low (< 2 mmol/L), and the MRS spectrum shows strong overlap with other metabolites. Therefore, traditional MRS is not optimal for detecting GABA. In light of this limitation, the spectral editing technique MEGA-PRESS (MEscher-GARwood Point RESolved Spectroscopy)<sup>[60]</sup> was designed to allow accurate detection of GABA. Results are encouraging and offer potential applications in the screening of neurodegenerative diseases, mental disorders, acute and chronic pain.

Functional magnetic resonance spectroscopy (fMRS) has been proposed for various applications<sup>[61-65]</sup>, especially in conditions involving psychological factors. MRS combined with fMRI is known as fMRS and can be used to detect changes in brain metabolites with high accuracy.

## CONCLUSION

Current BOLD-fMRI studies have confirmed changes in local cerebral blood oxygen concentrations in patients with CD. Therefore, it is hypothesized that the level of metabolites in functional brain areas will be changed accordingly. The correlation of these changes can be related with the pathogenesis of CD. Meanwhile, the progression of CD with respect to psychological factors and symptom of abdominal pain requires further investigation. Based on the brain-gut-enteric microbiota axis, fMRS can be used to study brain activity and biochemical concentrations of key neurotransmitters, particularly Glx and GABA, in patients with CD. fMRS studies therefore offer unpatrolled versatility for evaluation of patients with CD and serve to prevent further disease progression and relieve symptoms of abdominal pain for patients suffering from CD.

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## Perspectives of traditional Chinese medicine in pancreas protection for acute pancreatitis

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### Abstract

Acute pancreatitis (AP) is one of the most common diseases. AP is associated with significant morbidity and mortality, but it lacks specific and effective therapies. Traditional Chinese medicine (TCM) is one of the most popular complementary and alternative medicine modalities worldwide for the treatment of AP. The current evidence from basic research and clinical studies has shown that TCM has good therapeutic effects on AP. This review summarizes the widely used formulas, single herbs and monomers that are used to treat AP and the potential underlying mechanisms of TCM. Because of the abundance, low cost, and safety of TCM as well as its ability to target various aspects of the pathogenesis, TCM provides potential clinical benefits and a new avenue with tremendous potential for the future treatment of AP.

**Key words:** Acute pancreatitis; Traditional Chinese medicine; Alternative therapy; Pancreas protection; Anti-inflammatory

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**Core tip:** Specific and effective therapies for the treatment of AP are lacking. Traditional Chinese

medicine (TCM) exhibits beneficial, curative effects in basic research and clinical studies of AP treatment. Because of its abundance, low cost, safety and ability to target various aspects of AP pathogenesis, TCM provides a promising complementary and alternative therapy for the treatment of AP.

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## INTRODUCTION

Acute pancreatitis (AP) is characterized by the activation of trypsinogen and the establishment of a local inflammatory response in the pancreas, with the risk of developing into severe acute pancreatitis (SAP), characterized by systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS)<sup>[1-3]</sup>. The pathogenesis of AP is not clear. Research in recent decades has focused on trypsinogen activation, pancreatic microcirculation malfunction, calcium overload and inflammatory pathways<sup>[4]</sup>. Pancreatic exocrine secretion inhibitors, such as octreotide, demonstrate a modest preventative role in the treatment of AP<sup>[5]</sup>; however, specific and effective therapies are lacking. Given the limited treatment of option, patients seek additional therapies to improve the therapeutic effect, which leading many to focus on complementary and alternative therapies.

Based on the unique traditional Chinese medicine (TCM) theoretical system and effective treatment methods, people have used TCM to prevent and address diseases for centuries, and more attention has been directed to this medicinal approach in recent studies<sup>[6-9]</sup>. TCM has demonstrated its superiority in the management of AP and other inflammatory diseases in China for many years<sup>[10]</sup>. Under the guidance of TCM pharmaceutical theory, AP is categorized as epigastric pain, splenic precordial pain, splenopyretic disease and knotted chest disease. The principle of treatment in TCM is to clear away the heat-evil (heat as a pathogenic factor that causes heat pattern/syndrome) and expelling superficial evils, supplementing *qi* (vital energy) and nourishing *yin* (body fluid), activating blood circulation to dissipate blood stasis and inner communication and purgation.

The philosophical basis of TCM is influenced by a holistic view that aligns with a theory of organism balance regulation in modern medicine. The theory of TCM in AP treatment is localized not only in the pancreas but also in the integrity and functional regulation of the organism. TCM coincides with the concepts of modern medicine and has attracted

increasing attention as a combination therapy for AP<sup>[11]</sup>. As a complementary therapy, TCM that uses formulas and single herbs is increasingly considered to be effective and safe for treating AP<sup>[12]</sup>. Recently, the published guidelines for AP treatment in China stated that by using formulas and single herbs, TCM can be used as an alternative therapy for AP, and they acknowledged that TCM has exhibited good clinical effects. Accumulating evidence has demonstrated that TCM reduces the levels of serum and urinary amylase, decreases the permeability of capillaries, depresses the production of inflammatory cytokines, inhibits neutrophilic granulocyte activation and attenuates pancreatic injuries. These benefits block multiple steps in the development of AP according to experimental and clinical studies. Based on its characteristics of improved symptoms, reduced medical costs and increased satisfaction of AP patients, TCM appears to be a promising complementary and alternative therapy for the treatment of AP<sup>[7,13,14]</sup>. This review will provide a new understanding of the properties of TCM with an emphasis on the regulation of important molecular targets in AP in the context of basic and clinical research and the representative TCM approaches that can be combined with classic treatments of AP.

## TCM FORMULAS

A TCM formula is the combination of several types of medicinal herbs or minerals that can amplify the therapeutic efficacies of each agent. The theory of the compatibility of medicinal ingredients is the principle of formula prescriptions. A formula commonly comprises various medicines, which are usually named sovereign (*jun*), minister (*chen*), assistant (*zuo*) and messenger (*shi*) ingredient drugs because of their different roles in the formula; these ingredients affect multiple targets and exert synergistic therapeutic effects, which lead to maximal therapeutic efficacy with minimal adverse effects<sup>[15]</sup>. The frequently used formulas for AP treatment are liquid medicines that are termed "decoction", such as dachengqi decoction, qingyi decoction, yinchenchengqi decoction, chaiqinchengqi decoction, huoxueqingyi decoction and dahuangfuzi decoction.

### Dachengqi decoction

Dachengqi decoction, one of the famous formulas in China for purgation, comprises rheum, immature bitter orange, natni sulfas natura, and *Magnolia officinalis* bark. Dachengqi decoction was first recorded in the classic TCM masterpiece *Shang Han Lun* (*Treatise on Febrile Diseases*) and has been widely used for acute abdominal pain throughout China<sup>[16-18]</sup>. Animal experiments have shown that dachengqi decoction increases cell viability, induces pancreatic acinar cell apoptosis, reduces acinar necrosis and protects from injuries to the pancreas *in vivo* and *in*

*vitro*. The likely therapeutic mechanisms of action of dachengqi decoction may operate through reducing ROS generation and regulating the nitric oxide pathway in a rat SAP model<sup>[19]</sup>. Randomized controlled trials have reported that dachengqi decoction decreases serum resistin levels, significantly reduces mortality and exerts a beneficial therapeutic effect in SAP patients<sup>[20,21]</sup>. Concerning intestinal mucosal permeability, Chen *et al*<sup>[18]</sup> found that dachengqi decoction promoted the recovery of intestinal mucosal permeability and decreased the incidence of MODS and pancreatic infection in patients with SAP, which is supported by other studies<sup>[22]</sup>. Regarding abdominal compartment syndrome in SAP patients, Zhang *et al*<sup>[23]</sup> found that the combination of dachengqi decoction and conventional therapy decreased the intra-abdominal pressure of SAP patients and provided therapeutic effects on the abdominal compartment syndrome of SAP. Wan *et al*<sup>[24]</sup> investigated the effects of combined therapy with a modified dachengqi decoction and dexamethasone in the treatment of SAP patients on survival, acute respiratory distress syndrome (ARDS), renal failure, haemorrhage, sepsis, pancreatic pseudocyst, pancreatic abscess, operability, and days of hospitalization. Their data demonstrated that the modified dachengqi decoction combined with dexamethasone can decrease the risk of developing ARDS in SAP patients with SIRS, and it shortened their length of hospitalisation. This finding suggests that the modified dachengqi decoction is a safe and beneficial treatment option for SAP patients with SIRS.

### **Qingyi decoction**

Qingyi decoction is the most famous formula for AP treatment and consists of rheum, Chinese thoroughwort root, white peony root, baikal skullcap root, *Coptis chinensis* and other herbs. Qingyi decoction was invented by Doctor Boyu Zhang of the Shanghai University of Traditional Chinese Medicine and has exhibited good therapeutic effects for AP, as demonstrated in many preclinical and clinical studies. By targeting the inflammatory reaction in AP, qingyi decoction down-regulated the levels of serum endotoxin,  $\alpha$ -amylase and tumour necrosis factor- $\alpha$  and reduced the expression of inflammatory factors in SAP rats. Moreover, the formula can ameliorate AP-induced intestinal barrier injury and lung injury. The mechanism may operate through inhibiting the overexpression of intestinal- and lung-secreted phospholipase A2<sup>[25,26]</sup>. Concerning bacterial translocation, qingyi decoction increased the density, altitude and area of intestinal mucosa and up-regulated the level of intestinal histone, which inhibited intestine permeability and bacterial translocation from the intestine. Wu *et al*<sup>[27]</sup> found that qingyi decoction combined with sodium sulfate was significantly superior to sodium sulfate alone in improving clinical

symptoms; it also shortened hospitalizations and reduced the recovery time in SAP patients. Combined with enteral nutrition, qingyi decoction displayed improved clinical benefits in promoting the recovery of intestinal function and in regulating the balance of inflammatory cytokines<sup>[28]</sup>.

### **Other formulas**

Yinchenchengqi decoction is another frequently used formula in China for the treatment of AP. This decoction consists of *Artemisia scoparia*, *Gardenia jasminoides*, *Magnolia officinalis* bark, immature bitter orange, rheum and natni sulfas natura. Yinchenchengqi decoction may protect the pancreas by up-regulating Bax gene expression to induce apoptosis in the pancreatic acinar cells that are already injured; this treatment prevented cell necrosis in haemorrhagic necrotizing pancreatitis in rats<sup>[29]</sup>. In addition to the induction of apoptosis, this formula may down-regulate the expression of inflammatory mediators by inhibiting nuclear factor-kappa B (NF- $\kappa$ B) activation<sup>[30]</sup>.

Consistent with its use in AP therapy for thousands of years in China, chaqinchengqi decoction has been shown to inhibit the pancreatic enzymes and anti-inflammatory activity in patients with AP. Wang *et al*<sup>[10]</sup> found that chaqinchengqi decoction significantly decreased the level of the serum pro-inflammatory cytokine interleukin-6 (IL-6) within the first 48 h of AP onset; it also improved symptoms and shortened hospitalization times in 107 AP patients.

Huoxueqingyi decoction, which comprises *Salvia miltiorrhiza* and qingyi decoction, is a modified qingyi decoction that has been widely used in the treatment of AP. It has been demonstrated that huoxueqingyi decoction that is administered rectally, intragastrically or orally shortens the hospital stay, reduces hospitalization costs and decreases the duration of SIRS and hyperamylasemia in patients with SAP. Furthermore, the formula did not induce any adverse effects such as liver injury. Therefore, huoxueqingyi decoction provides an effective, safe and economic therapeutic option<sup>[31]</sup>.

Dahuangfuzi decoction, which consists of rheum, aconite, and *Asarum sieboldii*, is a famous traditional Chinese prescription with strong anti-inflammatory effects. Wu *et al*<sup>[32]</sup> found that dahuangfuzi decoction reduced the serum alanine aminotransferase (ALT) level and attenuated pancreas and liver injuries that are induced by SAP, and the anti-inflammatory mechanism of dahuangfuzi decoction operates by inhibiting the JAK/STAT signal pathway in SAP rats.

Although the decoctions have evident effects on AP, the applications of most decoctions are oral, which limits their utility because patients in the acute stage of AP have no access to food or water. In addition, the multiplicity of formulations also leads to issues with TCM standardization. This dilemma has led to the prevalence of studies on single Chinese medicinal



herbs and monomers.

## SINGLE CHINESE MEDICINAL HERB

Single Chinese medicinal herbs such as rheum, *Salvia miltiorrhiza*, natrii sulfas, baikal skullcap root, Saiko and *Gardenia jasminoides* have also been applied to AP treatment.

### Rheum

As a classic TCM purgative, rheum has been widely used and has commonly served as the principal component in many traditional Chinese formulas for AP treatment. Rheum has been found to trigger enterokinesia, prevent translocation of intestinal bacteria, regulate intestinal flora, repair the intestinal mucosal barrier, and have an obvious therapeutic effect in SAP rats. Furthermore, rheum can inhibit the intestinal inflammatory response and ultimately improve the prognosis and outcome in SAP rats by down-regulating the signalling of the toll-like receptors (TLR)-2 and -4<sup>[33]</sup>. Currently, rheum is widely used as an adjunctive treatment in China's guidelines for AP therapy and demonstrates good clinical effects. Wan *et al.*<sup>[34]</sup> investigated the effects of a combined therapy using early enteral nutrition (EEN) and rhubarb. A randomized controlled trial showed that combined EEN and rhubarb significantly decreased white blood cell counts, plasma C-reactive proteins and IL-6 levels and increased plasma IL-11 levels, thus inhibiting systemic inflammation. Furthermore, the trial found that the time of abnormal bowel movements, recuperation from high fever, periods in intensive care units and duration of hospital stays were all shortened in the combined EEN and rhubarb group. In addition, combined EEN and rhubarb can reduce abnormally high levels of plasma alanine aminotransferase, aspartate aminotransferase, and creatinine (Cr) and mitigates SAP-related liver and kidney dysfunction. Zhou *et al.*<sup>[35]</sup> investigated the combined effect of rhubarb and somatostatin in AP patients and found that it significantly reduced the total complications and APACHE II scores in patients with AP; this finding reveals that rhubarb can serve as an adjunctive therapeutic tool in AP treatment.

### Salvia miltiorrhizae

*Salvia miltiorrhizae* is a commonly used traditional Chinese herb to activate blood and remove stasis. It has been proven that *Salvia miltiorrhizae* has anti-inflammatory properties, and in SAP rats, it cleared reactive oxygen species, induced apoptosis, and improved microcirculation, thus demonstrating some protective effects<sup>[36]</sup>. *Salvia miltiorrhizae* can protect multiple organs, strengthen immune function and thereby decrease the mortality of SAP rats. The mechanism may be through a reduction in plasma endotoxin levels, the inhibition of intercellular cell adhesion molecule-1, TLR4, and NF-κB expression,

and the regulation of the protein levels of apoptosis-related gene Bax<sup>[37,38]</sup>.

### Natrii sulfas

Natrii sulfas plastering therapy is a common adjunctive treatment of AP therapy that has been used in recent years with a rheum application and conventional therapy. The data from clinical research that was conducted in 60 AP patients showed that conventional therapy combined with intragastric rhubarb administration and natrii sulfas plastering therapy can relieve the symptoms of abdominal pain and distention, decrease serum and urine amylase levels, and reduce the APACHE II score compared with a control group<sup>[39]</sup>. Because sodium sulfate is the major component of natrii sulfas and has diuretic, detumescent and anti-inflammatory properties, natrii sulfas plastering therapy can adsorb moisture from the abdomen and thus disperse the swelling of the abdominal wall and intestinal canal.

## TCM MONOMERS

### Emodin

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), an anthraquinone, with the molecular formula of C<sub>15</sub>H<sub>10</sub>O<sub>5</sub> and a molecular mass of 270.23, is isolated from the traditional Chinese herb of rheum. Emodin has exhibited excellent biological activities in inflammatory diseases, such as antibacterial, anti-inflammatory, antioxidant, antitumour and immunomodulatory properties, and it inhibits trypsinogen secretion and improves the microcirculation<sup>[40,41]</sup>. Regarding the systemic inflammatory responses in AP, researchers have investigated the anti-inflammatory pharmacological mechanism that is induced by emodin in AP rats. The data showed that emodin reduced serum trypsinogen, serum pro-inflammatory factor tumour necrosis factor-α (TNF-α), and IL-6 and IL-1β levels, and it inhibited NF-κB DNA-binding activity and enhanced peritoneal macrophage phagocytosis and apoptotic cell clearance. Emodin attenuated pancreatic damage through the inhibition of the TLR4 signal pathway, NF-κB and endoplasmic reticulum stress<sup>[42-45]</sup>. Emodin has also been reported to inhibit the abnormal metabolism of gadoleic acid and to improve pancreatic ischaemia in SAP. Concerning lung injury in AP, emodin intervention has been shown to up-regulate the expression of aquaporin-1, aquaporin-5, claudin-4, claudin-5 and occludin in lung tissue and decrease the histopathologic score. Emodin has also been reported to improve blood gas indexes, pulmonary oedema, vascular leakage, and alveolar epithelial barrier function, which ameliorated the acute lung injury that was induced by SAP<sup>[46,47]</sup>. Emodin has also been shown to up-regulate the mRNA expression of the apoptosis-related gene Bax, induce apoptosis in pancreatic acinar cells, and reduce cell necrosis in the pancreas. Its underlying mechanisms

may operate through the inhibition of the TLR2 and TLR4 signal pathways and immune inflammation regulation<sup>[48,49]</sup>. Moreover, emodin has induced NO liberation, improved microcirculation of the pancreas, promoted cell regeneration and prevented pancreatic fibrosis. Gong *et al.*<sup>[50]</sup> reported that emodin increased transforming growth factor  $\beta$ 1 and epidermal growth factor gene expression, which subsequently increased DNA synthesis and protein content and thereby accelerated pancreatic repair and regeneration<sup>[51]</sup>. In addition, Wang *et al.*<sup>[52,53]</sup> investigated the combined effect of emodin and EEN on SAP. Their data showed that the combination of emodin and EEN reduced the severity of experimental SAP in rats, and the combined strategy was rational, safe and more effective than the use of either EEN or emodin alone. In our previous study, we similarly confirmed the therapeutic effects of emodin *in vivo*<sup>[49,54]</sup>.

### Baicalein

Baicalein (5,6,7-trihydroxyflavone-7-O-D-glucuronic acid) is a flavonoid that is extracted from baikal skullcap root, a traditional Chinese herb. Baicalein has excellent antioxidant and anti-inflammatory activities and can be an anti-inflammatory agent<sup>[55]</sup>. In our previous study, we found that baicalein exerted an anti-inflammatory capability and showed a therapeutic effect in SAP rats. We investigated changes in pancreatic histopathology, ascites fluid and serum inflammatory mediators after baicalein treatment and found that baicalein was effective in decreasing the pancreatic histopathology score, reducing ascites fluid production and balancing the network between pro-inflammatory mediators and anti-inflammatory mediators. In addition, our study indicated that baicalein protected against pancreatic injury and led to improved survival in SAP rats<sup>[56]</sup>. Moreover, based on the theory of TCM, modern medicine, and the theory of the compatibility of medicinal ingredients, we have refined the classic qingyi decoction and selected baicalein and emodin for a combination treatment approach. We propose that the combined use of baicalein and emodin blocks multiple steps in the development of AP and exerts more profound therapeutic effects on pancreatic injuries in SAP rats without adverse effects<sup>[54,57]</sup>. The glycoside of baicalein that was mentioned above, which is called baicalin (5,6-dihydroxyflavon-7-yl  $\beta$ -D-glucopyranosiduronic acid), also has many biological properties, including antioxidant, anti-bacterial, antiviral, and anti-inflammatory effects<sup>[58]</sup>. Zhang *et al.*<sup>[59]</sup> showed that baicalin inhibited serum P-selectin expression, decreased serum inflammatory cytokine levels and induced apoptosis of thymocytes in SAP rats.

### Scutellarin

Scutellarin is extracted from the plants of the *Scutellaria* genus and has effective bioactivity. Scutellarin

has been reported to dilate blood vessels, improve cardiovascular and cerebrovascular ischaemia, and inhibit activation of NF- $\kappa$ B from acute lung injury in mice<sup>[60,61]</sup>. Chen *et al.*<sup>[62]</sup> investigated the pharmacological mechanisms of serum amylase inhibition and the protection of multiple organs (pancreas, liver, kidneys and lungs) by scutellarin in SAP rats. The data showed that scutellarin decreased serum ALT, Cr and amylase levels and relieved the pathologic changes of multiple organs. Furthermore, acute and subacute toxicity studies were performed to evaluate the safety of scutellarin. These data showed that scutellarin has a sufficient margin of safety for therapeutic use in rodents<sup>[63]</sup>.

### Ligustrazine

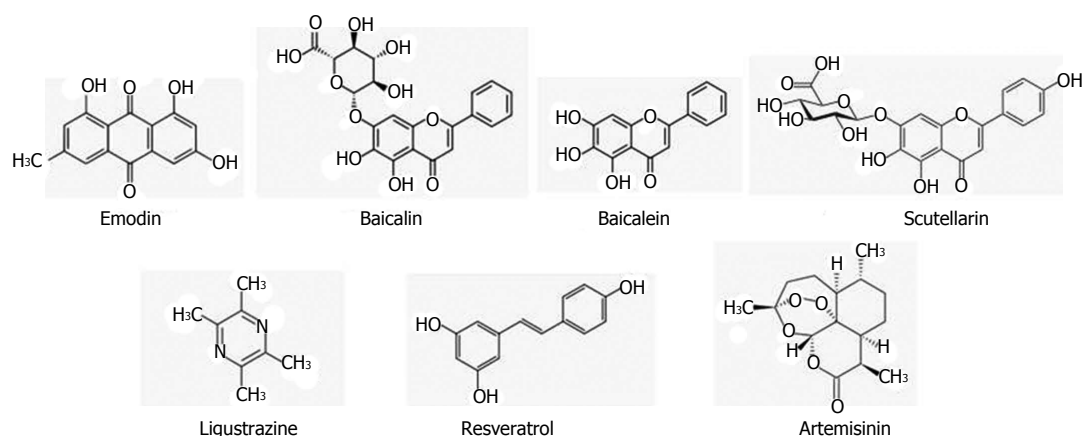
Ligustrazine is an alkaloid that is isolated from the traditional Chinese herb Szechuan lovage rhizome. Ligustrazine is a new type of calcium antagonist that has antiplatelet properties, improves microcirculation and enhances cerebral blood flow<sup>[64]</sup>. Ligustrazine has been shown to effectively induce pancreatic acinar cell apoptosis and prevent the apoptosis of cells in the liver and kidneys, which decreased the pathological score of these organs in SAP rats<sup>[65]</sup>. The mechanism of ligustrazine may operate by suppressing the p38 and ERK/MAPK pathways<sup>[66]</sup>. Moreover, ligustrazine has been shown to effectively decrease serum amylase levels and inflammatory cytokines and alleviate pathological changes in the pancreas, liver, kidney, small intestinal mucosa, thymus and spleen, which protect the body from multiple organ injuries<sup>[66,67]</sup>.

### Resveratrol

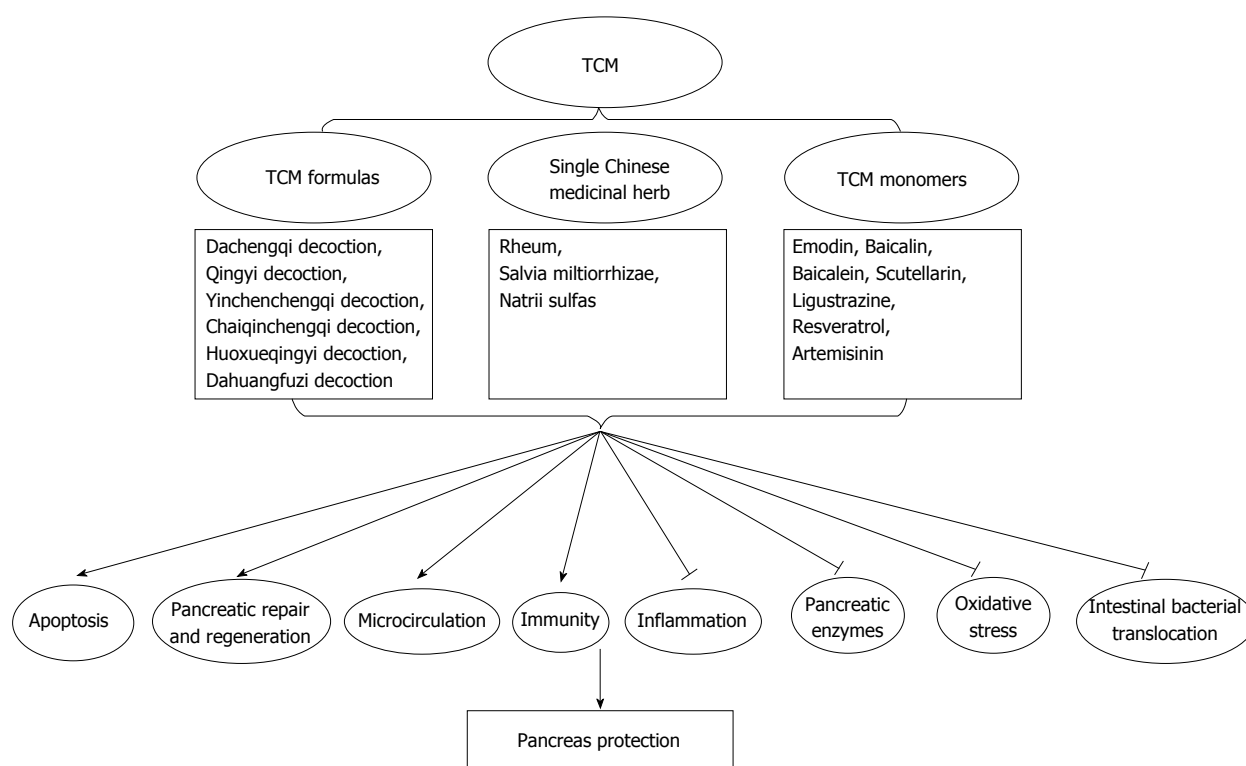
Resveratrol is a polyphenol that is isolated from the herb *Polygonum cuspidatum*, and it has high bioactivity, such as anti-inflammatory, antioxidative and anti-platelet aggregation activities. Resveratrol has been shown to effectively induce apoptosis in the pancreas, inhibit serum amylase release and inflammatory reactions, suppress microcirculatory disturbances, and alleviate pancreatic pathological injuries through the up-regulation of FasL expression and the down-regulation of the levels of angiotensin II, endothelin, nitric oxide and TNF- $\alpha$ <sup>[68-70]</sup>. In addition, the antioxidant and immunomodulatory properties of resveratrol may supply a promising chemopreventative approach in AP prevention<sup>[71]</sup>, which coincides with the core belief of TCM as a preventive treatment.

### Artemisinin

Artemisinin is a sesquiterpene that is isolated from the traditional Chinese herb sweet wormwood. Artemisinin is a specific antimalarial that also has antileukaemic and immunoregulation properties. Researchers have studied the effect of artemisinin on AP rats and found that artemisinin reduced trypsinogen excretion, inhibited the activation of neutrophilic granulocytes,



**Figure 1** Chemical structures of Traditional Chinese medicine monomers.



**Figure 2** Diagram showing proposed mechanisms of Traditional Chinese medicine for acute pancreatitis treatment. Traditional Chinese medicine (TCM) induces apoptosis, accelerates pancreatic repair and regeneration, improves pancreas microcirculation and strengthens the immunity function and results in the pancreas protection. In addition, TCM mediates inhibition of inflammatory reaction, pancreatic enzymes, oxidative stress and intestinal bacterial translocation, which may contributes to the pancreas protection in AP treatment.

and induced pancreatic acinar cell apoptosis. These results suggest that artemisinin alleviated the severity of AP through the caspase-3 signalling pathway and by inducing intrinsic apoptosis<sup>[72]</sup>.

The chemical structures of TCM monomers mentioned above are presented in Figure 1.

## CONCLUSION

Presently, TCM exhibits good curative effects in AP treatment (Table 1 and Figure 2). However, the ambiguity of the mechanism is an obstacle to the

internationalization and generalization of TCM. Better designed trials are needed to make significant advances in the management of AP. Both pre-clinical and clinical studies have shown promising uses for TCM as a complementary and alternative therapeutic strategy for the treatment of AP that can even supplement conventional treatments, but TCM remains an alternative therapy in AP treatment. The inconvenience of the decoctions has limited the application of TCM. In most formulas, the essential compounds have not been identified. The main obstacle to the internationalization of TCM is the difficulty of standardizing the large

**Table 1** Commonly used traditional Chinese medicine for AP treatment and their action targets/mechanisms

TCM	Targets/mechanisms	Ref.
Dachengqi decoction	Induce apoptosis, protect from pancreas injury, recover intestinal mucosal permeability	[16-24]
Qingyi decoction	Anti-inflammation, inhibit pancreatic enzymes, inhibit intestinal bacterial translocation, protect from organ injury	[25-28]
Yinchenchengqi decoction	Induce apoptosis	[29]
Chaiqinchengqi decoction	Inhibit pancreatic enzymes, anti-inflammation	[10]
Huoxueqingyi decoction	Shorten hospital stay, reduce hospitalization cost, decrease duration of SIRS, alleviate hyperamylasemia	[31]
Dahuangfuzi decoction	Anti-inflammatory, protect from organ injury	[32]
Rheum	Anti-inflammation, inhibiting intestinal bacterial translocation, protect from organ injury, accelerating pancreatic repair and regeneration	[33-35]
Salvia miltiorrhizae	Anti-inflammation, induce apoptosis, improve microcirculation, clean reactive oxygen species, protect from organ injury, strengthen the immunity function	[36-38]
Natrii sulfas	Relieve symptoms, inhibit pancreatic enzymes, reduce APACHE II score	[39]
Emodin	Anti-inflammation, inhibit endoplasmic reticulum stress, protect from organ injury, induce apoptosis, improve pancreas microcirculation, accelerate pancreatic repair and regeneration	[40-54]
Baicalin	Anti-inflammation	[54, 56-57]
Baicalein	Induce apoptosis	[58-59]
Scutellarin	Inhibit pancreatic enzymes, protect from organ injury	[60-63]
Ligustrazine	Induce apoptosis, anti-inflammation, inhibit pancreatic enzymes, protect from organ injury	[64-67]
Resveratrol	Induce apoptosis, anti-inflammation, inhibit pancreatic enzymes, antioxidant, immunoregulation	[68-71]
Artemisinin	Induce apoptosis, anti-inflammation, inhibit pancreatic enzymes	[72]

TCM: Traditional Chinese medicine.

number of herbs in one decoction. Along with the further exploration of the precise mechanisms of TCM action, it is hoped that a thorough understanding of the use of TCM in AP treatment strategies and its ability to target various aspects of the pathogenesis of AP will reveal the profound therapeutic benefits of TCM in the future.

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## Transition of pediatric to adult care in inflammatory bowel disease: Is it as easy as 1, 2, 3?

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### Abstract

Inflammatory bowel disease (IBD) is a heterogeneous group of chronic diseases with a rising prevalence in the pediatric population, and up to 25% of IBD patients are diagnosed before 18 years of age. Adolescents with IBD tend to have more severe and extensive disease and eventually require graduation from pediatric care to adult services. The transition of patients from pediatric to adult gastroenterologists requires careful preparation and coordination, with involvement of all key players to ensure proper collaboration of care and avoid interruption in care. This can be challenging and associated with gaps in delivery of care. The pediatric and adult health paradigms have inherent differences between health care models, as well as health care priorities in IBD. The readiness of the young adult also influences this transition of care, with often times other overlaps in life events, such as school, financial independence and moving away from home. These patients are therefore at higher risk for poorer clinical disease outcomes. The aim of this paper is to review concepts pertinent to transition of care of young adults with IBD to adult care, and provides resources appropriate for an IBD pediatric to adult transition of care model.

**Key words:** Inflammatory bowel disease; Adolescents; Young adults; Transition care; Transition to adult care

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**Core tip:** The process of transition of a young adult with inflammatory bowel disease (IBD) to adult care should be well coordinated and incorporate all key players to ensure proper collaboration and avoid interruption in care. An early, regular assessment of the adolescent readiness for transition is important.

The adolescent patient should be seen without the parent or caregivers in order to build self-reliance. Pediatric gastroenterologists need to discuss and introduce the concept of transition with the patient and family early and identify adult gastroenterologists with unique interests in young adults with IBD. The adult gastroenterologist should be prepared for the transition with advanced communication with the referring pediatric team, consider further training in adolescent health, and review health priorities and targets of care early with the young adult.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a heterogeneous group of diseases that includes both ulcerative colitis (UC) and Crohn's disease (CD). In the United States, nearly 1.5 million Americans are affected by this chronic disease, and approximately 30% of CD and 20% of UC patients have disease onset prior to the age of 20<sup>[1,2]</sup>. Population-based studies demonstrate a rising incidence of pediatric IBD over the last decade and the projected prevalence of pediatric onset of disease is about 10000 new cases annually<sup>[2,3]</sup>.

Similar to other chronic diseases such as cystic fibrosis (CF) and diabetes, children with IBD eventually require a transfer of care to an adult gastroenterologist. This transition from pediatric to adult care carries potential challenges, given the inherent differences between pediatric and adult health care models, disease characteristics and phenotype, and treatment strategies. There are two key elements to this healthcare transition. One is the transition of care from the primary caregiver or guardian to the patient, and the other is the transfer of responsibility from the pediatric gastroenterologist to an adult provider<sup>[4]</sup>. Without both elements, the process of transition will inevitably fail and result in poorer patient and disease outcomes.

This paper highlights the need for a structured transition process for IBD patients that graduate from pediatric to adult gastroenterology care and provides an overview of this process.

## DIFFERENCES IN PEDIATRIC AND ADULT HEALTH CARE MODELS

The pediatric healthcare model is family-focused and requires parental involvement for knowledge, understanding, guidance and consent<sup>[5,6]</sup>. Pediatric

specialists have also received baseline training in pediatric care and are appropriately sensitive to child-specific psychosocial needs. The delivery of care is usually designed to include a multi-disciplinary team of providers and medical staff.

Conversely, the adult care model promotes independence and individualized care<sup>[5,6]</sup>. Medical care is often carried out by a single provider. The focus of care is on the disease, with less attention on growth and puberty, and psychological development. The familiarity of the adult subspecialists on adolescent developmental milestones and its impact on disease is often limited.

Alongside these differences in models of health care, the pediatric patient is often also involved in other transitions, including possible graduation, new employment, financial independence, and moving away from family and home. These potential overlapping events may also affect health care models, including sudden new and unfamiliar assignment to a local adult healthcare provider, less oversight by caregiver or parent, economic barriers and access to healthcare and pharmacological therapies, and possible noncompliance or adherence to treatment.

## DIFFERENCES IN PEDIATRIC AND ADULT INFLAMMATORY BOWEL DISEASE

There are reported differences in pediatric and adult disease phenotype and behavior. Goodhand and colleagues<sup>[7]</sup> conducted a retrospective case-control of 100 adolescents matched with 100 adults with IBD, and described a more complex and aggressive disease phenotype among the adolescents. Specifically, adolescents with IBD were more likely to have extensive CD with ileocolonic involvement as compared to adults with disease restricted to only ileum or colon alone (69% vs 28%). Adolescents were also more likely to have perianal disease as compared to adults, 33% vs 16%. There was a higher incidence of pancolitis among adolescents with UC (67%) as compared to adults (39%) who had disease limited mostly to the left colon. There were also differences in therapies, with adolescents more likely than adults to require immunomodulator therapy (53% vs 13%) and biologic agents (20% vs 8%), which also suggests more severe disease.

Vernier-Massouille *et al*<sup>[8]</sup> described the natural history of patients with pediatric onset of disease and found that these patients were more likely to have upper gastrointestinal involvement, extraintestinal manifestations, and higher risk for steroid dependence and stricturing and penetrating disease. Among the pediatric IBD patients, 44% required surgery at some point from the time of diagnosis, with a 34% risk within the first 5 years of diagnosis. Adolescents were also more likely to require hospital admission (14%-46%) and more likely to miss appointments (0-20%)



compared to adults<sup>[7]</sup>. Hartman and colleagues reported that nutritional impairment and weight loss occurred in up to 85% of IBD children, which further affects patient growth and development<sup>[9]</sup>.

Mental health disorders are also more common and there is a lowered self-esteem, increased depression and anxiety, behavioral problems, body image distortion, and impaired social competence described among adolescents diagnosed with IBD<sup>[10]</sup>. In a meta-analysis review, Greenley *et al*<sup>[11]</sup> described higher rates of depressive disorders among young IBD patients compared to other chronic conditions. The negative impact of depression and anxiety on medical adherence has also been well described<sup>[12]</sup>.

Because of the more severe disease phenotype, need of chronic medications and multiple psycho-medical co-morbidities such as malnutrition, delayed growth, and underlying depression or anxiety, there needs to be uninterrupted care and management of IBD as the move toward adulthood is made, to ensure improved outcomes.

## TRANSITION OF CARE IN OTHER CHRONIC DISEASES

Transition of care should not be mistaken by a simple transfer of care, which is a “planned movement of patient and their medical records from one provider to another at a distinct point in time”<sup>[13]</sup>. The transition of care is a “purposeful planned movement of adolescents and young adults with chronic physical and medical conditions from child-centered to adult-oriented health care systems”<sup>[14]</sup>. The transition process should be well-coordinated and incorporate all key players to ensure proper collaboration and avoid interruption in care. Unlike a transfer, which is a single event, the transition is a gradual process that allows the young adult to acquire behavioral skills and knowledge to assume full responsibility for their health care needs and management of their disease.

The medical providers that are involved should understand the rationale for the transition process, have the knowledge and skills to facilitate this process, and also know when the transition is indicated. Because the transition period is a vulnerable time, it is important to have a structured program in place, which can have a positive effect on the clinical and medical, as well as psychological outcomes for the patient.

There are several examples of transition of care with other chronic diseases, such as cystic fibrosis (CF), type 1 diabetes and congenital heart disease. Pediatricians have long been aware of the need and challenges of ensuring that CF patients receive appropriate services as they move into adult care. CF pediatricians and other medical providers have been leaders in the development of models for transitional care for over 20 years<sup>[15]</sup>. Examples of different CF-transition models include: CF-trained pediatrician

to continue to provide the health service for life; pediatrician to transfer the care to adult physicians at “CF centers”; and lastly the pediatrician to transfer the care to both a designated local chest physician and with joint care at a designated CF center<sup>[15]</sup>. Regardless of the adopted model, the transition process is made clear to the patient and the provided care is appropriate for the needs of the patient. There is convincing evidence of improved survival of CF patients, despite no medical cure, and attributed in part to the improvement and proactive care provided during childhood and with the appropriate, purposeful transition to adult care<sup>[15]</sup>.

Similarly, among young adults with type 1 diabetes mellitus who underwent a structured transition of care as opposed to those only referred to an adult care provider by a referral letter or simple transfer, had improved attendance in clinic and hemoglobin A1c (HbA1c) levels<sup>[16]</sup>.

## TRANSITION OF CARE IN INFLAMMATORY BOWEL DISEASE

Emerging adulthood, defined as the first period of adult life from age 18 to 25, is often considered an unstable period between adolescence and full adulthood<sup>[13]</sup>. In IBD, as with other chronic diseases, “emerging adulthood” may be a longer period of time by default of the chronic disease. The emotional dynamics of the chronic disease and close dependence (excessive or appropriate) could contribute to the adolescents’ difficulty progressing to the age appropriate emotional maturity and independence. The emerging adults with IBD (EAI) coined by Trivedi and Keefer<sup>[13]</sup>, have an increased risk for disease progression, including stricturing and penetrating disease, and other complications of disease<sup>[17]</sup>. Studies have also demonstrated a higher economic burden among EAI, including increased all-cause total health care costs in both UC and CD<sup>[18,19]</sup> as well as the highest utilization of emergency services of any adult sub-population<sup>[20]</sup>. Poorer adherence to treatment plans and attendance to clinic visits are also reported to be the lowest among EAI<sup>[21]</sup>.

Although there are several described models of transition of care in IBD implemented in clinics in the United States and other Westernized countries, an ideal model for a structured transition program in IBD has yet to be defined. There is also suggestion that a “transition program may either be a physical clinic with a dedicated, multidisciplinary group of care providers or a conceptual element of the care provided in a more traditional clinic setting”<sup>[4]</sup>.

Hait *et al*<sup>[6]</sup> conducted a survey of adult gastroenterologists to determine which patient competencies are necessary for a successful transition to an adult practice. The study concluded that adult providers have identified a deficiency in the young adults’ knowledge

of disease, medical history and medications, and also reported that pediatric gastroenterologists need to improve communication with patients and referring providers. Less than half of the adult gastroenterologists in the survey reported competency with adolescent developmental and mental health issues, despite 89% who believed this was important. The study also concluded that adult gastroenterologists may benefit from additional formalized training in adolescent issues.

A United Kingdom study<sup>[22]</sup> of adult and pediatric gastroenterologists surveyed to identify the needs of adolescents with IBD and barriers to a successful transition, concluded that 80% of pediatric providers were more likely to consider a structured transition process to be very important, as compared to 47% among adult providers ( $P = 0.001$ ). Similar to the prior study, more adult vs pediatric gastroenterologists identified deficiencies in preparation of adolescents for transition of care, including lack of disease knowledge about disease condition and treatments (79% vs 42% respectively,  $P = 0.001$ ).

Trivedi and Keefer<sup>[13]</sup> describe three primary adult provider skill sets for the care of an EAI patient, which includes: "(1) understand the natural history, disease phenotype, complications and treatment options for IBD; (2) appreciate nutrition, growth, and radiation exposure concerns; and (3) recognize the convergence and divergence of traditional pediatric and adult care models in IBD". The authors also describe "expanded skill sets" which is crucial in the care and management of EAIs, and incorporates a good understanding of adverse effects of IBD treatments, implications of disease and therapies on sexual, fertility and reproductive systems, issues related to unemployment and disability, and understanding of health insurance and coverage plans<sup>[13]</sup>.

## ORGANIZING A TRANSITION PROGRAM IN INFLAMMATORY BOWEL DISEASE

The North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) has published recommendations regarding the transition of patients with childhood onset of IBD to adult care<sup>[23]</sup>. The NASPGHAN recommendation to the pediatric gastroenterologist is to begin the *process* of transition when the patient enters early to middle adolescence and: (1) see the adolescent patient without their parents or caregivers in order to build independence and self-reliance; (2) discuss and introduce the subject and the benefits of a transition to an adult gastroenterologist early on to the patient and family; (3) select an experienced adult gastroenterologist knowledgeable and interested in the unique needs of young adults with IBD; and (4) provide all appropriate medical records and summaries to the adult

gastroenterologist in advance, to ensure confidence in the patient and family that the pediatric and adult providers are working together. The NASPGHAN Medical Summary Form can be found in Appendix A of this medical position statement<sup>[23]</sup>.

Acquiring the skills necessary for transitioning to the adult setting should also not be limited to a single encounter and therefore a healthcare transition plan is needed. NASPGHAN has created a "Healthcare Provider Transitioning Checklist" for patients and providers that can be utilized to help evaluate the readiness of the adolescents to transition of care. It also provides information to the healthcare provider to better understand these goals and improve ways to help the adolescent best achieve appropriate readiness for transition. It is recommended that the process begins in early adolescence, age 12-14 years<sup>[24]</sup>.

A regular assessment of the adolescent readiness for transition is important. These assessments help identify areas where the adolescent may need further disease education to achieve independence and competence in transition-relevant skills.

The evaluation of readiness is difficult to measure and there have been several clinical instruments used to guide this process. One example is the Transition Readiness Assessment Questionnaire (TRAQ)<sup>[25]</sup>, which is a patient-reported assessment of health and skills needed for management of medications, tracking health issues and talking with healthcare providers. Benchimol *et al*<sup>[26]</sup> also developed a portable medical record for the patient called the MyHealth Passport for IBD, which allows patients and parents to answer questions about disease course, medical and treatment history. Both TRAQ and MyHealth Passport for IBD are available online for download and can be utilized in clinic and provided to all patients.

Morisky *et al*<sup>[27]</sup> Adherence Scale may also be used as a clinical instrument tool to identify patients with potential adherence problems. It can be used to monitor treatment adherence once therapies are initiated, as well as identify or address any potential adherence problems early and guide health care providers to provide reinforcement and clinical advice for better outcomes.

Seattle Children's Hospital Inflammatory Bowel Disease Center recently developed a readiness tool for the Pediatric-to-Adult IBD Transition Clinic. This form is available through the electronic medical record system and can be printed out at the time of the clinic encounter. The form contains separate age appropriate lists of questions to the adolescent patient, all pertaining to their diagnosis, treatment plan and general knowledge about IBD and patient's individual health care access. The goal of the structured interaction between the IBD team member and the patient is to emphasize the importance of knowing these answers firsthand and prepares the patient for

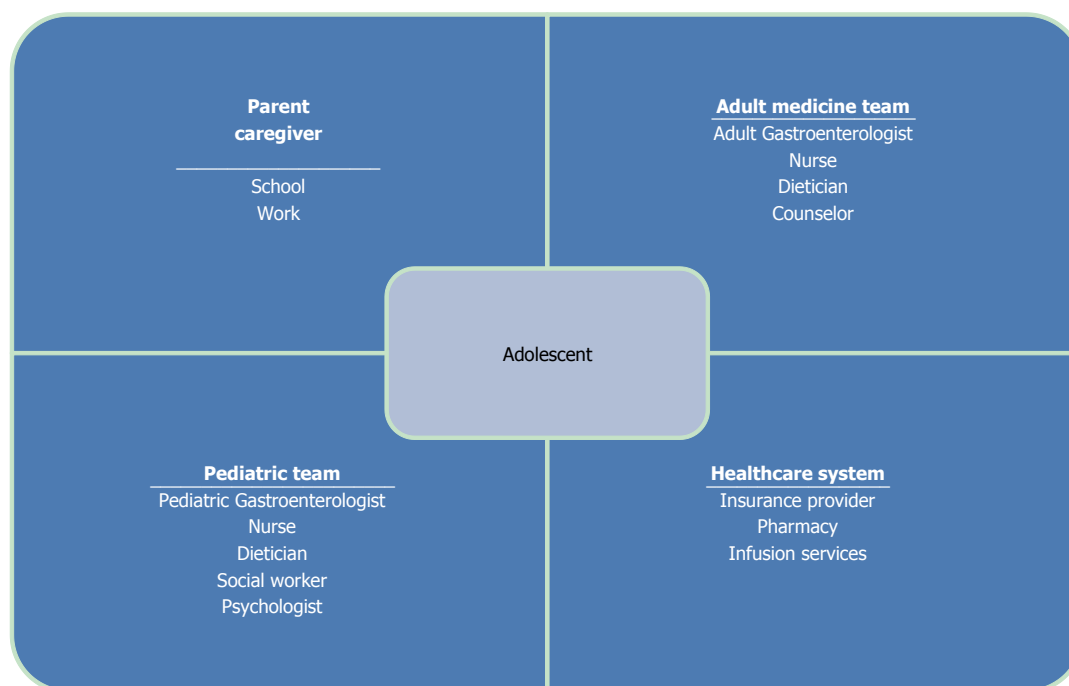


Figure 1 Transition of care involves multiple stakeholders invested.

the next age appropriate list of questions on future visits.

Lastly, the PHQ-9 Depression Screening Tool<sup>[28]</sup> is also another useful instrument that has incorporated the DSM-IV diagnostic criteria for depression and can be applied in clinic along with the transition readiness and adherence tools. Based on the severity score, treatment recommendations and appropriateness for anti-depressants, psychotherapy or support alone can be considered.

The transition of care is usually undertaken after completion of the developmental milestones and appropriate assessment for readiness of the adolescent. This is usually a transition target age of 18 or 19. The transition of care is not usually recommended when the adolescent is hospitalized or with acute bowel disease flare, but rather when the young adult is able to manage his or her disease largely independent of caregivers and professional staff, and is overall at baseline or stable with their disease and current treatment or management of disease. Certainly, there are exceptions to this particularly when the EAI has medical refractory disease and warrants special consideration such as enrollment into a clinical trial or options of treatment not yet FDA-approved for pediatric patients. Hait *et al.*<sup>[29]</sup> describe a suggested timeline, noting that this process is not a "rigid protocol" but rather a process that requires tailoring based on the developmental abilities of the adolescent and is more based on emotional and cognitive maturity and competency as opposed to chronological age alone.

## IDENTIFICATION OF KEY PLAYERS OR STAKEHOLDERS IN THE TRANSITION OF CARE

The transition of care involves multiple stakeholders invested in their respective roles in a seamless continuation of the adolescent's health care (Figure 1). Recognizing the important roles of the key players in this multidisciplinary team and enhancing timely communication between them is essential.

## GOALS AND BARRIERS IN THE PRACTICE FOR A SUCCESSFUL TRANSITION OF CARE

Escher JC identified three goals in the process of transition in IBD: "(1) to get the patient ready for transfer, having attained specific skills and knowledge; (2) to get the parents ready for transfer; and (3) to get the adult gastroenterologist ready and well informed at the time of transfer"<sup>[30]</sup>.

The Social-ecological Model of Adolescent and Young Adult Readiness to Transition (SMART) developed and validated by Schwartz and colleagues<sup>[31,32]</sup> has identified seven major inter-related components of patients, parents and providers which impacts transition readiness and potential targets of intervention including: patient development, knowledge, skills/efficacy to managing health, beliefs/expectations of the transition process, transition goals, relationships among patients, parents

and providers, and psychosocial functioning regarding conditions and emotions related to the transition process; as well as pre-existing factors which are less modifiable but may still influence the transition process including socio-demographics/culture, insurance/access, health status, risks and problems due to complications of disease, and neurocognition/IQ. These components are either barriers or facilitators of a successful transition of care and further validated in a different study that described that SMART components including patient developmental maturity, parental involvement and degree of provider support influenced the success of transition of care and outcomes that included healthcare utilization, health and quality of life<sup>[33]</sup>.

Achieving the goals of a successful transition of care program may be difficult due to limitations of resources or access to clinics with a structured program, poor readiness or preparation for the young adult to transition, limited communication between the referring pediatric gastroenterologists to the receiving adult providers, and suboptimal training in adolescent medicine for adult gastroenterologists<sup>[4,22,34-38]</sup>.

Other factors may also impede the success of IBD transition of care. As we assess and track the readiness of the patient for the transition, there may be reluctance by parent, caregiver or even the pediatric gastroenterology team. This reluctance or inability to "let go" by other key players involved in this process certainly also impacts the transition.

Because there is no standardized age for transition of care, a heterogeneity in milestones achieved by the young adult, such as high school or college graduation, vocation, employment, marriage or pregnancy to name a few, may also impact the transition process. A lack of IBD centers and few adult gastroenterologists in the local area with special interests in the care and management of IBD patients is another barrier towards a successful transition of care for a young adult. Leung and colleagues summarized practical guidelines to aide not only adolescents through the transition process, but recommendations were also outlined for both pediatric and adult gastroenterologists even when a structured IBD transition clinic is unavailable<sup>[39]</sup>.

Early preparation and good communication is essential in the transition process, but despite this, another limitation is the differences in health priorities among all key stakeholders<sup>[40]</sup>. These differences in priorities or targets of care needs to be identified and discussed early in the transition in order to help both the EAI and the adult gastroenterologist understand and best achieve them. It is also important to acknowledge that healthcare goals such as achieving clinical and mucosal remission, dysplasia surveillance and preventative health care, are usually different than pediatric care goals such as psychosocial development, growth and nutrition<sup>[13]</sup>. A close professional and educational relationship between pediatric and adult IBD providers is key to understanding the various

management goals, priorities and perhaps "styles" of practice, along the adolescent's continuum of IBD care.

Lastly, the variation in health care systems and access of care in the United States and other countries further complicates this process. Among both adult and pediatric gastroenterologists, lack of funding, time, support of services, training and too few of IBD transition of care patients were the top five obstacles experienced in their local setting which impacted the ability to deliver transition of care services<sup>[22]</sup>.

There are also additional logistical issues during the transition process and questions of concern include: Who is in charge for the management of the patient in transition? Where should the transition clinic be established - the pediatric or adult clinic? When can multiple providers all be available to meet with the patient and parents/caregivers at the same time? Finally, which gastroenterologist, pediatric or adult, can bill for the clinic visit?

## CONCLUSION

As we begin to consider IBD as a disease that begins in childhood and continues into adulthood, we face the same challenges for transitioning of care as other chronic diseases. This is a process that requires careful coordination and collaboration from key stakeholders of a multidisciplinary team including first and foremost the patient, as well as parents/caregiver and providers. Although a number of transition clinic models currently exist, there is paucity of data and gap in knowledge for the care and management of this vulnerable young population. The developmental maturity and core competency of the EAI, early preparation and good communication by the pediatrician and an expanded skill set and training for the adult gastroenterologist will allow for a smoother transition and best impact clinical outcomes.

Larger, prospective studies are needed to help standardize transition care practices. Data-driven assessment of strategies aimed to optimize competency and development, communication, education, and adherence will ultimately help improve clinical outcomes in the IBD transition of care.

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## Colorectal cancer population screening programs worldwide in 2016: An update

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### Abstract

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. The incidence and mortality show wide geographical variations. Screening is recommended to reduce both incidence and mortality. However, there are significant differences among studies in implementation strategies and detection. This review aimed to present the results and strategies of different screening programs worldwide. We reviewed the literature on national and international screening programs published in PubMed, on web pages, and in clinical guidelines. CRC Screening programs are currently underway in most European countries, Canada, specific regions in North and South America, Asia, and Oceania. The most extensive screening strategies were based on fecal occult blood testing, and more recently, the fecal immunochemical test (FIT). Participation in screening has varied greatly among different programs. The Netherlands showed the highest participation rate (68.2%) and some areas of Canada showed the lowest (16%). Participation rates were highest among women and in programs that used the FIT test. Men exhibited the greatest number of positive results. The FIT test has been the most widely used screening program worldwide. The advent of this test has increased participation rates and the detection of positive results.

**Key words:** Colorectal cancer; Colorectal cancer screening; Fecal occult blood test; Fecal immunochemical test; Colonoscopy

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**Core tip:** Colorectal cancer is the third most commonly diagnosed cancer worldwide. The incidence and mortality show wide geographical variations across the world. Screening is recommended to reduce both, however, there are significant differences among studies in implementation strategies and detection. This review aimed to present the results and strategies of different screening programs worldwide.

Navarro M, Nicolas A, Ferrandez A, Lanás A. Colorectal cancer population screening programs worldwide in 2016: An update. *World J Gastroenterol* 2017; 23(20): 3632-3642 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3632.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3632>

## INTRODUCTION

Colorectal cancer (CRC) is one the most commonly diagnosed cancers worldwide. Among men with cancer, CRC ranked third in prevalence (746000 cases, 10% of the total male population), after lung and prostate cancers. Among women with cancer, CRC ranked second in prevalence (614000 cases, 9.2% of the total female population), after breast cancer. CRC incidence and mortality show wide geographical variations across the world. When comparing age-standardized incidence rates (ASRIs) of CRC in different countries, we found the highest rates in Australia and New Zealand, and the lowest rates in Western Africa<sup>[1]</sup>.

Nearly 55% of CRC cases occur in developed regions, but CRC-related mortality is highest in less developed countries (including regions of Africa). This poor survival is probably due to the lack of available health resources. However, high- and low-income countries also show large variations in the proportion of the population included in CRC registries. These variations may arise from underdiagnoses, due to local medical and economic situations<sup>[2]</sup>.

In many regions, the risk of developing CRC is around 5%, and of those patients, 45% will die despite treatment<sup>[3]</sup>. According to GLOBOCAN data, 694000 individuals died in 2012 from CRC worldwide (374000 men and 320000 women). Mortality rates show less variability than incidence rates; the highest estimated CRC-related mortality rates in both sexes were found in Central and Eastern Europe, and the lowest were found in Western Africa<sup>[1]</sup>.

In Europe, there are huge variations in the ASRIs of CRC; the lowest incidences were observed among men and women in Bosnia Herzegovina (30 per 100000 and 19 per 100000, respectively) and in Albania (13 and 11 per 100000 respectively). Among men, the highest incidences were observed in Slovakia, Hungary, and the Czech Republic. Among women, the highest incidences were found in Norway, Denmark, and Holland<sup>[1]</sup>. Although mortality rates are generally

geographically similar to incidence rates, mortality is sometimes high in countries with relatively low incidence rates (Moldavia, Russia, Montenegro, Poland, and Lithuania)<sup>[4]</sup>. In North America, the ASRi of CRC was estimated to be 26.1 per 100000. In 2016, the American Cancer Society estimated that 134490 new CRC cases would be diagnosed in individuals of both sexes, and that 49190 individuals would die from CRC in the United States<sup>[5]</sup>. In the Eastern Mediterranean region, the highest CRC incidence was found in Israel (36 per 100000), followed by Jordan and Kazakhstan (26 and 23 per 100000, respectively). The highest mortality rates were found in Jordan, followed by Kazakhstan, Armenia, and Israel. In the Asian Pacific region, the incidence of CRC varies among regions. The highest incidence was reported in South Korea (ASRi: 45 per 100000). Singapore and Japan also had high incidence rates (ASRIs: 34 and 32 per 100000, respectively). Compared to those regions, other countries, like India, have much lower ASRIs (6 per 100000) and age-standardized mortality rates (ASRm: 5 per 100000)<sup>[4]</sup>.

CRC qualifies for screening according to the criteria established by Wilson and Jungner as the "gold standard of screening assessment"<sup>[6-8]</sup>. The criteria that CRC fulfills include its high incidence rate, its long preclinical phase, its recognizable and tractable precursor, and the correlation between the tumor stage and mortality rate. Although the value of the Wilson and Jungner criteria remains undisputed to this day, newer policy tools are now available (Table 1)<sup>[9]</sup>.

Screening for CRC appears to be cost-effective compared to no screening<sup>[10]</sup>. However, CRC screening programs must be adapted to the risk of each population. An average-risk population is defined as a population of individuals aged 50 years or older, with no additional risk factors. The recommended screening for the average-risk population is one of the following: an annual or biennial fecal immunochemical test (FIT); sigmoidoscopy every 5 years; or colonoscopy every 10 years. When subgroups are identified and characterized with a higher-than-average incidence of colorectal neoplasia, it is necessary to increase the screening frequency to achieve program cost-effectiveness<sup>[11]</sup>. Despite recommendations, screening is currently offered to only a small proportion of the population.

In this review, we evaluated the results of 17 screening programs. We obtained ASRIs and ASRms in the different countries reviewed from the website: [www.globocan.iarc.fr](http://www.globocan.iarc.fr). A literature search was conducted in PUBMED with the following keywords: Screening, Colorectal Cancer, Bowel cancer, guidelines, programmes, program, results, FIT, guaiac, first round, pilot, rounds, Europe/ United Kingdom/ Ireland/ The Netherlands/ Lithuania/ Italy/ Croatia/ Czech Republic/ Slovenia/ France/ Canada/ California/ USA/ Korea/ Australia/ Thailandia/ Taiwan/ Chile. CRC



**Table 1** New screening criteria (Adapted from: Andermann *et al.*<sup>[9]</sup>)

Emerging screening criteria proposed after Wilson and Junger principles
The screening programme should respond to a recognized need
The objectives of screening should be defined at the outset
There should be a defined target population
There should be scientific evidence of screening programme effectiveness
The programme should integrate education, testing, clinical services and programme management
There should be quality assurance, with mechanisms to minimize potential risks of screening
The programme should ensure informed choice, confidentiality and respect for autonomy
The programme should promote equity and access to screening for the entire target population
Programme evaluation should be planned from the outset
The overall benefits of screening should outweigh the harm

screening program characteristics were also collected from national governmental websites. To evaluate and compare screening programs, we used universally applicable CRC screening indicators.

## CRC SCREENING TESTS

### Stool tests

It has been established that CRC mortality could be reduced by screening with periodic fecal occult blood tests (FOBTs), followed by colonoscopy when the results were positive. A systematic review published in 2008, which included 4 clinical controlled trials involving over 300000 participants, found that screening with FOBT reduced the relative risk (RR) of CRC mortality by 16%, without adjustment, and by 25% after adjusting for screening attendance<sup>[12]</sup>. The follow-up of those patients showed that the effect of screening was reduced CRC-mortality, and this effect persisted for over 30 years<sup>[13]</sup>. A reduction in the RR of CRC incidence was also detected in follow-up, mainly due to the removal of adenomatous polyps. This effect was greater among individuals that received annual screenings (20% RR) than among those that received biennial (17%) screenings<sup>[14]</sup>.

Currently, there are two different tests available: the FIT and the guaiac fecal occult blood test (G-FOBT). The FIT achieved significantly higher detection rates for advanced adenomas and CRC than the G-FOBT. Although the FIT was more sensitive than the G-FOBT (61% vs 23.8%, respectively), its specificity was slightly lower (95.1% vs 97.7%, respectively)<sup>[15,16]</sup>. Participation rates appeared to be higher in screening programs that used FIT compared to those that used G-FOBT<sup>[15]</sup>. This issue is probably related to the facts that FIT does not require dietary restrictions (due to its specificity to human hemoglobin [Hb]) and that only one sample is needed in most screening programs<sup>[16]</sup>. Additionally, FIT offers quantitative results (ng Hb per mL buffer or µg Hb per gram feces) and an automated reading of the results. The cut-off value for the amount of Hb detected can be predetermined by the investigator. Several cut-off values have been used with different sensitivity and specificity rates. The investigator can adjust the

cut-off value to limit the number of colonoscopies required, and thus, avoid overextending the available endoscopic resources. An optimum cut-off value has not been established; therefore, the choice should be based on the availability of endoscopic resources, the epidemiology of CRC in the study population, and the expected participation in the program<sup>[17]</sup>. Values between 20 and 30 µg/g are recommended when the Health Care System can accommodate colonoscopies for approximately 5% (expected FIT positivity rate) of the population study (aged 50-74 years)<sup>[18]</sup>.

Based on current evidence, the FIT has been recommended as the first option for detecting fecal occult blood in CRC screening<sup>[19]</sup>. Most European countries with an organized screening program are currently using the FIT. This test has replaced the G-FOBT in screening programs in the United Kingdom since 2014 and in France since 2015<sup>[4]</sup>.

Other non-invasive techniques are available, such as the fecal DNA analysis. These tests identify molecular alterations in adenomas and CRC cells. However, these tests are currently underused, due to the high cost and relatively low cost-effectiveness<sup>[20]</sup>.

### Invasive techniques

Flexible sigmoidoscopy screening was shown to be effective in reducing the incidence and mortality rates of CRC<sup>[21,22]</sup>. It should be taken into account that, when a distal adenomatous polyp is detected in a sigmoidoscopy, a colonoscopy is required. This is necessary, because the characteristics of adenomas found in the rectum and sigma are correlated with the probability of presenting a proximal CRC<sup>[23,24]</sup>.

Colonoscopy screening is used in several programs. Data are scarce from randomized clinical trials that have attested to its effectiveness. However, several observational studies have reported that colonoscopy screening reduced CRC mortality and incidence, mainly due to its great capacity for detecting neoplasias and adenomas. In a case-control study, performing a colonoscopy, regardless of its indication, was associated with a large reduction in the risk of CRC in the following 10 years after the test. This effect was greater when the colonoscopy had been used as a screening test<sup>[25]</sup>. Several cohort studies have

confirmed this finding. One study showed that, in an average-risk population, performing a colonoscopy was associated with a 67% reduction in CRC incidence after an 8-year follow-up<sup>[26]</sup>. Another study demonstrated the long-term protective effect of a polypectomy. When a colonoscopy was performed with a polypectomy of at least one adenoma > 5 mm, the CRC incidence was reduced by 80% after a 10-year follow-up<sup>[27]</sup>.

Colonoscopy quality has varied in reports from different endoscopists. For this reason, over the last decade, a series of quality indicators for colonoscopy have been described. However, application of these indicators has not become well established in endoscopic practice<sup>[28]</sup>. Currently, the main quality indicator among endoscopists is the adenoma detection rate (ADR). The ADR is defined as the proportion of screening colonoscopies performed by a physician that detected at least one histologically confirmed colorectal adenoma or adenocarcinoma. The recommended ADR is  $\geq 25\%$  (men  $\geq 30\%$ , women  $\geq 20\%$ )<sup>[29]</sup>. Several studies have demonstrated a strong correlation between the ADR record of an endoscopist and the probability of diagnosing CRC within a given number of months of a colonoscopy (an interval CRC)<sup>[24]</sup>. Despite the clinical importance of this measure, large variations remain among endoscopists.

One study analyzed the results of colonoscopies performed in the US through an integrated health services organization, over a 12-year period. The association between the ADR and the risk of diagnosing CRC within 6 mo to 10 years after the first colonoscopy was evaluated, and the risk of death from cancer was calculated. They studied a total of 314872 colonoscopies performed by 136 gastroenterologists, with ADRs that ranged from 7.4% to 52.5%. They identified 712 interval colorectal adenocarcinomas and 147 associated deaths. The gastroenterologists were placed into quintiles, based on their ADRs (lowest ADR quintile  $\leq 19.06\%$  and highest ADR quintile  $\geq 33.51\%$ ). They found that patients examined by gastroenterologists in the lowest ADR quintile had almost double the risk of being diagnosed with an interval cancer compared to patients examined by gastroenterologists in the highest quintile. In addition, the risk of a fatal interval cancer was reduced by 62% among patients examined by gastroenterologists in the highest quintile. Each 1% increase in the ADR was associated with a 5% decrease in the risk of a fatal interval colorectal cancer<sup>[30]</sup>.

Although the colonoscopy is more effective than the FOBT for detecting neoplasias and adenomas, the FOBT is more readily accepted by participants in population screening programs. Thus, the higher FOBT participation rates may counteract its lower detection capacity<sup>[31]</sup>. The COLONPREV study hypothesized that biennial FIT screening would be non-inferior to a one-time colonoscopy, for reducing CRC-related mortality among subjects with average risk. They recruited more than 50000 asymptomatic participants between

ages 50 and 69 years, and randomly assigned them to undergo either a one-time colonoscopy or the biennial FIT. After the first round of FIT screening, they confirmed a similar CRC detection rate with both methods. However, advanced adenomas and other adenomas were detected at a higher rate in the colonoscopy than in the FIT groups. This result confirmed, once again, the superiority of the colonoscopy for detecting this type of lesion. Therefore, the colonoscopy has higher potential than the FIT for reducing the CRC incidence. Nevertheless, the higher participation rate in the FIT group (34.2% vs 24.6%), and the biennial periodicity of this test may reduce the apparent advantage of colonoscopy over the long term<sup>[32]</sup>. Final results from that study are expected in the next few years.

## CRC SCREENING PROGRAMS

### *CRC screening programs in Europe*

In 2003, based on compelling evidence, the Council of the European Union recommended that all Member States should establish early detection programs with CRC screening for men and women aged 50 to 74 years, with annual or biennial FOBTs, followed by colonoscopy, when the results were positive<sup>[33]</sup>. Following this recommendation, several CRC screening programs were launched in Europe, with wide variations in screening practices, probably due to different preexisting screening programs (pilots, opportunity-based, or organized) in several countries. Variations among different countries also arose due to differences in financial resources available for research and differences in colonoscopy capacities.

In 2015, 24 countries in the European Union had established or were preparing to organize country-wide CRC screening programs. For example, Finland, France, Slovenia, and the United Kingdom had completely implemented organized programs. In Belgium, the Netherlands, Denmark, Ireland, Italy, Malta, Poland, and Spain, programs were being launched. Norway, Portugal, and Sweden were in the pilot phase. In contrast, other countries, including Slovakia, with the highest CRC rate in Europe<sup>[1]</sup>, did not have a national screening program. Similarly, no screening programs existed in Bulgaria, Albania, Bosnia, Herzegovina, Kosovo, Macedonia, Montenegro, Romania, Serbia, and Russia<sup>[4]</sup>.

An analysis of different programs in several European countries showed that Croatia and the Czech Republic had the lowest participation rates ( $< 25\%$ ), and both countries reported high ASRms (18.7% and 15.4%, respectively), followed by France (participation rate 34.3%). The other countries achieved better participation rates (over 45%); the highest participation was observed in the Netherlands, followed by Slovenia. The Netherlands had the highest positive test rate (test positivity, 12.2%), but the lowest cut-off value for the FIT test: 15  $\mu\text{g/g}$ . The

**Table 2 Results of European Screening Programs**

Country	Netherlands	Italy	Ireland	Lithuania	Croatia	Czech Republic	Slovenia	England	France
ASRi	40.2	33.9	34.9	23.4	32.9	39.9	37	30.2	36.1
ASRm	13.4	10.8	12.2	13.7	18.7	15.4	16.2	10.7	12.9
Period	2014-2015	2007-2009	2008-2009	2009-2012	2007-2011	2000-2011	2009-2014	2006-2010	2008-2009
Age	55-75	50-69	50-74	50-74	50-74	> 50	50-69	60-69	50-74
Test	FIT	FIT	FIT	FIT	gFOBT	gFOBT/FIT	FIT	gFOBT	gFOBT
Participation, n (%)	129395 (68.2)	81619 (54.4)	9993 (51)	271396 (46)	210239 (19.9)	521429 (22.7)	152475 (60.43)	1079293 (52)	2964976 (34.3)
M, n (%)			2126 (42)				55.23%	510864 (49.6)	32.10%
F, n (%)			2937 (42)				65.53%	568429 (54.4)	36.20%
Positive test, n (%)	15802 (12.2)	(5.8)	514 (10)	19455 (7.2)	12477 (6.9)	31794 (6.1)	8108 (5.9)	21106 (2%)	82786 (2.8)
M, n (%)	14.50%		254 (5)				7.60%	12776 (2.5)	3.30%
F, n (%)	10.10%		260 (5)				4.70%	8330 (1.5)	2.40%
Colonoscopies performed	74.30%	92.50%	87%	66.10%	66%	95.70%	98.90%	83%	88.40%
Advanced adenomas, n (%)	3832 (33.5)	702	99 (24)	3.90%	41%	3077	1887 (25.16)	1721 (9.8)	14276
PPV Advanced adenomas	NA	30.20%	5%	NA	NA	16.80%	NA	NA	19.60%
CRC, n (%)	763 (6.7)	70	38 (9)	3.10%	472 (3.6)	829	159 (2.16)	1772 (10.1)	7.50%
PPV CRC	6.70%	3%	4%	NA	NA	4.50%	NA	NA	NA
CRC detection rate per 1000	5.9	1.6	3.3	0.2	NA	1	NA	NA	1.9

NA: Not available.

second highest test positivity was found in Ireland (10% test positivity, with a cut-off value 20  $\mu\text{g/g}$ ). In Italy, with the same cut-off value, test positivity was 5.8%, and in Slovenia, it was 5.9%. The lowest test positivity rates were found in England and France (2% and 2.8%, respectively), where the G-FOBT was used. In Croatia, Lithuania, and the Netherlands, the proportion of colonoscopies performed did not exceed 75%; but in the Czech Republic and Slovenia, the proportions were above 95%. In England, a very high CRC percentage was detected (10.1% of patients a with positive G-FOBT result), but only 2% of all patients had a positive G-FOBT. Ireland had a similar CRC percentage (9%), but also a greater number of patients had positive FIT results (10%). The highest detection rate was found in the Netherlands (5.9 per 1000 screenings), followed by Ireland (3.3 per 1000 screenings). More detailed results are described below and in Table 2.

**Spain:** Different screening programs have been implemented in all areas of Spain for the population aged 50-69 years. These programs mostly use the FIT. In 2014, participation rates varied among the regions, but the average was 49.2%. More women than men participated (51.41% vs 47.01%). On average, 6.56% of test results were positive, with a higher percentage found in men (8.2%) than in women (5.17%). The positive predictive value (PPV) for cancer was 4.70%. The CRC detection rate was 2.75 per 1000 screenings<sup>[34]</sup>.

**The Netherlands:** In 2011, the Netherlands decided to implement a national population screening program

for CRC. The program began in 2014, with the FIT test and a cut-off value of 15  $\mu\text{g/g}$ . This program achieved a 68.2% participation rate. Initially, it was necessary to increase the cut-off value (from 15  $\mu\text{g/g}$  to 47  $\mu\text{g/g}$ ), because the proportion of individuals with positive tests was higher than expected (12%). This proportion exceeded the number of false positives, and it surpassed the capacity to perform colonoscopies. With the first cut-off value, test positivity was 10.1% in women and 14.5% in men. Colonoscopies were performed in 74.3% of these patients; among these, CRC was detected in 763 (6.7%) and advanced adenoma was detected in 33.5%. The CRC detection rate was 5.9 per 1000 inhabitants and the PPV was 6.7%<sup>[35]</sup>.

**Ireland:** The first pilot program in Ireland was the Adelaide and Meath Hospital/Trinity College Dublin Colorectal Cancer Screening Program (TTC-CRC-SP). It included a population aged 50 to 74 years, and it applied the FIT (OC Sensor, cut-off value 20  $\mu\text{g/g}$ ). The participation rate was 51% (58% women and 42% men). The proportion of individuals with positive test results was 10%. Of the colonoscopies performed (87%), advanced adenomas were detected in 99 patients (24%) and CRC was detected in 38 (9%). The PPVs for CRC and advanced adenoma were 4% and 5%, respectively, and the CRC detection rate was 0.33%<sup>[36]</sup>. A second round was conducted, where they excluded patients that had changed residence, had been diagnosed with cancer, and had died. The participation rate was 48%, and 375 patients had positive test results (8%). Of the patients with a

**Table 3** Results of colorectal cancer screening program time-trend (adapted from Suchanek *et al.*<sup>[43]</sup>)

	2006	2007	2008	2009	2010	2011	Total
Examined patients ( <i>n</i> )	272658	320317	352595	414300	521429	NA	1881299
Positivity rate	3.6%	3.3%	4.1%	5.0%	6.1%	NA	4.6%
PPV for advanced adenoma	14.1%	13.5%	16.2%	16.6%	16.8%	16.7%	16.2%
PPV for CRC	6.3%	5.9%	6.0%	5.1%	4.5%	3.6%	4.8%

CRC: Colorectal cancer; PPV: Positive predictive value.

positive test, 87% underwent a colonoscopy. The PPV for CRC was similar to that of the first round (4%), and the CRC detection rate was 0.12%<sup>[37]</sup>.

**Italy:** Lombardy is a densely populated northern region of Italy with the highest incidence of CRC. The screening program began with the population aged 50-69 years, in 2005-2006. They applied the FIT with a cut-off value of 20 µg/g. The second round of the program had been completed in 2009. The PPVs for advanced adenoma were 29% in the first round, and 30.2% in the second round. The PPVs for CRC were 4% and 3%, respectively. The PPVs for advanced adenoma and CRC were higher in people aged 60-69 years and in males. The CRC detection rates in the first and second rounds were 2.5 and 1.6 per 1000 screened, respectively<sup>[38]</sup>.

**Croatia:** The Croatian screening program was implemented in 2007 for the population aged 50-74 years. They implemented the G-FOBT. Participation was low, reaching 19.9%. Positive tests were found in 6.9% cases, and of these, only 66% received a colonoscopy. CRC was identified in 472 patients (3.8%)<sup>[39]</sup>.

**Lithuania:** The Lithuanian National Screening Program began in 2009, for the population aged 50 to 74 years. The FIT was applied. The participation rate for 3 years was 46% (271396). A positive FIT was observed in 19455 participants (7.2%). Of these, 66.1% underwent a colonoscopy. High-grade neoplasia was detected in 3.9% of cases, and the rate of CRC was 3.1% among all colonoscopies. The rate of CRC detected with the program was 0.2%<sup>[40]</sup>.

**Slovenia:** The Slovenian National Screening Program (SVIT program) started in 2009. This program performed a biennial FIT for the population aged 50 to 69 years during 2014. The participation rate was 57.8% (53.2% men, 62.3% women). In this population, 6% had positive test results (7.6% men and 4.7% women). The colonoscopies detected 159 (2.12%) patients with CRC and 1887 (25.16%) patients with advanced adenomas<sup>[41]</sup>.

**England:** The English National Screening Program began in 2006 for the population aged 60 to 69 years. This program performed a biennial G-FOBT. The

first round was completed in 2010. Of the 2 million invitations sent, 49.6% of men and 54.4% of women responded. The overall participation rate was 52%. Positive test results were found in 2% (2.5% men, 1.5% women). Among the colonoscopies performed (83%), CRC was detected in 10.1% (*n* = 1772; detection rates of 11.6% in men and 7.8% in women). High risk adenomas were detected in 9.8% (*n* = 1721; detection rates of 12.2% in men and 6.2% in women)<sup>[42]</sup>.

**Czech Republic:** The Czech National Screening Program started in 2000 for individuals older than 50 years. They applied a biennial G-FOBT. By 2009, the FIT was introduced. The coverage of CRC screening in 2010 was 22.7%<sup>[43]</sup>. The results of the program from 2001 to 2011 are shown in Table 3.

**France:** The French National screening program started in 2008 for the population aged 50-74 years. They applied the G-FOBT. The participation rate was 34.3%. Positive test results were found in 2.8%. Of these, 88% underwent a colonoscopy. CRC was detected in 7.5% (detection was 9% in men and 5.8% in women). The advanced adenoma detection rate was 4.9 per 1000 screened; the CRC detection rate was 1.9%<sup>[44]</sup>.

### CRC screening programs in the Americas

**United States:** Currently in the US, screening programs have been established on an opportunistic basis. The average-risk population (50-75 years) are encouraged to undergo screening at 50 years of age, and participants choose among several options. The available options are: (1) annual G-FOBT or FIT, according to the manufacturer's recommendations for specimen collection; (2) multi-target stool DNA test every 3 years; (3) flexible sigmoidoscopy every 5 years; (4) colonoscopy every 10 years; (5) double-contrast barium enema every 5 years; or (6) CT colonography every 5 years. In about 90% of cases, colonoscopy is the preferred option<sup>[28]</sup>.

On the other hand, screening program evaluations have been conducted in several regions of the country. For example, the Kaiser Permanente Northern and Southern California program conducted 4 screening rounds in a population of 50 to 70 years. They used the annual FIT (cut-off value 20 µg/g). They achieved a 48.2% participation rate in the first round. A positive



**Table 4 Results of American, Western Pacific and East Asian screening programs**

Country	Canada	California (United States)	South Korea	Australia	Thailand	Taiwan	Chile
ASRi	35.5	25 (United States)	45	38	12.4	NA	15
ASRm	10.8	9.2 (United States)	12	9	7.3	NA	8.6
Period	2009-2011	2008	2004-2008	2002-2004	2011-2012	2004-2009	2007-2009
Age range	50-74	50-70	50-75	55-74	50-65	50-69	> 50
Test	G-FOBT/FIT	FIT	FIT	FIT	FIT	FIT	FIT
Participation, <i>n</i> (%)	104750 (16.1)	323349 (48.2)	984915 (21)	25840 (45.4)	80012 (62.9)	1160895 (21.4)	4938
M, <i>n</i> (%)			446590 (20.5)		57.8%	446290 (20.4)	
F, <i>n</i> (%)			538325 (21.9)		67.8%	714605 (25)	
Positive test, <i>n</i> (%)	4661 (4.4)	5%	73568 (7.5)	2308 (8.9)	873 (1.1)	4%	476 (9.6)
M, <i>n</i> (%)	5.9%		39233 (8.8)		1.2%	5%	
F, <i>n</i> (%)	3.4%		34335 (6.4)		1.1%	3.4%	
Colonoscopies performed	80.5%	NA	23117 (31.4)	1265 (54.8)	627 (71.8)	80%	279 (58.6)
Advanced adenoma, <i>n</i> (%)	NA	NA	NA	176 (13.9)	75 (12%)	4284	75 (16)
PPV Advanced adenoma	NA	NA	NA	NA	NA	NA	NA
CRC, <i>n</i> (%)	86		1.2%	67 (5.3)	23 (3.7)	2304	13 (1.1)
PPV CRC	4.4%	3.4%					
CRC Detection rate per 1000	1.8	NA	NA	2.59	0.29	2.5	2

FIT result was found in 5% of participants and the PPVs were 51.5% for adenomas and 3.4% for CRC<sup>[45]</sup>.

**Canada:** Canada organized a CRC screening program for average-risk individuals, aged 50-74 years, from January 2009 to December 2011. Five provincial programs were included (British Columbia, Saskatchewan, Manitoba, Nova Scotia, and Prince Edward Island). The results of the first round showed very low participation rates (16.1%)<sup>[46]</sup>. The test positivity rate was 4.4% (4.8% with FIT and 3.7% with G-FOBT). Positive test results were more frequent in men (5.9%) than in women (3.4%), and the frequency increased with age: positive tests were found in 5.7% of the 70-74 age group and 3.4% of the 50-54 age group. Compliance with a follow-up colonoscopy was 80.5%. The detection rates were 16.9 per 1000 screened for adenomas, and 1.8 per 1000 screened for CRC. The PPVs for adenoma were 35.9% with the G-FOBT and 50.6% with the FIT. The PPV for CRC was 4.4% in both tests.

**Chile:** In Chile, an organized screening program was launched between 2007 and 2009 in asymptomatic subjects, aged 50 years or older without risk factors. They applied the FIT test with a cutoff of 20 µg/g. The participation rate was 77%. Of 4938 participants, positive test results were found in 9.6%. Of these, a colonoscopy was performed in 58.6%. CRC detection rates were 2 per 1000 screenings<sup>[47]</sup>.

#### **CRC screening programs in the western pacific and east asia**

The Asian Pacific Colorectal Cancer Working Group has recommended organized screening in regions with the highest CRC incidence (> 30 per 100000)<sup>[48]</sup>. The programs target average-risk individuals, aged 50-75 years, and they preferably apply the FIT test. Several studies have investigated the barriers to

CRC detection in different cultural and socio-political contexts in the Asia-Pacific region. These barriers included poor understanding of the characteristics of screening and testing, lack of financial support, and lack of health insurance<sup>[49]</sup>. Several countries in East Asia have ongoing organized screening programs, including Japan, Korea, China, Hong Kong, Taiwan, and Bangkok<sup>[50]</sup>. The results for several of these CRC Screening Programs are described below and in Table 4.

**Japan:** In Japan, a CRC screening program has been in place since 1992 for beneficiaries of health insurance, aged 40-69 years. The program applies the FIT. In 2013, participation rates were 41.4% in men and 34.5% in women<sup>[51]</sup>.

**South Korea:** The South Korean National Screening Program was introduced in 2004. It targets the National Health Insurance population, aged over 50 years. They employ an annual FIT (qualitative or quantitative). Participation increased from 10.5% in 2004 to 21.1% in 2008 and to 25% in 2012<sup>[52]</sup>. In 2008, the FIT positivity rate was 7.5% (8.8% in men and 6.4% in women). A colonoscopy was performed in 31.4% of those with positive test results. The CRC detection rate was 1.2%<sup>[53]</sup>.

**Taiwan:** The Taiwanese National program began in 2004. They performed biennial FITs in individuals aged 50 to 69 years. In the first round, 1160895 individuals (21.4%) participated. The test positivity rate was 4%. Subsequent colonoscopies detected 4284 advanced adenomas (detection rate 4.6 per 1000) and 2304 CRCs (detection rate 2.5 per 1000). The PPVs of FITs were 11.7% for advanced adenoma, and 6.1% for CRC<sup>[54]</sup>.

**Thailand:** A Thai pilot screening program was implemented in 2011. The FIT was performed in the

population aged 50-65 years in Lampang Province. The participation rate (62.9%) was higher among women (67.8%) than men (57.8%). The test was positive in 1.1% (1.2% men, 1.0% women). Colonoscopy was performed in 72% of those with positive tests. Detection rates were 3.7% for CRC and 30.6% for adenomas<sup>[55]</sup>.

**Australia:** An Australian pilot program was conducted from 2002 to 2006 with the biennial FIT in the population aged 55-74 years. The participation rate was 45.4% (women 47.4%, men 43.4%). Positive FIT results were found in 9% of participants. Colonoscopy was performed in 54.8% of the individuals with positive FITs. Adenomas were found in 19.8% (13.9% advanced) and CRC was found in 5.3%<sup>[56]</sup>. In 2006, The National Bowel Cancer Screening Program was initiated with a biennial FIT for the population aged 55-65 years. The program will continue to expand until 2020. The program aims to apply biennial screening to the entire population aged 50-74 years<sup>[4]</sup>.

## COMPARISON OF THE RESULTS OF SEVERAL CRC SCREENING PROGRAMS

Most European countries have implemented a national organized screening program. However, some countries have not, despite high CRC incidence and mortality rates, such as Slovakia. Likewise, most countries of Central America, South America, the Middle East, and Africa, do not have organized screening programs. In most cases, the lack of organized programs could be explained by limited resources, including the limited availability of colonoscopy facilities, and the type of organization of the Health Care System. Most organized screening programs use non-invasive tests (FIT or G-FOBT); in contrast, most opportunistic programs rely on endoscopy. Colonoscopy remains the most commonly used screening test in North America, but FIT screening programs are beginning to be implemented in some areas, such as California<sup>[45]</sup>.

The efficacy of CRC screening is determined by the degree of participation and the diagnostic yield of the test. Studies have shown that FIT screening is superior to G-FOBT in both aspects<sup>[20,47]</sup>. The overall results showed that the highest participation rates were obtained in programs using FIT. In fact, in programs that used both tests (FIT and G-FOBT), participation rates increased after FIT was introduced, as observed in the Czech Republic<sup>[43]</sup>.

FIT screening also produced more positive tests than G-FOBT screening. Therefore, the lowest rates of test positivity in Europe were obtained in England and France, where screening was performed with the G-FOBT<sup>[42,44]</sup>. As mentioned, in the Czech Republic program, after the G-FOBT was replaced with the FIT, the PPV for advanced adenoma increased, and the PPV for CRC decreased<sup>[43]</sup>. This result implied

that, compared to the G-FOBT, the FIT had a higher sensitivity and PPV for advanced adenomas, but a lower PPV for CRC.

Among all the programs reviewed, only Korea used a qualitative FIT. Studies worldwide using different Hb thresholds have shown that defining a positive FIT result with a cut-off value of 100 ng/mL Hb (20 µg/g) provided high sensitivity, specificity, and PPV for detecting neoplasia<sup>[49,53,54]</sup>. Other studies reported a decline in specificity with cut-off values below 100 ng/mL Hb<sup>[5,45,46,48,53]</sup>. According to this information, a cutoff value between 75 and 100 ng/mL Hb might represent an optimum in most European populations, depending on the resources and availability of colonoscopy.

Some countries had to modify the cut-off value to align the need for colonoscopies with the limited capacity of endoscopic resources. For example, in the Netherlands, which had the highest rates of positivity among all international and European programs, the cutoff value was raised from 88 to 275 ng/mL Hb (15 µg/g to 47 µg/g) at the beginning of their program<sup>[35]</sup>. In addition, the Netherlands had the highest CRC detection rate in Europe; the detection rates were double those of several European and other countries. The program in Ireland also had a high percentage of positive tests (10%). In comparison, with the same cut-off value, Italy had almost half (5.8%) the proportion of positive tests. This suggested that there might be a relatively high incidence of adenomas in Ireland, and that lower cut-off values would be very difficult to manage with the current availability of colonoscopy resources in the country.

Another problem to consider is the low acceptance of colonoscopy in some countries. In South Korea, only 31.4% of individuals with a positive FIT had undergone a colonoscopy after. This reluctance could result in low detection rates<sup>[53]</sup>. In other European countries, like Croatia, Lithuania, and The Netherlands, the proportion of colonoscopies among individuals with positive test results did not exceed 75%<sup>[35,39,40]</sup>.

Participation rates in the screening programs were higher among women than men. This difference probably occurred because women had a greater awareness of preventive programs; in particular, women were likely to have had experience in breast and cervix screening. In addition, positive FIT rates were significantly higher in men than in women (except in Ireland and Thailand, where rates were similar between the sexes). The CRC detection rates were also higher in men than in women; in some programs, detection rates in men were double the rates in women. Special efforts should be made in all screening programs to increase both the overall participation and male participation rates.

The lowest participation rates were found in Canada, possibly because the data were published recently after the programs had been started.

In general, participation rates in the different

programs currently exceed the acceptable minimum of 45%, but they have not reached the desired target (> 65%). Screening programs must employ specific strategies to attract the target population and encourage participation in screening programs. A better understanding of the barriers and facilitators to participation is needed to design strategies that promote equity of access. It is important to monitor, record, and evaluate the minimum indicators and requirements of CRC Population Screening Programs, to ensure they meet the standards of the European Quality Control Guide.

## CONCLUSION

This review highlighted the large variations in CRC incidence and mortality around the world. Some regions with high CRC rates do not have screening programs, and other regions, like Europe, have widespread organized screening programs. Additionally, participation rates vary greatly between programs around the world. The highest rates were found in the Netherlands and the lowest were found in Canada. The most common test used as a screening tool in organized screening programs was the fecal occult blood test. In countries with screening programs that arose opportunistically, colonoscopy was most commonly used for screening. Between the two types of fecal occult blood tests, the most commonly used test was the FIT. Use of the FIT has increased participation rates, because it is user friendly; a single sample suffices, and no dietary restrictions are imposed prior to the test. Because the FIT is more sensitive than the G-FOBT, the number of false positives and the demand for invasive tests has increased. Consequently, the cut-off value of the test must be adapted to each region, taking into account the availability of endoscopic resources. The FIT also exhibited superior detection of advanced adenomas compared to the G-FOBT. This feature promotes treatment in early stages and prevents the formation of cancer. Participation rates were higher among women, possibly due to their increased awareness of the importance of other screening programs, such as breast cancer screening. Positive test results and CRC detection rates were higher in men than in women; therefore, men's awareness should be increased to encourage participation in screening programs.

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## Basic Study

# Urinary metabolic insights into host-gut microbial interactions in healthy and IBD children

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**Data sharing statement:** Data may be available upon request to Francois-Pierre Martin and Andreas Nydegger, subject in particular, to ethical and privacy considerations.

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## Abstract

### AIM

To identify metabolic signatures in urine samples from healthy and inflammatory bowel disease (IBD) children.

### METHODS

We applied liquid chromatography and gas chromatography coupled to targeted mass spectrometry (MS)-based metabolite profiling to identify and quantify bile acids and host-gut microbial metabolites in urine samples collected from 21 pediatric IBD patients monitored three times over one year (baseline, 6 and 12 mo), and 27 age- and gender-matched healthy children.

### RESULTS

Urinary metabolic profiles of IBD children differ significantly from healthy controls. Such metabolic differences encompass central energy metabolism, amino acids, bile acids and gut microbial metabolites. In particular, levels of pyroglutamic acid, glutamic

acid, glycine and cysteine, were significantly higher in IBD children in the course of the study. This suggests that glutathione cannot be optimally synthesized and replenished. Whilst alterations of the enterohepatic circulation of bile acids in pediatric IBD patients is known, we show here that non-invasive urinary bile acid profiling can assess those altered hepatic and intestinal barrier dysfunctions.

## CONCLUSION

The present study shows how non-invasive sampling of urine followed by targeted MS-based metabolomic analysis can elucidate and monitor the metabolic status of children with different GI health/disease status.

**Key words:** Pediatric; Metabolism; Phenotype; Growth; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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**Core tip:** Despite the limited number of subjects, the longitudinal experimental design with a healthy reference group reveals insights into childhood metabolic status in relation to growth and disease. Metabolite profiling of urine samples collected non-invasively enables identification and monitoring of biochemical signatures linked to metabolic and nutritional requirements in pediatric populations with inflammatory bowel disease (IBD). In the present study, a distinct biochemical process related to glutathione, glycine and bile acid metabolism distinguished children with IBD from healthy matched controls.

Martin FP, Su MM, Xie GX, Guiraud SP, Kussmann M, Godin JP, Jia W, Nydegger A. Urinary metabolic insights into host-gut microbial interactions in healthy and IBD children. *World J Gastroenterol* 2017; 23(20): 3643-3654 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3643.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3643>

## INTRODUCTION

The use of systems biology-oriented technologies (e.g., metabolomics, proteomics, genomics and microbiomics) redefines disease understanding and phenotyping of clinical characteristics in medical disorders such as in gastrointestinal deregulations<sup>[1,2]</sup>. This is particularly relevant for inflammatory bowel disease (IBD), where a systems-level approach of the pathogenesis can inform novel, integrated multiple pathway- therapies and contribute to the development of personalized disease management<sup>[2]</sup>.

The prevalence of IBD has markedly increased over the years, with 25% of the population developing the disease during childhood and adolescence<sup>[2,3]</sup>. In addition to the clinical and immunological features of

the disease, pediatric patients suffer from additional growth failure and delayed puberty<sup>[3-5]</sup>. During this critical period, IBD children can suffer from malnutrition, which is related to reduced energy intake, malabsorption and thus loss of nutrients, and inflammation<sup>[3,6-9]</sup>. These factors mainly contribute to delayed growth and physiological maturation. Moreover, there is a great lack of knowledge on how to address metabolic and nutritional requirements in these children and adolescents<sup>[5]</sup>. The use of omics-technologies for holistic molecular phenotyping over time can generate the data for a systems-level view of metabolic processes associated with growth and development of children in relation to the changing physiology in infancy and childhood<sup>[5]</sup>.

Amongst the omic technologies, metabolomics enables the study of physiological regulatory processes through the simultaneous analysis of a large range of biochemical species<sup>[2,10-13]</sup>. The technology is based on either targeted or untargeted profiling of small biomolecular compounds in tissues and biological fluids, such as blood, urine or saliva. Metabolites profiles can be generated through the use of proton nuclear magnetic resonance (<sup>1</sup>H NMR) spectroscopy or mass spectrometry (MS) combined eventually with chromatographic separation. Recently, we have applied <sup>1</sup>H NMR based profiling to urine samples from IBD and healthy matched children and highlighted differences related to central energy metabolism, as well as amino acid and gut microbial metabolism in relation to growth and disease activity<sup>[14]</sup>. In particular, we showed how specific metabolite patterns in urine could help monitoring metabolic status in relation to disease state. In the present study, we continue our metabolic exploration by targeting host-gut microbial metabolites in urine samples from the same children, including the aromatic amino acid and bile acid co-metabolites - two well-established examples of transgenomic metabolic cross-talk. For this purpose, we applied liquid chromatography (LC) and gas chromatography (GC) coupled to MS-based metabolite profiling. We here report urinary metabolic phenotypes specific to IBD children compared to healthy matched controls. Advanced clinical and anthropometric phenotyping was also performed on these IBD children over the course of one year of standard-care disease management (0, 6 and 12 mo) to identify relationships between biochemical processes and anthropometric phenotypes.

## MATERIALS AND METHODS

### Ethical considerations

This clinical study was approved by the Ethical Committee of the University of Lausanne, Switzerland (protocol 69/10), and conducted in the Pediatric Gastroenterology outpatient clinic of the University Hospital of Lausanne, Switzerland. Informed written consent was obtained from the patients and their parents.

**Recruitment of participants**

Eligible patients were aged between 10 and 18 years old, with a diagnosis of Crohn's disease (CD) or ulcerative colitis (UC), confirmed according to international criteria<sup>[15]</sup>. IBD subjects were assessed at baseline (T0), after 6 (T6) and 12 mo (T12), respectively. All patients were in remission and underwent therapeutical management of the disease according to recommended drugs. To be noted that none was treated with enteral nutrition and no endoscopy was performed to assess mucosal inflammation. Control healthy subjects were recruited among the general pediatric population. They were matched for age, pubertal stage and gender to the IBD subjects. They had neither chronic inflammatory disease nor family history of inflammatory bowel. Anthropometric and clinical data and urine samples for metabolic analyses were collected at each time point.

**Anthropometric assessment**

Body weight, height and BMI were measured under standard clinical practice, as previously reported<sup>[14]</sup>. Height velocity was calculated as the amount of growth in centimeters divided by the time interval between measurements in years. All values were expressed in z-scores<sup>[16,17]</sup>. Pubertal stage was assessed according to Tanner score<sup>[18]</sup>.

**Body composition**

Bioimpedance analysis (BIA) was performed using Body Impedance Analyser Akern (Florence, Italy), as previously reported<sup>[14]</sup>. Fat free mass in kg (FFM) was then calculated using the software BodyGram Pro<sup>®</sup> supplied by the manufacturer [which uses weight, age, and an impedance index ( $\text{height}^2/\text{resistance}$ )<sup>[19,20]</sup>. Percentage of FFM (%FFM) was calculated by dividing FFM with the body weight of the subject expressed in kg.

**Disease activity in patients with IBD**

Disease activity was scored using the Pediatric Crohn's Disease Activity Index (PCDAI)<sup>[21]</sup> for CD, a 100 point scale where a score > 30 indicates severe disease, and the Pediatric Ulcerative Colitis Activity Index (PUCAI)<sup>[22]</sup> for UC, a 85 points scale where a score > 35 indicates severe disease. Remission was defined as PCDAI or PUCAI score lower than 10. No endoscopic control was performed since all patients were in remission.

**Blood and stool markers**

Inflammatory markers [Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP)], urea, and growth factors [Insulin-like Growth Factor 1 and Insulin-like Growth Factor-Binding Protein 3, expressed in z-scores] were obtained after a fasting period of at least 6 h. Fecal calprotectin was measured and a cut-off value of 275  $\mu\text{g/g}$  was set to determine possible relapse of disease<sup>[23]</sup>.

**Dietary intake**

All subjects underwent a 24-h food recall with the help of a questionnaire showing pictures of different sizes of plates for the different foods with the same examiner (dietician TC). Qualitative and quantitative analyses were made using the software Prodi 5.8 Expert. Daily intake was expressed as kcal per day.

**Resting energy expenditure**

Resting energy expenditure (REE, kcal) was measured using Quark RMR (Cosmed, pulmonary function equipment, Delta Medical, Italy), as previously reported<sup>[14]</sup>.

**Sample collection**

Morning spot urine samples were collected at baseline for all subjects, at the 6-mo and 12-mo visit for IBD patients only. Urine samples (1 mL) were collected by means of sterile plastic tubes, and stored at -80 °C, prior to analysis.

**Bile acid analysis**

**Chemicals:** All of the 57 bile acid standards were obtained from Steraloids Inc. (Newport, RI) and TRC Chemicals (Toronto, ON, Canada), and 9 stable isotope- labeled standards were obtained from C/D/N Isotopes Inc. (Quebec, Canada) and Steraloids Inc. (Newport, RI).

Methanol (Optima LC-MS), acetonitrile (Optima LC-MS) and formic acid (Optima LC-MS) were purchased from Thermo-Fisher Scientific (FairLawn, NJ). Ultrapure water was produced by a Mill-Q Reference system equipped with a LC-MS Pak filter (Millipore, Billerica, MA).

**Sample preparation:** At the time of analysis, samples were thawed on ice-bath to diminish biochemical degradation. 100  $\mu\text{L}$  of urine or standard solution of a bile acid-free matrix was directly lyophilized to dry powder using a freeze dryer. The residue was reconstituted in 50  $\mu\text{L}$  of mobile phase B (acetonitrile/methanol = 95/5, v/v) and 25  $\mu\text{L}$  of mobile phase A (water with formic acid, pH = 3.25), and centrifuged at 13500 g and 4 °C for 20 min. The supernatant was transferred to a 96-well plate for LC-MS analysis and the injection volume was 10  $\mu\text{L}$ .

**Data generation and analysis:** An ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA) was used to quantitate bile acids in the human urine samples based on our previously published protocols<sup>[24,25]</sup>. The raw data was processed using the TargetLynx application manager (Waters Corp., Milford, MA) to obtain calibration equations and the measured concentration of each bile acid in the samples. The quality control samples were prepared with the addition of bile acid



stock solutions to blank matrix at a final concentration of 10, 100, or 1000 nmol/L. The quality control samples (four QCs in a 96-well plate) were prepared along with the real samples, and injected at regular intervals to allow evaluating overall process variability and monitoring platform performance. Each kit plate is ideal for the analysis of 84 samples, 8 calibrators (CAL0 = blank matrix), and 4 QCs (QC1 = 10 nmol/L, QC2 and QC3 = 100 nmol/L, and QC4 = 1000 nmol/L). The noise baseline was established from reagent blank measurement and any metabolite with signal to noise ratio  $\leq 3.0$  is rejected from statistical analysis. The relative standard deviation (RSD) for the lower concentration metabolites in the reference standard mixture was less than 30% and the RSD for the higher concentration metabolites was better than 15% for each batch of sample analysis (100 injections for LC-MS analysis).

### GC-MS based metabonomics

**Chemicals:** The derivatization reagents methyl and ethyl chloroformate, as well as HPLC grade solvents including methanol, ethanol, chloroform and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, United States). Sodium hydroxide, sodium bicarbonate and anhydrous sodium sulfate were of analytical grade and obtained from JT Baker Co. (Phillipsburg, NJ). All standard compounds were commercially purchased from Sigma-Aldrich and Nu-Chek Prep (Elysian, MN, United States). Ultrapure water was produced by Milli-Q system (Millipore, Billerica, MA).

### Extraction of metabolites from human urine:

Urine samples were thawed on ice and prepared as follows: urine sample (100  $\mu$ L) was added into a silanized glass vial and lyophilized directly under low temperature ( $-40^{\circ}\text{C}$ ) by a Labconco freeze-dryer. The solids from urine samples after lyophilization process were sealed and stored at  $-80^{\circ}\text{C}$  for subsequent automated derivatization assay.

### Automated chloroformate derivatization and GC/MS analysis:

The sample derivatization protocols with MCF and ECF was based on the method described by Villas-Bôas *et al.*<sup>[26]</sup> and our previously published procedures<sup>[27]</sup>, with minor modifications. For routine large-scale sample analysis, sample derivatization and all liquid handling were performed by a commercially available robotic workstation (GERSTEL MPS Autosampler).

### Gas chromatography/time-of-flight mass spectrometry analysis:

Samples were randomly analyzed by GC/TOFMS (Agilent 6890N gas chromatography coupled with a LECO Pegasus HT time-of-flight mass spectrometer) using our optimized conditions in this study. One microliter of each derivatized sample was injected splitless into a DB-5 ms capillary column

(30 m  $\times$  0.25 mm i.d., 0.25  $\mu$ m film thickness; (5%-phenyl)-methylpolysiloxane bonded and cross-linked; Agilent JW Scientific, Folsom, CA), with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The solvent delay time was set to 2.5 min. The optimized temperature gradient was the following:  $45^{\circ}\text{C}$  held for 1 min, then increased at a rate of  $20^{\circ}\text{C}/\text{min}$  up to  $260^{\circ}\text{C}$  and  $40^{\circ}\text{C}/\text{min}$  to  $320^{\circ}\text{C}/\text{min}$ , then held there for 2 min. The total time of analysis was 15.25 min. The temperature of injection, transfer interface and ion source were set to 270, 270 and  $220^{\circ}\text{C}$ , respectively. Electron impact ionization (70 eV) at full scan mode ( $m/z$  38-650) was used. The acquisition rate was 20 spectra/s.

**Data processing:** Non-processed MS files from GC/TOFMS analysis were exported in NetCDF format to ChromaTOF (v4.50, Leco Co., CA, United States) and subject to the following processing including baseline correction, smoothing, noise reduction, deconvolution, library searching, and area calculation. Compound identification was performed by comparing both MS and Kovats-RI with reference standards in author-constructed two alkyl chloroformates derivatives databases, with a similarity of more than 70%.

### Statistical analysis

Chemometric analysis was performed on clinical and metabonomics data using the software package SIMCA-P+ (version 12.0, Umetrics AB, Umeå, Sweden). Principal component analysis (PCA) and a modification of Partial Least Squares Regression (PLSR) that removes all information orthogonal to the response variable during the fitting process were employed. This variant, Orthogonal Projection to Latent Structures (O-PLS)<sup>[28]</sup> provides sparser models (improving their interpretability) with the same degree of fit as PLSR. For group comparison, multivariate data analysis was performed using O-PLS-discriminant analysis (OPLS-DA). To highlight the weight of individual variables in the model, Variable Importance in the Projection (VIP) was used, with a value above 1 used as a threshold by convention. In addition, a Pearson correlation coefficient with a p-value significant at 95% confidence interval (*e.g.*, with  $n = 46$ ,  $r = 0.40$ ). Metabolic pathway analysis was conducted by performing a metabolite set enrichment analysis, using the web-based MetaboAnalyst 3.0 tool<sup>[29]</sup>, to the list of influential metabolites obtained through multivariate data analysis.

## RESULTS

### Clinical and metabolite parameters of IBD and healthy subjects

The patient information has been reported previously<sup>[14]</sup>. Briefly, the study population was composed of 21 pediatric IBD patients and 27 age and gender

**Table 1** Urinary metabolite overview in healthy and inflammatory bowel disease children at baseline

Metabolites	Healthy	IBD baseline	P value
2-hydroxybutyric acid	324.2 ± 228.5	401.9 ± 306.8	0.335
3-(3-hydroxyphenyl)-3-hydroxypropanoic acid	85.1 ± 242.2	240.6 ± 488.6	0.168
3-aminoisobutanoic acid	7278 ± 8267.9	12302.4 ± 15388.8	0.166
3-hydroxyisovaleric acid	12675.2 ± 14366.2	13929.9 ± 6198.6	0.722
3-hydroxyphenylacetic acid	6618.8 ± 7906.4	3579.7 ± 4269.6	0.134
3-methyl-2-oxovaleric acid	12049.5 ± 5596.6	13764.2 ± 4303.2	0.269
4-hydroxybenzoic acid	1659.4 ± 835.8	1660.6 ± 735.9	0.996
4-hydroxycinnamic acid	1749 ± 2142.3	4655.3 ± 5127.8	0.013
Adipic acid	5757.5 ± 3488.3	6806.6 ± 6403.6	0.485
Alpha-hydroxyisobutyric acid	7514.4 ± 4562.7	10645.1 ± 5098.4	0.036
Aminoadipic acid	23462.2 ± 13757.6	24927 ± 13309.2	0.722
Beta-alanine	3554.8 ± 2690.1	3730.4 ± 2705.5	0.830
Butyric acid	594 ± 329.1	754.4 ± 551	0.230
Caproic acid	337.3 ± 393.0	423.4 ± 412.3	0.480
Caprylic acid	113.7 ± 233.6	114.2 ± 335.3	0.996
Cis-aconitic acid	126743 ± 94692.1	217610 ± 154442.2	0.019
Citraconic acid	1487.4 ± 719.3	1877.1 ± 529.1	0.051
Citramalic acid	13454 ± 11088.9	29200.5 ± 23835.6	0.005
Citric acid	1521627.7 ± 1117692.9	2010896.5 ± 1470323.3	0.211
Dodecanoic acid	76 ± 118.3	95.1 ± 230.1	0.718
Dopamine	25594.1 ± 15600.7	46132.8 ± 4070.5	0.000
Ethylmethylacetic acid	354.4 ± 161.6	300 ± 108.3	0.208
Fumaric acid	4447.5 ± 5258.8	8002.2 ± 5704.2	0.036
Gamma-aminobutyric acid	8011.9 ± 2433.5	9501.4 ± 2144.3	0.038
Glutaric acid	6350.9 ± 2956.9	8011.9 ± 3153	0.077
Glyceric acid	24294.2 ± 44555.4	34244.8 ± 54085.6	0.503
Glycine	90591.2 ± 73998.7	150330 ± 121868.4	0.048
Glycolic acid	85357.2 ± 57207.8	98641.7 ± 50868.8	0.424
Hippuric acid	579641.8 ± 409879.0	435904.9 ± 374381.5	0.234
Homogentisic acid	395731.6 ± 236443.5	706581.6 ± 61533.7	0.000
Indoleacetic acid	58737.6 ± 40343.9	107584.7 ± 107498.1	0.040
Isocitric acid	418285.5 ± 291898.4	767013.4 ± 476267.9	0.004
Isovaleric acid	96.7 ± 125.3	53.8 ± 103.4	0.228
Itaconic acid	7961.3 ± 6523.3	12949.6 ± 11408.9	0.071
L-2-hydroxyglutaric acid	14028.4 ± 7911.3	25645.4 ± 16050.4	0.003
L-alanine	26378.1 ± 18928.9	36445.6 ± 16618.3	0.070
L-alpha-aminobutyric acid	1777.6 ± 1093.0	1826.9 ± 1158.2	0.885
L-asparagine	16684.8 ± 15672.4	25642.5 ± 14904.9	0.059
L-aspartic acid	2801.2 ± 833.5	3708.1 ± 1242	0.005
L-cysteine	7833.5 ± 5012.7	12771 ± 4681.6	0.002
L-glutamic acid	18551.2 ± 13284.8	29044.7 ± 18203.4	0.030
L-histidine	300484.2 ± 313800.2	328182 ± 163648.8	0.726
L-isoleucine	1874.2 ± 954.0	3149.4 ± 1492.8	0.001
L-leucine	4028.1 ± 3120.4	5677.4 ± 4266.7	0.141
L-lysine	38409.7 ± 46071.6	46194.7 ± 36720.6	0.545
L-methionine	1236.4 ± 696.3	2195.1 ± 1206.4	0.002
L-phenylalanine	18545.8 ± 14394.6	26167 ± 13952.9	0.082
L-proline	1131.5 ± 512.2	2020.9 ± 1099.8	0.001
L-tryptophan	26500.2 ± 13737.3	33639.1 ± 12697.3	0.082
L-tyrosine	63971.6 ± 55716.2	84163.3 ± 63553.6	0.264
L-valine	4892.5 ± 2641.4	7343.2 ± 3296.4	0.008
Malonic acid	493 ± 486.4	663.3 ± 547.4	0.277
Methylsuccinic acid	1655.6 ± 861.4	2484.1 ± 1188.1	0.009
N-acetyltryptophan	82232.1 ± 58726.0	112567.4 ± 56382.5	0.088
Nicotinic acid	1293.3 ± 1311.8	2104.1 ± 1204	0.039
Ornithine	7948.8 ± 2592.9	11968.9 ± 4307.9	0.000
Ortho-hydroxyphenylacetic acid	1265.1 ± 770.7	1915.5 ± 1233.6	0.035
Oxoglutaric acid	23844.7 ± 20947.4	34260.9 ± 33676.2	0.209
Phenol	266.1 ± 168.1	282 ± 165.2	0.753
Phenylacetic acid	3055.4 ± 2506.3	5635.2 ± 5749.6	0.048
Phenyllactic acid	729.1 ± 1365.9	2726.7 ± 2958.9	0.004
P-hydroxyphenylacetic acid	8991.4 ± 5587.6	16438.3 ± 9973.0	0.003
Pimelic acid	2203 ± 1453.7	3343.5 ± 1728.2	0.021
Pyroglutamic acid	39396.7 ± 14482.8	52204.6 ± 19506.9	0.015
Salicyluric acid	3342.8 ± 1712.7	4135.9 ± 1498.5	0.112
Suberic acid	8538.8 ± 5594.7	12987.5 ± 5378.1	0.010
Succinic acid	23748.9 ± 18864.3	34383.7 ± 30189.9	0.154
Tartaric acid	5156.1 ± 9387.3	11376.5 ± 27499.5	0.291

Vanillic acid	2838.1 ± 5734.4	3091.8 ± 3028.7	0.861
12_ketoLCA	11.5 ± 2.4	13.5 ± 4.5	0.072
3_DHCA	13.4 ± 11.9	17 ± 15.7	0.396
7_DHCA	145.5 ± 276.1	267.1 ± 439.7	0.267
7_ketoLCA	10.9 ± 2.9	13.6 ± 7.2	0.093
Beta-CA	12.9 ± 10.4	26.1 ± 27.0	0.029
Beta-UCA	42.1 ± 93.8	114.8 ± 168.0	0.074
CA	182.3 ± 237.0	266 ± 322.5	0.327
CDCA	21.9 ± 9.7	24.9 ± 11.2	0.346
CDCA_24GLN	79.6 ± 49.1	168 ± 151.2	0.008
CDCA_3GLN	64.1 ± 45.2	149.1 ± 105.5	0.001
DCA	45.2 ± 28.7	40.2 ± 31.0	0.588
GCA	84.4 ± 43.2	151.8 ± 162.6	0.051
GCDCA	39.8 ± 20.6	51.3 ± 25.1	0.105
GDCA	34.5 ± 33.4	30.4 ± 15.9	0.627
GHCA	36.7 ± 17.6	49.4 ± 41.5	0.171
GHDCA	14.4 ± 2.6	12.3 ± 0.6	0.002
GLCA	8.4 ± 5.7	4.8 ± 4.2	0.026
GUDCA	15.5 ± 17.5	39.5 ± 47.9	0.025
HCA	10.3 ± 1.5	10.5 ± 1.5	0.768
HDCA	17.1 ± 35.3	15.2 ± 10.2	0.820
LCA	19.1 ± 6.6	22.4 ± 32.6	0.621
LCA_S	22.5 ± 11.9	22.6 ± 22.2	0.989
NORCA	154.3 ± 116.0	181.4 ± 133.4	0.476
NORDCA	9.2 ± 1.5	9.3 ± 0.4	0.679
TCA	24.6 ± 8.2	26 ± 11.9	0.652
TCDCA	23.4 ± 7.7	23.4 ± 5.6	0.99
THCA	13.7 ± 3.0	18.4 ± 11.4	0.052
TUDCA	8.2 ± 1.6	9 ± 1.2	0.099
UCA	108.2 ± 135.4	268.4 ± 310.0	0.024
UDCA	11.6 ± 1.4	13.1 ± 2.0	0.008

Metabolite data are reported as mean ± SD of their urinary concentrations, in ng/mL for GC-MS metabolites, and nmol/L for the bile acids. *P* value from standard *t*-test is reported as a qualitative indicator for metabolic variations between the two groups of children. 12-KetoLCA: 12-ketolithocholic acid; 3\_DHCA: 3-dehydrocholic acid; 7\_DHCA: 7-dehydrocholic acid; 7\_KetoLCA: 7-ketolithocholic acid; 3βCA: 3β-Cholic acid; βUCA: β-ursocholic acid; CA: Cholic acid; CDCA: Chenodeoxycholic acid; CDCA\_24GLN: Chenodeoxycholic acid 24-Acyl-β-D-glucuronide; CDCA\_3GLN: Chenodeoxycholic acid-3-β-D-glucuronide; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GDCA: Glycodeoxycholic acid; GHCA: Glycohyocholate; GHDCA: Glycohyodeoxycholate; GLCA: Glycolithocholate; GUDCA: Glycoursodeoxycholic acid; HCA: γ-muricholic acid/hyocholic acid; HDCA: α-hyodeoxycholic acid; LCA: Lithocholic acid; LCA\_S: Lithocholic acid 3 sulfate; NORCA: Nor cholic acid; NORDCA: 3α,12α-dihydroxynorcholelanate/23-nordeoxycholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; THCA: Taurohyocholate; TUDCA: Tauroursodeoxycholic acid; UCA: Ursocholic acid; UDCA: Ursodeoxycholic acid.

matched healthy children. IBD children were monitored over one year period, with a baseline visit, and follow-up visits after 6 and 12 mo. CD patients showed lower z-scores for body weight, height, BMI and resting energy expenditure at baseline and at follow-up visits<sup>[14]</sup>. Targeted MS metabolic profiling detected and quantified 69 metabolites by GC-MS and 30 bile acid species in urine. We report in Table 1 the urinary concentrations of all the metabolites at baseline for health and IBD children. Using this set of 99 metabolites, we have applied a Metabolite Set Enrichment Analysis<sup>[29]</sup> to summarize the main metabolic pathways captured by our systems approach. As reported in Figure 1, the analysis provides a comprehensive approach to generate insights in relation to protein biosynthesis, bile acid biosynthesis, aspartate, alanine, glutathione, urea, ammonia and citric acid (TCA) cycles.

#### **Urine biochemical composition illustrates metabolic differences between IBD and healthy children**

Due to sample dilution, the urine sample from one healthy control was discarded from analysis by MS

metabonomics. Initial inspection of the main source of variations between the samples was performed using principal component analysis (PCA). A model was generated using four principal components, explaining 30%, 8%, 6% and 4% of the total variance, respectively. The sources of variance captured by the third and fourth components highlighted a separation of the urine of healthy children from the IBD patients, irrespective of their time of collection.

Using OPLS-DA, we first assessed the occurrence of urinary signatures discriminating the urine samples from healthy subjects and IBD patients. A first OPLS-DA model generated on urine samples collected, with one predictive and one orthogonal component, described statistically robust metabolic differences at baseline. This is observed through the model parameters  $R^2X = 0.37$ ,  $R^2Y = 0.72$ ,  $Q^2Y = 0.47$ , where  $R^2X$  is the explained variance in the urine metabolic profile,  $R^2Y$  the explained group variance and  $Q^2Y$  an indicator of model robustness. We further tested the reproducibility of the metabolic differences by comparing the urine collected from IBD patients after 6 and 12 mo of standard disease management

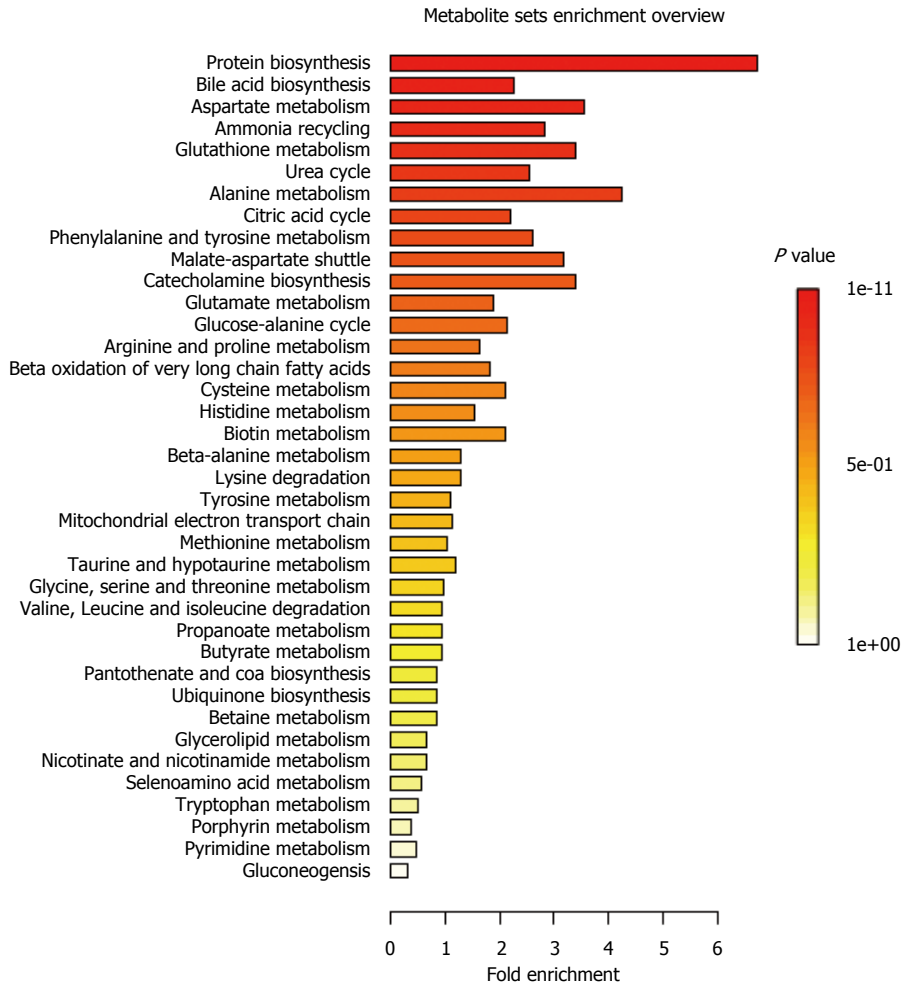


Figure 1 Summary plot for over representation analysis of urinary metabolites, using metabolite set enrichment analysis.

with the reference samples from the healthy children. The models generated with one predictive and one orthogonal component were significant at 6 mo ( $R^2X = 0.36$ ,  $R^2Y = 0.67$ ,  $Q^2Y = 0.41$ ) and at 12 mo ( $R^2X = 0.38$ ,  $R^2Y = 0.73$ ,  $Q^2Y = 0.47$ ).

Examination of the three models resulted in the identification of consistent urinary metabolic differences between the group of healthy subjects and IBD patients. In Table 2, we report metabolites that were significantly different in at least two of the models with  $VIP > 1.0$ , and their corresponding Pearson correlation coefficient. These differences described variations in relation to glutathione metabolism (glycine, glutamate, pyroglutamate and cysteine), urea (fumarate, arginine, alanine, aspartate, and ornithine) and TCA (fumarate, isocitrate, 2-hydroxyglutaric acid, and methylsuccinic acid) cycles, catecholamines (dopamine and tyrosine), several amino acids (e.g., phenylalanine, tyrosine, proline, and glutamate), aromatic acid and bile acid bacterial co-metabolism.

Whilst most of the metabolites appeared to be excreted in higher concentrations in IBD patients, only 3 metabolites showed reduced concentrations, namely hippurate, 3-hydroxyphenylacetate and the bile acid glyco-hydoxycholic acid (GHDCA).

We further assessed if some urinary metabolites were specific to the few UC patients. OPLS-DA models were generated to compare the subjects at the different time points. The models generated with one predictive and two orthogonal components were significant at baseline ( $R^2X = 0.42$ ,  $R^2Y = 0.94$ ,  $Q^2Y = 0.34$ ), at 6 mo ( $R^2X = 0.48$ ,  $R^2Y = 0.94$ ,  $Q^2Y = 0.55$ ) and at 12 mo ( $R^2X = 0.50$ ,  $R^2Y = 0.95$ ,  $Q^2Y = 0.55$ ). Only three metabolites showed pattern differentiating UC from CD patients consistently over time, including higher urinary concentrations of deoxycholic acid (DCA), 7-ketolithocholic acid and beta-ursocholic acid ( $\beta$ UCA) in urine from UC patients (Table 3).

Finally, we explored urinary metabolic signatures linked to clinical and anthropometric endpoints in the IBD population. An O-PLS regression approach was applied using cross-validation by subjects, since clinical and metabonomics data were generated from each subject at 3 different time points. Some relationships between urinary metabolite profiles and anthropometric features were modeled, but not in relation to disease index ( $Q^2Y < 0$ ) or inflammatory status (CRP, calprotectin,  $Q^2Y < 0$ ). Statistically significant associations were modeled in relation to BMI ( $R^2X = 0.40$ ,  $R^2Y = 0.82$ ,  $Q^2Y = 0.35$ ), BMI-z scores



**Table 2 Overview of influential metabolites discriminating healthy and inflammatory bowel disease patients**

Metabolic pathways	Metabolites	Comparison healthy - IBD subjects at					
		Baseline		6 mo		12 mo	
		VIP	r	VIP	r	VIP	r
Tca cycle	Methylsuccinic acid	1.3	0.4	1.2	0.2	1.0	0.1
	Fumaric acid	1.2	0.3	1.0	0.3	1.1	0.2
	2-hydroxyglutaric acid	1.4	0.5	1.3	0.4	1.3	0.3
	Isocitric acid	1.4	0.4	1.3	0.3	1.2	0.2
Glutathione metabolism	Cysteine	1.5	0.5	1.2	0.3	1.4	0.4
	Glycine	1.2	0.3	1.3	0.3	1.2	0.3
	Glutamic acid	1.2	0.3	1.3	0.3	1.2	0.3
	Pyroglutamic acid	1.3	0.4	1.3	0.3	1.0	0.1
Neurotransmitter metabolism	Gamma-Aminobutyric acid	1.2	0.3	1.2	0.3	1.0	0.1
	Dopamine	1.6	0.7	1.8	0.6	1.1	0.4
	Homogentisic acid	1.6	0.7	1.8	0.6	1.1	0.4
Bacterial metabolism	3-hydroxyphenylacetic acid	1.3	-0.5	1.2	-0.4	1.1	-0.3
	Hippuric acid	1.0	-0.3	1.2	-0.4	1.6	-0.5
	Indoleacetic acid	1.3	0.4	1.3	0.4	1.0	0.1
	4-hydroxyphenylacetic acid	1.3	0.5	1.1	0.3	1.0	0.2
	Phenyllactic acid	1.1	0.4	0.6	0.2	1.4	0.5
Other	Salicyluric acid	1.2	0.4	0.9	0.2	1.0	0.4
Dicarboxylic acid metabolism	Suberic acid	1.3	0.5	1.5	0.5	1.1	0.3
Bile acids	Beta-UCA	1.5	0.6	1.0	0.4	0.7	0.2
	GUDCA	1.3	0.5	1.5	0.5	1.9	0.7
	THCA	1.0	0.4	0.3	0.1	1.3	0.4
	UDCA	1.4	0.6	1.7	0.6	1.8	0.6
	GHDCA	1.3	-0.5	1.3	-0.5	1.0	-0.3
Amino acid metabolism	Aspartic acid	1.3	0.4	0.8	0.2	1.4	0.4
	Histidine	1.1	0.3	1.1	0.2	0.9	0.1
	Isoleucine	1.5	0.4	1.6	0.4	1.3	0.2
	Methionine	1.5	0.5	1.6	0.5	1.4	0.4
	Phenylalanine	1.3	0.3	1.2	0.2	1.1	0.1
	Proline	1.4	0.5	1.4	0.4	1.2	0.3
	Tryptophan	1.2	0.3	1.2	0.2	1.1	0.2
	Tyrosine	1.1	0.2	1.0	0.1	1.0	0.1
	Valine	1.3	0.3	1.3	0.3	1.1	0.1
	N-acetyltryptophan	1.2	0.3	1.2	0.2	1.1	0.2
	Ornithine	1.4	0.5	1.7	0.5	1.7	0.5
	Aminoadipic acid	1.0	0.0	1.2	0.3	1.1	0.1

IBD: Inflammatory bowel disease; TCA: Taurocholic acid;  $\beta$ UCA: Beta-ursocholic acid; GUDCA: Glycoursodeoxycholic acid; THCA: Taurohyocholate; UDCA: Ursodeoxycholic acid; GHDCA: Glycohyodeoxycholate.

**Table 3 Overview of influential metabolites discriminating ulcerative colitis and Crohn's disease patients**

Comparison UC - CD subjects at metabolites	Baseline		6 mo		12 mo	
	VIP	r	VIP	r	VIP	r
DCA	1.6	0.5	1.4	0.5	0.8	0.2
7-ketoLCA	1.1	0.3	1.2	0.4	1.8	0.5
Beta-UCA	0.3	0.1	2.0	0.7	1.0	0.3

UC: Ulcerative colitis; CD: Crohn's disease; DCA: Deoxycholic acid; 7\_KetoLCA: 7-ketolithocholic acid;  $\beta$ UCA: Beta-ursocholic acid.

( $R^2X = 0.40$ ,  $R^2Y = 0.83$ ,  $Q^2Y = 0.29$ ), body weight ( $R^2X = 0.43$ ,  $R^2Y = 0.74$ ,  $Q^2Y = 0.15$ ), % of fat mass ( $R^2X = 0.39$ ,  $R^2Y = 0.83$ ,  $Q^2Y = 0.36$ ). A conservative approach was applied for selecting the most influential metabolites, namely using the VIP threshold  $> 1.0$  and a  $r$  value significance at 95%CI (e.g.,  $r > 0.265$  or  $r < -0.265$ ). A consolidated data overview is reported in Table 4 for the relevant anthropometric parameters and metabolites. Similar urinary metabolite patterns

could be ascribed to the body composition parameters, including bile acids, amino acids and TCA cycle intermediates, as well as some dietary/gut microbial related metabolites (Table 4).

## DISCUSSION

Our MS based metabolomics approach allowed to characterize further metabolite differences between IBD and healthy children, by providing complementary metabolite readouts into the central energy metabolic pathways, bile acids, glutathione and glycine metabolism, which are discussed here after.

### Urinary bile acids as metabolic fingerprint of IBD phenotypes

IBD is often associated with some variations in the enterohepatic circulation of bile acids<sup>[2,30,31]</sup>. Recently, Duboc *et al.*<sup>[30]</sup> extensively discussed the connections between gut dysbiosis and bile acid metabolism in IBD,

**Table 4 Overview of metabolites associated with anthropometric parameters**

Metabolic pathways	Metabolites	Anthropometric parameters							
		Fat mass %		BMI		BMI z-score		Body weight	
		<i>r</i>	VIP	<i>r</i>	VIP	<i>r</i>	VIP	<i>r</i>	VIP
TCA cycle	L-2-hydroxyglutaric acid	0.41	1.57	0.22	1.23	0.29	1.36	0.14	1.07
	Oxoglutaric acid	0.48	1.66	0.29	1.32	0.35	1.41	0.13	0.91
	Succinic acid	0.39	1.50	0.17	1.12	0.24	1.24	0.10	0.98
Bacterial metabolism	3-hydroxyphenylacetic acid	0.44	1.49	0.27	1.20	0.29	1.22	0.23	1.07
	Hippuric acid	0.32	1.27	0.27	1.27	0.28	1.26	0.19	1.03
	Phenol	0.44	1.42	0.42	1.57	0.48	1.68	0.26	1.10
Bile acids	12_ketoLCA	0.34	1.13	0.41	1.53	0.40	1.40	0.39	1.48
	DCA	0.48	1.62	0.48	1.82	0.50	1.76	0.52	2.02
	GDCA	0.48	1.58	0.49	1.85	0.47	1.66	0.53	2.01
	GHDCA	0.38	1.28	0.49	1.81	0.42	1.47	0.45	1.69
	GLCA	0.19	0.87	0.32	1.28	0.30	1.17	0.43	1.69
	LCA	0.35	1.16	0.37	1.40	0.34	1.20	0.41	1.57
	NORDCA	0.37	1.25	0.45	1.65	0.44	1.54	0.57	2.13
	TUDCA	-0.12	0.81	-0.32	1.35	-0.31	1.27	-0.43	1.65
	UCA	-0.30	0.96	-0.38	1.39	-0.33	1.16	-0.35	1.42
	UDCA	-0.11	0.70	-0.42	1.60	-0.38	1.39	-0.44	1.65
Amino acid metabolism	Aminoadipic acid	0.36	1.41	0.22	1.20	0.25	1.23	0.17	1.06
	L-Asparagine	0.26	0.89	0.34	1.33	0.36	1.30	0.27	1.21
	L-Histidine	0.30	1.33	0.33	1.48	0.34	1.47	0.31	1.42
	L-Methionine	0.31	1.39	0.16	1.16	0.24	1.29	0.09	1.03
	L-Proline	0.30	1.35	0.15	1.13	0.23	1.27	0.10	1.06
	L-Tryptophan	0.32	1.39	0.29	1.41	0.34	1.50	0.22	1.21
	L-Tyrosine	0.27	1.29	0.23	1.26	0.26	1.32	0.15	1.07
	N-acetyltryptophan	0.32	1.42	0.28	1.41	0.33	1.50	0.22	1.23

12-KetoLCA: 12-ketolithocholic acid; DCA: Deoxycholic acid; GDCA: Glycodeoxycholic acid; GHDCA: Glycohyodeoxycholate; GLCA: Glycolithocholate; LCA: Lithocholic acid; NORDCA: 3 $\alpha$ ,12 $\alpha$ -dihydroxynorcholestanate/23-nordeoxycholic acid; TUDCA: Tauroursodeoxycholic acid; UCA: Ursocholic acid; UDCA: Ursodeoxycholic acid.

and sub-sequent implication for the management of inflammatory conditions. For instance, the alterations in gastrointestinal transit time link to altered bacterial metabolism of primary and secondary bile acids<sup>[32]</sup>. This is reflected through higher concentrations of conjugated bile acids in the stools from active IBD patients, and concomitant decreased concentrations of secondary bile acids<sup>[30]</sup>. Bile acid malabsorption has also been reported in children with CD<sup>[33]</sup>. In our study, we report higher urinary excretion of taurohyocholic acid (THCA, a product of TCDCA metabolism by CYP3A4), ursodeoxycholic acid (UDCA, a product of CDCA by the gut microbiota and the liver), tauroursodeoxycholic acid (TUDCA, a taurine conjugate of UDCA), and  $\beta$ UCA. This signature most likely reflects the alteration of the bile acid metabolism in the digestive tract, as well as modulation of the metabolism of the secondary bile acids by the liver. Previous reports showed an alteration in most circulating bile acids in CD and UC patients<sup>[31]</sup>. In particular, UC patients were reported with higher amount of LCA and DCA, two secondary bile acids, which may relate to their higher urinary excretion in the urine as noted here. We also observed a strong association between BMI z-scores and urinary levels in several bile acids, a trend that was previously reported as well in plasma in other cohort studies<sup>[34]</sup>. Interestingly, the secondary bile acids UDCA and TUDCA are associated with the lower BMI z-score, whilst bacterial metabolites hippurate

and 3-hydroxyphenylacetate are positively associated with the higher ones. Considering IBD children have overall higher urinary UDCA and TUDCA, lower urinary hippurate and 3-hydroxyphenylacetate, and lower BMI z-scores, the signature may be reflecting a higher degree of gastrointestinal deregulations with IBD.

#### ***Pediatric IBD patients have altered urinary excretion of signaling molecules***

Pediatric IBD patients also showed a higher urinary loss in dopamine, gamma-aminobutyric acid (GABA) and homogentisic acid. This pattern may be reflecting a disbalance in serotonin - dopamine metabolism, which is being studied in patient with CD<sup>[35]</sup>. Recent evidences tend to describe excessive synthesis with associated increased tissue levels of serotonin, leading to a perturbation of the molecular homeostasis with dopamine and catecholamines. In particular, a genetic defect of the OCTN1 and OCTN2 of the colon has been identified in patients with CD, resulting in altered transportation of monoamines of the serotonin-dopamine system and their precursors<sup>[35]</sup>. The higher concentration in dopamine, closely related to higher levels of homogentisic acid (another product of tyrosine metabolism) may reflect this disbalance, yet serotonin metabolism could not be captured with our analytical approach in urine. Furthermore, the higher urinary loss of GABA may be reflecting the higher activity in the TCA cycle towards alpha-ketoglutarate

(AKG) and glutamate, and altered colonic epithelial cell metabolism of serotonin-dopamine<sup>[36]</sup>.

### ***IBD in pediatric populations links to perturbations in glycine metabolism***

Another feature identified in the studied population is the occurrence of higher urinary levels of several metabolites involved in the glutathione metabolism, including pyroglutamic acid (PGA), glutamic acid, glycine and cysteine, which were significantly higher in IBD children in the course of the study. Since PGA is linked to glutathione turnover, the urinary pattern may reflect the higher requirement of the metabolism in glutathione to cope with oxidative stress and immune status<sup>[37,38]</sup>. In particular, higher urine PGA may indicate higher glutathione need concomitant to diminished glutathione utilization and/or deficient glutathione resynthesis. The higher urinary loss of cysteine, glycine and glutamic acid, the three main precursors for glutathione synthesis provides additional evidence that glutathione cannot optimally be resynthesized, and therefore is broken down and excreted in the urine via its constituent parts such as PGA. The observation of PGA may also point towards glycine deficiency<sup>[39]</sup>, whilst the higher urinary excretion of many essential and non-essential amino acid mainly reflected higher protein breakdown related to higher metabolic requirements in IBD children. However, no statistically significant associations could be found between urinary metabolites and inflammatory endpoints (*e.g.*, CRP, EST, calprotectin).

Moreover, it is well described, how before entering the urea cycle, glycine can be conjugated with benzoic acid to form hippurate, and be subsequently excreted in urine. An alternative pathway to excrete ammonia excess is through AKG to glutamic acid, glutamine and phenylacetylglutamine (PAG), which then exits the body via the urine. Glycine deficiencies would increase the demands on the urea cycle and the glutamate/AKG pathways. Moreover, the lower hippurate production may reflect this deficiency in glycine for specific metabolic processes. Previous studies reported that altered hippurate metabolic profile in IBD patients was not directly explain by variations in dietary intake or a deficit in the conjugation of benzoate to glycine in the kidney<sup>[40]</sup>. Moreover, our analysis describes a higher urinary excretion of salicyluric acid, a glycine conjugate of salicylic acid produced in the kidney via a pathway very similar to that of hippurate, suggesting selective requirement in glycine for metabolic functions. We previously discussed in the context of IBD<sup>[14]</sup>, that carbamyl phosphate synthetase (CAD) - rate-limiting enzyme for the urea cycle - was identified as negative regulator of NOD2 and might be a pharmacologic target for CD therapies<sup>[41]</sup>. Our observations may provide additional evidence on the essential role of this pathway in case of higher metabolic requirements for glycine associated to malnutrition, higher requirement

for glutathione, phase II metabolism and/or growth and development in pediatric populations.

### ***Children with IBD show different energy and protein metabolic requirements***

We previously reported and discussed how the pediatric IBD patients show the typical phenotype associated with growth failure and body weight loss<sup>[6,14,42]</sup>. In particular, we reported that our CD pediatric patients showed lower z-scores for body weight, body height, BMI and resting energy expenditure compared to healthy controls, which linked to lower concentrations in IGF-1 and IGFBP-3<sup>[14]</sup>. Our MS metabolomics approaches provided more detailed insights into the changes in protein and energy metabolites. IBD children showed a significant increase in the urinary content of several closely related intermediates of the TCA cycle (fumarate, isocitrate, 2-hydroxyglutaric acid, methylsuccinic acid). Our previous NMR analysis identified higher urinary PAG in IBD patients, which indicated a different energy metabolic status<sup>[14]</sup>. PAG is a major nitrogenous metabolite for which synthesis depends on the availability of glutamine, mainly generated in the Krebs cycle from AKG. Therefore the increase in isocitrate (precursor of AKG) may be functionally related to higher metabolic turnover for AKG. Moreover, we noted an increased excretion of a 2-hydroxyglutaric acid (Table 2), normally in molecular equilibrium with AKG, which brings further evidence for a specific increased metabolic flux towards AKG within TCA cycle. Whilst succinate does not show a significantly increased urinary excretion (in agreement with our previous NMR observations<sup>[14]</sup>), closely related metabolites, namely methylsuccinate and fumarate, had higher urinary levels which may describe a selective oxidation of aspartate, tyrosine and phenylalanine.

Furthermore, the overall increased excretion in most amino acids and the sulfur containing amino acids cysteine and methionine (Table 2), indicates a different protein metabolism and handling of nitrogen in pediatric IBD patients when compared to healthy matched controls. Urinary metabolite patterns could be ascribed to body composition parameters, such a BMI, body weight and fat mass. Moreover, most of our IBD patients were in clinical remission, and whilst the patients tend to have lower z-scores for body weight, height and BMI at baseline, the higher the BMI z-scores during follow-up the better growth catch-up for the patient. We here observed an association between higher BMI z-score and higher urinary content in key intermediates of the TCA cycle, namely succinate and AKG, amino acids (asparagine, histidine, methionine, tyrosine, proline). This signature may be associated with overall changes in protein and energy requirements related to body weight gain. Our observations with BMI z-scores are similar to recent observations in blood with the Framingham study<sup>[43]</sup>.

### Strengths and weaknesses of the study

One main limitation of our study is the small number of subjects. However, the longitudinal design and the use of reference group of age- and gender match healthy children provide novel insights into childhood metabolic status in relation to growth and disease.

### Future developments

We demonstrate that a direct analysis of urine - a biological sample that can be collected non-invasively and in a repeatable fashion - using targeted MS based metabolomics might provide metabolic insights complementary to more invasive blood and stool metabolic analysis, whilst offering an opportunity to monitor the metabolic status in relation to disease state. Here, we identified a peculiar metabolite signature related to bile acids, central energy and glutathione biochemical pathways, which should be further followed up in future studies.

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### COMMENTS

#### Background

The prevalence of inflammatory bowel disease (IBD) has been increasing over the year, with 25% of the population developing the disease during childhood and adolescence. In the pediatric population, in addition to the clinical and immunological features of the disease, patients suffer of additional growth failure and delayed puberty.

#### Research frontiers

There is a great lack of knowledge on how to address metabolic and nutritional requirements in children and adolescents. It is envisioned that the use of omics technologies can generate a system view of metabolic processes associated with the growth and the development of children in relation to the changing physiology. Metabolomics is a promising approach to explore physiological regulatory processes in human clinical research.

#### Innovations and breakthroughs

In the present study, they applied targeted mass spectrometry based metabolite profiling to explore bile acids and host-gut microbial metabolites in urine samples from IBD and healthy matched children. Metabolic differences encompass central energy, amino acids, and bile acid metabolism. The authors showed that non-invasive urine bile acid profiling reflect altered hepatic and intestinal barrier dysfunctions, as previously reported through blood or stool metabolic profiling. Moreover, levels of pyroglutamic acid, glutamic acid, glycine and cysteine were significantly higher in IBD children in the course of the study. This suggests that glutathione cannot be optimally synthesized and replenished, rather it is broken down to its constituent parts and excreted through the urine. In addition, a perturbation of the urinary levels of several glycine conjugated metabolites, may associate with differential metabolic requirements in children.

#### Applications

Non-invasive sampling of urine followed by targeted MS-based metabolomic analysis might elucidate and monitor the metabolic status of children in relation to disease state.

### Peer-review

The excellent study gives a very good data set reflecting metabolomics in IBD children. Understanding the metabolic characteristics of pediatric patients with IBD compared to control is a key factor in diagnosis. The authors stuck to their specific hypothesis and eloquently presented their material. This opens the door for future trials looking at predictive modeling utilizing metabolomics and modifying various therapies to target patients with specific metabolic characteristics.

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## Basic Study

# M2-like Kupffer cells in fibrotic liver may protect against acute insult

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## Abstract

### AIM

To investigate the mechanism of hepatoprotection conferred by liver fibrosis through evaluating the activation phenotype of kupffer cells.

### METHODS

Control and fibrotic mice were challenged with a lethal dose of D-GalN/lipopolysaccharide (LPS), and hepatic damage was assessed by histology, serum alanine transferase (ALT) levels, and hepatic expression of HMGB1, a potent pro-inflammatory mediator. The

localization of F4/80 (a surrogate marker of KCs), HMGB1, and type I collagen (Col-1) was determined by immunofluorescence staining. The phenotype of KCs was characterized by real-time PCR. KCs isolated from control or fibrotic mice were challenged with LPS or HMGB1 peptide, and HMGB1 translocation was analyzed.

## RESULTS

Liver fibrosis protected mice against D-GalN/LPS challenge, as shown by improved hepatic histology and reduced elevation of ALT compared with the normal mice treated in the same way. This hepatoprotection was also accompanied by inhibition of HMGB1 expression in the liver. Co-localization of F4/80, HMGB1, and Col-1 was found in fibrotic livers, indicating the close relationship between KCs, HMGB1 and liver fibrosis. KCs isolated from fibrotic mice predominantly exhibited an M2-like phenotype. *In vitro* experiments showed that HMGB1 was localized in the nucleus of the majority of M2-like KCs and that the translocation of HMGB1 was inhibited following stimulation with LPS or HMGB1 peptide, while both LPS and HMGB1 peptide elicited translocation of intranuclear HMGB1 in KCs isolated from the control mice.

## CONCLUSION

M2-like Kupffer cells in fibrotic liver may exert a protective effect against acute insult by inhibiting the translocation of HMGB1.

**Key words:** Liver fibrosis; Injury resistance; Kupffer cell activation; High-mobility group box 1; Translocation

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**Core tip:** The hepatoprotective effect conferred by liver fibrosis against acute liver injury is an interesting phenomenon, which has not yet been fully characterized. In the present study, we dissected the underlying mechanism of acute injury in the setting of liver fibrosis through investigating the correlation between KC activation and HMGB1 translocation. Our study showed that liver fibrosis protects mice against D-GalN/LPS challenge, and M2-like KCs in the fibrotic liver may exert a protective effect by inhibiting the translocation of HMGB1, a potent pro-inflammatory mediator.

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## INTRODUCTION

Liver fibrosis is the most clinically relevant consequence

of chronic liver diseases. It is characterized by the activation of hepatic stellate cells and the excessive accumulation of extracellular matrix<sup>[1]</sup>. Advanced fibrosis results in cirrhosis, portal hypertension and liver failure, and often requires liver transplantation<sup>[2-5]</sup>. On the other hand, liver fibrosis represents the wound healing response to liver injury due to a variety of etiologies<sup>[6,7]</sup>. Recently, increasing attention has been focused on the homeostatic and beneficial effects originating from fibrotic liver. Convincing evidence has shown that fibrosis results in increased liver resistance to subsequent acute insults, thus protecting hepatocytes against various toxic stimuli<sup>[8-10]</sup>. However, the mechanisms governing this hepatoprotection are largely unexplored.

Hepatic macrophages have central roles in maintaining homeostasis in the liver as well as in the pathogenesis of acute or chronic liver injury<sup>[11]</sup>. Macrophages are pleiotropic cells that assume diverse functions. The microenvironmental milieu is a key determinant of macrophage function during tissue inflammation<sup>[12]</sup>. Macrophages can undergo "classical" M1 activation when exposed to lipopolysaccharide (LPS), and interferon-gamma (IFN- $\gamma$ ) or "alternative" M2 activation when exposed to interleukin (IL)-4/IL-13<sup>[13-15]</sup>. M1- and M2-like macrophages exhibit distinct signatures and fulfill different functions. Kupffer cells (KCs), the resident macrophages in the liver, play pivotal roles in the progression and resolution of liver fibrosis<sup>[16]</sup>. Recently, KCs have been reported to exert a protective effect against tumor necrosis factor (TNF)- $\alpha$ -induced hepatocyte apoptosis<sup>[9]</sup>. Nevertheless, whether and how KC activation is involved in injury resistance in the setting of liver fibrosis is poorly understood.

HMGB1 is a ubiquitous protein present in the nucleus of most mammalian cells. HMGB1 has diverse functions which depend on its cellular localization. In the intracellular compartment, HMGB1 participates in gene transcription, DNA replication, and DNA repair. To function as a damage-associated molecular pattern (DAMP), HMGB1 can translocate from nucleus to cytoplasm and is subsequently released into the extracellular milieu. This process is implemented by two principal means: active secretion by innate immune cells (*e.g.*, macrophages) after hyperacetylation or passive release from necrotic cells. Extracellular HMGB1 activates pro-inflammatory signaling pathways by ligation of pattern recognition receptors including the receptor for advanced glycation end products or Toll-like receptor 4, leading to severe damage in multiple liver diseases<sup>[17-19]</sup>. As a biomarker of liver injury, HMGB1 is superior to serum alanine transferase (ALT) levels at identifying acetaminophen-induced acute liver injury and elevated HMGB1 in acute liver failure correlates well with poor outcome<sup>[20,21]</sup>.

In the present study, we investigated the roles of KCs, KC activation, and HMGB1 in the pathogenesis

of acute injury in the setting of liver fibrosis, and hypothesized that activated KCs in fibrotic liver protected against acute insult by inhibiting the translocation of HMGB1. We present herein evidence that liver fibrosis protects mice against D-GalN/LPS challenge and that M2-like KCs in fibrotic liver may exert a protective effect by inhibiting the translocation of HMGB1, a potent pro-inflammatory mediator.

## MATERIALS AND METHODS

### **Animals and treatments**

Male BALB/c mice (6-8-wk-old) were obtained from the Laboratory Animal Center, Academy of Military Medical Sciences, Beijing, China. The animal protocol was designed to minimize pain or discomfort in the mice. Mice were housed in a specific pathogen-free environment at 22-24 °C in a 12-h light-dark cycle. Animals were fed standard laboratory chow with free access to water. All animal care and experimental procedures performed in this study were in accordance with the guidelines for experimental animals approved by the Animal Care and Use Committee of Capital Medical University, China.

Mice were treated as follows: (1) induction of fibrosis: BALB/c mice received an intraperitoneal injection of carbon tetrachloride (CCl<sub>4</sub>) in mineral oil, twice weekly, for 6 wk. The initial dose of CCl<sub>4</sub> was 0.2 µL/g (2%), and the dose was gradually increased up to 3 µL/g (30%); and (2) acute challenge: Control and fibrotic mice received intraperitoneal injection of a lethal dose of hepatic toxins (1 mg/g D-GalN + 50 ng/g LPS; Sigma-Aldrich, St Louis, MO, United States). Sera and liver tissues were harvested 24 h after acute injury for analysis. A portion of the liver was fixed in 10% neutral-buffered formalin for histological analysis and immunostaining. The remaining liver was cut into pieces and snap-frozen for homogenization to extract total liver RNA.

### **Evaluation of liver injury**

Serum ALT levels were measured using a multi-parameteric analyzer (AU 5400; Olympus, Tokyo, Japan) according to an automated procedure. Formalin-fixed liver tissues were embedded in paraffin, sectioned and stained with hematoxylin-eosin for light microscopy.

### **Isolation of hepatic non-parenchymal cells and KCs**

Hepatic non-parenchymal cells (NPCs) were isolated from mice by collagenase digestion and differential centrifugation using a previously reported method with some modifications<sup>[22]</sup>. Briefly, *in situ* perfusion was applied through the portal vein and superior vena cava with 0.9% NaCl followed by DMEM/F12 (Gibco, Grand Island, NY, United States) containing 0.5% Pronase (Roche Diagnostics GmbH, Mannheim, Germany) and DMEM/F12 containing 0.04% type IV collagenase (Sigma-Aldrich). The liver was then harvested, excised

and digested with DMEM/F12 containing 10 µg/mL DNase (Sigma-Aldrich). Digested livers were passed through a 70 µm cell strainer (BD Falcon, Franklin Lakes, NJ, United States). The filtrate was centrifuged and washed. The pellets were re-suspended in DMEM (Hyclone, Logan, UT, United States), and then overlaid onto a Percoll (Amersham Pharmacia Biotechnology, Buckinghamshire, United Kingdom) gradient (40%-70%), and centrifuged at 1100 × *g* for 20 min. NPCs were collected from the interface for further purification.

To purify KCs, the liver NPC suspension was further overlaid onto the Percoll gradient (25%-50%), and centrifuged at 1800 × *g* for 30 min. The KC-enriched NPCs in the interface were harvested and washed. The isolated KCs were then cultured in DMEM medium containing 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Sigma-Aldrich) in a humidified chamber at 37 °C with 5% CO<sub>2</sub>. Following incubation for 2 h, the unattached cells were gently removed. The remaining adhered cells were further cultured for 24 h, and the phenotype of KCs was characterized by real-time PCR.

### **Reverse transcription and SYBR Green real-time quantitative PCR**

Total RNA was extracted from isolated KCs using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. Reverse transcription of the purified RNA (2.5 µg) was performed using random primers and the AMV retrotranscriptase system (TakaRa, Dalian, Liaoning, China) according to the manufacturer's protocol. SYBR Green real-time PCR was carried out using the ABI StepOne Plus (Applied Biosystems, Foster City, CA, United States). All reactions were performed in triplicate. In a final reaction volume of 20 µL, the followings were added: 1× SYBR Green (TakaRa), cDNA, 0.5 mmol/L of each primer, and ROX. The reaction conditions were as follows: 50 °C (2 min), 95 °C (5 min), followed by 40 cycles at 95 °C (15 s) and 60 °C (30 s). The primers used were designed with Primer 3.0 software and are listed in Table 1. The relative expression of target genes was calculated and normalized to the expression of the housekeeping gene GAPDH.

### **Treatment of isolated KCs with LPS or HMGB1 peptide**

KCs isolated from control and fibrotic mice were cultured in DMEM medium for 24 h. The KCs were then treated with LPS (10 ng/mL) or HMGB1 peptide (FKDPNAPKRLPSAFLFCSE) (30 µg/mL; SBS Genetech Co, Ltd, Beijing, China) for another 20 h. Translocation of HMGB1 in KCs was analyzed by immunofluorescence staining.

### **Immunofluorescence staining**

Frozen liver sections (or KCs) were fixed with 4%



**Table 1** Primer sequences used for reverse transcription-quantitative polymerase chain reaction analysis

Genes	Sense (5'-3')	Anti-sense (5'-3')
GAPDH	AACCTTGGCATTGTGGAAGG	ACACATIGGGGGTAGGAACA
IL-1 $\beta$	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGT
TNF- $\alpha$	GCCCTCTCTCATTCCTGCTTG	TGAGATCCATGCCGTTG
CD206	ATGCCAAGTGGGAAAATCTG	TGTAGCAGTGGCCTGCATAG
iNOS	CGGAGCCTTTAGACCTCAACA	CCCTCGAAGGTGAGCTGAAC
YM-1	ATCTATGCCTTGTGCTGGAATGC	TGAATGAATATCTGACGGTTCTGAG
CCL17	TGCTTCTGGGGACTTTTCTG	TGGCCTTCTTCACATGTTTG

paraformaldehyde for 30 min at room temperature. Slices (or KCs) were treated with 0.2% Triton X-100 for 5 min. The slices (or KCs) were then incubated with Tris-buffered saline (TBS) containing 5% fetal bovine serum for 30 min. Immunofluorescence staining was performed using the following primary antibodies: rat anti-mouse F4/80 antigen Alexa Fluor<sup>®</sup> 488 (clone BM8; eBioscience, San Diego, CA, United States), rabbit anti-mouse HMGB1 (Epitomics, Burlingame, CA, United States), and goat anti-mouse type I collagen (Col-1) (SouthernBiotech, Birmingham, AL, United States). For indirect immunofluorescence staining, FITC-conjugated (Santa Cruz Biotechnology, Inc, Dallas, TX, United States) or Cy3-conjugated anti-rabbit IgG (Sigma-Aldrich) for HMGB1, and Cy3-conjugated rabbit anti-goat IgG for Col-1 (Sigma-Aldrich) were used. A Nikon inverted fluorescence microscope ECLIPSE Ti and NIS-Elements F3.0 software (Nikon Corporation, Tokyo, Japan) were used for image capture.

#### HMGB1 immunohistochemical staining

After deparaffinization and rehydration, the embedded liver sections were treated with 3% H<sub>2</sub>O<sub>2</sub> for 15 min, followed by microwave antigen retrieval for a further 15 min in citrate buffer. The nonspecific proteins were blocked with 10% goat serum for 30 min. For HMGB1 staining, the specimens were incubated with a rabbit anti-mouse HMGB1 monoclonal antibody (Abcam, Cambridge, MA, United States) overnight at 4 °C, followed by 30 min incubation with horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The sections were incubated with diaminobenzidine as a chromogenic substrate and counterstained with hematoxylin, dehydrated, and stabilized with mounting medium. Images were captured using an Olympus Bx51 microscope (Olympus America, Melville, NY, United States) and CellSens standard 1.4.1 software.

#### Animal care and use statement

All animal care and experimental procedures performed in this study were in accordance with the guidelines for experimental animals approved by the Animal Care and Use Committee of Capital Medical University, China.

#### Statistical analysis

Results were expressed as mean  $\pm$  SE of the values obtained. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. Statistics and graphs were generated using Prism 5.0 software (GraphPad Software Inc, San Diego, CA, United States).  $P < 0.05$  was considered statistically significant. The statistical methods used in this study were reviewed by Dr. Jun-Feng Li from the First Affiliated Hospital of Lanzhou University, Lanzhou, China.

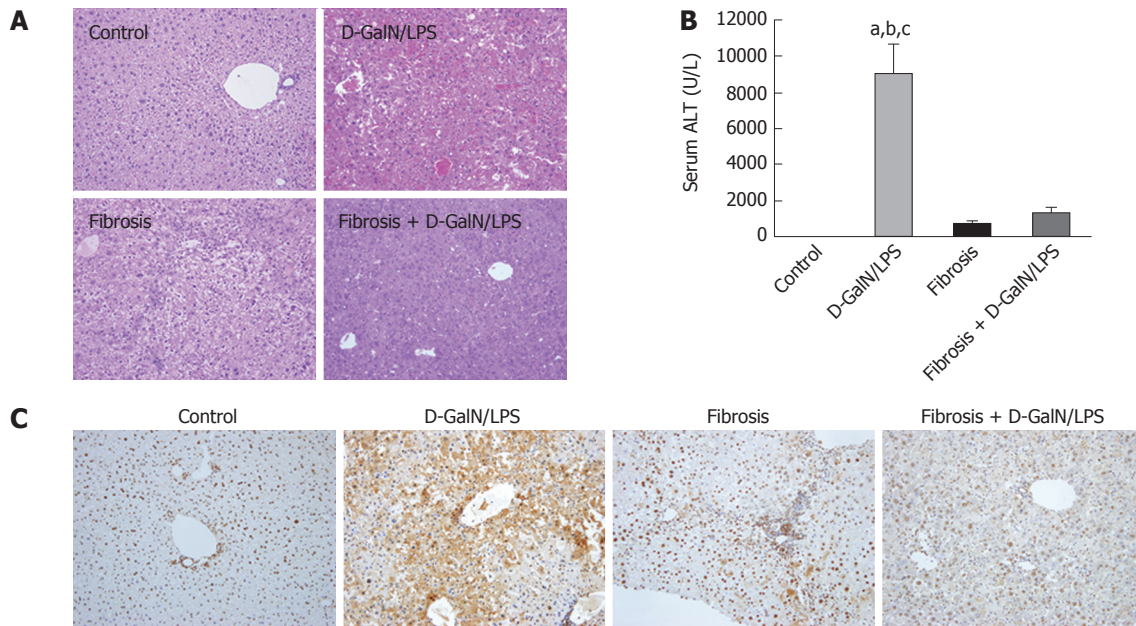
## RESULTS

#### *Inhibition of HMGB1 expression is accompanied by injury resistance in the setting of liver fibrosis*

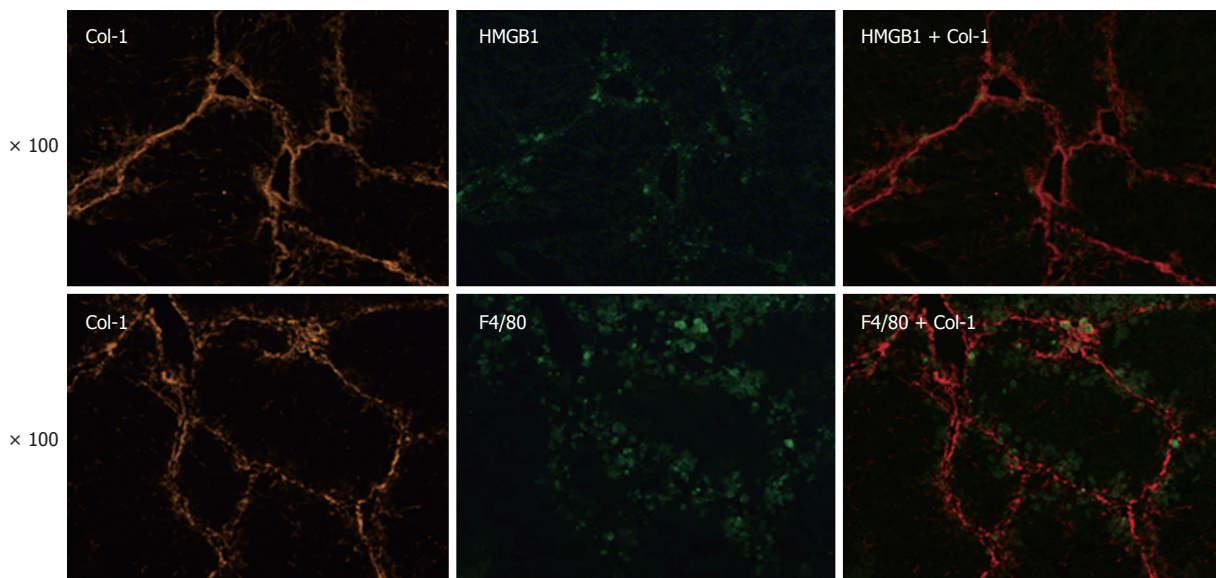
We first assessed hepatic injury in control and fibrotic mice with or without acute insult. As shown in Figure 1, the D-GalN/LPS challenge triggered a sharp increase in serum ALT levels in control mice, which corresponded well with the pathological findings. In contrast, fibrotic mice showed marked resistance to the same insult. In particular, hepatic damage was significantly alleviated in fibrotic mice following the D-GalN/LPS challenge compared with control mice treated in the same way, as shown by improved hepatic histology and reduced serum ALT levels (Figure 1A and B). HMGB1, a potent and classic pro-inflammatory mediator, was induced in acutely injured mice. However, the expression of HMGB1 was markedly inhibited in fibrotic mice, even under acute challenge (Figure 1C). These findings suggest that liver fibrosis protects mice against acute insult, which is accompanied by inhibition of HMGB1 expression.

#### *Kupffer cells may be involved in HMGB1-related injury resistance in the setting of liver fibrosis*

Kupffer cells are involved in the progression and resolution of liver fibrosis. Recently, KCs have been documented to possess protective effects against hepatocyte apoptosis. In light of this finding, we hypothesized that KCs are involved in HMGB1-related injury resistance in the setting of liver fibrosis. To explore this hypothesis, we analyzed the expression and distribution of KCs and HMGB1 in fibrotic liver



**Figure 1** Inhibition of High mobility group box 1 expression is closely associated with the injury resistance in the setting of liver fibrosis. Control and fibrotic mice (treated with CCl<sub>4</sub> for 6 wk) were challenged with a lethal dose of D-GalN (1 mg/g)/LPS (50 ng/g), and hepatic damage was assessed by histology (A: HE staining; original magnification,  $\times 200$ ) and serum ALT levels (B). <sup>a</sup> $P < 0.05$  vs the control group, <sup>b</sup> $P < 0.05$  vs the fibrosis group, <sup>c</sup> $P < 0.05$  vs the fibrosis + D-GalN/LPS group. The expression of HMGB1 was determined by immunohistochemical staining (C: original magnification,  $\times 200$ ). Data are expressed as mean  $\pm$  SEM. CCl<sub>4</sub>: Carbon tetrachloride; D-GalN: D-galactosamine; HMGB1: High mobility group box 1; LPS: Lipopolysaccharide.



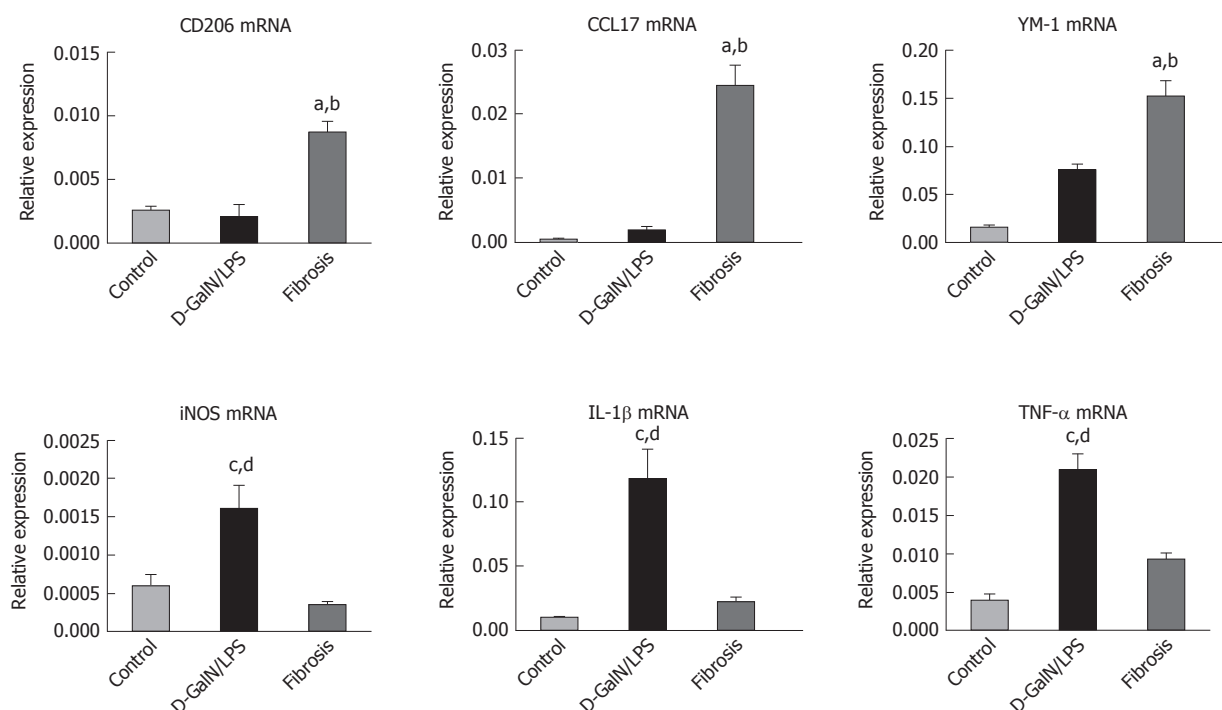
**Figure 2** Kupffer cells may be involved in High mobility group box 1-mediated injury resistance. The expression and localization of F4/80 (a surrogate marker of KCs), HMGB1, and Col-1 were determined by immunofluorescence staining (original magnification,  $\times 100$ ). Col-1: Type I collagen; HMGB1: High mobility group box 1; KCs: Kupffer cells.

by immunofluorescent staining. F4/80, a surrogate marker of KCs, was found to be co-localized with Col-1, supporting the key function of KCs in the pathogenesis of hepatic fibrosis (Figure 2). Interestingly, the co-localization of F4/80, HMGB1 and Col-1 was also found in fibrotic liver (Figure 2), which indicated functional interactions among KCs, the injury mediator HMGB1, and hepatic fibrosis. Thus, KCs may be involved in HMGB1-related injury resistance in the setting of

hepatic fibrosis.

#### **Kupffer cells in fibrotic liver exhibit a predominant M2-like activation**

We next sought to answer the question of how do KCs function in HMGB1-related injury resistance in the setting of liver fibrosis? It has been well established that macrophages are heterogeneous, and their functional plasticity is driven by microenvironmental



**Figure 3** Kupffer cells in the fibrotic liver exhibit predominantly a M2-like activation. KCs were isolated from the livers of normal, acutely injured (D-GalN/LPS) and fibrotic mice, and M1 and M2 gene signatures were then determined by quantitative real-time PCR. Data are expressed as mean  $\pm$  SEM. <sup>a</sup> $P < 0.05$  vs the control group, <sup>b</sup> $P < 0.05$  vs the D-GalN/LPS group, <sup>c</sup> $P < 0.05$  vs the control group, <sup>d</sup> $P < 0.05$  vs the fibrosis group. D-GalN: D-galactosamine; KCs: Kupffer cells; LPS: Lipopolysaccharide.

signals which shape their properties through a wide spectrum of phenotypes<sup>[23,24]</sup>. Thus, we characterized the phenotype of KCs by their representative markers. KCs were isolated from the livers of normal, acutely injured and fibrotic mice, and M1 and M2 gene signatures were then determined by quantitative real-time PCR. Fibrosis triggered marked up-regulation of M2 markers, including CD206, CCL17 and YM-1. However, macrophages from the acutely injured liver showed no increase or a moderate increase in these M2 signatures (Figure 3). On the other hand, iNOS, a marker of M1 activation, was substantially induced in KCs isolated from the acutely injured liver, but remained unchanged in KCs from the fibrotic liver (Figure 3). Therefore, KCs in fibrotic mice exhibit a predominantly M2-like phenotype.

#### **Translocation of HMGB1 is inhibited in M2-like KCs following LPS or HMGB1 peptide stimuli**

How do M2-like KCs and HMGB1 orchestrate injury resistance in the setting of hepatic fibrosis? We speculated that M2-like KCs in fibrotic liver may prevent the translocation of HMGB1 upon acute challenge, thereby hampering its critical function as a DAMP. To confirm our speculation, the translocation of HMGB1 was analyzed in KCs isolated from control and fibrotic mice and treated with LPS or HMGB1 peptide. Both LPS and HMGB1 peptide elicited the translocation of intranuclear HMGB1 in KCs isolated from control mice (Figure 4). Interestingly, HMGB1 was localized in the nucleus of the majority of M2-like KCs isolated

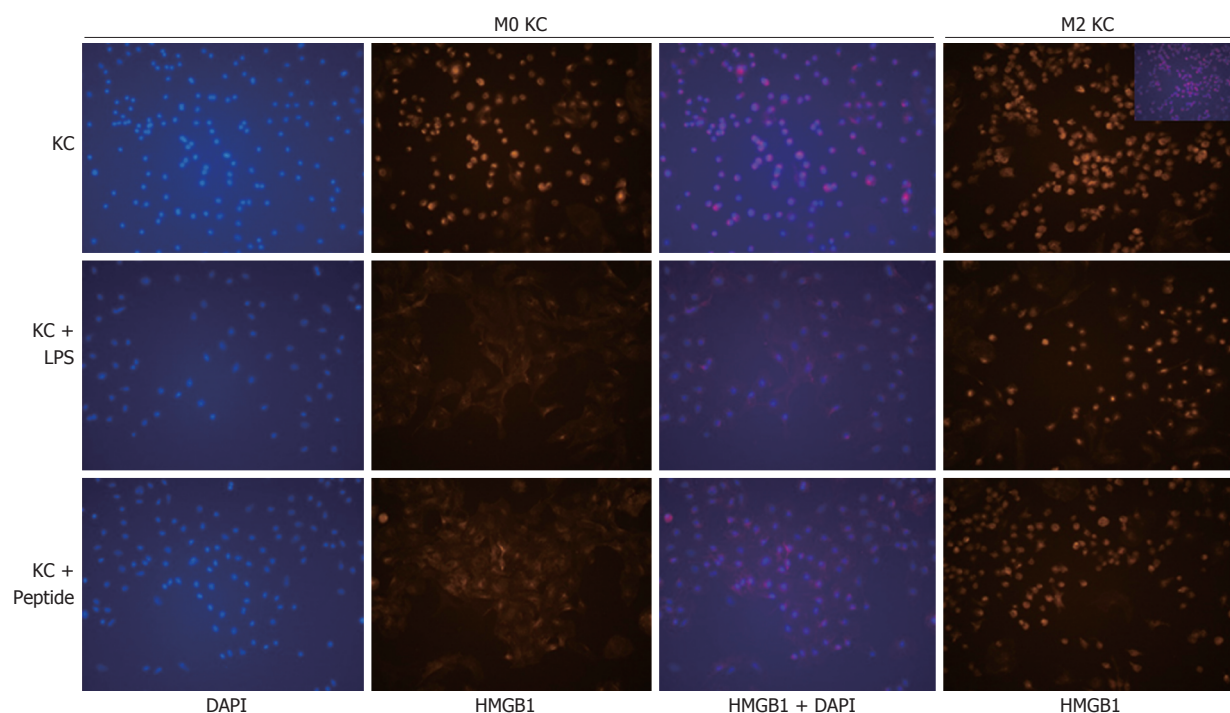
from fibrotic liver. Importantly, neither LPS nor HMGB1 peptide elicited the translocation of HMGB1 in M2-like KCs (Figure 4). Collectively, our *in vitro* data demonstrated that the translocation of HMGB1 was inhibited in M2-like KCs upon acute insult.

## **DISCUSSION**

In the present study, we found that liver fibrosis protects mice against D-GalN/LPS challenge, and M2-like KCs in fibrotic liver may exert a protective effect by inhibiting the translocation of HMGB1, a crucial pro-inflammatory mediator. Our findings may provide a potential explanation for the mechanism of injury resistance in the setting of liver fibrosis.

The deleterious effects resulting from liver fibrosis, including cirrhosis, liver failure and hepatocellular carcinoma, are widely accepted. Recent studies have reported the hepatoprotective effects conferred by liver fibrosis. In a mouse model of partial bile duct ligation (PBDL), injured ligated lobes exhibited improved tolerance to TNF- $\alpha$ - and Fas-induced hepatocyte apoptosis, compared with non-ligated lobes<sup>[10]</sup>. Similarly, thioacetamide-induced fibrotic liver was less vulnerable to acute injury<sup>[8]</sup>. In the present study, we found that CCl<sub>4</sub>-induced liver fibrosis conferred significant protection against a lethal challenge with D-GalN/LPS, as shown by improved hepatic histology and reduced serum ALT levels. Importantly, we linked HMGB1 with injury resistance in the setting of liver fibrosis. HMGB1 is an evolutionarily conserved non-





**Figure 4** Translocation of High mobility group box 1 triggered by lipopolysaccharide or High mobility group box 1 peptide is inhibited in M2-like Kupffer cells. KCs were isolated from the livers of control and fibrotic mice, and then treated with LPS (10 ng/mL) or HMGB1 peptide (30  $\mu$ g/mL). The translocation of HMGB1 in KCs was analyzed by immunofluorescence staining (original magnification,  $\times 200$ ). HMGB1: High mobility group box 1; KCs: Kupffer cells; LPS: Lipopolysaccharide.

histone nuclear protein with abundant expression in most mammalian cells. Recently, HMGB1 was identified as a potent pro-inflammatory mediator. Compared with ALT levels, HMGB1 is more sensitive at identifying acetaminophen-induced acute liver injury<sup>[20,21]</sup>. In our study, in comparison with acutely injured liver, the expression of HMGB1 was markedly inhibited in fibrotic liver following acute challenge, which corresponded well with a significant improvement in liver histology. These findings provide powerful evidence for injury resistance conferred by liver fibrosis.

Hepatoprotection in the setting of liver fibrosis is an interesting subject and remains to be fully elucidated. Bourbonnais *et al.*<sup>[8]</sup> attributed a hepatoprotective response to Col-1 produced during liver fibrosis, which significantly protected hepatocytes against toxic stimuli *via* activation of ERK1 signaling. The study by Osawa *et al.*<sup>[9]</sup> specifically addressed the role of KCs in cholestatic liver injury using PBDL mice. They found that KCs reduced liver damage, and induced hepatocyte survival and regeneration. These protective and regenerative effects require activation of AKT in hepatocytes and SphK in HSCs<sup>[9]</sup>. In the present study, KCs were co-localized with HMGB1 in fibrotic liver, indicating the critical role of KCs in HMGB1-related injury resistance in the setting of hepatic fibrosis.

We then explored the hepatoprotective effect of KCs in fibrotic mice from a new perspective, *i.e.*, the activation phenotype of KCs. Our hypothesis was based on the strong association between the function and phenotype of macrophages, as mentioned in the

introduction. According to our data, KCs exhibited a predominantly M2-like phenotype in the context of liver fibrosis, which is in line with the latest report on congenital hepatic fibrosis<sup>[25]</sup>. Finally, we examined the underlying mechanism concerning the potential protective effect of M2-like KCs on HMGB1-related injury resistance in the setting of hepatic fibrosis. As previously noted, nuclear-cytoplasmic translocation is regarded as a key step in HMGB1 functioning as a DAMP, which in turn, triggers inflammation. Accordingly, we speculated that M2-like KCs may inhibit the translocation of HMGB1 induced by acute insult. Hence, we compared HMGB1 translocation in KCs isolated from control and fibrotic mice challenged with LPS or HMGB1 peptide. Both LPS and HMGB1 peptide triggered the translocation of intranuclear HMGB1 in KCs from control mice. However, the translocation of HMGB1 was markedly inhibited in M2-like KCs, even under acute challenge. These results provide strong support for our hypothesis, that is, M2-like KCs in fibrotic liver may protect against acute insult by inhibiting the translocation of HMGB1, thereby suppressing HMGB1-mediated pro-inflammatory responses and attenuating hepatic injury<sup>[26]</sup>.

Post-translational modification seems to be a critical step in regulation of the translocation and secretion of HMGB1 during inflammatory responses. The acetylation of HMGB1 on lysine residues in response to IL-1 $\beta$ , TNF- $\alpha$ , and LPS is regarded as the most feasible and effective way<sup>[27]</sup>. A recent study has shown that ligand-activated peroxisome proliferator-



activated receptor (PPAR)- $\delta$  and PPAR- $\gamma$  modulate LPS-primed release of HMGB1 through SIRT-mediated deacetylation, which in turn, plays a critical role in the cellular response to inflammation<sup>[28]</sup>. Whether this modulation occurred in our model requires further investigation. Moreover, we cannot exclude the passive release of HMGB1 by injured hepatocytes, as reported by Kao *et al.*<sup>[29]</sup>. However, active secretion of HMGB1 by macrophages is also important in the context of fibrosis.

In conclusion, although the current findings are preliminary and may be speculative, our findings may shed new light on the pathogenesis of acute hepatic damage in the setting of liver fibrosis. We will elaborate the mechanisms underlying injury resistance by modulating the polarization and function of hepatic macrophages or by blocking HMGB1 signaling. From a clinical perspective, our study findings may pave the way for the treatment of liver failure, particularly acute-on-chronic liver failure.

## COMMENTS

### Background

Convincing evidence has shown that fibrosis confers increased liver resistance to subsequent acute insults, thus protecting hepatocytes against various toxic stimuli. The hepatoprotection conferred by liver fibrosis is intriguing and the mechanism of such protection remains to be fully elucidated.

### Research frontiers

Kupffer cells (KCs) have been reported to exert a protective effect against hepatocyte apoptosis in cholestatic liver disease, indicating the potential role of KCs in hepatoprotection. Nevertheless, whether and how the phenotype of KCs is involved in injury resistance in the setting of liver fibrosis is poorly understood.

### Innovations and breakthroughs

This study showed that liver fibrosis protects mice against D-GalN/lipopolysaccharide (LPS) challenge, and M2-like KCs in fibrotic liver may exert a protective effect by inhibiting the translocation of HMGB1.

### Applications

The findings from this study may provide a potential explanation for the mechanism of injury resistance in the setting of liver fibrosis. From a clinical perspective, these findings may pave the way for the treatment of liver failure, particularly acute-on-chronic liver failure.

### Terminology

Macrophages can undergo "classical" M1 activation when exposed to LPS and interferon- $\gamma$  or "alternative" M2 activation when exposed to interleukin (IL)-4/IL-13. M1- and M2-like macrophages exhibit distinct signatures and fulfill different functions. HMGB1 has diverse functions which depend on its cellular localization. In the intracellular compartment, HMGB1 participates in gene transcription, DNA replication, and DNA repair. To function in a damage-associated molecular pattern, HMGB1 can translocate from the nucleus to the cytoplasm and is subsequently released into the extracellular milieu. Extracellular HMGB1 activates pro-inflammatory signaling pathways by ligation of pattern recognition receptors, leading to severe damage in multiple liver diseases.

### Peer-review

This study is well designed, and the results are very interesting. In this study, the authors investigated the mechanism of hepatoprotection conferred by

liver fibrosis, evaluated the phenotype of KCs isolated from fibrotic liver. KCs isolated from the fibrotic mice exhibited predominantly an M2-like phenotype. *In vitro* experiments have shown that HMGB1 was localized in the nucleus of the majority of M2-like KCs and the translocation of HMGB1 was inhibited upon LPS or HMGB1 peptide stimuli, while both LPS and HMGB1 peptide could elicit the conspicuous translocation of intranuclear HMGB1 in KCs isolated from control mice.

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## Basic Study

# Sonographic appearance of anal cushions of hemorrhoids

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**Data sharing statement:** Technical appendix, statistical code, and dataset of this original article are available from the corresponding author at [mamutjan206@sina.com](mailto:mamutjan206@sina.com).

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## Abstract

### AIM

To evaluate the diagnostic value of different sonographic methods in hemorrhoids.

### METHODS

Forty-two healthy volunteers and sixty-two patients with grades I-IV hemorrhoids received two different sonographic examinations from January 2013 to January 2016 at the First and Second Hospitals of Xinjiang Medical University in a prospective way. We analyzed the ultrasonographic findings of these participants and evaluated the outcomes. Resected grades III and IV hemorrhoid tissues were pathologically examined. The concordance of ultrasonographic results with pathology

results was assessed with the Cohen's kappa coefficient.

## RESULTS

All healthy volunteers and all patients had no particular complications related to sonography. There were no statistically significant differences between the participants regarding age ( $P = 0.5919$ ), gender ( $P = 0.4183$ ), and persistent symptoms ( $P > 0.8692$ ). All healthy control participants had no special findings. However, 30 patients with hemorrhoids showed blood signals around the dentate line on ultrasonography. When grades I and II hemorrhoids were analyzed, there were no significant differences between transrectal ultrasound (TRUS), transperianal ultrasound (TPUS), and transvaginal ultrasound (TVUS) ( $P > 0.05$ ). Grades III and IV hemorrhoids revealed blood flow with different directions which could be observed as a "mosaic pattern". In patients with grades III and IV hemorrhoids, the number of patients with "mosaic pattern" as revealed by TRUS, TPUS and TVUS was 22, 12, and 4, respectively. Patients with grades III and IV disease presented with a pathologically abnormal cushion which usually appeared as a "mosaic pattern" in TPUS and an arteriovenous fistula in pathology. Subepithelial vessels of resected grades III and IV hemorrhoid tissues were manifested by obvious structural impairment and retrograde and ruptured changes of internal elastic lamina. Some parts of the Trietz's muscle showed hypertrophy and distortion. Arteriovenous fistulas and venous dilatation were obvious in the anal cushion of hemorrhoidal tissues. After pathological results with arteriovenous fistulas were taken as the standard reference, we evaluated the compatibility between the two methods according to the Cohen's kappa co-efficiency calculation. The compatibility (Cohen's kappa co-efficiency value) between "mosaic pattern" in the TPUS and arteriovenous fistula in pathology was very good ( $\kappa = 0.8939$ ). When compared between different groups, TRUS presented the advantage that the mosaic pattern could be confirmed in more patients, especially for group A. There was a statistical difference when comparing group A with group B or C ( $P < 0.05$  for both). There were obvious statistical differences between group A and group B with regard to the vessel diameter and blood flow velocity measured by TRUS ( $P < 0.05$ ).

## CONCLUSION

Patients with grades III and IV hemorrhoids present with a pathologically abnormal cushion which usually appears as a "mosaic pattern" in sonography, which is in accord with an arteriovenous fistula in pathology. There are clearly different hemorrhoid structures shown by sonography. "Mosaic pattern" may be a parameter for surgical indication of grades III and IV hemorrhoids.

**Key words:** Hemorrhoids; Anal cushion; Transperianal; Transrectal; Transvaginal; Sonography

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**Core tip:** There are still controversial opinions on the etiology of hemorrhoids. As to patients with grades III and IV hemorrhoids, a special signal of blood flow with different directions could be observed, as a mosaic pattern, which was confirmed as arteriovenous fistula in pathology. Mosaic pattern could be a parameter for surgical indication of grades III and IV hemorrhoids. If this abnormal cushion which appears as a "mosaic pattern" in sonography is confirmed, it could help to interpret important etiological aspects of hemorrhoids.

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## INTRODUCTION

The etiology of hemorrhoids remains somewhat controversial, with the main theories purported as either prolapse of anatomical anal cushions<sup>[1]</sup> or areas of abnormal vascular hyperplasia with an intrinsically hyperactive vascular sphincter<sup>[2,3]</sup>. The anal cushions, representing the anal mucosa and submucosa above the dentate line, have been implicated as important structures in hermetic closure of the anal canal, and Gibbons *et al.*<sup>[4]</sup> have shown that internal anal sphincter closure alone is insufficient to maintain anal occlusion at rest. There are several hypotheses, but no definite evidence regarding the etiology or origin of hemorrhoids. Most surgeons confirm the diagnosis and indication of hemorrhoids according to their anal examination result. Ultrasound is an inexpensive, safe technique that can dynamically and noninvasively evaluate the anorectal area using transperianal, transrectal, and transvaginal examinations. In addition, these techniques are helpful for diagnosing rectal cancer, fecal incontinence, anal sphincter abnormalities, sphincter atrophy, and perianal fistula. However, there have been fewer reports concerning hemorrhoid diagnosis or abnormalities using endoanal sonography. Several different techniques of ultrasonography were used for the evaluation of the functional anatomy of the pelvic floor, of which transperianal ultrasound (TPUS), transvaginal ultrasound (TVUS), and transrectal ultrasound (TRUS) are frequently used<sup>[5]</sup>. As a noninvasive method, TPUS was initially used in 1983 as an examination for the anorectal area in newborn babies with imperforate anus; more recently, other studies have reported the importance of this examination for the assessment of the anorectal



area<sup>[6,7]</sup>, and TRUS can clearly show the morphological characteristics of the hemorrhoid vascular network and pathological anal cushion. Therefore, there is clinical importance attached to the selection of treatment options. Endoanal ultrasound (EUS) or TRUS was introduced 25 years ago by urologists to evaluate the prostate<sup>[8]</sup>. Later, TRUS was used to stage rectal tumors<sup>[9]</sup> and to diagnose benign disorders of the anal sphincters and pelvic floor<sup>[10,11]</sup>. Sultan *et al.*<sup>[12]</sup> first reported an abnormal manifestation of female hemorrhoids using TVUS to visualize the anal canal, and it was used to measure the hemorrhoid cushions by Nicholls *et al.*<sup>[13]</sup>. The purpose of this prospective study was to measure the anal cushion area using sonographic examination in a group of patients with hemorrhoids of different grades (I-IV) and to compare the results with a control group of age-matched healthy volunteers. This research also aimed to evaluate the diagnostic value of sonographic examination of the hemorrhoid cushion *via* different sonographic methods to obtain early detection and early intervention. In this study, we analyzed color Doppler ultrasound characteristics of 62 symptomatic patients with hemorrhoids, with the aim to investigate the clinical value of ultrasound and to improve the early diagnostic ability of ultrasound for hemorrhoids.

## MATERIALS AND METHODS

### Study subjects

Forty-two healthy control volunteers and sixty-two patients with grades I-IV hemorrhoids received two different sonographic examinations (TRUS and TPUS for males, and an additional TVUS for female patients) from January 2013 to January 2016 at the First and Second Hospitals of Xinjiang Medical University. The participants ranged in age from 21 to 76 years with a mean age of 53.6 years. We have prospectively performed ultrasonography for all these participants. All included cases for analysis were followed for at least 4 wk. Rectal cancer, hemorrhoid thrombosis, anal fissure, anal fistula, fecal incontinence, ulcerative colitis, Crohn's disease and any bleeding risk condition were excluded. Study participants were divided into three groups. Group A consisted of patients with stages III and IV disease (38 patients, 26 males and 12 females); the mean age was  $42 \pm 2.4$  years, and the duration of symptoms was  $7.8 \pm 2.4$  mo. Group B consisted of patients with stages I and II disease (24 patients, 14 males and 10 females); the mean age was  $42 \pm 2.4$  years, and the duration of symptoms was  $6.6 \pm 3.8$  mo. Group C consisted of normal healthy participants (42 volunteers, 24 males and 18 females), with a mean age of  $42 \pm 2.4$  years. TRUS was considered as the standard modality to determine abnormal findings of hemorrhoids. Resected grades III-IV hemorrhoid

tissues were pathologically examined.

### Diagnostic criteria

Hemorrhoids were diagnosed based on the diagnostic criteria established previously<sup>[14]</sup>. Grade I hemorrhoids are characterized by prominent vasculature with engorgement but no prolapse. Grade II hemorrhoids prolapse only with straining but spontaneously reduce. Grade III hemorrhoids prolapse beyond the dentate line with straining and require manual reduction. Grade IV hemorrhoids prolapse beyond the dentate line with straining but cannot be reduced manually.

### Instruments and ultrasound procedures

Patients received a cleansing enema 1 h before examination. The patients were placed in the left lateral position. Performance of the examination was accomplished using either a linear 12- to 7-MHz transducer or a transvaginal 8- to 4-MHz probe (ATL 3000 or 5000; ATL Ultrasound, Bothell, WA, United States), which was placed against the patient body. TPUS was used to provide a standardized image of the anal canal displaying the mucosa and submucosa, the hypoechoic internal anal sphincter, and the hyperechoic external anal sphincter, and the images provided are comparable with those obtained with an endoluminal probe. The anal cushion area was measured as described by Nicholls *et al.*<sup>[13]</sup>. Generally, the measurement was made in the mid-anal canal where the image of the anal canal is most enduring and reproducible measurements can be made. Before TRUS and TVUS, the rigid probes were covered for hygienic reasons with a condom filled with ultrasound gel. Then, during the endoluminal process, the condom was covered with a gel on the outside and gently introduced into the rectum for a distance of about 3 to 5 cm. Landmarks used are the prostate, vagina, and puborectalis (PR) muscle. Then the probe was slowly withdrawn and entered the anal canal. Pressure of the probe and gravity can lead to different thickness measurements (that is, lying on the left side, left thickness measurements may be smaller). In female patients, the transvaginal probe was placed low in the vagina and angled posteriorly to image the anal canal as described by Stewart and Wilson<sup>[15]</sup>. Then, the ultrasound probe was used to examine the rectal cavity in a clockwise direction. We analyzed the ultrasonographical findings of these participants and evaluated the outcomes. After finding the irregular widening of the hypoechoic area in the second layer, the probe was placed in the five-layer longitudinal muscle of the rectal wall, and we used color Doppler ultrasound to examine the size, shape, distribution, and number of blood vessels, and the vessel shape and vascular hemodynamic index of the hemorrhoid. To achieve maximum sensitivity, Doppler settings were set at low frequency and filter, and the

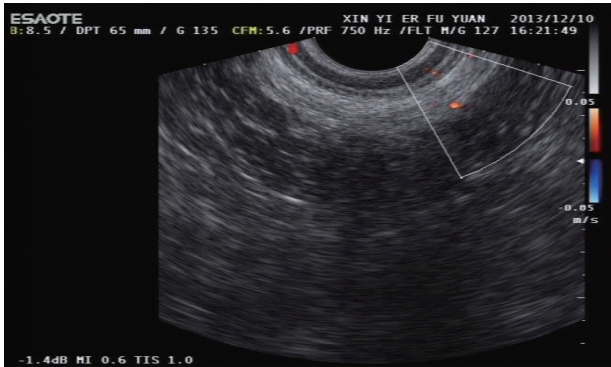


Figure 1 Normal five-layer structure of a healthy participant.

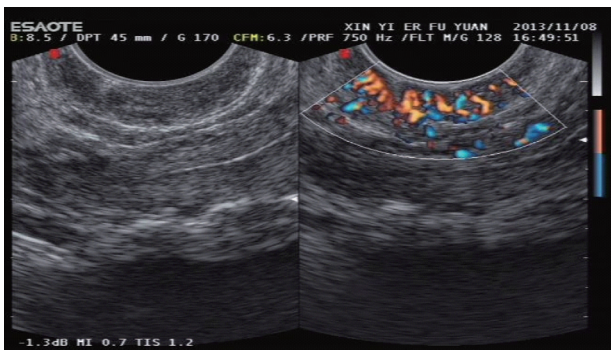


Figure 2 Typical blood flow in patients with stage I or II (left) and stage III or IV (right) hemorrhoids.

artifact in the image was removed. Starting from the highest level (4-6 cm above the anorectal junction) and continuing to the anal verge (1 cm below the line of the internal sphincter muscle), the transanal probe was slowly pulled distally. Doppler window was placed at the haemorrhoidal artery, and the baseline and pulse repetition frequency were changed according to blood flow velocity to obtain continuous blood flow images for measurement of peak systolic velocity and resistance index. Each artery diameter was measured three times, and mean value was calculated. Parameters of power Doppler settings were: frequency, 6.1 MHz; power Doppler gain, 40 dB; dynamic power Doppler setting parameter: range, 20 dB; edge, 1; time smooth, 7; special smooth, 2; color map, 5; filter, 5; pulse repetition frequency, 21.5 kHz; scale, 3.8 m/s. The vessel diameter and blood flow velocity were detected by TRUS in groups A and B.

### Ethic and informed consent

This research was approved by the Ethic Committee of Human Subject Research of the First and Second Affiliated Hospitals of Xinjiang Medical University. All healthy participants and patients signed an informed consent form before this study. There were no commercial conflicts or other problems related to the participants. All sonographic examinations were free to all the participants.

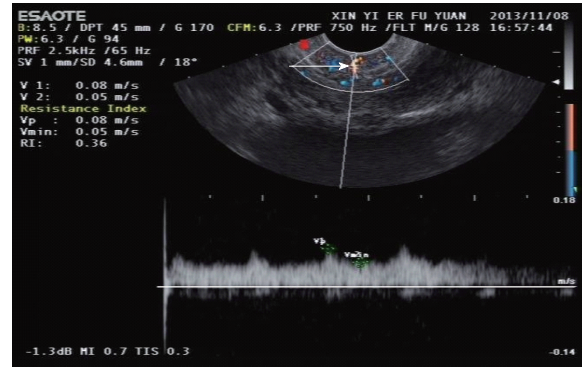


Figure 3 Mosaic pattern in the anal cushion of stages III and IV hemorrhoids. Blood flow with different directions could be observed as a “mosaic pattern” (white arrow). High-speed low-resistance arterial flow spectrum and arterialized venous spectrum could be observed as a bright colored area.

### Statistical analysis

Statistical analyses were performed using SPSS software version 19.0. The intention-to-treat principle was applied in this study. Numerical data are expressed as median and ranges. Student’s *t*-test was used to compare the treatment results, and the chi square ( $\chi^2$ ) test was used for the comparison of proportions. A *P*-value < 0.05 was considered statistically significant. The diagnostic reliability of sonography in determining significant mosaic pattern (more than 70%) was assessed. Pathological results with arteriovenous fistulas were taken as the standard reference. To evaluate the compatibility between the two methods in grading the mosaic pattern, Cohen kappa co-efficiency [ $\kappa$  very good ( $\kappa > 0.8$ ), good ( $\kappa = 0.61-0.8$ ), moderate ( $\kappa = 0.41-0.6$ ), low ( $\kappa = 0.21-0.4$ ), and very low ( $\kappa \leq 0.2$ )] was calculated.

## RESULTS

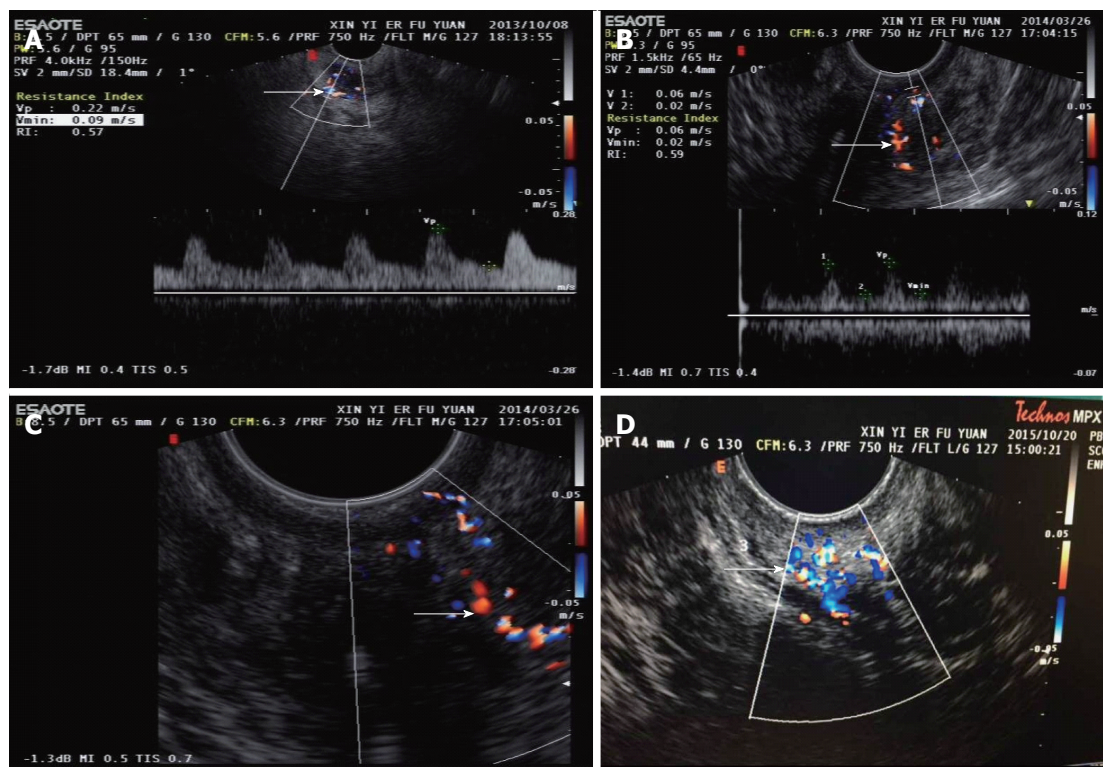
### Comparison of baseline data

All healthy participants and all patients had no particular complications related to sonography. There were no statistical differences between the three groups with regard to age and gender, or between the two patient groups with regard to persistent symptoms (*P* > 0.05). All healthy participants had no special findings on sonography (Figures 1 and 2). The general characteristics of the three groups showed no significant differences (*P* > 0.05) (Table 1).

### “Mosaic pattern” in TPUS

TPUS was considered as the standard modality to determine abnormal findings of hemorrhoids. Grades III and IV hemorrhoids revealed blood flow with different directions which could be observed as a “mosaic pattern” (Figures 3 and 4, Table 2). In patients with grade III or IV hemorrhoids, color Doppler ultrasound showed multidirectional, turbulent flow consistent with an arteriovenous flow pattern, which seemed similar to the mosaic pattern mentioned in other studies of





**Figure 4** Mosaic pattern in the pathological anal cushion. A: “Mosaic pattern” was a special blood flow with different directions (white arrow); B: Special blood flow of “mosaic pattern” (white arrow); C: Special blood flow of “mosaic pattern” instructed with red and blue color (white arrow); D: Special blood flow of “mosaic pattern” instructed with multiple colors (white arrow).

Table 1 General characteristics of the three groups				
	Total No.	Gender, male/female	Age (yr)	Duration of symptoms (mo)
Group A (stages III and IV)	38	26/12	42 ± 2.4	7.8 ± 2.4
Group B (stages I and II)	24	14/10	44 ± 2.5	6.6 ± 3.8
Group C (healthy controls)	42	24/18	41 ± 2.2	-
$\chi^2/t$ -value	1	0.28751	1	
	2	0.65502	2	
	3	0.02713	3	
P value	1	0.59191	1	
	2	0.41832	2	
	3	0.86923	3	

<sup>1</sup>Comparison between group A and group B; <sup>2</sup>Comparison between group A and group C; <sup>3</sup>Comparison between group B and group C.

arteriovenous malformation<sup>[16,17]</sup>. In patients with grade III and IV hemorrhoids, the number of patients with the “mosaic pattern” as revealed by TRUS, TPUS, and TVUS was 22, 12, and 4, respectively. When compared between different groups, TRUS presented the advantage that the “mosaic pattern” could be confirmed in more patients, especially for group A. Patients with grade III and IV disease presented with a pathologically abnormal cushion which usually appeared as a “mosaic pattern” in TPUS and an arteriovenous fistula in pathology. The compatibility (Cohen’s kappa co-efficiency value) between the TPUS and pathology was calculated as 0.8939 (Table 3). The vessel diameter measured by TRUS in group A and group B was 2.6

Table 2 The mosaic pattern according to different methods for the three groups					
	TRUS	TPUS	TVUS in female	Pathology	P value
Group A (stages III and IV)	22/38	12/38	4/12	20/38	0.0379 <sup>4</sup>
Group B (stages I and II)	4/24	2/24	2/10	-	0.2488 <sup>5</sup>
Group C (healthy controls)	0/42	0/42	0/18	-	0.8093 <sup>6</sup>
$\chi^2/t$ -value	8.6449 <sup>1</sup>	3.3142 <sup>1</sup>	0.0477 <sup>1</sup>	-	-
	30.6981 <sup>2</sup>	13.2252 <sup>2</sup>	4.3389 <sup>2</sup>	-	-
	4.8117 <sup>3</sup>	1.3305 <sup>3</sup>	1.4479 <sup>3</sup>	-	-
P value	0.0033 <sup>1</sup>	0.0687 <sup>1</sup>	0.8270 <sup>1</sup>	-	-
	0.0000 <sup>2</sup>	0.0003 <sup>2</sup>	0.0373 <sup>2</sup>	-	-
	0.0283 <sup>3</sup>	0.2487 <sup>3</sup>	0.2289 <sup>3</sup>	-	-

<sup>1</sup>Comparison between group A and group B; <sup>2</sup>Comparison between group A and group C; <sup>3</sup>Comparison between group B and group C; <sup>4</sup>Comparison between TRUS and TPUS; <sup>5</sup>Comparison between TRUS and TVUS; <sup>6</sup>Comparison between TPUS and TVUS. TRUS: Transrectal ultrasound; TPUS: Transperianal ultrasound; TVUS: Transvaginal ultrasound.

± 0.4 and 0.6 ± 0.4, respectively. Blood flow velocity measured by TRUS in group A and group B was 56.4 ± 4.3 and 20.8 ± 3.4, respectively (Table 4). There were obvious statistical differences between group A and group B with regard to the vessel diameter and blood flow velocity measured by TRUS ( $P < 0.05$ ).

**Table 3** Cohen's kappa co-efficiency between mosaic pattern in transrectal ultrasound and arteriovenous fistula in pathology in stages III and IV hemorrhoids

Pathology	Mosaic pattern in transrectal US		Total
	Positive	Negative	
Positive	20	0	20
Negative	2	16	18
Total	22	16	38

The compatibility (Cohen's kappa co-efficiency value) between the transperineal US and pathology was calculated as 0.8939. The distribution of the Cohen kappa ( $\kappa$ ) co-efficiency values: very high ( $\kappa > 0.8$ ), high ( $\kappa = 0.61-0.8$ ), moderate ( $\kappa = 0.41-0.6$ ), low ( $\kappa = 0.21-0.4$ ), very low ( $\kappa \leq 0.2$ ).

**Table 4** Vessel diameter and blood flow velocity detected by transrectal ultrasound

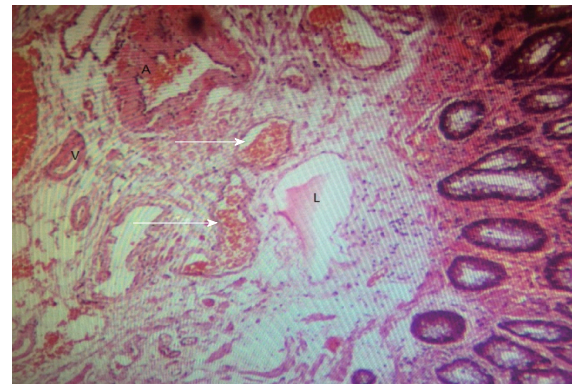
	<i>n</i>	Vessel diameter (mm)	Blood flow velocity (cm/s)
Group A (stages III and IV)	22	2.6 ± 0.4	56.4 ± 4.3
Group B (stages I and II)	20	0.6 ± 0.4	20.8 ± 3.4
$\chi^2/t$ -value		16.1835	29.5566
<i>P</i> value		0	0

#### "Arteriovenous fistulas" in pathology

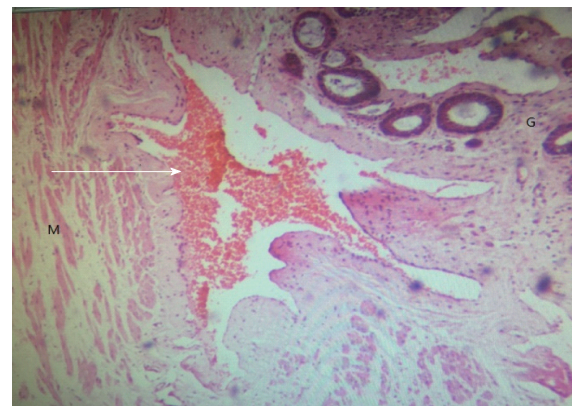
Subepithelial vessels of resected grades III-IV hemorrhoid tissues were manifested by obvious structural impairment and retrograde and ruptured changes of internal elastic lamina. Some parts of the Trietz's muscle showed hypertrophy and distortion. Arteriovenous fistulas and venous dilatation were obvious in the anal cushion of hemorrhoidal tissues (Figures 5-7). After pathological results with arteriovenous fistulas were taken as the standard reference, we evaluated the compatibility between the two methods according to the Cohen's kappa co-efficiency calculation. The compatibility (Cohen's kappa co-efficiency value) between the "mosaic pattern" in TPUS and arteriovenous fistula in pathology was very good (Table 3).

#### Comparison of findings of the three different sonographic modalities

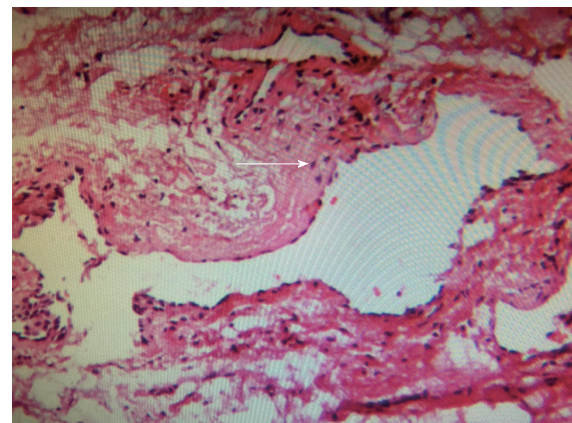
There was no special findings in sonography of healthy participants (Figure 1). A pilot series of experiments enabled the sonographers to attain technical expertise in all methods before commencement of the study. Ultrasonographic measurements using a 2-sonographer protocol were obtained for all 104 study patients; the examinations were blinded and paired and used transperineal and transrectal techniques combined with transvaginal technique in female patients. No patients were excluded from the analysis. We calculated the number of patients in which the "mosaic pattern" could be observed in each group, and we analyzed the outcomes between the different groups of patients, and between different techniques (Table 2). For patients with stages III and IV hemorrhoids, TRUS seemed to be the most effective technique.



**Figure 5** Subepithelial vessels of resected grades III and IV hemorrhoid tissues were manifested by obvious structural impairment and retrograde and ruptured changes of internal elastic lamina. Arteriovenous fistulas and venous dilatation were obvious in the anal cushion of hemorrhoidal tissues (white arrow, magnification × 80); A: Artery, V: Vein, L: Lymphatic duct.



**Figure 6** Blood cells in the arteriovenous fistula of anal cushion were seen (white arrow, magnification × 80). M: Muscle; G: Glands.



**Figure 7** Arteriovenous fistula of anal cushion was obvious (white arrow, magnification × 400). Some parts of the Trietz's muscle showed hypertrophy and distortion.

When compared with TPUS, there was a significant difference ( $P < 0.05$ ). But between TRUS and TVUS, and between TPUS and TVUS, there was no significant difference ( $P > 0.05$  for both). When stages I and II hemorrhoids were examined, there were no significant



differences between TRUS, TPUS, and TVUS ( $P > 0.05$ ). When compared between different groups, the technique of TRUS presented advantages; the "mosaic pattern" could be confirmed in more patients, especially in group A, and there was a statistical difference when comparing group A with group B or C ( $P < 0.05$  for both). A statistical difference using TPUS was also observed between group A and group B or C, but group A and B had no statistical difference between the results of the TVUS examination. In patients with hemorrhoids, the submucosa may be thickened and show a more echolucent appearance, suggesting the presence of fluid (*i.e.*, blood). The amount of fluid was correlated with the degree of severity of hemorrhoids. Treatment of hemorrhoids with rubber band ligation or infrared coagulation did not alter anal configuration. The mucosal hemorrhoidal plexus was better visualized by vaginal endosonography. Thirty patients with hemorrhoids had blood signals around the dentate line on ultrasonography, and on the same scanning plane, blood flow with different directions could be observed as a mosaic pattern in grades III and IV hemorrhoids. There were underlying characteristics of the sonographic changes of hemorrhoids (Figures 1-4). Patients with grade III and IV hemorrhoids presented with a pathologically abnormal cushion which usually appeared as a "mosaic pattern" in sonography and an arteriovenous fistula in pathology (Figures 1-6). Two-dimensional ultrasonography revealed point sheet hypoechoic areas and intertwined flowing fine punctate anechoic areas; these were observed inside the inner layer of the thickened internal sphincter, which resembled a mixed cystic and solid honeycomb echo area. Color Doppler ultrasonography indicated that the anal cushion area of hemorrhoids contained blood signals. On the same scanning plane, blood flow with different directions could be observed as a "mosaic pattern". High-speed low-resistance arterial flow spectrum and arterialized venous spectrum could be observed as a bright colored area (Figures 3 and 4).

## DISCUSSION

Hemorrhoids are a commonly occurring human disease, and the causes are not well known to date. There are still controversial opinions on the etiology of hemorrhoids. There are several hypotheses, but no definite evidence regarding the etiology or origin of hemorrhoids. Ultrasonographic examination is an accepted technique for local staging of both benign anorectal disease and disorders, and malignant anal and perianal neoplasms<sup>[18,19]</sup>. Endoanal ultrasound (EAUS) of the anal sphincters is achieved by the simple expedient of replacing the balloon system used for rectal scanning with a hard cone<sup>[20]</sup>. The normal rectal wall is 2 to 3 mm thick and is composed of a 5-layer longitudinal muscle, joint longitudinal muscle and external sphincter, as is the rest of the digestive tract<sup>[21]</sup>. The anal canal is 2 to 4 cm long and is closed

in the normal situation. Therefore, excellent images can be obtained by TRUS, as the anus lies tight around the probe. It is very important that the rectum is empty and clear prior to examination because residual stool can cause a deterioration of the image quality and impede interpretation<sup>[22]</sup>. The external anal sphincter is a voluntary muscle arising from the levator ani and puborectalis muscle to form a circular structure around the anal canal. The anatomy of the external anal sphincter remains controversial and is usually described as having three parts: a deep part joining with the puborectalis muscle, a superficial part attached to the superficial transverse perineal muscle, and a subcutaneous part continuing below the internal anal sphincter. The length of the anal canal, corresponding to the posterior longitudinal length of the external anal sphincter, was substantially greater in men than in women (3.5 cm vs 3.05 cm, on average), consistent with numerous previous reports<sup>[23,24]</sup>. The anatomical gap produced by this asymmetrical configuration would explain, on the basis of a lower resistance of the anterior rectal wall, the greater incidence of functional female pelvic floor disorders<sup>[5,25]</sup>. This anatomical description cannot always be observed using TRUS<sup>[26-28]</sup>. Vaginal endosonography, used to visualize the perianal area and especially the perineum, is an alternative when rectal endosonography is not possible because the anus is asymmetrical, causing air artifacts, extreme anal stenosis, or pain<sup>[29]</sup>. TPUS is also used to image the perianal area in patients with perianal fistula<sup>[30,31]</sup> or imperforate anus<sup>[31]</sup>. However, results using EUS seem somewhat better<sup>[32,33]</sup>.

The highlight of this study was a new finding of a "mosaic pattern" in sonography which may be concordant with an arteriovenous fistula in pathology. Patients with grades III and IV hemorrhoids presented with a pathologically abnormal cushion which usually appeared as a "mosaic pattern" in sonography and an arteriovenous fistula in pathology. "Mosaic pattern" was a special blood flow with different directions. This special pattern was firstly reported by Aslan H<sup>[16]</sup>, and described as "clover-leafed shape" in a pilot study reported by Zbar *et al*<sup>[34]</sup>. Color Doppler ultrasonography also indicated that "mosaic pattern" in the anal cushion area of hemorrhoids contained special blood signals. On the same scanning plane, blood flow with different directions can be observed as a "mosaic pattern". High-speed low-resistance arterial flow spectrum and arterialized venous spectrum can be observed as a bright colored area. This result is encouraging. This new finding of a pathologically abnormal cushion, observed as "mosaic pattern" in sonography and an arteriovenous fistula in pathology, was confirmed in our study. After pathological results with arteriovenous fistulas were taken as the standard reference, we evaluated the compatibility between the two methods according to the Cohen's kappa co-efficiency calculation. The compatibility (Cohen's kappa co-efficiency value) between "mosaic pattern" in the

transperineal US and arteriovenous fistula in pathology was very good ( $\text{Kappa} = 0.8939$ ). It could help to interpret important etiological aspects of hemorrhoids, and could influence traditional surgical methods. The procedure of prolapse to hemorrhoids (PPH) or tissue selecting therapy (TST) for hemorrhoid treatment could be replaced by sonographic vascular therapy or other related techniques which are safer and less expensive.

TPUS is a simple, accessible, inexpensive, safe, and painless technique that dynamically and noninvasively evaluates anorectal structures. A previous study showed that two-dimensional TPUS had a specificity and sensitivity of 85% and 64%, respectively<sup>[35]</sup>. In addition, TRUS has been used for almost every possible disease in the anal region, and by delineating the anatomy it has increased insight into anal pathology. Clinical indications for TRUS in benign anorectal diseases are fecal incontinence for the detection of defects and atrophy, perianal fistulas, and abscesses for the demonstration of fistula tracts. Thekkinkattil *et al.*<sup>[36]</sup> reported differences of the anal cushion area for some anorectal diseases using TVUS. These types of anal cushion differences were also confirmed in this study. We suggest that abnormalities and pathological changes of the anal cushion are one of the important reasons for symptomatic hemorrhoids.

Ultrasound can provide high-resolution images that can show hemodynamic information, and allow one to observe morphological and hemodynamic changes through a unique perspective. Therefore, ultrasound can play an important role in the diagnosis and differential diagnosis of hemorrhoids. The researcher can observe the rectal cavernous region and blood supply to the hemorrhoid through color endosonography, perineal sonography, and even transvaginal sonography. The vascular plexus full of blood flow can also be observed<sup>[37]</sup>. The reduction of the hemorrhoid blood supply could reduce blood reflux burden, symptoms of bleeding, and swelling. In recent years, hemorrhoid artery ligation has already been used as a minimally invasive surgery<sup>[38]</sup>. Color biplane endosonographic imaging studies have shown that the vast majority of hemorrhoidal arteries are located within the anorectal junction at 2 cm above the rectal mucosa, and this is regarded as the best area for hemorrhoid artery ligation<sup>[39]</sup>. Ten years before, Aigner *et al.*<sup>[40]</sup> concluded that increased caliber and arterial blood flow of the terminal branches of the superior rectal artery are correlated with the appearance of hemorrhoids. They suggested that the hypervascularization of the anorectum contributes to the growth of hemorrhoids rather than being a consequence of hemorrhoids. Their observations confirmed that morphological changes are clearly detectable with the use of transperineal color Doppler ultrasound in patients with symptomatic hemorrhoids. Aigner F believed that transperineal color Doppler ultrasound is an appropriate method to assess

these findings in patients with hemorrhoids. Four years before, Miyamoto *et al.*<sup>[41]</sup> reported encouraging results using power Doppler imaging transanal ultrasound and three-dimensional power doppler angiography to visualize the haemorrhoidal plexus and the course of the haemorrhoidal artery *in vivo*. They found that blood flow significantly increased following advancement of the grade of haemorrhoid, and they also concluded that the distribution of haemorrhoidal arteries varies widely in both the number and the position. In their research, they demonstrated that the median number of haemorrhoidal arteries was five (range, 3-9) and that they were found in various positions, being located, in 62.1% of patients, at 1 o'clock but more commonly at the 3, 7 and 11 o'clock positions. Around the hemorrhoid area, using color biplane endosonographic imaging, we found this new abnormal structure characterized as a "mosaic pattern" inside the abnormal anal cushions, and we found an arteriovenous vascular plexus or fistula using pathology. We also observed a specific sonographic appearance characterized as arterialized venous blood flow of the arteriovenous fistula under pulsed sonography. The hemorrhoid in the same patient may have a single or several vessels in certain anal points or may have specific characteristics of an arteriovenous fistula at other points. Hemorrhoid vessels may directly penetrate the submucosa in other patients. It is unclear if these characteristics define the severity of the hemorrhoid, and further research is needed to confirm this. The discovery of an arteriovenous fistula in pathological anal cushions provides important diagnostic, therapeutic, and etiologic information for hemorrhoids. We strongly believe that the "mosaic pattern" inside the abnormal anal cushion on endosonography was most likely to fit with a pathological diagnosis of an arteriovenous fistula. However, we need further investigation and research to clarify these changes.

In our study, arteriovenous fistulas and venous dilatation were very obvious in the anal cushion of hemorrhoidal tissues. After pathological results with arteriovenous fistulas were taken as the standard reference, we evaluated the compatibility between the two methods according to the Cohen's kappa co-efficiency calculation. The compatibility (Cohen's kappa co-efficiency value) between "mosaic pattern" in the transperineal US and arteriovenous fistula in pathology was very good. We believe that correctly identifying the hemorrhoidal artery and arteriovenous vascular plexus and avoiding blind treatment at the 3, 7, and 11 o'clock positions has become an urgent problem when dealing with bleeding anal diseases. The aim of this study was to provide a highly sensitive and accurate method for positioning vessels in the hemorrhoid area. We believed that not all the grades III and IV hemorrhoids should receive surgery. We considered that mosaic pattern should be a key parameter in determining surgical operation. In addition, we hope

to guide clinicians in their preoperative assessment by selecting minimally invasive, safe and effective treatment strategies and decreasing surgical complications and the recurrence rate. At the same time, we aimed to provide a broader range of ideas for reasonable treatment, the innovation of new surgical techniques, and drug development which targets hemorrhoids. However, ultrasonography still has its limitations, although TVUS of the anorectum is well tolerated and can accurately detect abnormalities of the anal sphincter and surrounding structures<sup>[42]</sup>. Limitations for the application of EAUS include strictures and acute painful conditions. These caveats are largely caused by the size of regular rectal probes which have an average diameter of 17 to 20 mm. The introduction of EAUS probes with a diameter below 1 cm (actually 7 mm) represents a major improvement, particularly for imaging all layers of the anal canal even under acute pain<sup>[43]</sup>. The second pitfall of EAUS, also caused by the size of the probes, relates to changes in the anatomy of the anal canal resulting from the stretching of the anal canal and compression of the mucosal tissues. Thus, the EAUS picture of the anal canal does not reflect the anatomical situation, which makes it difficult to judge the status of the anoderm and to locate the hemorrhoidal tissue. The use of a small endoanal probe of only 7 mm in diameter enabled us to overcome these limitations. The limitation of EAUS vs endoanal magnetic resonance imaging, however, is the poor inherent contrast on images, which makes characterization of the external anal sphincter difficult, but it could be overcome by using a higher frequency transducer (10 MHz)<sup>[44]</sup>.

We can conclude from this prospective study that there were clearly different structures of hemorrhoids observed by sonography compared with sonography of the control group. Findings of special mosaic pattern in sonography greatly influence clinical decisions in the treatment of hemorrhoids. Sonography may be useful for the early detection and early intervention of hemorrhoids. Mosaic pattern can play a key role in determining the best hemorrhoids management. Mosaic pattern could be a parameter for surgical indication of stages III and IV hemorrhoids. If our new finding of a pathologically abnormal cushion, which appeared as a "mosaic pattern" in sonography and an arteriovenous fistula in pathology, is confirmed in the future, it could help to interpret important etiological aspects of hemorrhoids and would influence traditional surgical methods. The PPH or TST for hemorrhoids could be replaced by sonographic vascular therapy or other related techniques which are safer and less expensive.

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## COMMENTS

### Background

There are still controversial opinions on the etiology of hemorrhoids. Most surgeons confirm the diagnosis and indication of hemorrhoids according to their anal examination result. Ultrasound is an inexpensive, safe technique that can dynamically and noninvasively evaluate the anorectal area, using transperianal, transrectal, and transvaginal examinations. However, there have been few reports concerning hemorrhoid diagnosis or abnormalities using endoanal sonography. There are clearly different hemorrhoid structures shown by sonography in patients.

### Research frontiers

The main purpose of this prospective study was to measure the anal cushion area using sonographic examinations in a group of patients with hemorrhoids of different grades (I-IV) and to compare them with a control group of age-matched healthy volunteers. This research also aimed to evaluate the diagnostic value of different sonographic methods in hemorrhoids.

### Innovations and breakthroughs

The authors found the special structure in hemorrhoid patients by ultrasound and it was confirmed by pathology. Patients with grade III and IV disease presented with a pathologically abnormal cushion which usually appeared as a mosaic pattern in transperianal US and an arteriovenous fistula in pathology.

### Applications

Mosaic pattern could be a parameter for surgical indication of stages III and IV hemorrhoids. Mosaic pattern can play a key role in determining the best hemorrhoid management. If our new finding of a pathologically abnormal cushion, which appeared as a "mosaic pattern" in sonography and an arteriovenous fistula in pathology, is confirmed in the future, it could help to interpret important etiological aspects of hemorrhoids and would influence traditional surgical methods.

### Terminology

"Mosaic pattern" is a special blood flow with different directions. Patients with grades III and IV hemorrhoids present with a pathologically abnormal cushion which usually appears as a "mosaic pattern" in sonography and an arteriovenous fistula in pathology.

### Peer-review

It is a very interesting paper. These data could be very useful for the diagnosis and choice of treatment for hemorrhoids.

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## Basic Study

# Effect of NDC80 in human hepatocellular carcinoma

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**Informed consent statement:** All tissue samples were taken from the patients after informed consent.

**Conflict-of-interest statement:** All authors declare that they have no competing interests related to this study.

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## Abstract

### AIM

To investigate the role of nuclear division cycle (NDC)80 in human hepatocellular carcinogenesis.

### METHODS

*NDC80* gene expression was analyzed by real-time reverse transcription polymerase chain reaction in 47 paired hepatocellular carcinoma (HCC) and adjacent tissues. The HCC cell line SMMC-7721 was transfected with lentivirus to silence endogenous *NDC80* gene expression, which was confirmed by real-time polymerase chain reaction and western blotting. The effects of *NDC80* silencing on SMMC-7721 cell proliferation were evaluated by Cellomics ArrayScan VTI imaging. Cell cycle analysis and apoptosis were detected with flow cytometry. Colony formation was assessed by fluorescence microscopy.

### RESULTS

*NDC80* expression levels in HCC tissues were significantly higher than those in the adjacent tissues. Functional studies demonstrated that *NDC80* silencing significantly reduced SMMC-7721 cell proliferation and colony formation. Knockdown of *NDC80* resulted in increased apoptosis and cell cycle arrest at S-phase. *NDC80* contributed to HCC progression by reducing apoptosis and overcoming cell cycle arrest.

## CONCLUSION

Elevated expression of NDC80 may play a role in promoting the development of HCC.

**Key words:** NDC80; Cell proliferation; Apoptosis; Cell cycle; Hepatocellular carcinoma

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**Core tip:** Nuclear division cycle (NDC)80 is a member of the NDC80 kinetochore complex and is highly expressed in cancer. NDC80 is a newly identified gene that is overexpressed in hepatocellular carcinoma (HCC). We analyzed the biological function of NDC80 in the proliferation and apoptosis of HCC cells, and provided new reference data and experimental support for HCC-targeting gene therapy.

Ju LL, Chen L, Li JH, Wang YF, Lu RJ, Bian ZL, Shao JG. Effect of NDC80 in human hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(20): 3675-3683 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3675.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3675>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and the third most frequent cause of cancer mortality, following lung and stomach cancers<sup>[1,2]</sup>. Especially in China, which has a high incidence of hepatitis B, the magnitude of the problem should never be underestimated<sup>[3]</sup>. HCC has received considerable attention in recent years because of its rapidly increasing incidence<sup>[4,5]</sup>. Patients diagnosed with HCC have a poor prognosis because of the aggressive features of the disease. Only 10%-15% of HCC patients are suitable candidates for curative treatments, including surgical resection and liver transplantation. Surgical resection, ablation therapy, and liver transplantation are effective but only at an early stage of HCC development<sup>[5-8]</sup>. However, HCC is diagnosed at advanced stages in most patients when only limited therapeutic options are available. High metastasis and recurrence rates have become the major obstacles to improving long-term survival of HCC patients<sup>[8]</sup>. The molecular mechanisms of HCC development are not fully understood; thus, it is of importance to elucidate further the mechanisms of HCC and explore effective treatment. Gene therapy has emerged as a promising intervention against HCC. In this study, we investigated the role of a gene that has been previously associated with human HCC cells - nuclear division cycle (NDC)80 - in order to ascertain better its role in human hepatocellular carcinogenesis.

NDC80 (also called Hec1), a core component of the outer kinetochore and a mitotic regulator, is of

particular interest because it clearly has an association with cancer progression. NDC80 forms a dumbbell-like heterotetramer with Nuf2, Spc24, and Spc25 to form the NDC80 complex<sup>[9-12]</sup>. NDC80 complexes are important to the spindle assembly checkpoint and participate in the regulation of mitosis<sup>[13-15]</sup>. NDC80 plays essential roles in chromosome segregation by mediating the spindle assembly checkpoint signaling and chromosome alignment. The spindle assembly checkpoint is a mechanism that ensures faithful chromosome segregation by monitoring the kinetochore-microtubule attachment<sup>[13,16]</sup>. Chromosome segregation dysfunction is one of the causes of chromosome instability. Chromosome instability is a common feature of tumor cells, and may be an important mechanism in tumor formation. In this study, we analyzed the biological function of NDC80 in the proliferation and apoptosis of HCC cells, and provided new reference data and experimental support for HCC-targeting gene therapy.

## MATERIALS AND METHODS

### Cell lines and clinical samples

We used HepG2, Huh-7, SMMC-7721 and Hep3B cell lines, which are commonly used in HCC research, purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum at 37 °C with 50 mL/L CO<sub>2</sub>. Forty-seven pairs of HCC and matched adjacent tissues were provided by Nantong Third People's Hospital Affiliated to Nantong University. All patients had primary HCC and had not received any preoperative radiotherapy or chemotherapy. The diagnosis of all HCC patients was confirmed histopathologically. The pathological stage of HCC was determined according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) Classification. The characteristics of the participants are summarized in Table 1. All tissues samples were frozen immediately after resection and stored in liquid nitrogen until use.

### Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from tissue samples and HCC cell lines using TRIzol (Takara Bio, Dalian, China). RNA purity and concentration were determined by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). RNA was reversely transcribed using a Prime Script™ RT Reagent Kit (Takara Bio). The reactions were performed in a Bio-Rad Real-Time polymerase chain reaction (PCR) Detection System using a SYBR® Green Master Mix (Vazyme Biotech, Nanjing, China). The primers used were as follows: NDC80: forward, 5'-CCTCTCCATGCAGGAGTTAAGA-3',

**Table 1 Clinical characteristics of hepatocellular carcinoma and paired adjacent tissues *n* (%)**

Clinical variable	No. of patients
No. of patients	47
Age, yr, mean $\pm$ SD	56 $\pm$ 11
Sex	
Female	18 (38.3)
Male	29 (61.70)
Serum AFP, ng/mL	
$\leq$ 400	36 (76.60)
> 400	11 (23.40)
HBV infection	
Positive	38 (80.9)
Negative	3 (19.1)
Largest tumor diameter, cm	5.7 $\pm$ 3.4
TNM stage	
I	2 (4.25)
II	30 (63.83)
III	15 (31.92)
Lymph node metastatic	
Positive	20 (42.55)
Negative	27 (47.45)

reverse, 5'-GGTCTCGGGTCCTTGAT TTTCT-3'; GAPDH: forward, 5'-TGACTTCAACAGCGACACCCA-3', reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. After a pre-denaturation step at 95 °C for 5 min, 40 cycles of PCR were performed as follows: 10 s denaturation at 95 °C and 30 s annealing at 60 °C. The fold amplification for each gene was calculated using the  $2^{-\Delta\Delta C_t}$  method.

#### Lentiviral transfection of SMMC-7721 cells

The SMMC-7721 cell suspension was seeded onto six-well plates at  $5 \times 10^4$  cells/well and incubated at 37 °C in 50 mL/L CO<sub>2</sub> until 30% confluence was reached. Two experimental groups were constructed: NDC80-siRNA, which was transfected with NDC80-siRNA green fluorescent protein (GFP) lentivirus; and control group, which was transfected with empty GFP lentivirus. An appropriate amount of lentivirus was added according to the multiplicity of infection (MOI). The cells were repeatedly cultured in normal culture medium after 12 h. GFP-tagged gene expression was observed under a fluorescence microscope at 3 d after transfection, and cells with a transfection efficiency > 80% were selected for subsequent analyses.

#### Western blotting

The cultured cells were lysed in RIPA lysis buffer containing phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology, Shanghai, China), and the protein concentrations were determined. Protein extracts were separated on 10% SDS-PAGE and then electrophoretically transferred to PVDF membranes at 100 V for 90 min. The membranes were incubated with antibody against NDC80 (1:1000; Abcam, Cambridge, MA, United States). The target proteins were examined using an electrochemiluminescence system (Thermo Fisher Scientific) and visualized with

X-ray films. GAPDH was used as the control.

#### Cell counts

NDC80-siRNA and control cells were removed using 0.25% trypsin-EDTA and resuspended in standard medium after achieving logarithmic growth. Cells were seeded in five wells at 1000 cells/well, followed by further incubation at 37 °C and 50 mL/L CO<sub>2</sub>. A Cellomics ArrayScan VT1 (Thermo Fisher Scientific) was used to continuously measure GFP expression in each well over a 5-day period. In this study, statistical data were mapped and cell proliferation curves were drawn.

#### Colony-formation assay

NDC80-siRNA and control cells were digested in 0.25% trypsin to reconstitute the single cell suspension at  $5 \times 10^4$  cells/mL. A hemocytometer was used to assess the cell count, and cell suspensions were transferred into six-well plates at 800 cells/well. After approximately 2 wk incubation, colonies containing > 50 cells were scored as surviving colonies. Colonies were visualized under a fluorescence microscope (MicroPublisher 3.3RTV; Olympus, Tokyo, Japan). The supernatants were discarded, and cells were washed with phosphate-buffered saline (PBS) and fixed with paraformaldehyde (Sangon, Shanghai, China) for 30 min. All wells were washed with PBS and stained with 500  $\mu$ L Giemsa solution (ECM550; Chemicon, Temecula, CA, United States) for 20 min. The cells were washed several times with deionized distilled water and allowed to air dry at room temperature. Colonies were counted and images were captured by a digital camera under light microscopy. The assay was repeated three times.

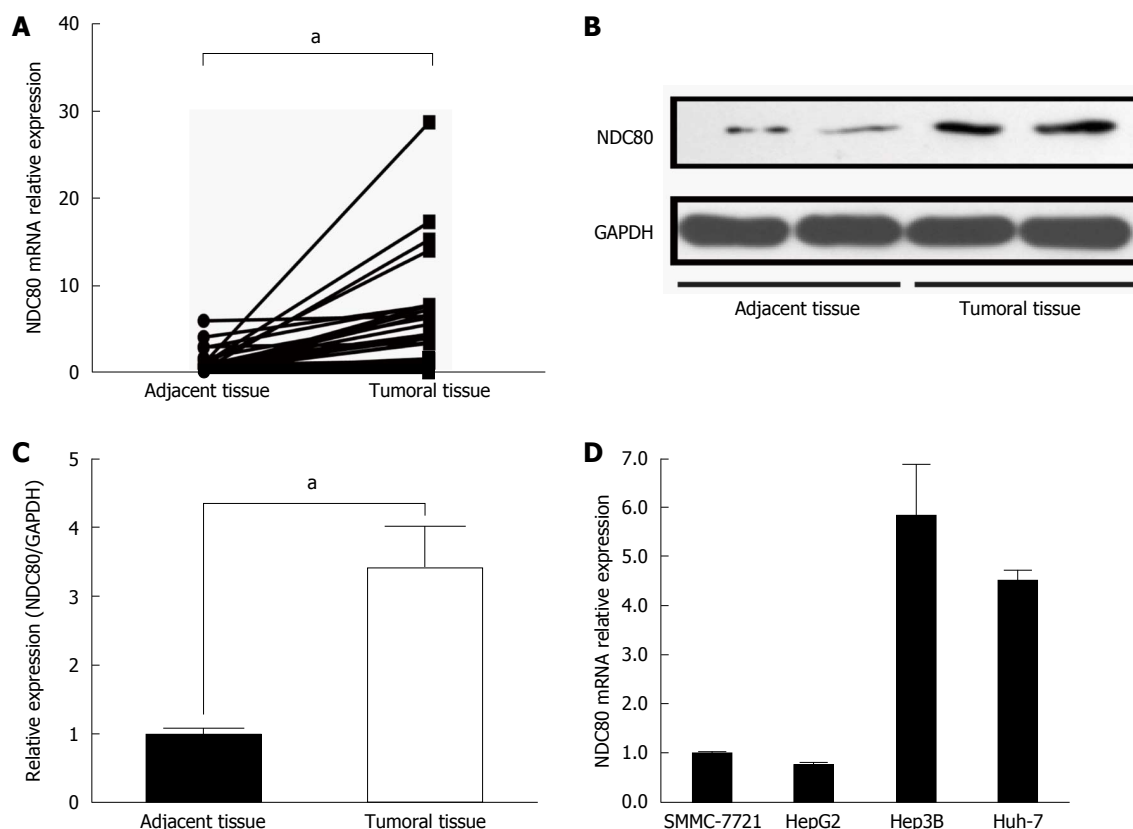
#### Cell cycle analysis

SMMC-7721 cells were plated in six-well plates under standard culture conditions. After treatment, cells were washed with PBS, fixed in 70% ice-cold ethanol and stored at -20 °C overnight. After being washed with PBS, the cells were incubated in 500  $\mu$ L sample buffer containing 50  $\mu$ g/mL propidium iodide (PI) and 0.25 mg/mL RNase A for 30 min at room temperature. Analysis of the apoptotic cells was performed by flow cytometry. The cells with sub-G1 DNA content were considered as apoptotic cells.

#### Apoptosis analysis

The cells were harvested with 0.25% trypsin and washed once with ice-cold PBS. After the cells were washed, the Annexin V-APC Apoptosis Detection Kit and PI (eBioscience, San Diego, CA, United States) were applied to assess apoptosis. Cells were centrifuged and resuspended in 500  $\mu$ L binding buffer  $10^6$  cells/mL. And, 100  $\mu$ L of the cell suspension was incubated with 10  $\mu$ L PI and 5  $\mu$ L Annexin V-FITC in a dark environment for 15 min at room temperature.





**Figure 1 Reverse transcription polymerase chain reaction results for NDC80 mRNA expression.** A: Expression levels of NDC80 mRNA in HCC ( $n = 47$ ) and paired adjacent tissue samples ( $n = 47$ ); B: NDC80 protein expression in HCC and paired adjacent tissue samples was determined by western blotting; C: Gray value analysis of western blot experiments, and data was normalized against GAPDH; D: NDC80 mRNA expression varied among SMMC-7721, HepG2, Hep3B and Huh-7 cell lines. GAPDH was used as an internal control. Statistical significance was assessed by paired  $t$  tests. Error bar indicates SD ( $^{\#}P < 0.001$  vs control).

Analysis of the apoptotic cells was performed by flow cytometry.

### Ethical considerations

All tissues samples from the patients were taken after informed consent. The study was reviewed and approved by the Nantong Third People's Hospital Affiliated to Nantong University Institutional Review Board.

### Statistical analysis

GraphPad Prism version 6.0 was used for data analysis. Statistical significance was defined as  $P < 0.05$ . All data were presented as the mean  $\pm$  SD. All the experiments were repeated at least three times.

## RESULTS

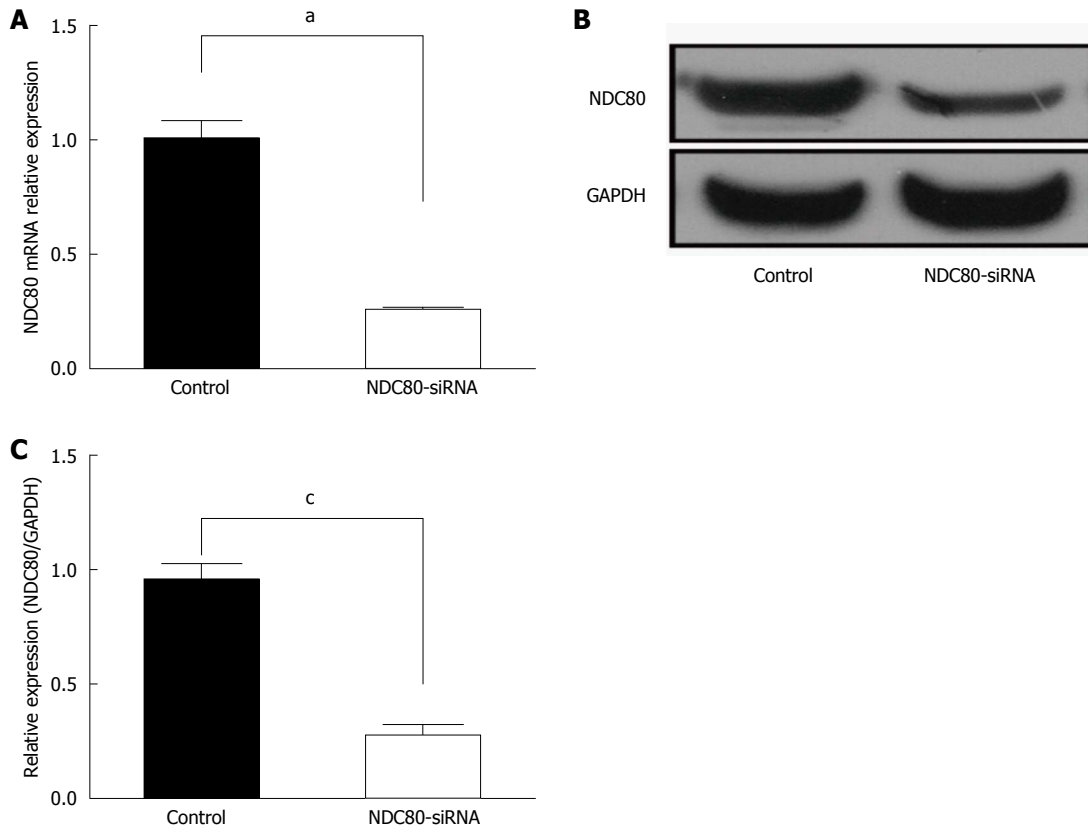
### NDC80 was overexpressed in HCC tissues and cell lines

To investigate whether NDC80 expression was altered in HCC tissues, we detected its expression level by qRT-PCR in 47 paired tumor and adjacent tissues. NDC80 mRNA expression levels in the tumor tissues were drastically increased compared with those in the adjacent tissues (Figure 1A). A similar trend in NDC80 protein levels was observed by western blot analysis (Figure 1B and C). To reveal the potential role of

NDC80 in HCC, we also examined NDC80 expression in four HCC cell lines: SMMC-7721, HepG2, Hep3B and Huh-7 (Figure 1D). The cell line must be lentivirus-friendly ( $\text{MOI} < 10$ ) and enjoy a vigorous proliferation. Hence, the SMMC-7721 cell line was selected for future investigation. The NDC80 complex is comprised of NDC80, Nuf2, Spc24 and Spc25, which together form a dumbbell-like heterotetramer. We then detected the expression levels of Nuf2, Spc24 and Spc25 mRNA by qRT-PCR. The expressions of Nuf2 and Spc24 were significantly enhanced in HCC tissues compared with paired adjacent tissues (Supplementary Figure 1A and B). However, the expression of Spc25 mRNA was not changed between HCC tissues and adjacent tissues (Supplementary Figure 1C).

### NDC80 silencing inhibited SMMC-7721 cell proliferation

After NDC80-siRNA lentiviral transfection, RT-PCR analysis showed that NDC80-siRNA diminished the expression of the endogenous NDC80 mRNA by up to 80% ( $P = 0.0001$ ) (Figure 2A). Correspondingly, the protein expression of NDC80 in NDC80-siRNA-treated cells was also suppressed ( $P = 0.0023$ ) (Figure 2B and C). After transfection, cell proliferation was significantly inhibited in NDC80-siRNA-silenced cells relative to control cells, as shown by GFP-based Cellomics



**Figure 2** Interference efficiency 72 h after transfection. A: After lentiviral transfection, relative NDC80 mRNA expression was significantly inhibited in the SMMC-7721 NDC80-siRNA silenced cells as compared to SMMC-7721 negative control cells by RT-PCR; B: Western blotting of NDC80-depletion efficiency in SMMC-7721 cells; C: Gray value analysis of western blotting, and data were normalized against GAPDH. GAPDH was used as an internal control. Statistical significance was assessed by two-tailed Student's *t* test. Error bar indicates SD (\**P* < 0.01 vs control; \*\**P* < 0.001 vs control).

ArrayScan VTI imaging (Figure 3A). Cell numbers were monitored for 5 consecutive days. The number of cells and the fold-change in proliferation were markedly reduced in the NDC80-siRNA-silenced cells (Figure 3B). Accordingly, the results suggested that the silencing of NDC80 was associated with cell proliferation.

#### **NDC80 silencing reduced SMMC-7721 cell colony formation**

Silencing of NDC80 reduced the anchorage-independent growth of SMMC-7721 cells in soft agar (Figure 4A). The number of cell clones was significantly decreased in SMMC-7721 cells infected with NDC80-siRNA (*P* = 0.0005) (Figure 4B). The colony formation experiment confirmed that the silencing of NDC80 reduced the proliferative potential of SMMC-7721 cells.

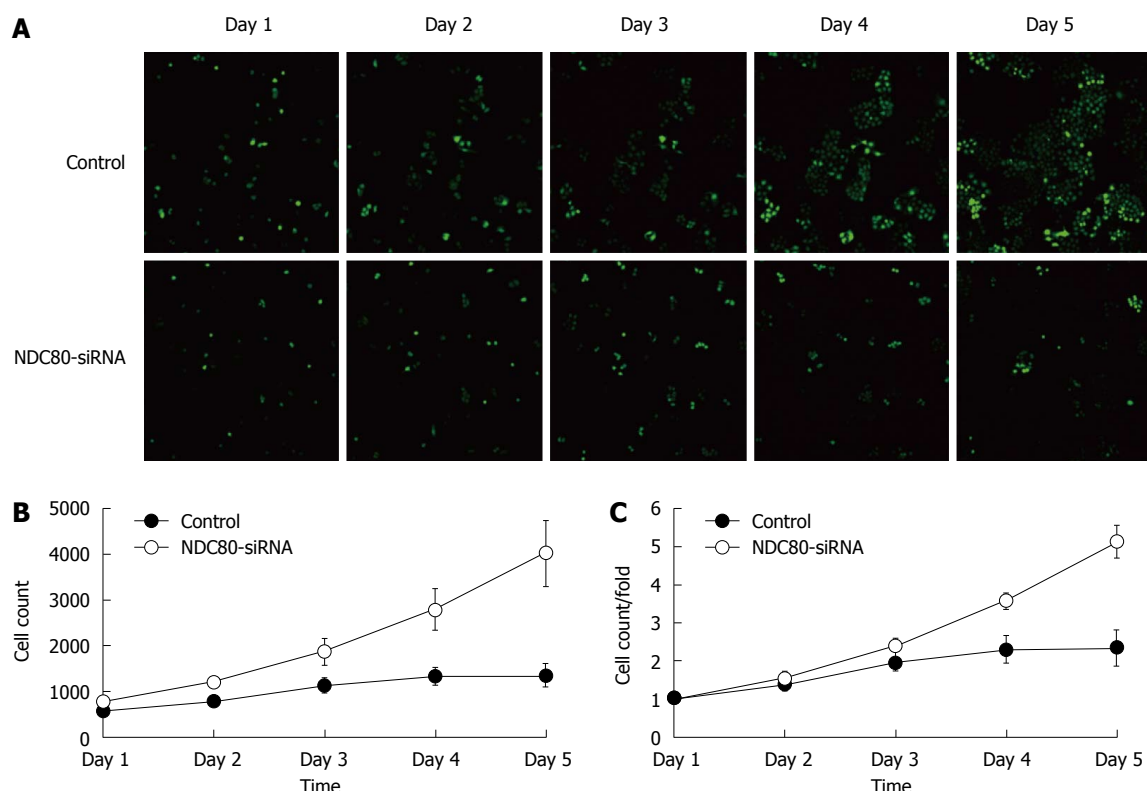
#### **NDC80 silencing induced cell cycle progression and the cell apoptosis process**

To elucidate further the growth-suppressing effect of NDC80-siRNA on SMMC-7721 cells, cell cycle distribution was analyzed by flow cytometry. In comparison with the control group, NDC80-siRNA significantly increased the fraction of S-phase cells, but decreased G1- and G2/M-phase cells in NDC80-siRNA group (Figure 5A). The result demonstrated that the silencing of NDC80 might induce cell cycle arrest at

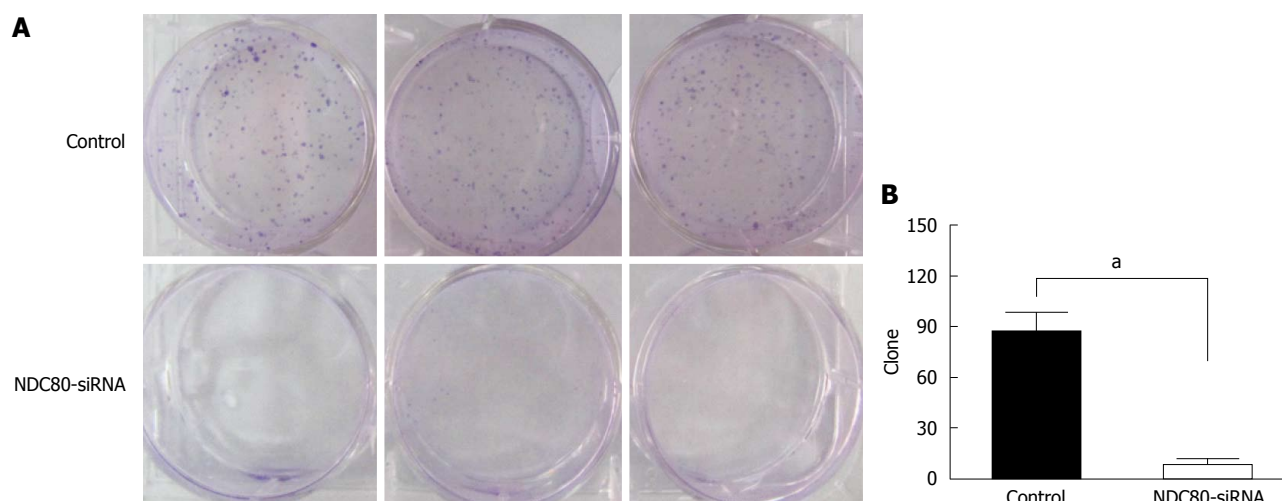
S-phase and that the effect of NDC80 on the cell cycle was time-dependent. Whether the silencing of NDC80 was related to the level of apoptosis in SMMC-7721 cells was further investigated. The apoptotic rate was assessed by flow cytometry using the Annexin V-APC Apoptosis Detection Kit. The proportion of apoptotic cells was significantly higher in NDC80-silenced cells than in the control cells (Figure 5B). These data suggested that the silencing of NDC80 interrupted cell cycle progression and affected cell survival.

## **DISCUSSION**

Chromosomal instability has long been suggested to be a driving force for tumor development and progression. Accurate chromosome segregation requires that sister kinetochores of each mitotic chromosome interact with microtubules connected to opposite spindle poles, so that separated sister chromatids migrate toward opposite directions at anaphase onset<sup>[17-19]</sup>. NDC80 is a core component of the outer kinetochore, the function of which is intricately involved in the establishment of appropriate microtubule attachments. NDC80 protein maintains chromosome stability, and spindle checkpoint dysfunction, abnormal chromosome separation and cell cycle disorder may occur in cells with NDC80 overexpression, which perhaps leads to



**Figure 3** Cell proliferation analysis by green fluorescent protein-based imaging and MTT assay. A: After lentiviral transfection of SMMC-7721 cells, cell proliferation was significantly inhibited in NDC80-siRNA-silenced cells as compared to the control cells according to green fluorescent protein-based Cellomics ArrayScan VTI imaging; B: After lentiviral transfection of SMMC-7721 cells, MTT assays were performed at the days indicated to show the proliferation of SMMC-7721 cells. The MTT value ratio was significantly reduced in the NDC80-siRNA-silenced cells as compared to the control cells.

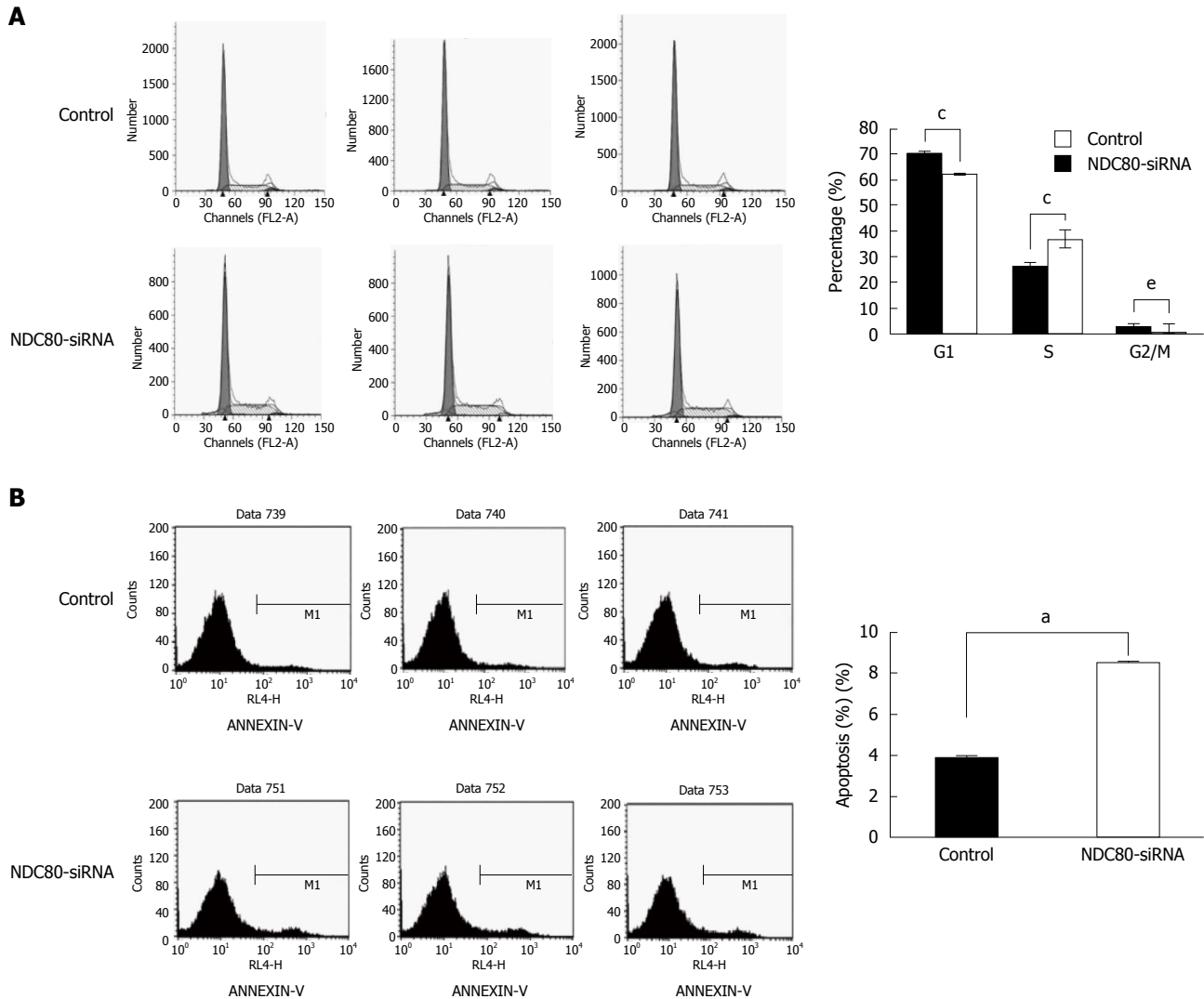


**Figure 4** Effects of the silencing of NDC80 on SMMC-7721 cell colony formation. A: After lentiviral transfection of SMMC-7721 cells, the NDC80-siRNA-silenced cells displayed a significantly reduced number of cell colonies compared to control cells. Colonies were stained with crystal violet. The whole plate fields were photographed and presented. The number of cell colonies of triplicate values in a representative experiment was counted; B: Statistical significance was assessed by two-tailed Student's *t* test. Error bar indicates SD ( $^aP < 0.001$  vs control).

tumor initiation<sup>[18,20-22]</sup>.

Expression of the mitotic regulator NDC80 is increased in various human malignancies, including HCC. Considering the significant overexpression of NDC80 in multiple human malignancies<sup>[23-27]</sup>, studies on the mechanisms of NDC80 overexpression are significant. Liu *et al.*<sup>[28]</sup> also reported that the

expression level of NDC80 was remarkably up-regulated in HBV-related HCC tissues. In this study, we investigated the significance of the increased NDC80 expression in carcinogenesis in general and HCC in particular, by both loss and gain of function analysis. This study was conducted to ascertain the role of NDC80 in human hepatocellular carcinogenesis.



**Figure 5** Effects of the silencing of NDC80 on SMMC-7721 cell cycle distribution and apoptosis. A: After lentiviral transfection of SMMC-7721 cells, cell cycle assessment showed that knockdown of NDC80 in SMMC-7721 cells induced accumulation in S-phase. The percentages of cells in different phases are shown as the mean  $\pm$  SD of three independent experiments; B: Apoptotic rates were analyzed by Annexin V-FITC/PI assay. Apoptosis was significantly increased in NDC80-siRNA-silenced cells as compared to control cells. Statistical significance was assessed by two-tailed Student's *t* test. Error bar indicates SD (<sup>a</sup>*P* < 0.05; <sup>c</sup>*P* < 0.01; <sup>e</sup>*P* < 0.0001, vs control).

Lentiviral packaging has become an ideal genetic engineering technology after years of optimization and improvement<sup>[29,30]</sup>. In this study, lentivirus-mediated siRNA provided an attractive approach to suppress NDC80 gene expression. We monitored the lentiviral infection efficiency by fluorescence microscopy, and confirmed the target gene knockdown by western blotting and real-time RT-PCR, which provided a basis for the continued observation of the role of NDC80 in SMMC-7721 cells. Real-time RT-PCR analysis showed that the NDC80 expression levels in the tumor tissues were significantly increased compared with those in adjacent tissues.

We found that cell proliferation and cell colony formation were significantly inhibited in NDC80-silenced HCC cells. Moreover, apoptosis was significantly increased in NDC80-silenced HCC cells. We performed a cell-cycle assay to illustrate the mechanism by

which NDC80 promotes cell proliferation. We found that attenuation of NDC80 expression in carcinoma cells delayed cell-cycle progression through S-phase, signifying arrest at this phase. This suggests that abnormal NDC80 expression leads to severe disruption of cell-cycle progression. The present study also demonstrated that NDC80 knockdown induced HCC apoptosis. The apoptosis caused by NDC80 knockdown might be due to incomplete mitosis caused by severe mitotic spindle checkpoint dysfunction. Hence, NDC80 plays a significant role in maintaining the growth of HCC cells.

These findings strongly suggest that NDC80 contributes to the pathogenesis of HCC through its proliferative and anti-apoptotic effects. Our data suggest that NDC80 overexpression may be an early event in hepatocellular carcinogenesis. Therefore, NDC80 may represent a new target for HCC gene



therapy. The results of this study provide a new theoretical basis for gene therapy in HCC.

In conclusion, the present study evaluates the association between NDC80 expression and HCC. NDC80 is highly expressed in HCC tissues relative to adjacent tissues. NDC80 significantly promotes HCC cell proliferation and colony formation and significantly inhibits apoptosis by arresting cells at the S phase. Therefore, we determined that NDC80 plays critical roles in tumor growth and HCC development. Although detailed mechanisms remain to be elucidated, the critical role of NDC80 in HCC development may provide evidence for development of novel therapeutics against NDC80 for the early detection and treatment of HCC.

## ACKNOWLEDGMENTS

We would like to thank Nantong Third People's Hospital Affiliated to Nantong University for providing the HCC tissue samples.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and the third leading cause of cancer mortality. High metastasis and recurrence rates have become the major obstacles to improving long-term survival of HCC. The molecular mechanisms of HCC development are not fully understood; thus, it is of importance to elucidate the mechanisms of HCC and explore effective treatment.

### Research frontiers

Nuclear division cycle (NDC)80 plays essential roles in chromosome segregation by mediating the spindle assembly checkpoint signaling and chromosome alignment. Chromosome instability is a common feature of tumor cells, and may be an important mechanism in tumor formation.

### Innovations and breakthroughs

This is the first study which shows that NDC80 contributes to the pathogenesis of HCC through its proliferative and anti-apoptotic effects. The authors hypothesized that the critical role of NDC80 in HCC development could provide evidence for development of novel therapeutics against NDC80 for the early detection and treatment of HCC.

### Applications

NDC80 may represent a new target for HCC gene therapy. The results of this study provide a new theoretical basis for gene therapy in HCC.

### Peer-review

The authors demonstrated that NDC80 expression levels in the tumor tissues were significantly increased compared with those in the adjacent tissues. NDC80 significantly promotes HCC cell proliferation and colony formation and significantly inhibits apoptosis by affecting cell cycle S-phase arrest. However, the detailed mechanisms about the critical role of NDC80 in HCC development have not been elucidated.

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## Basic Study

# Animal experimental studies using small intestine endoscope

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## Abstract

### AIM

To assess the feasibility and safety of a novel enteroscope, negative-pressure suction endoscope in examining the small intestine of a porcine model.

### METHODS

*In vitro* experiments in small intestinal loops from 20 pigs and *in vivo* experiments in 20 living pigs were conducted.

### RESULTS

In *in vitro* experiments, a negative pressure of > 0.06 MPa was necessary for optimal visualization of the intestine, and this pressure did not cause gross or histological damage to the mucosa. For satisfactory

examination of the small intestine *in vivo*, higher negative pressure ( $> 1.00$  MPa) was required. Despite this higher pressure, the small intestine did not show any gross or microscopic damage in the suctioned areas. The average time of examination in the living animals was  $60 \pm 7.67$  min. The animals did not experience any apparent ill effects from the procedure.

### CONCLUSION

Small intestine endoscope was safely performed within a reasonable time period and enabled complete visualization of the intestine in most cases.

**Key words:** Small intestine endoscope; Endoscope; Animal experiment; Endoscopic examination; Negative-pressure suction

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**Core tip:** The main component of endoscopes is an ultrafine tubular endoscope, with an added external propeller, to assist in the migration of the endoscope through the intestine.

Liu JH, Liu DY, Wang L, Han LP, Qi ZY, Ren HJ, Feng Y, Luan FM, Mi LT, Shan SM. Animal experimental studies using small intestine endoscope. *World J Gastroenterol* 2017; 23(20): 3684-3689 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3684.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3684>

### INTRODUCTION

Endoscopic examination of the small intestine remains a challenge. Endoscopes currently used in the diagnosis and treatment of gastrointestinal diseases include capsule gastroscope<sup>[1]</sup>, duodenoscope, double-balloon endoscope, single-balloon enteroscope, and colonoscope. The detection range of these instruments is from the mouth to the duodenum and from the anus, retrogradely, to the cecum<sup>[2-5]</sup>. Negative-pressure suction small intestine endoscope has been proposed as a solution to this problem. Its working principle is similar to the functions of suction cups of parasites, and its operating principles are similar to those of double-balloon and single-balloon endoscopes. The main component of negative-pressure endoscopes is an ultrafine tubular endoscope, with an added external propeller, to assist in the migration of the endoscope through the intestine. The endoscope is intended to enable the collection of intestinal fluids and tissue samples as well as to aid in the diagnosis and treatment of small intestinal diseases.

Small intestine endoscopes have been developed independently in China, with regard to the intellectual property rights. Three patent licenses from the Chinese

State Intellectual Property Office, as well as two patent licenses from the Japanese State Intellectual Property Office, have been granted for these endoscopes<sup>[2-6]</sup>.

In the present study, we aimed to assess the feasibility and safety of a negative-pressure suction endoscope in examining the small intestine of pigs.

### MATERIALS AND METHODS

#### Materials

The materials used included freshly excised small intestines, with the complete mesentery, from 20 live pigs (150 kg; 2 years of age); a negative-pressure suction small intestine endoscope (Figure 1); four electric suction units (Yuyue, 7A-23D; Nanjing, China), with pressures ranging from 0.06 to 1.00 Mpa; the cardinal machine; heatless light source; monitor (Jiangsu matt phillips photoelectric technology Co., LTD); computer; fixative solution; refrigerator; tissue sectioning instrument; and microscope.

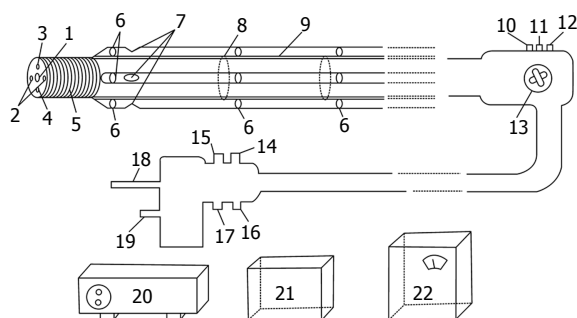
#### In vitro experiments

The cardinal machine, heatless light source, monitor, and negative-pressure suction device were switched on. Under the control of an operator, the working component of the endoscope was inserted into the prepared pig small intestine, the suction tubes on the slider were pulled, and the suction cup-like inlets on the front at the sides of the endoscope were examined to determine whether they properly sucked the small intestine. The areas surrounding the inlets were smeared with methylene blue, the suction force was increased, and the suction tubes on the sliders were pulled, thus causing the small intestine to pile up behind the working component of the endoscope. After the operation, the suctioned areas, which were stained with methylene blue, were biopsied for microscopic examination.

#### In vivo experiments

All animal experiments were conducted according to the institutional guidelines for the care and use of animals. The animal protocol was designed to minimize pain or discomfort to the animals. The ethical standards of experiments were in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Animals were fasted for 2 d prior to being anesthetized with ketamine hydrochloride injection (5 mg/kg). The negative-pressure suction device, the cardinal machine, heatless light source, and monitor were switched on. The endoscope was inserted into the mouth of the pigs, and if necessary, a small amount of air was introduced before the examination of the digestive tract. During the examination, the suction tubes on the slider were pulled, which gradually caused the intestine to pile up like sleeves around the main lens. The intestinal surface was examined as the





**Figure 1** Schematic diagram of negative-pressure suction small intestine endoscope. 1: Observation eyehole; 2: Windows for outgoing light; 3: Clamp tube; 4: Nozzle; 5: Rotating section; 6: Suction tube retainers; 7: Suction hole; 8: Slider retainer; 9: Slider; 10: Button for water and air inlet; 11: Button for water and air suction inlets; 12: Biopsy sample inlet; 13: Button for rotation of external propeller; 14: Air inlet; 15: Water inlet; 16: Air and water suction inlet; 17: Ground wire inlet; 18: Input terminal of heatless light source; 19: Image output terminal; 20: Main engine; 21: Monitor; 22: Air compressor. The length of the negative-pressure suction small intestine endoscope is 2.5 m. The main endoscope diameter is 12 mm. The imaging system used is Medical CMOS. The power system involves negative-pressure suction, and the light used is cold light.

suction tubes were being pulled. An attempt was made to reach the ileocecal junction in each animal. The diet, activity, and defecation of the pigs were monitored for 2 wk after the operation.

### Statistical analysis

Data were analyzed using SPSS version 12.0 for windows (SPSS Inc., Tokyo). All data are expressed as mean  $\pm$  SD. Continuous data were compared using *t* tests ( $n = 20$ ), with *P*-values  $< 0.05$  considered statistically significant.

## RESULTS

### *In vitro* experiments

The operator used the propelling arm of the endoscope to grasp the small intestine by applying negative-pressure suction from the electric suction unit. When the suction force was not sufficiently strong, the endoscope could not grasp the small intestine firmly; the initial pressure required for the endoscope to properly function was greater than 0.06 MPa. During the operation, the small intestine appeared to pile up in a sleeve-like manner around the main lens. Through the image acquisition window at the front of the main lens, the backward movement of the small intestine could be followed and the inner intestinal walls could be examined. In total, only 80% of the ileocecal junction were observed in four pigs, whereas a 4-m length of the small intestine was visualized in all 20 pigs. The examination required an average of  $40 \pm 5.47$  min (95%CI: 38.8-41.1). No histological damage to the small intestine was evident in the areas where negative-pressure suction had been applied.

### *In vivo* experiments

On the basis of the results from the *in vitro* experiments,

the initial pressure for the *in vivo* examinations was set at  $> 0.06$  MPa. When the pressure was greater than 1.00 MPa, no slippage was seen. Small intestine endoscopy was successful in all the 20 pigs, and the procedure required an average of  $60 \pm 7.67$  min (95%CI: 56.41-63.59). The average depth of insertion was 2.0 m. After the examination, all pigs were able to eat; 18 were able to defecate by the second day, and 2 by the third day. During the 2-week observation period after endoscopy, no pigs showed signs of abdominal distension, bloody stool, constipation, or other complications, and their food intake, activity levels, sleep patterns, defecation patterns, and urination were normal.

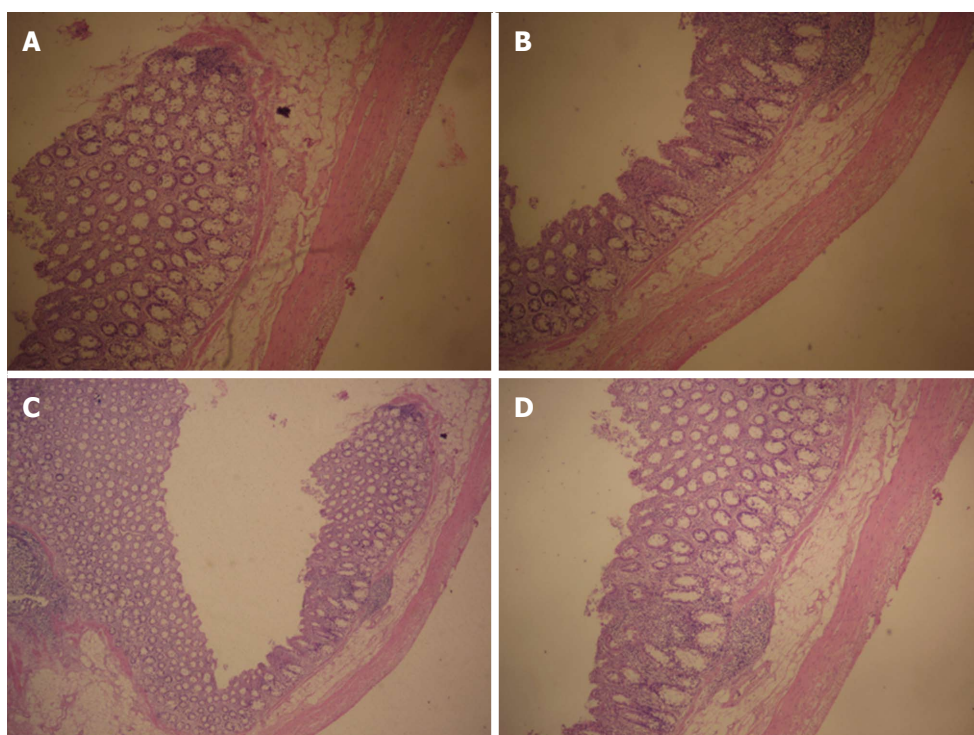
Examinations of histological sections from areas of the small intestine that were sucked by negative pressure did not show any breakage, shedding of large areas of the intestinal villi, tissue displacement, deformation, or damage to the glandular structures in the submucosal layer. The normal orderly arrangement of smooth muscle layers was maintained, and the muscle cells were clearly outlined (Figure 2).

## DISCUSSION

Endoscopic examination of the small intestine has always presented a tough challenge. Small intestine endoscopy has been proposed as a technique that might help overcome this challenge. Therefore, in the present study, we evaluated the feasibility and safety of this approach through *in vitro* and *in vivo* experiments in a porcine model.

In the *in vitro* experiments, we established that the pig small intestine was tolerant to negative pressures that were acceptable for endoscopic examination of the intestinal wall, and indeed tolerant to even much higher pressures. At a pressure of  $> 0.06$  MPa, the endoscope functioned smoothly, and 4-m segments of the small intestine were successfully examined in the intestines from all 20 animals studied. No visual damage was noted in the areas of the small intestine that were repeatedly sucked. Moreover, tissue biopsy specimens from those areas showed no signs of submucosal damage or breakage in the muscle layers. Therefore, we concluded that the negative pressure exerted by the suction endoscope was within the tolerance level of the small intestine. Indeed, we found that the intestine had an extremely high level of tolerance to negative pressure; even when 6 MPa of negative-pressure was exerted, no apparent damage to the sucked areas was noted. The positive results of these *in vitro* experiments encouraged us to proceed with *in vivo* experiments.

In live pigs, we found that somewhat higher negative pressure was needed, as compared to the *in vitro* condition, in order to prevent slippage of the bowel. Thus, we used a pressure of  $> 1.00$  MPa, which permitted successful examination of the intestines of all 20 animals. The average time of completing the *in*



**Figure 2** Light microscopic images of a tissue section from an area of the small intestine to which endoscopic negative-pressure suction had been applied *in vivo*. A: No damage to the smooth muscle is evident; B: No histologic damage is evident; C: No submucosal or glandular damage is present; D: No submucosal damage is present. Hematoxylin-eosin staining,  $\times 400$  magnification.

*in vivo* examinations was  $60 \pm 7.67$  min.

The time for this procedure was considerably shorter than that reported for double-balloon endoscope performed in Fuji, Japan, which took an average of 3-4 h per examination. The Japanese researchers also found that slippage of the intestine occurred when the two balloons were alternated, whereas we found no slippage when the working pressure level was set as  $> 1.00$  MPa.

We believe that it is encouraging that the negative-pressure suction endoscope did not histologically damage the small intestine, and that the animals had no evident adverse effects associated with the procedure; indeed, they resumed normal defecation within 2-3 d after the operation and had no signs of discomfort during the 2 postoperative weeks. This study in pigs indicated that small intestine endoscopy is feasible and can be performed safely in a reasonable length of time. The entire small intestine was visualized in a high percentage of animals, and neither gross nor microscopic damage to the intestine was noted. We are hopeful that this promising technique for examination of the small intestine will soon be evaluated in human subjects. In addition, we are planning an experiment on human subjects, which will enable the commercial application of this type of endoscope in the small intestine.

The small intestine endoscope had four power modes, and the examination speed was greater than that with the double-balloon and single-balloon endoscope. The curve formed by the endoscope is

not associated with any serious effects, and can avoid pain.

This endoscope is designed based on the ultrafine endoscope, and has four power modes. It is inserted into the cavity through the mouth while the patient is under general anesthesia, thus enabling the small intestine to pile up at the proximal end along the primary eyepiece, while the detector at the distal end can perform detection or treatment. Experimental results on animals confirmed that endoscopic examination using our newly designed endoscope has the following advantages compared to the Japanese double-balloon endoscope: (1) quicker examination; (2) high operability and ease of mastery; (3) low cost; and (4) short slippage distance<sup>[7-15]</sup>. Moreover, endoscopic examination using our newly designed endoscope is more advantageous than capsule endoscopy in terms of (1) clearer images; (2) uninterrupted examination; (3) ability to collect pathological samples or conduct endoscopic treatment; (4) short examination duration; and (5) not causing ileus<sup>[16-30]</sup>. The most important feature of small intestine endoscopy is its rapid examination speed; it only takes an average of  $60 \pm 7.67$  min to complete the examination of a 4-m-long small intestine with complete mesentery, which is 2 h less than the average examination time required for Japanese Fujinon double-balloon endoscope, as reported in the literature. In the present study, we provided evidence (Figure 3) to support the manufacture of this endoscope, which will be beneficial for patients, alleviate pain, and help ensure that



**Figure 3 Animal experiments using small intestine endoscope.** A: Animals were fasted for 2 d prior to being anesthetized with ketamine hydrochloride injection (5 mg/kg); B: The endoscope was inserted into the mouth of the pig; C: An attempt was made to reach the ileocecal junction; D: A picture obtained by using the small intestine endoscope.

no blind spots are present during examination and treatment with the small intestine endoscope.

## COMMENTS

### Background

Endoscopic examination of the small intestine remains a challenge. Endoscopes currently used in the diagnosis and treatment of gastrointestinal diseases include capsule gastroscope, duodenoscopy, double-balloon endoscopy, single-balloon enteroscopy, and colonoscopy.

### Research frontiers

The small intestine endoscope has four power modes, and the examination speed is greater than that with the double-balloon and single-balloon endoscope. The curve formed by the endoscope is not associated with any serious effects, and can avoid pain.

### Innovations and breakthroughs

Small intestine endoscopes have been developed independently in China, with regard to the intellectual property rights. Three patent licenses from the Chinese State Intellectual Property Office, as well as two patent licenses from the Japanese State Intellectual Property Office, have been granted for these endoscopes.

### Applications

In live pigs, they found that somewhat higher negative pressure was needed, as compared to the *in vitro* condition, in order to prevent slippage of the bowel. Thus, they used a pressure of > 1.00 MPa, which permitted successful examination of the intestines of all 20 animals. The average time of completing the *in vivo* examinations was  $60 \pm 7.67$  min.

### Terminology

Small intestine endoscope is a medical device for the diagnosis and treatment of small intestine diseases.

### Peer-review

This is an interesting study about the small intestine enteroscopy. In this study, Liu *et al* assessed the feasibility and safety of a novel enteroscopic technique, negative-pressure suction enteroscopy, for examining the small intestine in a porcine model. Experiments in small intestinal loops from 20 pigs, and *in vivo* experiments in 20 living pigs, were conducted. The authors found that the enteroscopy was safely performed within a reasonable time period and enabled complete visualization of the intestine in most cases.

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## Retrospective Cohort Study

# Radiological response and inflammation scores predict tumour recurrence in patients treated with transarterial chemoembolization before liver transplantation

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## Abstract

### AIM

To investigate the prognostic value of the radiological response after transarterial chemoembolization

(TACE) and inflammatory markers in patients affected by hepatocellular carcinoma (HCC) awaiting liver transplantation (LT).

### METHODS

We retrospectively evaluated the preoperative predictors of HCC recurrence in 70 patients treated with conventional ( $n = 16$ ) or doxorubicin-eluting bead TACE ( $n = 54$ ) before LT. The patient and tumour characteristics, including the static and dynamic alpha-fetoprotein, neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio (PLR) measurements, were recorded. Treatment response was classified according to the modified Response Evaluation Criteria in Solid Tumours (mRECIST) and the European Association for the Study of the Liver (EASL) criteria as complete response (CR), partial response (PR), stable disease or progressive disease. After examination of the explanted livers, histological necrosis was classified as complete (100% of the cumulative tumour area), partial (50%-99%) or minimal ( $< 50\%$ ) and was correlated with the preoperative radiological findings.

### RESULTS

According to the pre-TACE radiological evaluation, 22/70 (31.4%) and 12/70 (17.1%) patients were beyond Milan and University of San Francisco (UCSF) criteria, respectively. After TACE procedures, the objective response (CR + PR) rates were 71.4% and 70.0% according to mRECIST and EASL criteria, respectively. The agreement between the two guidelines in defining the radiological response was rated as very good both for the overall and target lesion response (weighted  $k$ -value: 0.98 and 0.93, respectively). Complete and partial histological necrosis were achieved in 14/70 (20.0%) and 28/70 (40.0%) patients, respectively. Using histopathology as the reference standard, mRECIST criteria correctly classified necrosis in 72.9% (51/70) of patients and EASL criteria in 68.6% (48/70) of cases. The mRECIST non-response to TACE [Exp(b) = 9.2,  $P = 0.012$ ], exceeding UCSF criteria before TACE [Exp(b) = 4.7,  $P = 0.033$ ] and a preoperative PLR  $> 150$  [Exp(b) = 5.9,  $P = 0.046$ ] were independent predictors of tumour recurrence.

### CONCLUSION

The radiological response and inflammatory markers are predictive of tumour recurrence and allow the proper selection of TACE-treated candidates for LT.

**Key words:** Liver transplantation; Recurrence-free survival; Hepatocellular carcinoma; Radiological response; Locoregional therapies; Inflammatory markers; Selection criteria

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**Core tip:** The response to loco-regional therapy and biological markers appear to stratify the prognosis of hepatocellular carcinoma patients awaiting liver

transplantation (LT) better than morphological criteria; however, their role in the selection scheme still needs validation. We analysed a homogeneous cohort of 70 patients treated exclusively by transarterial chemoembolization (TACE) prior to LT; the radiological response was assessed by two different enhancing methods (mRECIST and EASL criteria) that provided an accurate preoperative estimation of histological necrosis. We also demonstrated that a lack of response to TACE and a high platelet-to-lymphocyte ratio before surgery are strongly predictive of tumour recurrence, independently of the Milan criteria status at referral.

Nicolini D, Agostini A, Montalti R, Mocchegiani F, Mincarelli C, Mandolesi A, Robertson NL, Candelari R, Giovagnoni A, Vivarelli M. Radiological response and inflammation scores predict tumour recurrence in patients treated with transarterial chemoembolization before liver transplantation. *World J Gastroenterol* 2017; 23(20): 3690-3701 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3690.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3690>

### INTRODUCTION

Liver transplantation (LT) is the best chance of a cure for patients with hepatocellular carcinoma (HCC)<sup>[1]</sup>, removing both the tumour and underlying cirrhosis, known to be a premalignant condition. Over the past two decades, several dimensional criteria, based on tumour size and number, have been validated to identify patients with a lower risk of recurrence after LT. The Milan Criteria (MC)<sup>[2]</sup> are currently considered the gold-standard for selecting patients for LT at most transplant centres worldwide<sup>[3]</sup>. Tumour features including microvascular invasion (mVI), poor histopathological differentiation and gene expression profiles are universally recognized as strong prognostic indicators<sup>[4,5]</sup>; however, these features are rarely assessable at the pre-operative diagnostic work-up.

Locoregional treatments (LRTs) are used in patients awaiting LT to help prevent progression while on the waiting list (bridging therapy) and as neoadjuvant treatment to downstage HCC according to the commonly accepted MC or University of San Francisco criteria (UCSF)<sup>[6]</sup>.

It has been shown that applying the MC to baseline imaging may incorrectly stage a patient with HCC in over 30% of cases compared with assessing the explanted histopathological specimen<sup>[7,8]</sup>. Furthermore, two prospective studies have demonstrated favourable outcomes in patients with advanced HCC treated with neoadjuvant therapy with successful down-staging prior to LT<sup>[9,10]</sup>. These findings suggest that reliable selection criteria for LT candidates require more than static dimensional criteria based on preoperative liver imaging.

Transarterial chemoembolization (TACE) is the

most extensively studied and widely used neoadjuvant therapy in current clinical practice<sup>[11,12]</sup>. To define the treatment response, the modified Response Evaluation Criteria In Solid Tumours (mRECIST)<sup>[13]</sup> and European Association for the Study of the Liver (EASL)<sup>[14]</sup> criteria are enhancement methods used in the transplant<sup>[15-17]</sup> and non-transplant settings<sup>[18,19]</sup>. Although the post-LT prognosis can be predicted using mRECIST response dynamics following TACE<sup>[15,20]</sup>, the accuracy of this criterion remains controversial compared with the histopathological standard of reference<sup>[16,21,22]</sup>.

Regarding serum tumour markers, alpha-fetoprotein (AFP) levels<sup>[23-25]</sup> and their fluctuation during the waiting list (WL) period<sup>[26-28]</sup> have been shown to predict tumour recurrence in many clinical experiences<sup>[23-25]</sup>. However, there is no universally agreed upon cut-off value that would make a patient ineligible for LT<sup>[26-28]</sup>. Besides AFP, a growing number of recent studies have emphasized the association between tumour biological aggressiveness and the "so-called" inflammatory markers, namely the neutrophil-to-lymphocyte ratio (NLR)<sup>[29-31]</sup> and platelet-to-lymphocyte ratio (PLR)<sup>[32]</sup>. These laboratory markers are inexpensive and readily available, but their prognostic role in HCC recurrence and WL dropout remains widely debated<sup>[33]</sup>.

We evaluated the radiological response and pre-operative inflammation scores as prognostic variables to predict post-transplant HCC recurrence, through comprehensive multivariate analysis of preoperative risk factors in 70 consecutive patients treated with TACE at our institution. The accuracy of mRECIST and EASL criteria for the prediction of histological necrosis was also evaluated.

## MATERIALS AND METHODS

The study population was retrieved from the institutional, prospectively entered database of the Hepatobiliary and Transplant Unit of Polytechnic University of Marche, Ancona. Between August 2005 and December 2014, 114 liver transplants were performed in patients with histologically confirmed HCC in the background of liver cirrhosis. Patients who did not receive neoadjuvant treatment ( $n = 24$ ), who received a type of neoadjuvant therapy other than TACE ( $n = 15$ ) or who were transplanted before the scheduled post-TACE imaging evaluation ( $n = 5$ ) were excluded from the analysis. The final study population included 70 patients who exclusively underwent one or more TACE sessions prior to LT.

The preoperative diagnosis of HCC was based on EASL guidelines<sup>[1]</sup>. Thoracic and abdominal contrast-enhanced computed tomography (CT) or multiphasic contrast-enhanced MRI imaging was performed before TACE to exclude intra-abdominal or pulmonary tumour spread, lymphatic metastasis or macrovascular invasion of the portal branches. In line with our institution's policy, TACE was performed in patients

with a greater tumour burden than described by the MC at initial imaging, or in patients fulfilling MC with an expected waiting list time of longer than 2 mo. The patient eligibility for LT was discussed during the weekly local multidisciplinary liver transplant committee meeting, considering all aspects of the pre-operative work-up and staging test results.

The patient demographics, aetiology of cirrhosis, Child-Pugh and Model for End-Stage Liver Disease (MELD) scores, and imaging and pathological records were collected for each patient. Laboratory factors related to tumour biology such as serum AFP and inflammation-based scores were recorded at two different well-defined time points: the day of admission to perform the first TACE and immediately before surgery. The NLR is expressed as the ratio between the absolute blood count of neutrophils and lymphocytes. The PLR is the ratio between the absolute blood count of platelets and lymphocytes. The prognostic value of both static and dynamic (difference between initial and final values divided by the time lapse between the two referral points) AFP, NLR and PLR values were evaluated.

The transplant procedures were performed using deceased donor allografts. The immunosuppressive schedule included mammalian target of rapamycin inhibitors (Everolimus) in association with low-dose calcineurin inhibitor (Tacrolimus) in most patients. Steroids were gradually tapered and discontinued after 3 mo in all patients.

Screening for tumour recurrence involved serial AFP measurements, 3 monthly abdominal ultrasounds and annual contrast-enhanced CT chest/abdomen imaging.

### *TACE procedures and assessment of the radiological response*

All patients underwent baseline celiac and superior mesenteric arteriography using femoral artery puncture. Conventional TACE was performed by administering 50 mg of epirubicin in an emulsion with Lipiodol followed by embolization with gelatin sponge particles. Doxorubicin-eluting bead TACE (DEB-TACE) was performed using DC beads impregnated with 75 mg of doxorubicin in each vial. When the radiologic findings demonstrated residual viable tumour in the treated nodules or new lesions, patients with a low risk of decompensation underwent further TACE therapy.

Two radiologists (Agostini A and Giovagnoni A, with 5 and 25 years of experience, respectively, in liver imaging) evaluated the baseline contrast-enhanced imaging (dynamic MRI or CT) and last available imaging before LT to define the tumour response according to mRECIST and EASL guidelines. Both mRECIST and EASL guidelines define viable tumour as the area of enhancement during the arterial phase. For each measurable lesion, the largest diameter and largest perpendicular diameter were recorded; the first was used to assess the target lesion

response according to mRECIST (based on the sum of unidimensional measurements), and the product of both measurements was used to assess the response of measurable lesions according to EASL. Both criteria define a complete response (CR) as the absence of arterial enhancement within a lesion. mRECIST defines a partial response as at least a 30% decrease in the sum of the longest diameter of target lesions, taking as the reference the baseline sum longest diameter; progressive disease (PD) is defined as an increase of at least 20% of the sum of the longest diameter of the target lesions. EASL defines a PR as a decrease of at least 50% of the sum of cross products of the enhancing diameters, while PD is defined as an increase of at least 25%. Stable disease (SD) occurs when neither PR nor PD is assigned with both criteria. New lesions denote PD for mRECIST and EASL. Overall, the patient response is a result of the combined assessment of target lesions, non-target lesions, and new lesions. Patients obtaining a CR or PR after TACE cycles are defined as objective responders (OR).

### **Evaluation of the explanted livers**

A dedicated liver pathologist performed the analysis of all explanted livers that were serially cut into sections of approximately 0.5-cm thick. Tumour grade according to the Edmonson and Steiner classification was assessed except when complete necrosis of the tumour was achieved. The presence of satellite lesions and mVI were also reported. Nodule necrosis was expressed as the percentage of necrotic tissue within the whole area of the nodule. In patients with multiple lesions, the necrosis of the cumulative tumour area (% of necrosis on CTA) was calculated, including that of non-treated tumours, as a mean of necrosis rates weighted on nodular areas. To explore the accuracy of radiological criteria in predicting the histological outcome, we assumed that the CR corresponds to 100% of necrosis on CTA (no viable cells detected), whereas PR corresponds to 50%-99% of necrosis on CTA and SD or PD correspond to < 50% necrosis on CTA. For the radio-histological correlation of target lesions, the percentage of necrosis on CTA was re-calculated considering only nodules  $\geq 1$  cm in diameter at pathology. Adherence to MC or UCSF criteria was re-assessed at pathological examination using only the viable portion of each nodule.

### **Statistical analysis**

Categorical variables are reported as numbers and percentages and were compared with Fisher's exact test. Continuous variables are reported as medians and interquartile ranges (IQRs); the Mann-Whitney *U* test was applied to compare continuous variables in different subgroups of patients, whereas any difference in laboratory values before and after TACE therapy was investigated using the Wilcoxon test. Continuous variables such as AFP serum levels, AFP slope, NLR, and

PLR were dichotomized according to previously reported threshold values (400 ng/mL for the static AFP value and 15 ng/mL/mo for the AFP slope; 4 for NLR and 150 for PLR)<sup>[23,31-37]</sup>. Third-quartile values, corresponding to 0.24 and 3.04/mo, were used for the NLR and PLR slopes, respectively. The rank correlation test for nonparametric continuous variables (Kendall's tau) was applied to investigate the relationship between the amount of necrosis and different categories of the radiological response. The agreement in defining the radiological response between mRECIST and EASL criteria was explored by the k-cohen test. The impact of each individual variable in determining HCC recurrence-free survival (RFS) was assessed by the Kaplan-Meier method and was compared by the log-rank test. A multivariate Cox proportional regression model (stepwise method) was designed to investigate risk factors independently related to HCC recurrence, considering only preoperative variables that proved to be significant (*P* value < 0.05) after univariate analysis. Statistical significance was set at a *P* value < 0.05.

## **RESULTS**

### **Patient characteristics**

The demographics and clinical characteristics of the 70 patients included in the study are summarized in Table 1. The median post-LT follow-up was 38.4 (IQR: 24.8-68.6) mo. At the initial radiological evaluation, multifocal HCC was detected in 30 (42.9%) patients, and the median diameter of the largest nodule was 2.6 (IQR: 2.0-3.4) cm. Twenty-two (31.4%) patients were beyond MC, and 12 (17.1%) were also outside the UCSF criteria.

Globally, 124 TACE procedures were performed in 70 LT candidates; 37 (52.8%) patients underwent two or more TACE sessions. The number of treatments did not significantly differ between the patients classified within or beyond MC according to the initial imaging (*P* = 0.1546). The median time interval between the first TACE and LT was 6.9 (IQR: 3.7-11.0) mo. DEB-TACE was employed more frequently (77.1% of patients) than c-TACE (22.9%).

### **Radiological response to TACE and comparison with pathology**

According to mRECIST criteria, the OR rate was 71.4%, with 24 (34.3%) patients achieving CR and 26 (37.1%) patients achieving PR (Table 2). When EASL criteria were applied to define the response to TACE, a CR was seen in 24 (34.3%) patients, a PR in 25 (35.7%), SD in 11 (15.7%) and PD in 10 (14.3%). The agreement between the two guidelines in defining the radiological response was rated as very good both for the overall and target lesion response (weighted k-value 0.98 and 0.93, respectively).

Considering only the viable portion of the nodules,



**Table 1** Clinical, radiological and laboratory characteristics of the study population at the initial evaluation *n* (%)

Variable	All treated patients ( <i>n</i> = 70)
Demographics and indications	
Age at LT (yr) [median (IQRs)]	57 (51-62)
Male gender	62 (88.6)
Biochemical MELD Score [median (IQRs)]	11 (7-15)
Child-Pugh class A/B/C	29 (41.4)/27 (38.6)/14 (20.0)
Virus B-related cirrhosis	15 (21.4)
Virus C-related cirrhosis	41 (58.6)
Pre-TACE radiological evaluation	
Type of imaging technique (CT/MR)	49 (70.0)/21 (30)
Exceeding Milan criteria	22 (31.4)
Exceeding UCSF criteria	12 (17.1)
Number of nodules [median (IQRs)]	1 (1-2)
Single/multiple	40 (57.1)/30 (42.9)
Sum of nodule diameters (cm) [median (IQRs)]	3.35 (2.1-5.4)
Sum of nodule diameters > 5 cm	22 (31.4)
Diameter of the largest nodule (cm) [median (IQRs)]	2.6 (2.0-3.4)
Diameter of the largest nodule > 5 cm	7 (10.0)
Pre-TACE laboratory evaluation	
<sup>1</sup> AFP (ng/mL) [median (IQRs)]	12.5 (5.8-52.0)
AFP > 400 ng/mL	5 (8.1)
<sup>2</sup> NLR [median (IQRs)]	2.0 (1.4-3.1)
NLR > 4	13 (20.3)
<sup>3</sup> PLR [median (IQRs)]	67.2 (44.6-84.0)
PLR > 150	2 (3.1)
AST/ALT (U/L) [median (IQRs)]	69 (43.7-108.7)/56 (34.0-88.2)
WBC ( $\times 10^3$ /mmc) [median (IQRs)]	4.7 (3.7-5.8)
Characteristics of TACE and time-intervals between procedures	
Number of treatments [median (IQRs)]	2 (1-2)
Repeated TACE	37 (52.9)
Type of TACE (DEB/conventional)	54 (77.1)/16 (22.9)
Interval of last imaging-LT (mo) [median (IQRs)]	1.4 (0.7-2.7)
Interval of last TACE-LT (mo) [median (IQRs)]	3.9 (2.1-7.4)
Interval of first TACE-LT (mo) [median (IQRs)]	6.9 (3.7-11.0)

<sup>1</sup>The AFP value was missing in 8 patients; <sup>2</sup>TACE NLR was missing in 6 patients; <sup>3</sup>PLR was missing in 6 patients. LT: Liver transplantation; IQR: Interquartile range; MELD: Model for End-Stage Liver Disease; TACE: Transarterial chemoembolization, CT: Computed tomography; MR: Magnetic resonance, UCSF: University of California San Francisco; AFP: Alpha-fetoprotein; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cell; DEB: Doxorubicin-eluting bead.

12 (17.1%) patients were beyond MC at pathological examination. The median percentage of necrosis on CTA was 62.5% (IQR: 23.3-95.4); 14 (20.0%) patients exhibited complete necrosis. Histological necrosis significantly differed across the different response categories as defined by mRECIST and EASL. Using mRECIST, the patients classified as CR, PR or SD/PD showed a percentage of necrosis on CTA of 86.6%, 64.1% and 16.4%, respectively (Kendall's Tau: 0.66,  $P < 0.0001$ ). Similarly, patients assigned to CR, PR or SD/PD as defined by EASL showed a % of necrosis on CTA of 86.6%, 60.5% and 22.9%, respectively (Kendall's Tau: 0.61,  $P < 0.0001$ ). No correlation was found between the amount of histological necrosis and time interval between the last radiological assessment and LT or the interval between the last TACE and LT.

The accuracy of the mRECIST overall response was 72.9% (51 patients). In 17 (24.3%) cases, mRECIST overestimated histopathological necrosis, and in 2 (2.9%) cases, mRECIST underestimated tumour necrosis as seen on imaging before LT. When EASL criteria were applied, the accuracy was slightly

lower (48 patients; 68.6%), with 18 (25.7%) cases of overestimation and 4 (5.7%) cases of underestimation (Table 3). Overall, the accuracy in discriminating responders from non-responders was 85.7% and 81.4% for mRECIST and EASL criteria, respectively.

### Biological markers

Before TACE, 5 (8.1%) patients had an AFP value higher than 400 ng/mL, and 13 (20.3%) patients had an NLR > 4. The median PLR initial value was 67.2 (IQR: 44.6-84.0) (Table 1). Considering the initial radiological evaluation, no differences were found in terms of AFP, NLR or PLR between patients fulfilling or exceeding MC ( $P = 0.5148$ ,  $P = 0.2672$  and  $P = 0.3780$ , respectively). The pre-LT values of biological markers and their fluctuation during the observation period are displayed in Table 2. An increase in AFP of more than 15 ng/mL/mo was seen in 6 (10.2%) patients. At the time of LT, patients who did not respond to TACE (SD or PD) did not have significantly higher levels of AFP, NLR or PLR compared with those who achieved an OR (CR or PR). With respect to their pre-TACE values,

**Table 2 Radiological and laboratory characteristics of the study population after transarterial chemoembolization procedures and tumour histopathological data *n* (%)**

Variable	All treated patients ( <i>n</i> = 70)
Pre-LT radiological evaluation	
mRECIST overall response	
Complete/partial response	24 (34.3)/26 (37.1)
Stable/progressive disease	10 (14.3)/10 (14.3)
EASL overall response	
Complete/partial response	24 (34.3)/25 (35.7)
Stable/progressive disease	11 (15.7)/10 (14.3)
Number of enhancing nodules [median (IQRs)]	1 (0.0-2.0)
None/single/multiple	24 (34.3)/22 (31.4)/24 (34.3)
Sum of enhancing diameters (cm) [median (IQRs)]	1.4 (0.0-3.3)
Sum of enhancing diameters > 5 cm	8 (11.4)
Diameter of the largest enhancing nodule (cm) [median (IQRs)]	1.3 (0.0-2.1)
Diameter of the largest enhancing nodule > 5 cm	1 (1.4)
Pre-LT laboratory evaluation	
<sup>1</sup> AFP (ng/mL) [median (IQRs)]	13.5 (5.3-65.0)
AFP > 400 ng/mL	6 (9.1)
<sup>2</sup> AFP increase > 15 ng/mL per month	6 (10.2)
NLR [median (IQRs)]	2.6 (1.8-3.8)
NLR > 4	15 (21.4)
<sup>3</sup> NLR increase > 0.24	16 (29.6)
PLR [median (IQRs)]	62.9 (49.7-85.9)
PLR > 150	5 (7.1)
<sup>3</sup> PLR increase > 3.04	16 (29.6)
AST/ALT (UI/L) [median (IQRs)]	68 (43-100)/49 (32-76)
WBC ( $\times 10^3$ /mmc) [median (IQRs)]	4.7 (3.7-5.8)
Tumour histopathological characteristics	
Number of viable nodules [median(IQRs)]	1 (1-3)
Number of viable nodules > 3	11 (15.7)
Tumour differentiation ( <sup>4</sup> Gx/G1-G2/G3-G4)	14 (20.0)/48 (68.6)/8 (11.4)
Microvascular invasion	8 (11.4)
Exceeding Milan criteria	12 (17.1)
Exceeding UCSF criteria	11 (15.7)
% of necrosis on cumulative tumour area (100/99-50/< 50)	14 (20.0)/28 (40.0)/28 (40.0)

<sup>1</sup>The AFP value was missing in 4 patients; <sup>2</sup>The AFP progression value was missing in 11 patients due to the lack of pre-TACE or pre-LT value; <sup>3</sup>NLR and PLR modification was missing in 6 patients due to the lack of pre-TACE values; <sup>4</sup>Tumour grading was not available in 14 patients with complete histological necrosis at the explant examination. LT: Liver transplantation; mRECIST: Modified Response Evaluation Criteria in Solid Tumours; EASL: European Association for the Study of the Liver; IQR: Interquartile range; AFP: Alpha-fetoprotein; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cell; UCSF: University of California San Francisco.

a significant increase in the median NLR was seen in patients who experienced an OR after LRT (1.9 vs 2.9,  $P = 0.0025$ ); this increment was less evident in patients who did not respond to TACE (2.0 vs 2.4,  $P = 0.0539$ ).

### Predictors of HCC recurrence

Eight (11.4%) patients in the entire cohort developed HCC recurrence after a median period of 16.9 mo (IQR: 14.2-23.4, range: 9.7-38.3). The three-year patient and recurrence-free survival rates were 79.9% and 86.4%, respectively. Five patients were found to have recurrence in the liver, one patient had spinal cord recurrence in addition to liver lesions, a further patient had an adrenal gland deposit, and the final patient was found to have recurrent pulmonary disease. Two of eight patients were alive with recurrence at the time of data extraction.

On multivariate Cox regression analysis, mRECIST non-response to TACE at the last imaging before LT [Exp(b) = 9.2, CI: 1.6-51.3,  $P = 0.0119$ ] was the

strongest predictor of HCC recurrence. The lack of fulfilment of UCSF criteria before TACE [Exp(b) = 4.7, CI: 1.1-19.3,  $P = 0.0331$ ] and an increased (> 150) PLR before LT [Exp(b) = 5.9, CI: 1.0-33.9,  $P = 0.0458$ ] were also independently associated with tumour recurrence (Table 4).

The presence of multiple nodules at initial imaging and an AFP level greater than 400 ng/mL prior to performing TACE were significantly associated with HCC recurrence after univariate analysis but were not confirmed as independent prognostic factors after multivariate analysis. Notably, the MC status at initial radiological evaluation was not significantly associated with HCC recurrence in our cohort ( $P = 0.0736$ ).

Stratifying the entire cohort according to the risk factors that proved statistically significant after multivariate analysis (pre-LT PLR > 150, mRECIST non response and exceeding UCSF criteria before TACE), patients beyond MC at the initial radiological evaluation with at least one risk factor (15 patients) experienced the worst outcome in terms of recurrence (3-year RFS

**Table 3** Correlation analysis between histological necrosis and radiological response according to modified Response Evaluation Criteria in Solid Tumours and European Association for the Study of the Liver criteria *n* (%)

	Patients ( <i>n</i> )	Overall response			Patients ( <i>n</i> )	Target lesion response		
		% of necrosis on CTA				% of necrosis on CTA <sup>1</sup>		
		100	50-99	< 50		100	50-99	< 50
mRECIST								
Complete response	24	13 (54.2)	8 (33.3)	3 (12.5)	29	16 (55.2)	8 (27.6)	5 (17.2)
Partial response	26	1 (3.8)	19 (73.1)	6 (23.1)	25	2 (8.0)	16 (64.0)	7 (28.0)
Stable/progressive disease	20	0 (0.0)	1 (5.0)	19 (95.0)	16	0 (0.0)	0 (0.0)	16 (100.0)
EASL								
Complete response	24	13 (54.2)	8 (33.3)	3 (12.5)	29	16 (55.2)	8 (27.6)	5 (17.2)
Partial response	25	1 (4.0)	17 (68.0)	7 (28.0)	25	1 (4.0)	15 (60.0)	9 (36.0)
Stable/progressive disease	21	0 (0.0)	3 (14.3)	18 (85.7)	16	1 (6.3)	1 (6.3)	14 (87.5)

<sup>1</sup>For radio-histological correlation of the target lesion response, the percentage of necrosis on CTA was re-calculated considering only nodules  $\geq 1$  cm in diameter. CTA: Cumulative Tumour Area; mRECIST: Modified Response Evaluation Criteria in Solid Tumours; EASL: European Association for the Study of the Liver.

**Table 4** Univariate and multivariate analysis of the preoperative risk factors related to tumour recurrence

Risk factors	Univariate analysis			Multivariate analysis	
	3-yr RFS rate (%)	HR (95%CI)	Log-rank <i>P</i> value	Exp(b) (95%CI)	<i>P</i> value
Pre-TACE radiological and laboratory evaluation					
Exceeding Milan criteria	77.7 vs 91.2	3.41 (0.78-14.92)	0.074		
Exceeding UCSF criteria	60.6 vs 92.8	5.06 (0.78-32.74)	0.011	4.69 (1.14-19.30)	0.033
Multiple nodules	77.2 vs 93.4	4.33 (1.06-17.72)	0.049	1.77 (0.27-11.48)	0.550
AFP > 400 ng/mL	80.0 vs 85.3	1.96 (0.13-30.81)	0.520		
NLR > 4	100 vs 80.4	NA	0.185		
PLR > 150	50.0 vs 88.6	5.98 (0.06-573.48)	0.059		
Pre-LT radiological and laboratory evaluation					
mRECIST non response	71.2 vs 94.3	6.96 (1.54-31.50)	0.006	9.19 (1.65-51.30)	0.012
EASL non response	76.8 vs 91.6	3.67 (0.82-16.34)	0.056		
AFP > 400 ng/mL	83.3 vs 89.0	4.74 (0.29-77.77)	0.034	1.43 (0.23-9.10)	0.703
AFP increase > 15 ng/mL/mo	41.7 vs 87.7	3.89 (0.30-50.72)	0.072		
NLR > 4	80.8 vs 87.9	1.54 (0.25-9.41)	0.594		
NLR increase > 0.24/mo	83.3 vs 100	NA	0.114		
PLR > 150	50.0 vs 89.1	5.32 (0.28-101.01)	0.022	5.95 (1.04-33.95)	0.046
PLR increase > 3.04	80.8 vs 88.7	1.48 (0.24-9.04)	0.636		

RFS: Recurrence-free survival; HR: Hazard ratio; TACE: Transarterial chemoembolization; UCSF: University of California San Francisco; AFP: Alpha-fetoprotein; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; LT: Liver transplantation; mRECIST: Modified Response Evaluation Criteria in Solid Tumours; EASL: European Association for the Study of the Liver.

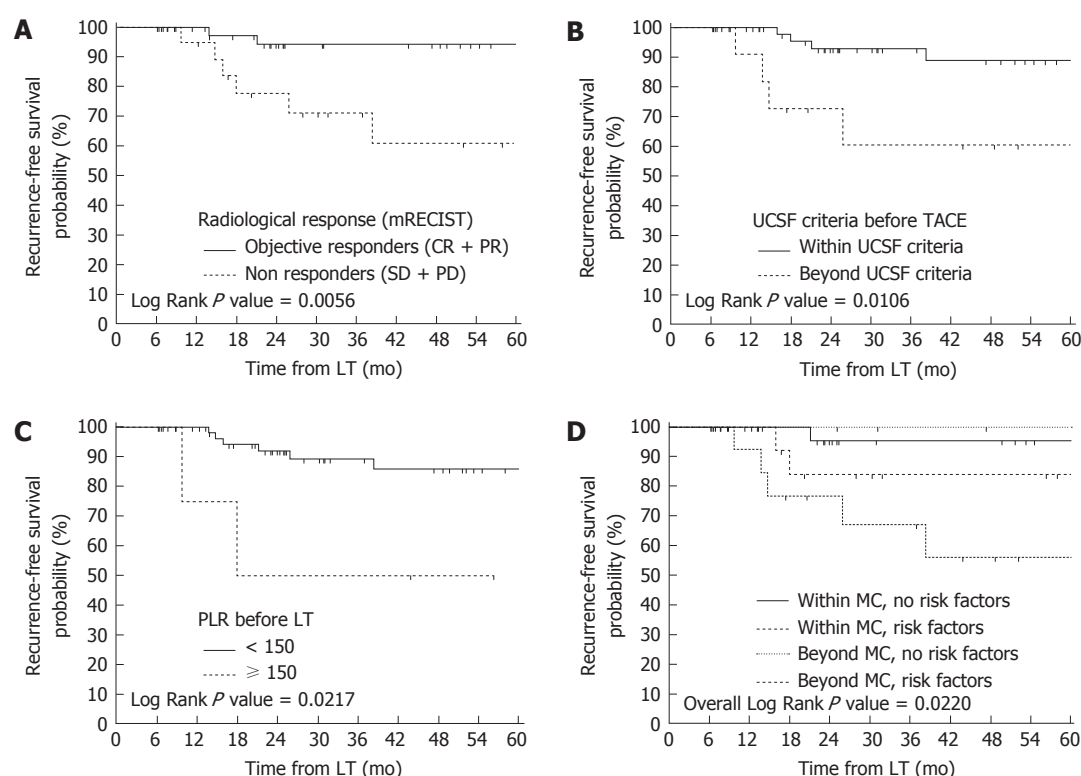
= 67.3%) (Figure 1). Interestingly, patients initially classified as MC-OUT without risk factors (7 patients) achieved excellent 3-year RFS rates (100%), similar to MC-IN patients without risk factors (95.5%) and even slightly better than patients fulfilling MC with risk factors (83.9%).

## DISCUSSION

Dimensional selection criteria, in particular MC, are widely used to define eligibility for LT in HCC patients in many transplant centres. There is increasing evidence to show that additional variables may play an important role in patient prognosis because it is recognized that some patients falling within the MC have a poor outcome following LT, whereas some patients falling outside of the criteria demonstrate a good outcome. There are several factors that affect

a patient's prognosis; in particular, a significant association has been demonstrated between the presence of mVI, poorly differentiated tumour grading and HCC recurrence<sup>[4,5]</sup>. Unfortunately, the usefulness of these variables for selecting patients for LT is limited because they usually cannot be assessed in the pre-operative setting<sup>[38,39]</sup>. Moreover, the radiological staging for HCC at referral ("inside" or "outside" MC) may be unreliable, under-staging or over-staging up to 25% of cases compared with surgical histopathology<sup>[7,8]</sup>. In the context of LRT, this discrepancy is confirmed when considering the last imaging available before LT, with incorrect radiological staging in up to 26% of patients<sup>[40]</sup>. This suggests that further variables, in addition to lesion size and number, should be considered when selecting patients for LT.

TACE is the most commonly used technique<sup>[41]</sup> for bridging or down-staging patients with HCC prior to



**Figure 1** Recurrence-free survival probabilities according to the preoperative risk factors. A: Comparison of recurrence-free survival probabilities according to the radiological response (mRECIST criteria) after transarterial chemoembolization (TACE); B: Comparison of recurrence-free survival probabilities according to the University of California, San Francisco (UCSF) criteria at imaging before TACE; C: Comparison of recurrence-free survival probabilities according to the platelet-to-lymphocyte ratio (PLR) before liver transplantation (LT); D: Risk stratification of tumour recurrence according to the Milan criteria (MC) at imaging before TACE and the presence of the independent prognostic factors identified after multivariate analysis (pre-LT PLR > 150, mRECIST non response and exceeding UCSF criteria before TACE).

LT, and the treatment response has been suggested as a surrogate marker of tumour biology and predictor of post-LT outcome<sup>[9,12,42-45]</sup>. Recently, Lai *et al.*<sup>[20]</sup> conducted a European multicentre study looking at the radiological response to LRT in 422 patients with HCC. The authors demonstrated that an AFP slope of more than 15 ng/mL per month and radiological PD according to mRECIST were unique independent risk factors for tumour recurrence and death, both in patients classified within or beyond MC before LRT. Similarly, Kim and colleagues identified that nonresponse to TACE (SD or PD) and a tumour size greater than 3 cm were preoperative predictors of recurrence in 173 patients treated with TACE before LT<sup>[15]</sup>.

However, the reproducibility of these study findings is affected by treatment heterogeneity, the centre-specific criteria for LT and the reported outcome measures (post-LT recurrence or survival, achievement of tumour necrosis and classification of radiological response). Thus, some authors stratified the radiological response according to traditional RECIST criteria, or “self-established” radiological criteria, which probably lack reproducibility in other patient cohorts. For example, Otto and colleagues defined “any progression” as any increase in the sum of the diameter of target lesions (even if less than 20%),

in contradiction to mRECIST or EASL guidelines<sup>[40]</sup>. Furthermore, the team from UCLA, who have reported on the largest single-institution series of HCC patients undergoing LRT and LT, defined the radiological response by the arterial enhancement of treated lesions [absent, possible or definite viable tumour (or new tumours)]. This seems to be an oversimplification of the commonly accepted mRECIST criteria.

The use of validated and well-defined parameters such as mRECIST or EASL guidelines has been shown to be the most accurate method of evaluating treatment response; in contrast to the RECIST criteria, they consider intratumoural necrosis when estimating a decrease in the tumour burden and not only the reduction in the overall tumour size. In our analysis, we applied these two criteria to explore their prognostic performance, inter-method agreement and accuracy in predicting different subcategories of necrosis. The agreement between the two guidelines was rated as very good for both the overall and target lesion responses; a non-response according to mRECIST criteria, namely SD or PD, was seen to be an independent prognostic factor for recurrence. This important point should be highlighted because there is currently no consensus regarding the radiological response and association with disease recurrence or patient drop-out from WL. In particular,



some authors have previously suggested that only radiological tumour progression is predictive of post-LT tumour recurrence<sup>[20,40,46]</sup>; in keeping with these findings, patients classified as SD following LRT, with no evidence of progression, may be prioritized for LT. However, in the absence of a radiological response, these patients with SD could be at a higher risk of recurrence.

Accurate preoperative radiological estimation of tumour necrosis is essential, particularly in light of recent evidence. In two large series of patients, complete or nearly complete histological response to LRT was shown to improve long-term survival after LT<sup>[47,48]</sup>. At the per-patient level, the accuracy of the mRECIST overall response was 72.9%, whereas EASL criteria correctly defined histological necrosis in 68.6% of our patients. Other studies reporting on the reliability of radiological criteria *via* a pathological-radiological correlation have reported accuracies ranging from 57% to 74.3% for EASL criteria<sup>[21,49,50]</sup> and from 67.4% to 76.3% for mRECIST criteria<sup>[16,51]</sup>. Noticeably, the unique prospective study in this field reported that mRECIST has a low accuracy (56.2%) 1 mo following radioembolization<sup>[52]</sup>. The authors concluded that neither EASL nor mRECIST criteria correctly predict the pathological necrosis.

Recently, several reports have demonstrated that increased systemic inflammation is related to the poor prognosis of various types of cancers, promoting angiogenesis and tumour invasion through the upregulation of cytokines<sup>[53-56]</sup>. The prognostic performance of NLR and PLR, two simple and easily accessible serum parameters of systemic inflammation, were tested in the clinical scenario of LT, leading to controversial results<sup>[29-31,36,37,57-59]</sup>. An intention-to-treat study by Lai *et al.*<sup>[32]</sup> demonstrated that NLR is a good predictor for dropout from the waiting list, while PLR is a good predictor of post-LT recurrence. Parisi *et al.*<sup>[33]</sup> did not confirm these findings in a study involving 150 patients fulfilling the MC. To the best of our knowledge, our study is the first of its kind to look at the combination of inflammatory markers and the tumour radiological response as competitive risk factors using multivariate analysis. As reported by other authors<sup>[32,60]</sup>, a high pre-LT PLR value was an independent risk factor for tumour recurrence in our cohort.

The definition of a well-established “upper limit” when considering patients with HCC for a down-staging protocol remains controversial. Our results suggest that applying radiological UCSF criteria before beginning TACE can provide significant prognostic information; when patients exceed the criteria, the risk of recurrence is unacceptably high. This was shown to be independent from the other static and dynamic variables included in the regression model.

This study has the following limitations: first, despite the data extraction being prospective, the

analysis was retrospective. Second, the relatively small number of patients and events (*i.e.*, recurrences) may affect the power of the study; in particular, some relevant preoperative variables (AFP and AFP slope) failed to reach statistical significance after univariate analysis. Third, as an intention-to-treat analysis of the entire WL population was not performed, the prognostic ability of the radiological and biological parameters in relation to the risk of drop-out was not tested.

However, as advised by Lai *et al.*<sup>[20]</sup>, we chose to analyse a homogeneous cohort of patients treated with only one type of LRT (*i.e.*, TACE) at a single centre to eliminate bias derived from the different treatments’ efficacy, timing and modality. The radiological response was assessed rigidly following two enhancing criteria, namely mRECIST and EASL guidelines, to make the results reproducible, and an accurate radiological-pathological analysis of explanted livers was conducted to explore the reliability of these criteria in predicting histological necrosis. Our regression analysis of risk factors for post-LT recurrence was performed considering radiological (morphological and response to therapy) and biological variables (AFP, NLR and PLR, including their slopes) at two well-defined time-points (before starting TACE therapy and immediately before LT).

In conclusion, our data suggest that patients who experience an OR to TACE according to mRECIST criteria or not exceeding a pre-LT PLR value of 150 can achieve optimal results in terms of tumour-free survival, independent from their MC status at the initial evaluation. Further studies involving larger cohorts of patients are required to validate these new parameters as selection criteria in TACE-treated candidates for LT.

## COMMENTS

### Background

Liver transplantation (LT) is the best chance of cure for patients with hepatocellular carcinoma (HCC). Transarterial chemoembolization (TACE) is widely used in HCC patients awaiting LT, to prevent tumor progression whilst on the waiting list or to downstage the tumor within the commonly accepted Milan or University of San Francisco criteria. Recently, the radiological response to TACE and others biological markers, as alpha-fetoprotein (AFP) and inflammatory markers, have been demonstrated to predict the risk of post-LT tumor recurrence better than morphological criteria; however, the use of these parameters as selection criteria for LT is still to validate.

### Research frontiers

The authors analyzed a single-center homogeneous cohort of 70 patients treated exclusively by TACE prior to LT; noticeably, 31.4% of them were beyond Milan criteria at the initial radiological evaluation. Radiological response to TACE was assessed rigidly following two enhancing criteria, namely modified Response Evaluation Criteria in Solid Tumors (mRECIST) and the European Association for the Study of the Liver (EASL) criteria, to make the results reproducible. An accurate radiological-pathological analysis of explanted livers was conducted to explore the reliability of these criteria in predicting histological necrosis. Multivariate analysis of competitive risk factors for post-LT recurrence was performed taking in account radiological (morphological and response to therapy) and biological variables at two well-defined time-points (before starting

TACE therapy and immediately before LT).

### Innovations and breakthroughs

In this manuscript, we demonstrated that a lack of response to TACE and a high platelet-to-lymphocyte ratio before surgery are strongly predictive of tumor recurrence, independently from the Milan criteria status at referral. The overall diagnostic accuracy in predicting histological necrosis was 72.9% and 68.6% for mRECIST and EASL criteria, respectively.

### Applications

This study highlights the prognostic role of 'biological' and 'dynamic' tumor parameters in HCC recurrence after LT. These preoperative factors should be integrated in the selection algorithm to increase the number of transplantable patients and to improve the recurrence-free survival rates in TACE-treated candidates for LT.

### Terminology

TACE is an image-guided, endovascular procedure that is used to treat malignant lesions in the liver, by injecting selectively small embolic particles into an artery directly supplying the tumor. These particles both block the blood supply and induce cytotoxicity by releasing chemotherapeutic drugs. Neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) have been used as markers to evaluate the systemic inflammatory responses; the theoretical background for their possible predictive value in cancer-related prognosis lies in the close association of chronic inflammation and carcinogenesis.

### Peer-review

The retrospective study was the first of its kind to look at the combination of inflammatory markers and tumor radiological response as competitive risk factors using multivariate analysis. The regression analysis of risk factors for post-LT recurrence was performed taking in account radiological (morphological and response to therapy) and biological variables (AFP, NLR and PLR, including their slopes) at two well-defined time-points (before starting TACE therapy and immediately before LT). The results are reliable and convincing, and better reference value to predict the tumor recurrence and allow a proper selection of TACE-treated candidates for LT.

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## Retrospective Study

# Surgical management of liver diseases invading the hepatocaval confluence based on IH classification: The surgical guideline in our center

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**Informed consent statement:** We conducted interventions with the consent of patients and their families. The informed consent of usage of medical records was acquired from the hospital.

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## Abstract

### AIM

To investigate the short-term outcomes and risk factors indicating postoperative death of patients with lesions adjacent to the hepatocaval confluence.

### METHODS

We retrospectively analyzed 54 consecutive patients who underwent hepatectomy combined with inferior vena cava (IVC) and/or hepatic vein reconstruction (HVR) from January 2012 to January 2016 at our liver surgery center. The patients were divided into 5 groups according to the range of IVC and hepatic vein involvement. The patient details, indications for surgery, operative techniques, intra- and postoperative outcomes were compared among the 5 groups. Univariate and multivariate analyses were performed to explore factors predictive of overall operative death.

### RESULTS

IVC replacement was carried out in 37 (68.5%) patients

and HVR in 17 (31.5%) patients. Type I2H2 had the longest operative blood loss, operative duration and overall liver ischemic time (all,  $P < 0.05$ ). Three patients of Type I3H1 with totally occluded IVC did not need IVC reconstruction. Total postoperative morbidity rate was 40.7% (22 patients) and the operative mortality rate was 16.7 % (9 patients). Factors predictive of operative death included IVC replacement ( $P = 0.048$ ), duration of liver ischemia ( $P = 0.005$ ) and preoperative liver function being Child-Pugh B ( $P = 0.025$ ).

### CONCLUSION

IVC replacement, duration of liver ischemia and preoperative poor liver function were risk factors predictive of postoperative death. We should be cautious about IVC replacement, especially in Type I2H2. For Type I3H1, it was unnecessary to replace IVC when the collateral circulation was established.

**Key words:** Hepatectomy; Inferior vena cava; Hepatic vein; Reconstruction

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**Core tip:** The proposed IH classification, which divided the patients into 5 groups according to the range of vascular invasion, may be meaningful in selecting procedures for patients with hepatocaval confluence infiltration. Inferior vena cava replacement, duration of liver ischemia and preoperative poor liver function were risk factors predictive of postoperative death for patients with lesions adjacent to the hepatocaval confluence.

Li W, Han J, Wu ZP, Wu H. Surgical management of liver diseases invading the hepatocaval confluence based on IH classification: The surgical guideline in our center. *World J Gastroenterol* 2017; 23(20): 3702-3712 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3702.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3702>

### INTRODUCTION

Liver malignancies including hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and colorectal liver metastases, combined with liver parasitic diseases like alveolar echinococcosis (AE), often show an infiltrative growth pattern. If major vessels such as the inferior vena cava (IVC) and hepatic vein adjacent to its caval confluence are invaded by these lesions, combined liver and IVC resection followed by IVC and/or hepatic outflow reconstruction with other materials is necessary to achieve R0 resection<sup>[1-3]</sup>. As a consequence of recent advances in perioperative management and surgical technique, liver and IVC resection combined with major

vascular reconstruction has become a reasonably safe treatment option with acceptable short- and long-term survival.

Preoperative portal vein embolism, associating liver partition with portal vein ligation for staged hepatectomy (commonly referred to as ALPPS), systemic chemotherapy (mainly for colorectal liver metastases) and other innovative treatments increase the tumor resectability<sup>[4-6]</sup>. Total vascular exclusion (TVE) and other vascular exclusion techniques offer chances of resection for tumor with major vascular involvement. *In situ* perfusion technique can be applied in patients with TVE longer than 60 min. Moreover, the utilization of anti-situm and *ex vivo* technique makes it easier to acquire a better operative field and obtain tumor-free surgical margins<sup>[7-9]</sup>. Venovenous bypass (VVB) is necessary in some patients under TVE with drastic hemodynamic fluctuations<sup>[7]</sup>.

Though technically challenging, hepatectomy combined with major vascular resection and reconstruction has been performed in many centers<sup>[7-10]</sup>. However, due to the lack of surgical protocols, different standards have been used in different centers. Here, we present our surgical guideline and outcomes for the combined liver and IVC resection in 54 patients with different kinds of liver lesions invading the hepatocaval confluence. The "IH classification" outlined herein was established based on our experience, and was the surgical guideline in our center.

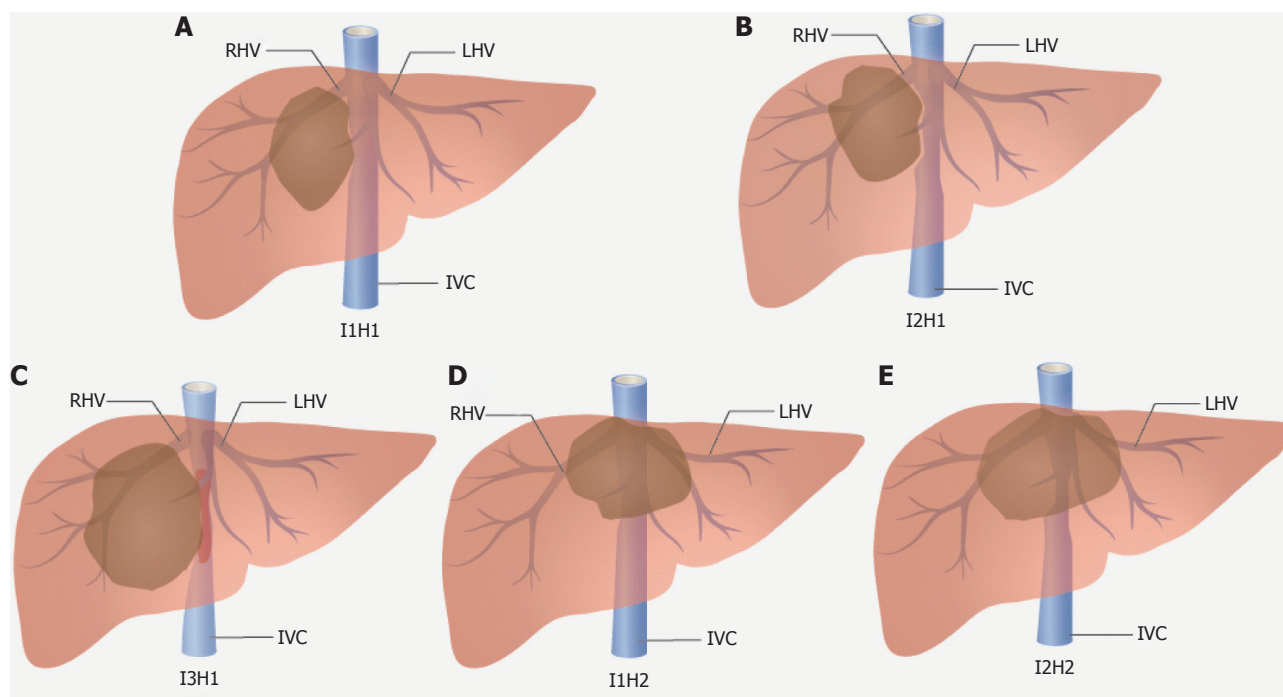
### MATERIALS AND METHODS

We retrospectively analyzed 54 consecutive patients who underwent liver resection combined with IVC resection and reconstruction from January 2012 to January 2016 at our liver surgery center at the West China Hospital, Sichuan University. Cases with IVC involvement that could be detached primarily without reconstruction were not included in this study. Patients with tumor thrombus in the IVC or hepatic veins were also excluded. The final diagnoses were confirmed by histopathological examinations after surgery. We have established classifications for this challenging situation based on our experience and the patients were divided into 5 groups according to the classifications (Figure 1). The indications for surgery of these patients are summarized in Table 1. All procedures described in this study were approved by the Ethics Committee of West China Hospital, Sichuan University.

#### Classifications for liver diseases invading the hepatocaval confluence

##### Classification based on varying degrees of IVC

**infiltration:** I1: Less than 50% of IVC circumference is involved and the IVC is not totally occluded; I2: More than 50% of IVC circumference is involved and the IVC is not totally occluded; and I3: The encroached IVC is totally occluded.



**Figure 1** Classifications of liver lesions. A: Type I1H1; B: I2H1; C: I3H1; D: I1H2; E: I2H2. RHV: Right hepatic vein; LHV: Left hepatic vein; IVC: Inferior vena cava.

**Table 1** Patients undergoing combined liver and inferior vena cava resection

Indications for surgery	n	Sex (M:F), n	Classifications				
			I1H1	I2H1	I1H2	I2H2	I3H1
Hepatocellular carcinoma	11	6:05	2	5	1	3	0
Cholangiocarcinoma	26	18:08	3	10	4	6	3
Colorectal metastases	8	5:03	1	5	0	1	1
Alveolar echinococcosis	9	5:04	3	3	0	2	1

**Classification based on hepatic outflow conditions:** H1: The hepatic outflow of the residual liver is not involved; and H2: The hepatic outflow of the residual liver is involved (3 hepatic veins are all infiltrated).

#### Preoperative management

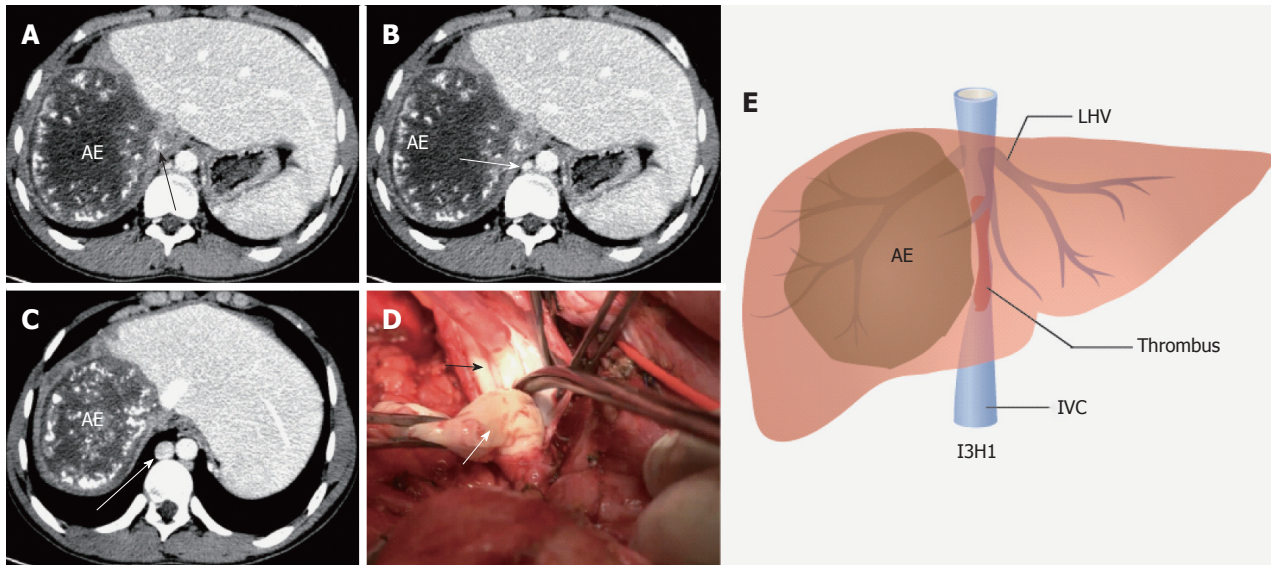
The ultrasonography and contrast computed tomography (CT) scan or magnetic resonance imaging of the abdomen were performed to evaluate the number and extent of lesions, gross type, liver volume, presence of major vascular infiltration, and regional or distant metastasis. Our standard indication for hepatectomy was Child-Pugh grade A or B, or indocyanine green retention rate at 15 min < 10%. Some patients with metastatic colorectal cancer received systemic chemotherapy after evaluation in the cancer center of our hospital, and all of them underwent colonoscopy before surgery. Our policy for indication of portal vein embolism is when the predicted future liver remnant is less than 40% of the total non-tumorous functional liver volume<sup>[11]</sup>.

#### Surgical procedures

The procedures for hepatectomy have been reported elsewhere<sup>[11,12]</sup>. Our preferred abdominal incision was J-shaped thoracoabdominal incision. After mobilization, the intraoperative ultrasound was performed routinely to confirm the number and location of lesions as well as to evaluate the relation of tumor to major vessels. The other major procedures before hepatectomy included: portal pedicle division and ligation, exposing and encircling the infra- and supra-hepatic IVC (the supradiaphragmatic IVC was encircled if the diaphragm was invaded), and dividing and ligating the short hepatic veins if possible. Liver parenchyma transaction (Pringle's maneuver was used if necessary) was carried out with the Kelly crush technique or other instruments including CUSA (Valleylab Corp., Somerville, NJ, United States) or Harmonic scalpel (Johnson & Johnson Corp., Princeton, NJ, United States). The anterior approach was used if bulky lesions resided in the right lobe of the liver.

When the critical remaining parenchyma and vascular structures were exposed, various vascular





**Figure 2** One patient with alveolar echinococcosis in the right lobe of liver. A: The IVC wall was totally occluded (longer black arrow); B and C: The azygos vein was dilated gradually (longer white arrows); D: The retrohepatic IVC was totally occluded, filled with organized thrombus (shorter white arrow). The shorter black arrow: IVC; E: Classification of this patient was I3H1. LHV: Left hepatic vein; IVC: Inferior vena cava; AE: Alveolar echinococcosis.

control techniques were applied. For type I1H1, we clamped the IVC tangentially without IVC exclusion or clamped IVC below the hepatic vein (CIBH) of the remnant liver without hepatic outflow exclusion. In our experience, if IVC involvement was less than 30% of the IVC circumference and 2 cm of the length, the defect was usually sutured transversely after removing the invaded IVC wall. If IVC involvement was 30% to 50% of IVC circumference and longer than 2 cm, we used autogenous veins such as great saphenous vein patches or expanded polytetrafluoroethylene (ePTFE; Gore-Tex, Flagstaff, AZ, United States) patches for IVC repair. As for type I1H2, IVC reconstruction was similar to type I1H1. TVE (clamping the infra-hepatic IVC, portal triad and supra-hepatic IVC sequentially) was utilized for IVC and hepatic vein reconstruction. In this type, 3 hepatic veins were involved, and the remaining stump of hepatic vein was reimplanted directly into the vena cava, or an interposed reinforced ePTFE graft. With respect to type I2H1, we used TVE or CIBH (if there was enough room below the hepatic vein) for blood control. And, if longitudinal infiltration was longer than 3 cm, IVC replacement was performed. Regarding type I2H2, TVE was necessary to complete tumor resection and vascular reconstruction. In this type, vascular reconstruction included hepatic outflow and IVC reconstruction. IVC was replaced with ePTFE tube graft and the hepatic vein was reimplanted into the ePTFE tube graft if it was totally invaded. Otherwise, if hepatic vein of the residual liver was partially involved, we used autogenous vein patches or ePTFE patches for hepatic vein plasty. With respect to I3H1, if collateral circulation including ascending lumbar veins, hemiazygos vein, and azygos vein were dilated and compensated portal hypertension and caval flow effectively, we only performed liver and IVC

resection without IVC replacement (Figure 2).

#### **Ex vivo, in situ perfusion and anti-situm technique**

*Ex vivo, in situ* perfusion and anti-situm technique were predominately used in type I2H2. *In situ* hypothermic perfusion as described by DuBay *et al.*<sup>[13]</sup> can be performed when TVE lasts longer than 60 min. When TVE was utilized, the patient's hemodynamic condition was carefully monitored and VVB (installed from the inferior mesenteric vein, and the right femoral vein to the left internal jugular vein) was applied when the patient could not tolerate the hemodynamic fluctuation. *Ex vivo* technique, which we have reported elsewhere<sup>[14]</sup>, is easier to obtain tumor-free surgical margins and reconstruct the vessels. However, given the higher complications (including bile leakage, bile duct stricture and prosthetic graft infection caused by bile leakage) associated with biliary tract anastomosis<sup>[15,16]</sup>, *ex vivo* was performed only on patients with the IVC, hepatic vein confluence, and/or portal structures infiltrated extensively. VVB was needed for most of the patients who underwent *ex vivo*. Anti-situm technique, first introduced by Pichlmayr *et al.*<sup>[17]</sup> 20 years ago, did not need to divide the portal structures. After cutting off the supra-hepatic IVC, the liver together with the IVC was rotated to the anterior position, away from their anatomic location. Then, hepatectomy could be achieved rather easily with infra-hepatic IVC and the portal triad exclusion, hypothermic hepatic perfusion and percutaneous VVB.

#### **Postoperative management**

All patients were treated with low-molecular weight heparin sodium anticoagulation solution (1 mg per kg bodyweight) from 2 d after surgery, with close monitoring. After discharge from hospital, the



patients were given warfarin (2.5 mg, qd, po) for 3 mo. Enhanced abdominal CT or ultrasonography was performed every 7 d in the first mo postoperatively to detect the patency of reconstructed vessels. For hepatitis B virus-infected patients, anti-viral drugs were applied.

Postoperative mortality was defined as death within 90 d of operation. Clavien-Dindo classification was used to classify all general complications occurring at any time during the hospital stay<sup>[18]</sup>. Liver failure was defined as peak bilirubin concentration > 7 mg/dL, peak international normalized ratio > 2.0, refractory ascites, or encephalopathy<sup>[19]</sup>. Bile leakage was defined as a drain fluid-to-serum total bilirubin concentration ratio  $\geq 3.0$ <sup>[20]</sup>. Renal insufficiency was defined as increase of serum urea and/or creatinine level (50% above the baseline). Clinically significant ascites was defined when abdominal drainage was more than 500 mL/d for longer than 3 d.

### Statistical analysis

The clinicopathologic characteristics and short-term surgical outcomes of these patients were compared among the 5 groups. Categorical variables were expressed as number and tested by chi-square test or Fisher's exact test. Continuous variables were summarized as mean (range) and tested by one-way ANOVA (Student-Newman-Keuls test was used when ANOVA was significant) or Kruskal-Wallis H rank test when necessary. The prognostic significance of the variables in predicting operative death was performed by univariate and multivariate binary logistic regression analysis. All statistical analyses were 2-tailed and *P* values < 0.05 were regarded as statistically significant. All analyses were performed by SPSS 19.0 statistical software (IBM Corp., Armonk, NY, United States).

## RESULTS

Fifty-four patients (34 males, 20 females) underwent hepatectomy combined with vascular resection and reconstruction, with a mean (range) age of 49.7 (39-72) years. The indications for surgery were: ICC (*n* = 26), HCC (*n* = 13), colorectal metastases (*n* = 8) and AE (*n* = 9) (Table 1). The intra- and postoperative data for the different types of liver lesions treated by hepatectomy combined with IVC and/or HVR are summarized in Table 2. The resection concerned 4.7 liver segments medially (range, 1-6 segments). IVC replacement was performed in 37 (68.5%) patients and HVR in 17 (31.5%) patients.

Type I2H2 had the longest operative blood loss, operation duration and overall liver ischemic time than the other 4 types (all, *P* < 0.05). The other clinical characteristics of the 5 types including tumor size, postoperative liver function, and hospital stay were listed in Table 2 in detail. Type I2H2 had the most complex procedure, which needed IVC replacement

and hepatic vein plasty (*n* = 3; with autogenous vein patches in 2 and ePTFE patches in 1) or reimplantation (*n* = 9; reimplant to ePTFE graft in 5 and to residual IVC in 4). Anti-situm (*n* = 2), *ex vivo* (*n* = 6) and *in situ* perfusion (*n* = 5) were mainly utilized in I2H2. Three patients of type I3H1 with totally occluded IVC did not need IVC reconstruction. The other 2 patients underwent IVC resection and replacement due to the uncompensated collateral circulation. The surgical procedures for the other 3 types were described in Table 2.

Total postoperative morbidity rate was 40.7% (22 patients) and the operative mortality rate was 16.7% (9 patients) (Table 3). Total morbidity and mortality rates of type I2H2 were higher than for type I1H1 (both, *P* < 0.05). Artificial graft infection (*n* = 4; 2 in type I2H2 and 2 in type I2H1), liver failure (*n* = 4; 2 in type I2H1 and 2 in type I2H2) and thrombosis of reconstructed vessels (*n* = 1; 1 in type I2H2) were the main reasons leading to postoperative death. Univariate analysis of factors predictive of death were Child-Pugh B (*P* = 0.004), IVC replacement (*P* = 0.044), duration of ischemia (*P* < 0.001) and duration of operation (*P* < 0.001) (Table 4). Factors predictive of operative death in multivariate analysis included IVC replacement (*P* = 0.048), duration of liver ischemia (*P* = 0.005) and preoperative liver function being Child-Pugh B (*P* = 0.025) (Table 5).

The median follow-up time was 20 mo (range, 2-48 mo). No patient was lost during follow-up. A total of 8 patients (3 in type I2H2, 2 in type I2H1, 2 in type I1H2, and 1 in type I1H1) died from tumor recurrence within 6 mo after the operations. Overall 1- and 3-year actuarial survival rates for HCC were 60% and 45% and for ICC were 55% and 38%. Twenty-five patients developed recurrence. Local recurrence in the liver occurred in 16 patients, in brain in 3, and in lung in 4, and abdominal cavity metastasis was detected in 2. Disease-free 1- and 3-year survival rates for patients with HCC were 18% and 8% respectively, and for patients with ICC were 16% and 9%. All AE patients were alive without recurrence and metastasis at the last follow-up.

## DISCUSSION

In the present study, 54 patients who underwent liver resection combined with IVC and/or HVR were included. ICC, HCC, AE and colorectal metastasis were the main causes leading to IVC encroachment. Undoubtedly, when liver diseases have involved the hepatocaval confluence, resection and reconstruction of the vascular structures remain technically difficult. A variety of vascular exclusion techniques, IVC reconstruction strategies, and other innovative surgical methods have brought hope for patients in this late stage<sup>[21,22]</sup>.

Due to the high postoperative morbidity and

**Table 2** Intra- and postoperative results

Variables	I1H1 (n = 9)	I2H1 (n = 23)	I1H2 (n = 5)	I2H2 (n = 12)	I3H1 (n = 5)	
					IVC resection and replacement (n = 2)	Only IVC resection (n = 3)
RL:REL:RT:LLE:LT	3:2:2:1:1	4:6:8:2:3	0:1:2:2:0	2:4:3:2:1	1:0:1:0:0	1:2:0:0:0
Tumor size (cm)	7.2 (2.9-14.3)	8.7 (7.1-15.4)	9.2 (3.9-9.9)	9.6 (7.2-16.1)	9.4 (6.6-12.2)	8.3 (7.1-10.2)
Operative blood loss (mL)	460 (310-950)	740 (450-1250)	570 (450-1050)	1020 (550-1700) <sup>a</sup>	680 (550-810)	450 (350-560)
Need for blood transfusion	1	8	0	7	1	0
Transfusion volume (mL)	400	550 (200-950)	0	600 (300-850) <sup>c</sup>	400	0
Operation duration (min)	290 (210-420)	592 (480-800)	520 (250-860)	750 (310-1150) <sup>a</sup>	580 (490-670)	320 (240-440)
No. of patients using TVE	2	23	3	12	2	0
No. of patients using PM	5	17	2	8	2	2
Duration of TVE (min)	50 (40-60)	62 (46-90)	48 (35-68)	73 (37-89) <sup>a</sup>	49 (39-57)	0
Duration of PM (min)	25 (10-35)	36 (15-45)	22.5(20-25)	38 (10-50)	30 (15-15)	30 (15-15)
Reconstruction detail	IVCR: direct suture in 4, with a patch in 5; HVR: no	IVCR: replacement; HVR: no	IVCR: with a patch in 5; HVR: reimplant to ePTFE in 1, to residual IVC in 4	IVCR: replacement; HVR: with a patch in 3, reimplant to ePTFE in 5, to residual IVC in 4	IVCR: replacement; HVR: no	IVCR: no; HVR: no
Surgical technique						
Anti-situm	0	0	0	2	1	0
<i>Ex vivo</i>	0	0	0	6	0	0
<i>In situ</i> perfusion	0	2	1	5	0	0
Postoperative liver function						
Serum maximum AST (IU/L)	460 (220-870)	557 (240-1240)	490 (230-590)	630 (330-1350)	520 (370-670)	665 (265-768)
Serum maximum ALT (IU/L)	565 (345-1350)	695 (230-1510)	520 (280-1020)	710 (340-1405)	610 (410-810)	685 (210-830)
Serum maximum PT (s)	14.1 (12.2-16.3)	15.4 (13.3-16.9)	14.8 (12.8-15.9)	15.4 (13.4-17.5)	16.4 (15.5-17.3)	15.5 (13.7-16.7)
Serum maximum TB (mmol/L)	33.4 (28.5-44.7)	36.8 (29.4-56.9)	33.7 (31.2-47.7)	45.0 (34.1-55.6)	35.0 (27.0-43.0)	33.1 (28.0-56.1)
Hospital stay (d)	11 (7-17)	15 (9-24)	12 (8-22)	19 (13-28)	14 (11-17)	16 (13-19)

Data are shown as median (range) or *n*. <sup>a</sup>*P* < 0.05 *vs* each other type; <sup>c</sup>*P* < 0.05 *vs* each other type except for I2H1. RL: Right lobectomy; REL: Right extended lobectomy; RT: Right tri-segmentectomy; LLE: Left extended lobectomy; LT: Left tri-segmentectomy; TVE: Total vessel exclusion; PM: Pringle maneuver; IVCR: Inferior vena cava reconstruction; HVR: Hepatic vein reconstruction; ALT: Alanine transaminase; AST: Aspartate transaminase; PT: Prothrombin time; TB: Total bilirubin.

**Table 3** Postoperative complications

Variable	I1H1 (n = 9)	I2H1 (n = 23)	I1H2 (n = 5)	I2H2 (n = 12)	I3H1 (n = 5)	
					IVC resection and replacement (n = 2)	Only IVC resection (n = 3)
Total number	1	8	2	8 <sup>a</sup>	2	1
Biliary leak	1	1		1		
Liver failure		2		2		
Ascites	1	4	1	2	1	1
Jaundice				1		
Hemorrhage requiring reoperation			1			
Thrombosis of reconstructed vessels						
Hepatic vein				1		
Inferior vena cava		1				
Intraabdominal abscess				1		
Reconstructed vessel infection		2		2	1	
Wound infection						
Respiratory complication		1		1		
Clavien-Dindo classification						
Grade I - II	1	7	2	7	1	1
Grade III-IV	1	3	0	4	1	0
Grade V	0	1	0	0	0	0
90-d mortality	0	4	0	5 <sup>a</sup>	0	0

Data are shown as *n*. <sup>a</sup>*P* < 0.05 *vs* I1H1; Liver failure: peak bilirubin concentration > 7 mg/dL, peak international normalized ratio > 2.0, refractory ascites, encephalopathy. Ascites: > 500 mL/d lasting longer than 3 d. IVC: Inferior vena cava.

mortality rates, though technically feasible, it remains controversial as to whether or not we should perform radical resection with vascular reconstruction for lesions invading IVC and other major vessels.

However, prognosis of malignant tumor involved IVC is unfavorable when performing hepatectomy without IVC reconstruction<sup>[10]</sup>. R0 resection combined with IVC reconstruction may have a better short- and long-

**Table 4 Univariate analysis of factors predictive of death**

	All patients ( <i>n</i> = 54)	Operative death		<i>P</i> value
		Yes ( <i>n</i> = 9)	No ( <i>n</i> = 45)	
Age (yr)	49.7 (39-72)	53(45-72)	49 (39-67)	0.249
Sex ratio (M:F)	34:20	6:3	28:17	0.801
Preoperative chemotherapy	4	1	3	1.000
Preoperative PVE	9	2	7	1.000
Tumor type				
Colorectal metastases	8	2	6	0.864
Hepatocellular carcinoma	11	2	9	1.000
Cholangiocarcinoma	26	4	22	1.000
Alveolar echinococcosis	9	1	8	1.000
Preoperative TB > 34 μmol/L	6	1	5	1.000
ICG-R15 over 10%	9	3	6	0.327
Child-Pugh B	6	4	2	0.004
No. of segments resected	4.7 (3-6)	5.1 (4-6)	4.6 (4-6)	0.189
Classifications				0.157
I1H1	9	0	9	0.328
I2H1	23	5	18	0.623
I1H2	5	0	5	0.576
I2H2	12	4	8	0.188
I3H1	5	0	5	0.576
IVC replacement				
Yes (I2 + 2 cases in I3)	37	9	28	0.044
No (I1 + 3 cases in I3)	17	0	17	
Hepatic vein reconstruction				
Yes (H2)	17	4	13	0.600
No (H1)	37	5	32	
Duration of ischemia (min)	68.7 (0-112)	87.7 (62-112)	64.9 (0-106)	< 0.001
Operative blood loss (mL)	721.5 (310-1250)	769.0 (550-1250)	712.3 (310-780)	0.389
Blood transfused amount (mL)	174.1 (0-950)	219.4 (0-950)	165.8 (0-850)	0.501
Duration of operation (min)	554.8 (210-1150)	709.6 (310-1150)	523.7 (210-860)	< 0.001
R0 resection	49	7	42	0.401
Tumor size	8.7 (2.9-16.1)	9.5 (8.8-16.1)	8.6 (2.9-15.4)	0.062

Data are shown as median (range) or *n*. PVE: Portal vein embolization; TB: Total bilirubin; ICG-R15: Indocyanine green retention rate at 15 min; IVC: Inferior vena cava.

**Table 5 Multivariate binary logistic regression analysis of factors predictive of death**

	OR (95%CI)	<i>P</i> value
IVC replacement	37.56 (1.46-945.32)	0.048
Duration of ischemia	1.65 (1.02-2.58)	0.005
Child B or C	1.82 (1.14-2.89)	0.025

IVC: Inferior vena cava.

term prognosis than cases which only underwent hepatectomy or conservative treatment, but further prospective studies are needed to investigate it.

In Table 6, we summarized the morbidity and mortality rates of the patients who underwent liver resection and IVC reconstruction in previous reports. In the present study, total postoperative morbidity rate was 40.7% (22 patients) and the operative mortality rate was 16.7% (9 patients; Table 3). Artificial vascular graft was the most commonly used material due to the shortage of xenogenous vessels and a larger surgery injury when utilizing autogenous vein<sup>[23]</sup>. Though graft infection was a life-threatening complication of artificial tube graft, many studies including ours showed that graft infection rate after artificial graft replacement was

< 10%<sup>[15,24,25]</sup>. For type I2H2, postoperative mortality rate was higher than the other types, which may be related to the longer operation time, longer ischemic time, more blood loss and higher postoperative morbidity rate. Consequently, for patients in type I2H2, it was still controversial about whether we should perform such an extensive operation.

Most of the previous studies demonstrated that it was difficult to assess IVC involvement preoperatively relying on imaging technique<sup>[15,24,25]</sup>. Though intraoperative ultrasonography and cavography were performed to help confirm the IVC invasion, the true IVC invasion rate confirmed by pathological examinations after surgery was only 60% in our study (data not shown). For malignant infiltrative-growth diseases, including ICC and AE (characterized by tumor-like growth), R0 resection was a primary goal of treatment. IVC resection was necessary when it was infiltrated or embraced by the lesions which cannot be divided totally. For HCC and colorectal liver metastases (the tumor usually compress rather than encroaches the vessels), sometimes we could not achieve R0 resection when the IVC was surrounded by the tumor; thus, IVC replacement was performed in some of these patients. Multi-organ infiltration was

**Table 6 Literature review of the reported series of hepatectomies combined with inferior vena cava resection**

Ref.	Hospital mortality	Hospital morbidity	No. alive/total (follow-up time)
DuBay <i>et al</i> <sup>[13]</sup>	11.1% (1 of gastrointestinal bleeding and multiple organ failure)	22.2%	6/9 (2-33 mo)
Malde <i>et al</i> <sup>[15]</sup>	11.4% (4 of multiple organ failure)	40.0%	16/35 (1-140 mo)
Azoulay <i>et al</i> <sup>[31]</sup>	4.5% (1 of sepsis and multiple organ failure)	64.0%	11/22 (7-84 mo)
Madariaga <i>et al</i> <sup>[21]</sup>	11.0% (1 of liver failure)	22.2%	6/9 (3-156 mo)
Giordano <i>et al</i> <sup>[25]</sup>	4.0% (1 of liver failure)	39.1%	16/23 (1-33 mo)
Hemming <i>et al</i> <sup>[24]</sup>	8.3% (liver failure and multiple organ failure)	43.0%	46/60 (median 31 mo)
Yamamoto <sup>[29]</sup>	28.6% (1 of sepsis, 1 of liver failure)	28.6%	2/7 (2-72 mo)
Lodge <i>et al</i> <sup>[10]</sup>	25% (1 of sepsis and multiple organ failure, 1 of respiratory and renal failure)	87.5%	7/8 (0.5-30 mo)

not a surgical contraindication for AE. Given the lack of alternative curative approaches, a radical operation with complete removal of the parasitic lesions was the best beneficial way to achieve radical treatment<sup>[26-28]</sup>. However, IVC resection combined with reconstruction in AE patients was still controversial considering the severe complications related to the IVC replacement.

Moreover, multivariate analysis in the present study showed that IVC replacement was a prognostic factor predictive of operative death ( $P = 0.048$ ); thus, indications of IVC replacement should be controlled strictly. In our experience, we have established the IH classification according to the range of tumor invasion. According to the extent of caval involvement, the IVC was reconstructed using a tube graft (I2), direct suture or with patches (I1). For I3 (IVC was totally occluded), if there were no symptoms and life-threatening complications associated with caval obstruction and portal hypertension (Figure 2), the IVC was removed without replacement (empirically, when renal vein pressure was  $< 40$  mmHg, the kidney function was not affected). Once the collateral circulation could not compensate the IVC stricture or occlusion, IVC replacement was necessary. In our study, 3 patients with AE were given IVC resection without reconstruction and had good short- and long-term survival. As for H1, we protected the hepatic vein of the residual liver during the operation and HVR was unnecessary. If 3 hepatic veins were involved (H2), hepatic vein plasty (with autogenous vein graft or ePTFE patches) or reimplantation (to the tube graft or residual IVC) was carried out to recover hepatic outflow. However, the criteria of IVC reconstruction in different centers are not identical due to the small sample size and patient heterogeneity (Table 7).

Vascular exclusion methods, including intermittent Pringle maneuver, TVE and CIBH, are all widely utilized in different centers<sup>[24,25,29-31]</sup>. In our study, multivariate analysis showed that duration of liver ischemia was a factor predictive of operative death ( $P = 0.005$ ). When the duration of anticipated TVE was longer than 60 min, hypothermic hepatic perfusion (University of Wisconsin solution, chilled to  $4^{\circ}\text{C}$ ) was applied to acquire an extended period of time (the longest was 102 min in our study) and protect the remnant liver. Kim *et al*<sup>[32]</sup> used a new technique of extracorporeal

hepatic venous bypass to avoid hypothermic perfusion successfully. They sutured a part of cryopreserved iliac vein to the hepatic vein stump of the remnant liver and a cannula for hepatic venous bypass was placed in it to drain the blood to the internal jugular vein. When we carried out *ex vivo* and anti-situm, consistent with some of the previous reports<sup>[10,21,29]</sup>, we used VVB if hemodynamic intolerance and splanchnic congestion occurred. Our criterion was: a decrease in mean arterial pressure  $> 30\%$  and/or a decrease in cardiac index  $> 50\%$ . However, Zhang *et al*<sup>[33]</sup> have performed *ex vivo* liver resection and liver autotransplantation without VVB in order to shorten anhepatic time. After removing en bloc liver and IVC, they replaced the IVC transiently with a tube graft before reconstructing the IVC with autogenous veins. In one of our patients, we also utilized synthetic caval graft to replace the resected part of IVC combined with transient portacaval shunt reconstruction. A vena cava vessel made by autogenous veins was applied to replace the IVC eventually. This technique is feasible and it could take place of VVB in selected patients.

If the lesions involved 3 hepatic veins at the hepatic vein confluence (H2), then *ex vivo*, *in situ* perfusion and anti-situm technique were applied. In these cases, hepatic vein reconstruction of the remnant liver should be done<sup>[17]</sup>. *In situ* perfusion and anti-situm technique were preferable for protection of the portal structures. However, if the portal triads were also involved, *ex vivo* technique had to be used. We have performed *ex vivo* liver resection followed by autotransplantation on several patients with advanced AE. The IVC were replaced using autogenous vein graft or artificial graft. We propose that AE may be a specific indication for *ex vivo* technique, with better prognosis than in malignant cancers.

In conclusion, liver resection combined with IVC and/or HVR is technically feasible with acceptable short-term survival. However, IVC replacement should be prudent as it was a risk factor related to postoperative death. In addition, preoperative liver function should be given special attention and intraoperative liver ischemia time should be shortened to reduce postoperative mortality. The proposed IH classification, which divided the patients into 5 groups according to the range of vascular invasion, may be meaningful in selecting procedures



**Table 7** Surgical technique of reported series of hepatectomies combined with inferior vena cava reconstruction

Ref.	Year	No. of cases	Indication	IVC repair type			Hepatic vein reconstruction	VVB	Perfusion	Technique	IVC reconstruction criteria
				Tube	Patch	Suture					
DuBay <i>et al</i> <sup>[13]</sup>	2009	9	IVC leiomyosarcoma = 4; ICC = 2; PCC = 1; Metastases = 1; Malignant schwannoma = 1	7	0	0	Into the native IVC = 1; Into the graft = 5; Primary repair = 1	Not described	9	<i>In situ</i> perfusion	Not described
Malde <i>et al</i> <sup>[15]</sup>	2011	35	metastasis = 21; HCC = 6; ICC = 3; Other conditions = 5	11	2	22	Not described	Not described	12	<i>In situ</i> perfusion = 13; Anti situm = 3; <i>Ex vivo</i> = 6	< 2 cm: direct suture; > 2 cm: with patches; > 50% of the circumference and longitudinally infiltration: replacement < 30% circumference: longitudinally suture; 30%-50% circumference: transversely suture; > 50% circumference: replacement
Azoulay <i>et al</i> <sup>[31]</sup>	2006	22	Metastasis = 9; ICC = 8; HCC = 2; Other cancers = 3	10	4	8	Into the native IVC = 4; Into the graft = 2	12	9	<i>In situ</i> perfusion = 9; Anti situm and <i>ex vivo</i> = 0; TVE only = 12; Others = 1	circumference: longitudinally suture; 30%-50% circumference: transversely suture; > 50% circumference: replacement
Madariaga <i>et al</i> <sup>[21]</sup>	2000	9	Metastasis = 1 IVC leiomyosarcoma = 3; ICC = 3; other cancers = 2	8	0	1	Into the graft = 1; Primary repair = 1	1	0	<i>In situ</i> perfusion, Anti situm and <i>ex vivo</i> = 0; TVE only = 3	Not described
Giordano <i>et al</i> <sup>[25]</sup>	2011	23	Metastases = 13; ICC = 3; HCC = 4; Others = 3	7	0	16	Into the graft = 1	4	4	<i>In situ</i> perfusion = 4; Anti situm = 0; <i>Ex vivo</i> = 0	< 30% of the circumference: suture; > 50% of the circumference: replacement
Hemming <i>et al</i> <sup>[24]</sup>	2012	60	ICC = 26; HCC = 16; Metastases = 13; Others = 5	38	14	8	Into the graft = 4	6 ( <i>ex vivo</i> )	8	<i>In situ</i> perfusion = 8; <i>Ex vivo</i> = 6; Anti situm = 0	< 3 cm longitudinally: end-to-end anastomosis; > 5 cm sections of the anterolateral wall: with patches; 3-8 cm longitudinally : replacement > 50% of the circumference: replacement < 60°
Yamamoto <sup>[29]</sup>	2012	7	ICC = 2; HCC = 5	4	1	2	Into the graft = 4	0	7	Anti-situm = 7	circumferentially and < 2 cm longitudinally: clamp tangentially
Lodge <i>et al</i> <sup>[10]</sup>	1999	8	Metastasis = 8	3	4	1	Into the native IVC = 1; Into the graft = 3	6 (4 <i>ex vivo</i> and 2 TVE)	Not described	<i>Ex vivo</i> = 4; TVE only = 4; Anti situm = 0	

PCC: Perihilar cholangiocarcinoma; IVC: Inferior vena cava; HCC: Hepatocellular carcinoma; ICC: Cholangiocarcinoma.

for patients with hepatocaval confluence infiltration. However, due to the small sample size and patient heterogeneity in the present study, this classification still needs to be investigated in more studies. For example, IVC replacement and HVR must be applied in type I2H2 patients to achieve R0 resection. Nevertheless, such an aggressive treatment is controversial for colorectal liver metastasis and HCC because alternative treatment approaches with lower morbidity and mortality could be

applied. Consequently, the proposed IH classification describes anatomic issues but may not have identical significance in guiding surgical approach and indicating postoperative prognosis in different liver diseases.

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## COMMENTS

### Background

Though technically challenging, hepatectomy combined with major vascular resection and reconstruction has been performed in many centers because it was the only way to achieve R0 resection. However, the surgical indications and protocols were different and controversial in different centers.

### Research frontiers

The authors investigated the short-term outcomes and risk factors predictive of postoperative death for patients with lesions adjacent to the hepatocaval confluence.

### Innovations and breakthroughs

The authors established the "IH classification" dividing the patients into 5 groups according to the range of vascular invasion, which was meaningful in selecting procedures for patients with hepatocaval confluence infiltration.

### Applications

In this study, the authors present their surgical guideline and outcomes about the combined liver and inferior vena cava (IVC) resection in 54 patients with different kinds of liver lesions invading the hepatocaval confluence. The IH classification was established based on our experience, which can be a reference for other surgeons.

### Peer-review

This is an interesting paper reviewing the center's experience with a very challenging group of patients with liver malignancies and IVC or hepatic vein involvement.

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## Observational Study

# Study on the value of serum miR-106b for the early diagnosis of hepatocellular carcinoma

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**Author contributions:** Shi BM, Lu W and Wang XY performed the majority of experiments; Shi BM, Lu W, Ji K, Wang YF, Xiao S and Wang XY provided vital reagents and analytical tools and were also involved in editing the manuscript; Wang XY coordinated and provided the collection of all the human material in addition to providing financial support for this work; Shi BM, Lu W and Wang XY designed the study and wrote the manuscript; Shi BM and Lu W contributed equally to this study.

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## Abstract

### AIM

To analyze the incidence of hepatocellular carcinoma (HCC) in a population that underwent health checkups and had high serum miR-106b levels.

### METHODS

A total of 335 subjects who underwent checkups in the Digestive and Liver Disease Department of our hospital were randomly selected. RT-PCR was used to detect the level of miR-106b in serum samples. Laboratory and imaging examinations were carried out to confirm the HCC diagnosis in patients who had a > 2-fold change in miR-106b levels. Ultrasound-guided biopsy was also used for HCC diagnosis when necessary. On this basis, the clinical data of these subjects, including history of hepatitis virus infection, obesity, long-term history of alcohol use and stage of HCC, were collected. Then, the impact of these factors on the level of miR-106b in serum was analyzed. Furthermore, receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic efficacy of miR-106b for HCC.

### RESULTS

A total of 35 subjects had abnormal serum miR-106b



levels, of which 20 subjects were diagnosed with HCC. *t*-test revealed that the difference in serum miR-106b level in terms of sex, age, history of hepatitis virus infection, obesity and long-term history of alcohol use was not statistically significant. However, serum miR-106b levels in patients with advanced HCC (stage III/IV) was higher than in patients with early HCC (stage I/II), and the difference was statistically significant ( $P = 0.000$ ). Moreover, the ROC curve revealed that the area under the curve value for miR-106b was 0.885, which shows that serum miR-106b level has a certain clinical value for HCC diagnosis.

## CONCLUSION

The random sampling survey shows that serum miR-106b level is a valuable diagnostic marker for HCC. However, the diagnostic threshold value needs to be further researched.

**Key words:** MiR-106b; Hepatocellular carcinoma; ROC curve; Random sampling survey

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**Core tip:** Primary hepatocellular carcinoma (HCC) is the cause of a large number of patient deaths each year, and presents a heavy financial burden to the community and family. MicroRNAs (miRNAs) have been proven to be involved in the development of various cancers, as well as during the development process. Among these miRNAs, miR-106b has been shown to be a potential diagnostic marker for early HCC. We randomly selected the sera of medical examiners and detected their miR-106b level and further verified the diagnostic value of miR-106b, and provided more data to support the clinical application of miR-106b in evidence-based medicine.

Shi BM, Lu W, Ji K, Wang YF, Xiao S, Wang XY. Study on the value of serum miR-106b for the early diagnosis of hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(20): 3713-3720 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3713.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3713>

## INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, is the cause of a large number of patient deaths each year and presents a heavy financial burden to the community and families<sup>[1,2]</sup>. The incidence of HCC in China has shown an increasing trend year by year, and the main risk factor of hepatitis B infection has gradually transformed into alcohol and other non-viral infectious factors<sup>[3-7]</sup>. For the occult onset of HCC, many patients with advanced HCC obtain their initial diagnosis after

the best treatment window for HCC has closed<sup>[8-11]</sup>. Therefore, there is an urgent clinical need for effective diagnostic indicators for the early diagnosis of HCC.

At present, B ultrasound and serum alpha-fetoprotein (AFP) are mainly used by medical personnel for initial screening. However, these two methods have the shortcoming of poor sensitivity, and are insufficient for early warning in high-risk groups<sup>[12]</sup>. MicroRNAs (miRNAs) are a class of 18-24 nucleotide short-chain non-coding RNAs that have been proven to be involved in the development of various cancers, as well as during the normal organismal development process. Tumor cells may also be able to release miRNA into the bloodstream, and such circulating miRNAs have become a hot topic in recent years.

Serum miRNA has a high stability, is resistant to RNase hydrolysis, and its contents relatively remain at a constant level after repeated freezing and thawing. Among these miRNAs, miR-106b has been shown to be a potential diagnostic marker for early HCC<sup>[13-19]</sup>. However, such studies involved mostly targeted subjects and were not conducive for evaluating the true diagnostic efficacy of miRNA. Hence, there is a need to conduct a random sampling survey of the physical examination blood samples of patients with digestive and liver disease, in order to determine whether miR-106b can be used as an early warning indicator of HCC.

In this study, we randomly selected the sera of medical examinees and detected their miR-106b level. The level of miR-106b in serum of patients with HCC was further analyzed. Furthermore, the significance of miR-106b levels in the diagnosis of HCC was further analyzed. The results of this study further verified the diagnostic value of miR-106b, and provided more data to support the clinical application of miR-106b in evidence-based medicine.

## MATERIALS AND METHODS

### Inclusion and exclusion criteria

Inclusion criteria: (1) randomly selected serum samples from outpatients who underwent physical examination in our hospital between January 2011 and August 2015; (2) clinical information is complete; and (3) time within 1 mo after the serum test and willing to accept further physical examination and follow-up. Exclusion criteria: (1) liver cancer patients; (2) incomplete information; and (3) refusal to accept further physical examination and follow-up.

### General information

A total of 335 serum samples were selected at different time intervals, and the mean age of the subjects was  $55.4 \pm 10.7$  years. The relative content of miR-106b was significantly increased two-times or more in 35 subjects. Among these 35 subjects, 21 were male and 14 were female. This study was approved by the

Ethics Committee, and all patients signed an informed consent form.

### Methods

**Clinical research methods:** Real-time quantitative PCR detecting system (qPCR) was used to detect miRNA levels in serum samples. MiR-106b was amplified by selecting cel-miR-39 as an internal reference. The relative expression of miR-106b in the serum sample was calculated using the  $2^{-\Delta\Delta Ct}$  method. When the relative levels of miR-106b were significantly more than double, the notification to participate in the group was further reviewed. The patients were diagnosed with HCC after follow-up and were divided into two groups: non-HCC group and HCC group. Follow-up records included the patient's age, sex, obesity status, history of hepatitis, and long-term drinking history. Patients diagnosed with HCC were actively treated.

### Detection of serum miR-106b relative content:

Serum total RNA extraction was performed. First, blood samples were collected using vacuum blood collection tubes, and were left at room temperature for 40 min. Then, the supernatants were collected by centrifugation (3000 rpm at 4 °C) and stored at -80 °C until use. The extraction of total RNA in serum was performed according to kit instructions (miRNeasy Serum/Plasma Kit; Qiagen, Hilden, Germany).

Serum samples were supplemented with 1000  $\mu$ L of QIAzol (lysate). After standing for 5 min, 3.5  $\mu$ L of miRNeasy Serum/Plasma Spike-In Control was added (concentration of  $1.6 \times 10^8$  copies/L) as a control for RNA purification yield and amplification efficiency. This was followed by the addition of chloroform at room temperature for 3 min. The 600  $\mu$ L supernatant was mixed well with 900  $\mu$ L of absolute ethanol, and transferred to an RNeasy MinElute spin column and centrifuged at 12000 rpm at 4 °C. This was followed by RWT, RPE buffer and ethanol elution phenol, and other organic reagents, and finally with 14  $\mu$ L of DEPC water-eluting RNA. The resultant products were stored at -80 °C until use.

The RT miRNA tailing method was performed next. The tailing method was used to reverse transcribe miRNA into cDNA, and the operation was carried out according to the instructions of the reverse transcription kit (MiScriptII RT Kit; Qiagen). Briefly, the RNA was extracted according to instructions, and the other solutions were prepared for use in the reverse transcription system. Then, the reaction system was placed on the PCR instrument for amplification. The resulting cDNA was stored at -80 °C until use.

Real-time fluorescence quantitative PCR was performed finally. The relative content of miR-106b was measured according to miRNA SYBR Green PCR detection kit instructions (Qiagen). Reactions were performed on an ABI 7500 Real Time PCR instrument. According to the relevant literature, cel-miR-39 was

selected as the internal reference. MiR-106b was amplified. Then, the relative expression of miR-106b in serum samples was calculated using the  $2^{-\Delta\Delta Ct}$  method ( $\Delta Ct = Ct_{(miR-106b)} - Ct_{(cel-miR-39)}$ ).

### Retrospective evaluation of HCC patients with abnormal serum miR-106b levels:

When the relative levels of miR-106b significantly more than doubled, the patient was followed-up and notified to further confirm the suspected population prevalence of HCC. Further diagnosis of suspected patients was conducted according to the Chinese Ministry of Health guidelines issued in 2011 for the diagnosis and treatment of primary liver cancer patients. Clinical symptoms, blood biochemical examination [aspartate aminotransferase (AST) or glutamic oxaloacetic transaminase], tumor marker detection (serum AFP and its heteroplasm), and imaging studies were applied on the risk assessment and diagnosis of suspected cancer patients. B-ultrasound and computed tomography imaging were used as basis for the early diagnosis of liver cancer. The imaging diagnosis process was completed by an ultrasound physician and associate professors, in order to ensure accuracy of the diagnostic results.

### Analysis of the effect of common factors on serum miR-106b

History of hepatitis, obesity, drinking and other factors were evaluated to determine whether these also caused any change in serum miR-106b. Furthermore, related medical history and clinical signs were collected and analyzed statistically.

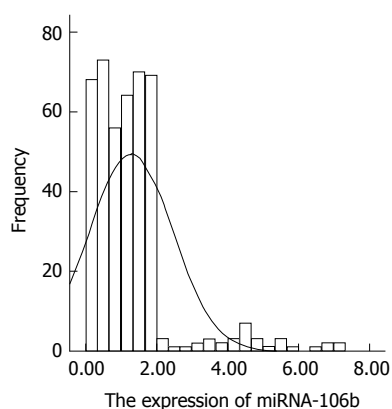
### Statistical methods

SPSS 19.0 software was used to analyze the data. Measurement data were expressed as mean  $\pm$  SD. Subjects were divided into two groups according to clinical examination results: early HCC group and non-HCC group. *t*-test was used to compare the basic clinical data between the two groups. On this basis, the curve derived from the patient (receiver operating characteristic (ROC) curve) was used to evaluate the diagnostic efficacy of miR-106b.  $P < 0.05$  was considered statistically significant.

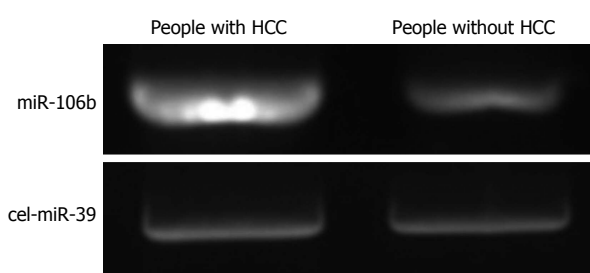
## RESULTS

### Serum levels of miRNA-106b

The distribution of miRNA-106b in serum samples obtained from patients is shown in Figure 1. The Kolmogorov-Smirnov test revealed normal distribution of the data ( $P = 0.000$ ). The relative serum miRNA-106b expression level of all the subjects was  $1.12 \pm 0.89$  times. MiRNA-106b increased more than two-times as an early warning indicator; a total of 35 subjects were suspected during the review, and results revealed that 25 of these subjects were diagnosed



**Figure 1** Serum miRNA-106b levels of patients that underwent health checkups.



**Figure 2** Results of qPCR.

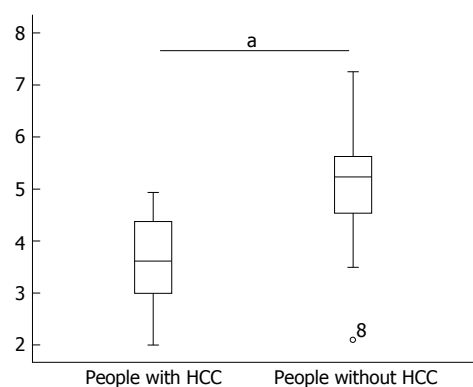


**Figure 3** Early hepatocellular carcinoma B-ultrasound results.

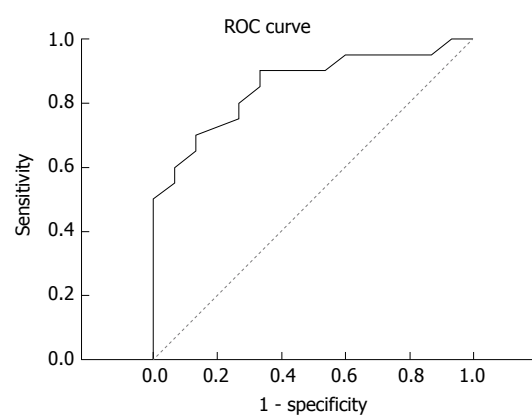
with HCC. The qPCR results of typical case samples are shown in Figure 2. The difference in cel-miR-39 in these two typical patients was not statistically significant, while the content of miRNA-106b in patients with hepatitis B was significantly higher than that in normal patients. Serum miRNA-106b increased in some of the suspected patients during the review as shown by ultrasound results (Figure 3). Early liver cancer lesions < 3 cm could be observed through ultrasound. There is a need for such to be carefully identified before they can be located.

#### **Analysis of the diagnostic efficacy of serum miR-106b**

Patients were divided into two groups according to the



**Figure 4** Difference in miRNA-106b expression levels between the two groups were statistically significant ( $^aP < 0.001$ ).



**Figure 5** Receiver operating characteristic curve analysis of the differences in miRNA-106b expression.

review results, and the difference in serum miR-106b levels between these two groups is shown in Figure 4.

The *t*-test results revealed that patients with HCC had higher serum miR-106b levels than patients without HCC, and the difference was statistically significant ( $P = 0.000$ ).

On this basis, the diagnostic efficacy of serum miRNA-106b was analyzed through the ROC curve. Results revealed that the area under the curve was 0.855, and sensitivity and specificity were 90.0% and 66.7%, respectively (Figure 5).

#### **Clinical parameter analysis of subjects with elevated serum miRNA-106b levels**

In clinical practice, obesity, viruses and alcohol cause liver damage, as well as changes in serum markers. Therefore, analyzing whether miR-106b is affected by these factors is of great significance for the diagnosis of HCC. The clinical data of the two groups of subjects are shown in Table 1. Age, sex, obesity, history of hepatitis and history of alcohol consumption had no significant effect on miRNA-106b expression ( $P > 0.05$ ). However, serum miRNA-106b levels were significantly higher in patients with higher tumor stages (III/IV) than in patients in stage I/II ( $P = 0.000$ ).

**Table 1 Clinical parameters of suspected subjects with elevated miRNA-106b levels**

Clinical parameter	n (%)	miRNA-106b level	t value	P value
History of hepatitis				
Have	20 (57.14)	4.62 ± 0.91	0.532	0.598
None	15 (42.86)	4.45 ± 0.97		
Long-term drinking history				
Have	24 (68.57)	4.72 ± 0.92	1.378	0.177
None	11 (31.43)	4.29 ± 0.69		
Obesity				
Have	16 (45.71)	4.62 ± 0.90	0.986	0.331
None	19 (54.29)	4.35 ± 0.72		
Liver cancer stage				
I / II	33 (94.26)	4.64 ± 0.86	-4.200	0.000
III / IV	2 (5.74)	7.23 ± 0.02		

## DISCUSSION

HCC is one of the most common types of malignant tumors<sup>[11,20-22]</sup>. A survey has shown that HCC ranks second among deaths caused by diseases annually. Risk factors for liver cancer vary in different regions. In China, there is a high prevalence of liver cancer caused by hepatitis B.

Hence, liver cancer has become a major threat to national health. According to statistics, the number of patients with liver cancer in China has reached up to 50% of the cases worldwide<sup>[9,23-29]</sup>. At present, with tumor resection surgery, interventional techniques and the development of liver transplantation technology, the treatment of liver cancer has made remarkable progress.

However, even so, the survival rate of patients with liver cancer was not significantly improved. Furthermore, according to relevant studies on liver cancer patients, the overall 5-year survival rate is approximately 5%-9%<sup>[30-35]</sup>. The main reason for this startling statistic is that liver cells have good compensatory function, so that no obvious imaging and serological changes are found in early liver cancer patients during routine physical examination. Obvious clinical signs often manifest in the advance stage.

Therefore, clinically, there is an urgent need to develop high sensitivity and specificity diagnostic indicators for patients who may be suffering from liver cancer, in order to provide early warning of the disease in this population. At present, medical examiners mainly rely on B-ultrasound and serum AFP levels during the physical examination of high-risk groups for early warning. However, the accuracy of B-mode ultrasonography is directly related to the patient's experience. Furthermore, the sensitivity and specificity of serum AFP diagnosis is low, which limits the diagnosis of early liver cancer and early treatment, and affects the prognosis of patients.

MiRNA is a non-coding small RNA with a length of approximately 22 nucleotides. MiRNAs can adjust

the level of physiological functions in the cell after transcription, including that of cell proliferation, differentiation and apoptosis. Research has shown that the growth of tumor cells is subject to the corresponding miRNA regulation, such as miR-26a/b and miR-146b-5p, which affects the growth of tumor cell cycle regulation. MiR-7 inhibits tumor growth by regulating the PI3K/AKT/mTOR pathway during the migration process. Furthermore, miR-1826 can affect angiogenesis by down-regulating VEGFC.

More studies have found that tumor cells can release miRNA into the circulatory system. Furthermore, miRNA induces changes in blood, leading to tumor occurrence; hence, this development has important relevance<sup>[36-43]</sup>. In patients with liver cancer, miR-21, miR-122, miR-1, miR-25, miR-92a, miR-206, miR-106b and let-7f have been found to have potential as diagnostic indicators of liver cancer, with diagnostic sensitivity of  $\geq 80\%$ <sup>[13]</sup>. However, since many studies have involved targeted selected subjects, this resulted in the existence of certain bias in the selection process, which is not conducive to determining the true diagnosis of the evaluation of miRNA efficacy.

Therefore, in this study, we selected miR-106b as the research focus due to its high diagnostic value. Through the random screening of blood samples conducted by the medical staff, the efficacy of serum miR-106b in the diagnosis of liver cancer was analyzed, with a view of making some preliminary experiments for the entry of miRNA into routine laboratory diagnosis.

### Survey results and the detection rate of liver cancer

The miR-106b gene is located on human chromosome 7, as well as in glioma, prostate cancer and other tumors<sup>[44]</sup>. In HCC tissues, a study found that miR-106b can inhibit the APC gene and thereby promote cancer cell growth; and it can also significantly increase the serum miRNA of patients with liver cancer<sup>[44]</sup>. There are some controversies regarding the diagnostic limits of miR-106b in different studies. However, the relative serum miR-106b expression of patients with HCC was more than double in most studies<sup>[44-46]</sup>. Therefore, in order to include as many potential HCC patients as possible, in this study, relative serum miR-106b expression elevated above two-times was selected as the indicator. Furthermore, in this study, among the 35 subjects suspected to have miR-106b abnormalities, 20 were diagnosed with late stage HCC. The relative miR-106b expression level in subjects in the HCC group was significantly higher than that in the non-HCC group. These results suggest that miR-106b may serve as a potential biomarker for HCC.

### Evaluation of the diagnostic efficacy of miR-106b

In each study, the serum miRNA diagnosis threshold differs greatly depending on the subjects studied. In the study conducted by Choo *et al.*<sup>[47]</sup>, the sensitivity



and specificity of serum miRNA for the diagnosis of HCC was 81% and 97%, respectively. In the present study, the ROC curve revealed that the area under the curve of miR-106b was 0.855, and sensitivity and specificity were 90.0% and 66.7%, respectively. This suggests that it has a certain diagnostic value. However, there may be a need to diagnose with other diagnostic indicators, in order to improve the specificity of liver cancer diagnosis. Although the present study was carried out with a large-scale sample survey, only 20 people were diagnosed with liver cancer among the 35 suspected subjects (accuracy rate: 57.14%). Therefore, the limits of the diagnostic value of miR-106b needs to be further studied with an expanded sample size.

In the study of the accuracy of AFP as a routine physical examination index, results revealed that serum miRNA significantly increased in these patients, and the difference in AFP was not significant. Furthermore, if 400 ng/mL were used as a diagnostic threshold for conventional diagnostic criteria, this would result in the missed opportunity to detect 6 patients with liver cancer. For early screening examinations, B-ultrasound is a routine diagnostic. However, in the absence of other high-risk diagnostic indicator warnings, physicians in the actual inspection process can easily misdiagnose the location of hidden and small ranges of early liver cancer.

If the presence of diagnostic markers predicts the risk of HCC, higher-resolution B-mode ultrasonography can be used to increase the detection rate in patients with early HCC. Taking into account that blood sample collection is a routine physical examination, as well as the high stability of miRNA in serum samples and other characteristics, miR-106b is suitable for use with AFP and other serum markers associated with early warning indicators for liver cancer. However, further studies are needed to determine the warning limits for miR-106b.

### **Analysis of influencing factors of serum miR-106b levels**

Liver cancer is an occult onset process. Furthermore, it is not easy to distinguish patients with early liver cancer from with patients with chronic liver disease through routine blood biochemistry and laboratory tests. In addition, obesity-induced fatty liver, virus-induced hepatitis and alcohol can induce liver damage, and at the same time can cause serological AFP, AST and other related enzymatic indicators to change<sup>[48-52]</sup>. Therefore, analyzing whether miR-106b is affected by these factors is of great significance in the diagnosis of HCC.

By comparing the miR-106b level of the suspected subjects, we found that in addition to tumor stage, hepatitis, obesity and long-term drinking history would not cause significant differences in miR-106b level. However, if liver cancer patients had a higher tumor stage, this significantly increased the serum miR-106b

level; this finding is consistent with relevant literature reports, suggesting that in the future appropriate research should be conducted on the relationship between miRNA and tumor staging, in order to further analyze the value of serum miRNA for evaluating the condition of patients.

At the same time, this subject matter also has certain limitations. First of all, it is limited by objective conditions. In this study, only one miRNA was included in the assessment, and a single miRNA study does not accurately assess the value of the entire miRNA family for diagnosis. Hence, more representative miRNAs should be included in the study. At the same time, since the truncation values for miR-106b remain controversial, referral indicators used in this study may not be the optimal diagnostic threshold. This would induce the detection rate in subjects with suspected liver cancer to decline. Hence, these optimal diagnostic thresholds also need to be determined in larger clinical studies.

In summary, in this study, miR-106b was used as the research object, and the value of miRNAs in the diagnosis of HCC was evaluated by random sampling. miR-106b has the potential to become an early serologic diagnostic marker for HCC.

## **COMMENTS**

### **Background**

Primary hepatocellular carcinoma (HCC) is the cause of a large number of patient deaths each year, and presents heavy financial burden to the community and family. MicroRNAs (miRNAs) have been proven to be involved in the development of various cancers, as well as during the normal organismal development process. Among these miRNAs, miR-106b has been shown to be a potential diagnostic marker for early HCC.

### **Research frontiers**

Although the treatment of liver cancer has made remarkable progress, the survival rate of patients with liver cancer has not significantly improved. miRNA is a non-coding small RNA with a length of approximately 22 nucleotides. Moreover, studies have shown that tumor cells can release miRNA into the circulatory system, which means that they have the potential to forecast the occurrence of HCC.

### **Innovations and breakthroughs**

In this study, miR-106b was chosen as the research object due to its high diagnostic value. The authors randomly selected the sera of medical examinees and detected their miR-106b level and further verified the diagnostic value of miR-106b, and provided more data to support the clinical application of miR-106b in evidence-based medicine.

### **Applications**

This survey shows that serum miR-106b level is a valuable diagnostic marker for HCC. However, the diagnostic threshold value needs to be further researched.

### **Peer-review**

Due to its high diagnostic value, serum miR-106b was chosen as the focus of this research. This study further showed that serum miR-106b level is a valuable diagnostic marker for HCC. However, more research should be carried to find out the diagnostic threshold value.

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## Prospective Study

# Clinical significance of expression of proliferating cell nuclear antigen and E-cadherin in gastric carcinoma

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## Abstract

### AIM

To investigate the expression of proliferating cell nuclear antigen (PCNA) and E-cadherin in gastric carcinoma and to analyze their clinical significance.

### METHODS

A total of 146 patients were selected for this study, including 38 patients with intestinal metaplasia, 42 with dysplasia, and 66 with primary gastric cancer. In addition, 40 patients with normal gastric tissues were selected as controls. The expression of PCNA and E-cadherin was detected by immunohistochemistry. Differences in PCNA and the E-cadherin labeling indexes among normal gastric mucosa, intestinal metaplasia, dysplasia, and gastric carcinoma were compared. Subjects with normal gastric tissues were assigned to a normal group, while gastric cancer patients were assigned to a gastric cancer group. The difference in PCNA and E-cadherin expression between these two groups was compared. The relationship between expression of PCNA and E-cadherin and clinicopathological features was also explored in gastric cancer patients. Furthermore, prognosis-related factors, as well as the expression of PCNA and E-cadherin, were analyzed in patients with gastric cancer to determine



the 3-year survival of these patients.

## RESULTS

The difference in PCNA and the E-cadherin labeling indexes among normal gastric mucosa, intestinal metaplasia, dysplasia, and gastric carcinoma was statistically significant ( $P < 0.05$ ). During the transition of normal gastric mucosa to gastric cancer, the PCNA labeling index gradually increased, while the E-cadherin labeling index gradually decreased ( $P < 0.05$ ). The PCNA labeling index was significantly higher and the E-cadherin labeling index was significantly lower in gastric cancer than in dysplasia ( $P < 0.05$ ). The expression of PCNA was significantly higher in the gastric cancer group than in the normal group, but E-cadherin was weaker ( $P < 0.05$ ). There was a negative correlation between the expression of PCNA and E-cadherin in gastric carcinoma ( $r = -0.741$ ,  $P = 0.000$ ). PCNA expression differed significantly between gastric cancer patients with and without lymph node metastasis and between patients at different T stages. E-cadherin expression also differed significantly between gastric cancer patients with and without lymph node metastasis ( $P < 0.05$ ). High T stage and positive PCNA expression were risk factors for the prognosis of patients with gastric cancer ( $RR > 1$ ), while the positive expression of E-cadherin was a protective factor ( $RR < 1$ ). The sensitivity, specificity, and accuracy of PCNA positivity in predicting the 3-year survival of patients with gastric cancer were 93.33%, 38.89%, and 0.64, respectively; while these values for E-cadherin negativity were 80.0%, 41.67%, and 0.59, respectively. When PCNA positivity and E-cadherin negativity were combined, the sensitivity, specificity, and accuracy were 66.67%, 66.67%, and 0.67, respectively.

## CONCLUSION

Combined detection of PCNA and E-cadherin can improve the accuracy of assessing the prognosis of patients with gastric cancer.

**Key words:** Proliferating cell nuclear antigen; E-cadherin; Gastric cancer; Gastric mucosa

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**Core tip:** The expression of proliferating cell nuclear antigen (PCNA) and E-cadherin was detected by immunohistochemistry in gastric tissues of 186 patients. During the transition of normal gastric mucosa to gastric cancer, the PCNA labeling index gradually increased, while the E-cadherin labeling index gradually decreased ( $P < 0.05$ ). There was a negative correlation between the expression of PCNA and E-cadherin in gastric carcinoma. High T stage and positive PCNA expression were risk factors for the prognosis of patients with gastric cancer ( $RR > 1$ ), while the positive expression of E-cadherin was a protective factor ( $RR < 1$ ). Combined detection of PCNA and E-cadherin can improve the accuracy of assessing the prognosis of patients with gastric cancer.

Hu L, Li HL, Li WF, Chen JM, Yang JT, Gu JJ, Xin L. Clinical significance of expression of proliferating cell nuclear antigen and E-cadherin in gastric carcinoma. *World J Gastroenterol* 2017; 23(20): 3721-3729 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3721.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3721>

## INTRODUCTION

Gastric cancer is a common digestive system malignancy that progresses rapidly<sup>[1-3]</sup>. Its occurrence and development are a very complex process that involves the dysregulation of a variety of oncogenes and tumor suppressor genes<sup>[4-7]</sup>. At present, an increasing number of scholars have focused their attention on exploring protein and gene markers, in order to help clinicians early and accurately diagnose gastric cancer and assess its prognosis.

E-cadherin has been known as an epithelial cell adhesion molecule. A decrease in E-cadherin expression allows tumor cells to easily transfer and invade. Hence, E-cadherin has been identified as a metastatic suppressor of cancer cells<sup>[8,9]</sup>. Gastric cancer is a malignant tumor that originates from gastric epithelial cells. It has been reported that E-cadherin expression decreases in gastric cancer tissues, and that decreased E-cadherin expression correlates with high degree of malignancy and poor prognosis in patients with gastric cancer<sup>[8]</sup>.

Proliferating cell nuclear antigen (PCNA) is a cell proliferation-associated protein. PCNA expression is associated with metastases of breast cancer, liver cancer and other malignancies, as well as tumor infiltration<sup>[10-13]</sup>. However, the expression of PCNA in gastric cancer and its clinical significance remain to be further studied.

In the present study, we detected the expression of PCNA and E-cadherin in gastric tissues of patients with gastric precancerous lesions or gastric cancer. We also evaluated the correlations of PCNA and E-cadherin expression with clinicopathological features and survival in patients with gastric cancer, with an aim to determine their clinical and prognostic significance in this malignancy.

## MATERIALS AND METHODS

### Patients

One hundred and forty-six patients who underwent gastric surgery at our hospital from March 2012 to September 2013 were included in this observational study. These patients were pathologically diagnosed with intestinal metaplasia ( $n = 38$ ), dysplasia ( $n = 42$ ), or primary gastric cancer ( $n = 66$ ). Forty patients with normal gastric tissues, who underwent gastrectomy during the same period, were included as controls. The inclusion criteria were: (1) patients who did not

receive preoperative radiotherapy, chemotherapy, or other anti-cancer treatments; (2) patients with a clear pathological diagnosis; (3) patients without other malignancies; (4) patients who were followed for > 3 years (the deadline for the follow-up was the time of death) and had complete medical records. Among the patients with primary gastric cancer, 50 were male and 16 were female, with a mean age of  $61.1 \pm 11.2$  years (range: 32-83 years). Among patients with intestinal metaplasia, 28 were male and 10 were female, with a mean age of  $62.3 \pm 10.6$  years (range: 33-84 years). Among patients with dysplasia, 31 were male and 11 were female, with a mean age of  $60.8 \pm 10.9$  years (range: 30-82 years). Among control subjects with normal gastric tissues, 29 were male and 11 were female, with a mean age of  $61.4 \pm 11.2$  years (range: 32-81 years). There was no significant difference in age, gender or other demographic data between these four groups ( $P > 0.05$ ). Informed consent was obtained from all patients enrolled in this study.

### **Immunohistochemical staining**

Tissue specimens were fixed in 10% formalin, embedded in paraffin, and sectioned into 3- $\mu$ m thick sections. The sections were then dewaxed in xylene and hydrated in graded ethanol solutions (100%, 95% and 75%). After antigen retrieval with citrate buffer and inactivation of endogenous peroxidase with hydrogen peroxide, the slides were incubated with a primary antibody overnight at 4 °C, followed by incubation with a secondary antibody at 37 °C for 30 min. Sections were visualized using DAB solution, counterstained with hematoxylin, mounted with neutral gum, and observed under a microscope.

### **Evaluation of immunohistochemical staining**

Immunohistochemical staining was evaluated by two pathologists in a double-blind manner. Using a high-power microscope, five fields of vision were randomly selected from each slice, with 100 cells counted in each field. The number of positive cells and the intensity of staining were then scored. The number of positive cells was scored as follows: 0 points, < 5% of stained cells; 1 point, 5%-20%; 2 points, 21%-50%; 3 points, 51%-75%; and 4 points, > 75%. The calculated percentage of positive cells was referred to as the labeling index. Staining intensity was scored as: 0 points, no staining; 1 point, light yellow; 2 points, brown yellow; and 3 points, tan. Protein expression was graded based on the product of scores for the percentage of stained cells and staining intensity: 1-3 points, negative (-); 4-5 points, weakly positive (+); 6-7 points, positive (++);  $\geq 8$  points, strongly positive (+++).

### **Analysis of associations of PCNA and E-cadherin expression with clinicopathological features, prognosis, and survival in patients with gastric cancer**

Differences in E-cadherin and PCNA labeling indexes

were compared among normal gastric mucosa, intestinal metaplasia, dysplasia, and gastric cancer tissues. The expression of PCNA and E-cadherin was compared between subjects with normal gastric tissues (normal control group) and patients with gastric cancer (gastric cancer group). Association of E-cadherin and PCNA expression with clinicopathological features in patients with gastric cancer, including gender, age, degree of differentiation, lymph node metastasis, and T stage, were also analyzed. Factors that may affect the survival of patients were assessed, in order to identify whether PCNA and E-cadherin expression influences the prognosis of patients with gastric cancer. The survival curve of gastric cancer patients was drawn, and the accuracy of PCNA and E-cadherin in predicting 3-year survival of patients with gastric cancer was also assessed.

### **Statistical analysis**

SPSS18.0 software was used for statistical analyses. Analysis of variance was used to analyze the difference in PCNA and E-cadherin labeling indexes among normal gastric mucosa, intestinal metaplasia, dysplasia, and gastric cancer tissues, and pairwise comparisons were performed using the Student-Newman-Keuls test. The expression of PCNA and E-cadherin between the gastric cancer group and normal control group was compared using the Mann-Whitney rank sum test, and Spearman's correlation analysis was used for correlation assessment. The relationship between PCNA and E-cadherin expression and clinicopathological features of patients was assessed using the  $\chi^2$ -test (Fisher's exact test). Log-rank analysis and Cox regression model were used to identify the factors that influence the survival of patients with gastric cancer, and the survival curve of gastric cancer patients was plotted.  $P$ -values < 0.05 were considered statistically significant.

## **RESULTS**

### **Expression of PCNA and E-cadherin in normal gastric tissues and gastric lesions**

Analysis of variance was used to compare the PCNA and E-cadherin labeling indexes in normal gastric mucosa, intestinal metaplasia, dysplasia, and gastric cancer tissues, and significant differences in the PCNA and E-cadherin labeling indexes were observed among these groups ( $P < 0.05$ ). During the transition from normal gastric mucosa to intestinal metaplasia, dysplasia, and gastric cancer, the PCNA labeling index gradually increased and the E-cadherin labeling index gradually decreased. The PCNA labeling index was significantly higher and the E-cadherin labeling index was significantly lower in gastric cancer than in dysplasia ( $P < 0.05$ ; Table 1, Figure 1).

### **Comparison of PCNA and E-cadherin expression in normal gastric tissues and gastric cancer**

The expression of PCNA in gastric cancer was signi-

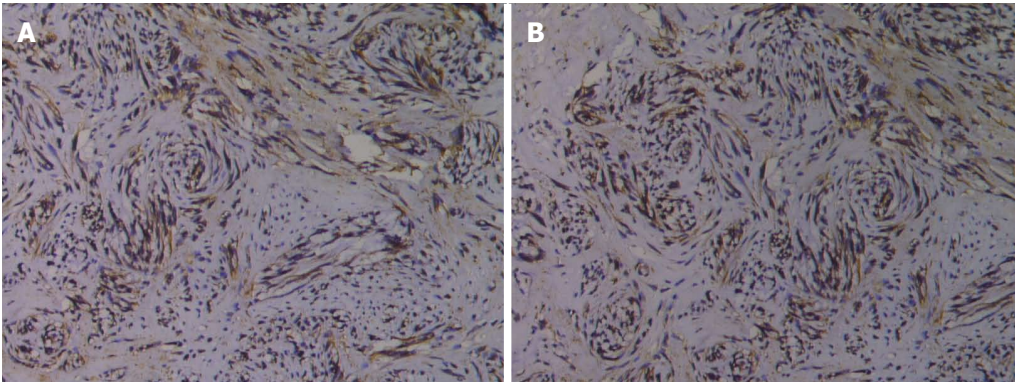


Figure 1 Representative images of immunohistochemical staining for proliferating cell nuclear antigen and E-cadherin in gastric cancer.

Table 1 Proliferating cell nuclear antigen and E-cadherin labeling indexes in normal gastric tissues and diseased tissues			
Tissue type	Number of cases	PCNA labeling index	E-cadherin labeling index
Normal gastric tissue	40	1.37 ± 0.32	22.34 ± 6.23
Intestinal metaplasia	38	11.53 ± 3.38	13.92 ± 4.34
Dysplasia	42	14.34 ± 4.71	7.84 ± 2.08
Gastric cancer	66	44.50 ± 9.85	1.68 ± 0.47
F-value	-	4.170	5.181
P-value	-	0.018	0.002
Comparison of gastric cancer and dysplasia			
Q-value		-18.519	23.204
P-value		0.000	0.000

PCNA: Proliferating cell nuclear antigen.

Table 2 Proliferating cell nuclear antigen and E-cadherin expression in the two groups									
Group	No. of cases	PCNA				E-cadherin			
		-	+	++	+++	-	+	++	+++
Normal group	40	29	6	4	1	5	15	11	9
Gastric cancer group	66	16	11	17	22	45	11	7	3
Z-value	-	-5.231				-4.982			
P-value	-	0.000				0.000			

PCNA: Proliferating cell nuclear antigen.

ificantly higher than that in normal gastric tissues ( $Z = -5.231$ ,  $P = 0.000$ ), while the expression of E-cadherin was significantly lower in gastric cancer than in normal gastric tissues ( $Z = -4.982$ ,  $P = 0.000$ ) (Table 2). Spearman’s correlation analysis showed that PCNA expression was negatively correlated with E-cadherin expression in gastric cancer ( $r = -0.741$ ,  $P = 0.000$ ).

**Association of E-cadherin and PCNA expression with clinicopathological features in patients with gastric cancer**

Among the 66 patients with gastric cancer, PCNA expression was positive in 50 cases and negative in 16 cases, while E-cadherin expression was positive in 21 cases and negative in 45 cases. The expression

Table 3 Relationship between proliferating cell nuclear antigen and E-cadherin expression and clinicopathological characteristics of gastric cancer patients					
Clinicopathological characteristic	No. of cases	PCNA		E-cadherin	
		Positive	P value	Positive	P value
Gender			0.073		0.340
Male	45	37		16	
Female	21	13		5	
Age (yr)			0.319		0.140
≥ 60	40	32		10	
< 60	26	18		11	
Lymph node metastasis			0.039		0.000
Yes	42	36		7	
Non	24	14		14	
Degree of differentiation			0.278		0.065
Medium and low differentiation	42	30		10	
High differentiation	24	20		11	
T stage			0.003		0.568
T1/T2	25	14		9	
T3/T4	41	36		12	

PCNA: Proliferating cell nuclear antigen.

of PCNA was not significantly associated with gender, age, or degree of differentiation ( $P > 0.05$ ), but was significantly correlated with lymph node metastasis and T stage ( $P < 0.05$ ). E-cadherin expression was not significantly correlated with gender, age, degree of differentiation, or T stage ( $P > 0.05$ ), but was significantly associated with lymph node metastasis ( $P < 0.05$ ) (Table 3).

**Prognostic significance of E-cadherin and PCNA expression**

Log-rank analysis was performed to identify factors that influence the survival of patients with gastric cancer, with gender, age, lymph node metastasis, degree of differentiation, T stage, PCNA expression, and E-cadherin expression analyzed. It was found that lymph node metastasis, T stage, PCNA expression, and E-cadherin expression were correlated with the prognosis of patients ( $P < 0.05$ ). These indexes were then included in the Cox regression model



**Table 4** Analysis of clinicopathological factors that influence the prognosis of patients with gastric cancer

Variable	Log-rank univariate analysis <i>P</i> value	Cox regression multivariate analysis	
		<i>P</i> value	RR (95%CI)
Gender	0.285	-	-
Age	0.128	-	-
Lymph node metastasis	0.000	0.055	4.369 (0.967-19.733)
Degree of differentiation	0.268	-	-
T stage	0.004	0.000	17.556 (5.343-57.680)
PCNA	0.003	0.000	28.786 (5.088-162.853)
E-cadherin	0.021	0.005	0.174 (0.051-0.598)

**Table 5** Evaluation of gastric cancer patient survival by proliferating cell nuclear antigen and E-cadherin

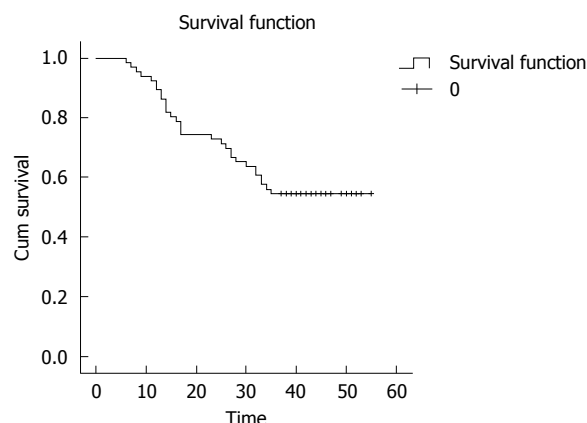
Indicators	Survival time < 3 yr	Survival time > 3 yr	Sensitivity	Specificity	Accuracy
PCNA			93.33%	38.89%	0.64
Positive	28	22			
Negative	2	14			
E-cadherin			80.0%	41.67%	0.59
Positive	6	15			
Negative	24	21			
The combination of both (PCNA[+] and E-cadherin[-])			80.0%	66.67%	0.73
Positive	24	12			
Negative	6	24			

PCNA: Proliferating cell nuclear antigen.

multivariate analysis, which revealed that T stage and the expression levels of E-cadherin and PCNA were independent prognostic factors in gastric cancer ( $P < 0.05$ ). Among these factors, high T stage and positive PCNA expression were risk factors for the prognosis of patients with gastric cancer ( $RR > 1$ ), while the positive expression of E-cadherin was a protective factor ( $RR < 1$ ) (Table 4).

#### Significance of E-cadherin and PCNA expression in predicting 3-year survival of patients with gastric cancer

The 3-year survival rate of the 66 patients with gastric cancer was 60.1% (40/66), and the survival curve is shown in Figure 2. The significance of E-cadherin and PCNA expression in predicting 3-year survival rate of gastric cancer patients was then assessed. It was found that PCNA positivity had a sensitivity, specificity, and accuracy of 93.33%, 38.89%, and 0.64, respectively, while the sensitivity, specificity, and accuracy of E-cadherin negativity were 80.0%, 41.67%, and 0.59, respectively. When combining these two indexes (PCNA positivity and E-cadherin negativity), the sensitivity, specificity, and accuracy were 80.0%, 66.67%, and 0.73, respectively (Table 5).

**Figure 2** Survival curve of patients with gastric cancer.

## DISCUSSION

### Significance of PCNA and E-cadherin expression in gastric cancer

The development of gastric cancer is a gradual process of evolution controlled by a variety of oncogenes and tumor suppressor genes, and this multistep and sequential process evolves from normal gastric mucosa to intestinal metaplasia, dysplasia, and gastric cancer<sup>[14-18]</sup>. At present, TNM staging of gastric cancer has been applied clinically to assess the prognosis of patients. However, the TNM stage does not fully reflect the prognosis of patients with gastric cancer<sup>[19-24]</sup>. For some gastric cancer patients with the same TNM stage, their response to treatment and prognosis are different<sup>[25,26]</sup>. The wide use of endoscopy and other technologies has allowed to obtain lesion samples from patients at an earlier stage. Simultaneously, these tissues can also be sent for more molecular testing to assess the nature of these lesions and evaluate the prognosis. Therefore, more experts and scholars have focused on the study of molecular changes in gastric cancer and the prognostic value of TNM staging in patients with gastric cancer<sup>[27-32]</sup>.

Strong proliferation is an important characteristic of malignant tumors. PCNA, as a cell cycle-related protein, is closely related to DNA synthesis. PCNA is rarely expressed in the G0 phase of the cell cycle, but begins to increase in the G1 phase, reaches a peak in the S phase, and decreases in the G2-M phase. Thus, PCNA can be a good indicator of cellular proliferation and be used to assess invasive lesions. However, its expression and clinical significance in the development of gastric cancer remains to be further studied<sup>[33-35]</sup>. The invasion and metastasis of malignant tumors involve the tumor cell itself and the interaction between tumor cells and their microenvironment, in which cell adhesion changes play an important role. E-cadherin, as a marker of epithelial cells, can mediate the adhesion between cells. A decline in E-cadherin expression would cause cells to lose its



polarity, decrease cell junction stability, and contribute to the invasion and metastasis of tumor cells<sup>[36,37]</sup>. Previous studies have confirmed that E-cadherin is a cancer metastasis inhibitory molecule, and that decreased expression of E-cadherin may be used as a molecular marker to evaluate the malignant degree of gastric cancer<sup>[8,36]</sup>. Therefore, in this study, the expression of PCNA and E-cadherin in gastric cancer tissues was detected, with E-cadherin as a malignancy assessment indicator, in order to analyze changes in PCNA expression in the occurrence and development of gastric cancer, and determine its prognostic significance in patients with gastric cancer.

#### **PCNA and E-cadherin expression and their correlation**

The degree of malignancy in the progression of normal gastric tissues to gastric cancer gradually increased. In order to understand the changes in PCNA expression during this process, we detected the expression of PCNA and E-cadherin in normal gastric mucosa, intestinal metaplasia, dysplasia, and gastric cancer, and significant differences in the E-cadherin and PCNA labeling indexes were found among these four phases. The expression of PCNA had a gradually increasing trend and that of E-cadherin exhibited a decreasing trend, and the differences were statistically significant between dysplasia and gastric cancer. In order to clarify whether the expression of PCNA and E-cadherin in gastric cancer is different from that in normal gastric tissues, we further conducted a detailed analysis on the expression of E-cadherin and PCNA in gastric carcinoma and normal gastric tissues. The expression of PCNA in the gastric cancer group was stronger than that in the normal group, while the expression of E-cadherin was weaker. Furthermore, there was a negative correlation between the expression of PCNA and E-cadherin in gastric carcinoma. With the gradual evolution of normal gastric mucosa toward gastric cancer, the degree of malignancy increased. During this progression, the proliferation rate of malignant cells was significantly higher than that in normal tissues<sup>[38-40]</sup>. Previous studies have demonstrated that p53 is associated with the progression of gastric cancer, while PCNA is a downstream regulatory target of p53, suggesting that the expression of PCNA is associated with the progression of gastric cancer<sup>[41-43]</sup>. The occurrence and development of gastric cancer and gastric epithelial hyperplasia are correlated, and malignant cell proliferation also enables the number of cells entering the cell cycle to significantly increase. In the G1 and S phases, cells express large amounts of PCNA. Therefore, we found that as the degree of malignancy increased in tissues, PCNA expression gradually increased<sup>[44-47]</sup>. We also found that changes in the expression of PCNA and E-cadherin exhibited a contradictory trend, and there was a negative correlation between them. E-cadherin is known as a tumor suppressor. It is important in maintaining

the number of cells and the interconnection between normal cells. The occurrence of tumor suppressor gene mutations and other changes affect the expression of E-cadherin, which thus weakens the connection between tumor cells and promotes cancer cell activity and invasion<sup>[48,49]</sup>. Therefore, we speculate that PCNA may reflect the degree of malignancy in the occurrence and development of gastric cancer, and increased expression of may PCNA suggest the increased malignancy of tissues.

#### **Relationship between expression of E-cadherin and PCNA and clinicopathological characteristics**

In order to further understand whether PCNA has good value in assessing the malignancy and prognosis of gastric cancer, we further analyzed the relationship of PCNA and E-cadherin expression with the clinicopathological characteristics of gastric cancer. We found that there were significant differences in the expression of PCNA between patients with and without lymph node metastasis, and between patients at different T stages. In addition, the expression of E-cadherin in patients with and without lymph node metastasis was also significantly different. Since T stage and lymph node metastasis are important prognostic factors and are closely related to the prognosis of patients with gastric cancer, we hypothesized that the expression of PCNA in patients who present with these prognostic factors may also be affected. Therefore, we analyzed the survival time of gastric cancer patients. Results revealed that high T stage and positive PCNA expression are risk factors for the prognosis of patients with gastric cancer, while the positive expression of E-cadherin was a protective factor. Thus, high PCNA expression may be associated with tumor proliferation and invasion ability, and is a risk factor for the prognosis of patients with gastric cancer<sup>[50,51]</sup>. Since E-cadherin is an inhibitor of cancer cell metastasis, the normal expression of E-cadherin reflects the good adhesion between cells, and in this condition cancer cells from tumor tissues could not easily metastasize. Therefore, E-cadherin expression is a protective factor for the prognosis of patients with gastric cancer<sup>[8]</sup>.

#### **PCNA and E-cadherin are used to evaluate the prognosis of patients with gastric cancer**

In order to further understand the value of PCNA and E-cadherin expression in evaluating the survival of patients with gastric cancer, we analyzed the accuracy of PCNA and E-cadherin expression in predicting the 3-year survival of patients with gastric cancer. It was found that PCNA expression had a high sensitivity but a low specificity in predicting the 3-year survival. This may be associated with the presence of non-cancerous cells in such cases of benign proliferation. In addition, the proliferation of normal cells also produces PCNA protein. The expression of E-cadherin had a slightly lower sensitivity but a higher specificity than

that of PCNA. Taking into account the use of tissue immunohistochemical detection for conducting multiple molecular tests, it is feasible to evaluate the degree of malignancy by combining multiple molecules. We further combined both PCNA positivity and E-cadherin negativity to evaluate the prognosis of gastric cancer patients, and found that although the sensitivity of the combined detection was slightly lower than that of PCNA alone, the specificity and accuracy were higher than those of PCNA alone. Therefore, we believe that when assessing the prognosis of patients with gastric cancer, PCNA can be first detected, and the positive of E-cadherin can be further detected, in order to help improve the accuracy of prognostic evaluation.

However, the development of gastric cancer is the result of a variety of genetic variations and abnormal proteins. In this study, only PCNA and E-cadherin were detected and analyzed. Future research may consider combining Ki67, Oct4, and other molecular indicators of tumor invasion and metastasis, in order to evaluate the prognosis of patients with gastric cancer. Furthermore, extending the follow-up time may also be considered, in order to obtain a more comprehensive understanding of their prognostic value for patients.

In summary, PCNA expression in gastric cancer tissue increases, and the expression of E-cadherin decreases. The detection of both indicators can help assess tumor proliferation and metastasis activity. Furthermore, the combined application of these two indicators can improve the accuracy of assessing the prognosis of patients with gastric cancer.

## COMMENTS

### Background

Gastric cancer is a common digestive system malignancy that progresses rapidly. Its occurrence and development are a very complex process that involves the dysregulation of a variety of oncogenes and tumor suppressor genes. At present, an increasing number of scholars have focused their attention on exploring protein and gene markers, in order to help clinicians early and accurately diagnose gastric cancer and assess its prognosis.

### Research frontiers

It has been reported that E-cadherin expression decreases in gastric cancer tissues, and that decreased E-cadherin expression correlates with high degree of malignancy and poor prognosis in patients with gastric cancer. Proliferating cell nuclear antigen (PCNA) is a cell proliferation-associated protein. PCNA expression is associated with metastases of breast cancer, liver cancer and other malignancies, as well as tumor infiltration. However, the expression of PCNA in gastric cancer and its clinical significance remain to be further studied. Therefore, this study investigated the clinical significance of expression of PCNA and E-cadherin in gastric carcinoma, with an aim to help explore more molecular markers for assessing gastric cancer.

### Innovations and breakthroughs

The wide use of endoscopy and other technologies has allowed to obtain lesion samples from patients at an earlier stage. The use of immunohistochemical method has made it simple and convenient to detect the expression of PCNA and E-cadherin. PCNA, as a cell cycle-related protein, is closely related to DNA synthesis. It can be a good indicator of cellular proliferation and be used to assess invasive lesions. E-cadherin is a cancer metastasis inhibitory molecule,

and decreased expression of E-cadherin may be used as a molecular marker to evaluate the malignant degree of gastric cancer. Therefore, detecting the expression of PCNA and E-cadherin in gastric cancer tissues can help evaluate the prognosis of patients with gastric cancer.

### Applications

This study demonstrated that as normal gastric mucosa transitioned into gastric cancer, the PCNA labeling index gradually increased, while the E-cadherin labeling index gradually decreased. There was a negative correlation between the expression of PCNA and E-cadherin in gastric carcinoma. Combined detection of PCNA and E-cadherin improves the accuracy of assessing the prognosis of patients with gastric cancer. Therefore, combined detection of PCNA and E-cadherin is recommended to evaluate the tissue malignancy and the prognosis of patients.

### Peer-review

This is an interesting study about the expression and detection value of PCNA and E-cadherin in gastric carcinoma. This study is well designed and the results are very interesting. In this study, the authors investigated the expression and detection value of PCNA and E-cadherin in gastric carcinoma. Approximately 146 patients were selected for this study, including 38 cases with intestinal metaplasia, 42 with severe atypical hyperplasia, and 66 with primary gastric cancer.

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## Different techniques for harvesting grafts for living donor liver transplantation: A systematic review and meta-analysis

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### Abstract

#### AIM

To perform a systematic review and meta-analysis on minimally *vs* conventional invasive techniques for harvesting grafts for living donor liver transplantation.

#### METHODS

PubMed, Web of Science, EMBASE, and the Cochrane Library were searched comprehensively for studies comparing MILDH with conventional living donor hepatectomy (CLDH). Intraoperative and postoperative outcomes (operative time, estimated blood loss, postoperative liver function, length of hospital stay, analgesia use, complications, and survival rate) were

analyzed in donors and recipients. Articles were included if they: (1) compared the outcomes of MILDH and CLDH; and (2) reported at least some of the above outcomes.

## RESULTS

Of 937 articles identified, 13, containing 1592 patients, met our inclusion criteria and were included in the meta-analysis. For donors, operative time [weighted mean difference (WMD) = 20.68, 95%CI: -6.25-47.60,  $P = 0.13$ ] and blood loss (WMD = -32.61, 95%CI: -80.44-5.21,  $P = 0.18$ ) were comparable in the two groups. In contrast, analgesia use (WMD = -7.79, 95%CI: -14.06-1.87,  $P = 0.01$ ), postoperative complications [odds ratio (OR) = 0.62, 95%CI: 0.44-0.89,  $P = 0.009$ ], and length of hospital stay (WMD): -1.25, 95%CI: -2.35-0.14,  $P = 0.03$ ) significantly favored MILDH. No differences were observed in recipient outcomes, including postoperative complications (OR = 0.93, 95%CI: 0.66-1.31,  $P = 0.68$ ) and survival rate (HR = 0.96, 95%CI: 0.27-3.47,  $P = 0.95$ ). Funnel plot and statistical methods showed a low probability of publication bias.

## CONCLUSION

MILDH is safe, effective, and feasible for living donor liver resection with fewer donor postoperative complications, reduced length of hospital stay and analgesia requirement than CLDH.

**Key words:** Living donor hepatectomy; Graft harvesting; Minimally invasive techniques; Conventional invasive approaches; Meta-analysis

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**Core tip:** Minimally invasive procedures have been increasingly used in liver resection, as they are considered safe and effective. Concerns have been raised, however, about the feasibility and donor safety of minimally invasive living donor hepatectomy. We analyzed 13 articles, containing 1592 patients, to compare two techniques for harvesting grafts for living donor liver transplantation. Finally, we concluded that minimally invasive procedures are safe, effective, and feasible for living donor liver resection, with fewer donor postoperative complications and reduced length of hospital stay and analgesia requirement than conventional approaches.

Li H, Zhang JB, Chen XL, Fan L, Wang L, Li SH, Zheng QL, Wang XM, Yang Y, Chen GH, Wang GS. Different techniques for harvesting grafts for living donor liver transplantation: A systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(20): 3730-3743 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3730.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3730>

## INTRODUCTION

Since the first reported successful human liver transplantation in 1967<sup>[1]</sup>, this technique has gained worldwide acceptance, becoming the best and most common treatment for patients with end-stage liver disease. Because of the shortage of deceased donor organs, especially in East Asian countries, living donor liver transplantation (LDLT) has become an established treatment modality for patients with end-stage liver disease<sup>[2]</sup>. In 1990, the first successful pediatric LDLT, using a left lateral section graft from a mother to her son, was reported in Australia<sup>[3]</sup>. Since then, the feasibility and safety of pediatric LDLT have been well documented<sup>[4]</sup>. Donor safety is considered paramount, as donor hepatectomy is a major surgery for healthy individuals<sup>[5]</sup>. However, the large permanent abdominal incision scar resulting from conventional open surgery may cause mental and physical stress among some putative living donors, especially young unmarried women, resulting in hesitation or unwillingness to donate liver tissue<sup>[4,6]</sup>.

Although conventional living donor hepatectomy (CLDH) is safe, approximately 40% of donors have experienced postoperative complications<sup>[7-9]</sup>. Minimally invasive liver surgery has been widely used to treat patients with various liver diseases. Although laparoscopic liver surgery has resulted in lower rates of surgical morbidity and reduced postoperative pain and recovery time when compared with standard liver surgery<sup>[10,11]</sup>, minimally invasive approaches to living donor hepatectomy are not generally performed. Minimally invasive living donor hepatectomy (MILDH), involving either a laparoscopic approach or a hybrid technique, has been compared with CLDH in several centers.

Although studies have compared outcomes following MILDH and CLDH, most of these studies were small series with unclear results<sup>[12-15]</sup>. Thus, their relative benefits for donors have not been investigated. This systematic review and meta-analysis analyzed studies comparing MILDH with CLDH to evaluate the safety, efficacy, and potential advantages of MILDH.

## MATERIALS AND METHODS

### Objective and groups

This meta-analysis was performed to compare the feasibility and donor safety of MILDH with CLDH, including evaluations of recipient survival rates. Outcomes compared included perioperative complications, estimated blood loss (EBL), requirement for analgesics, overall survival, operative time, postoperative liver function and hospital costs. MILDH in this study included fully laparoscopic and laparoscopy-assisted approaches, upper midline incision with or without laparoscopic assistance, and a hybrid approach

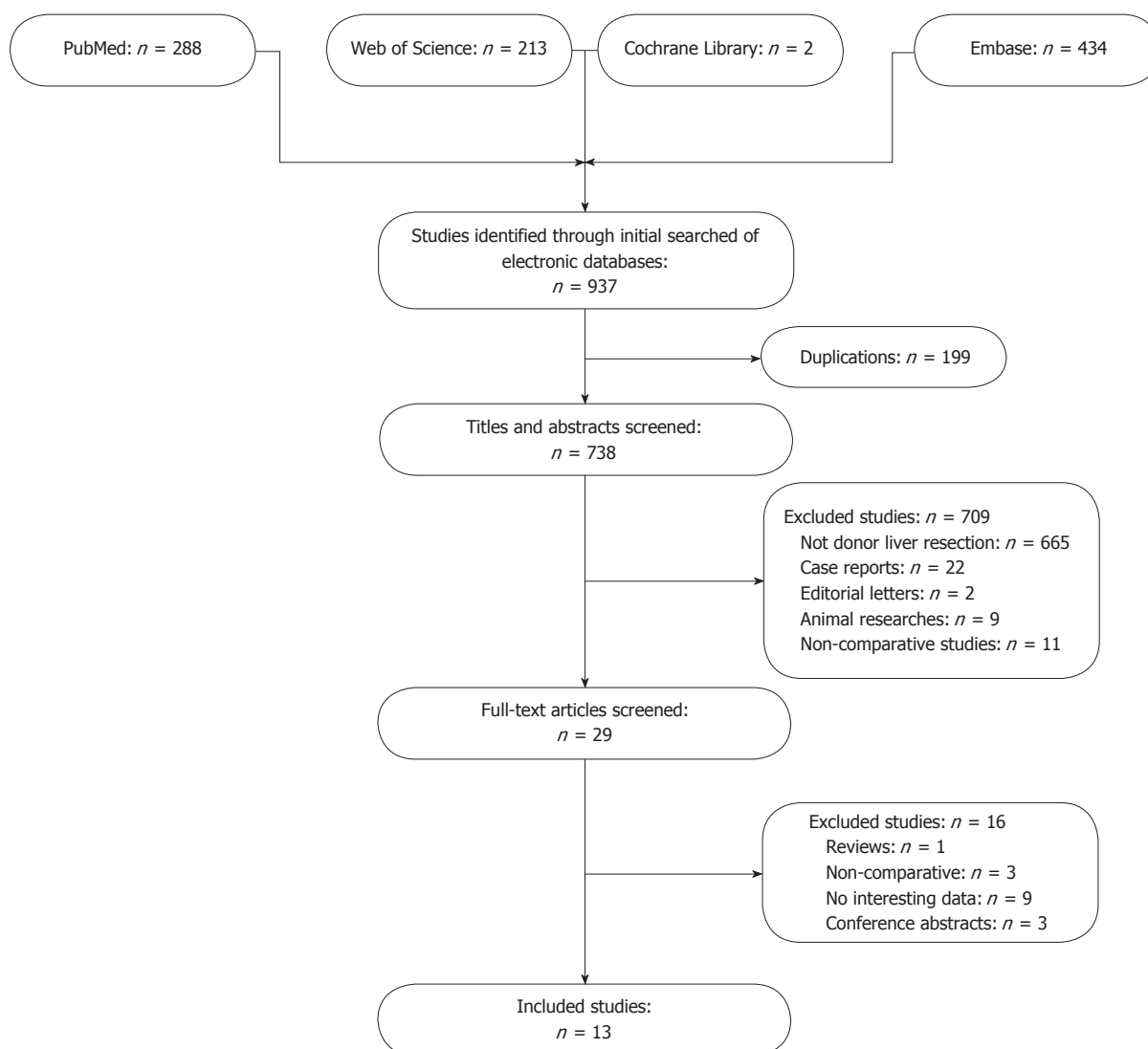


Figure 1 Flow diagram of study identification, inclusion and exclusion.

with incision length  $\leq 15$  cm. CLDH included standard open donation with a large subcostal incision, Mercedes incision, L-shaped incision, and a large J-shaped or midline skin incision.

### Search strategy and criteria

PubMed, EMBASE, the Web of Science, and Cochrane Library were searched for studies comparing MILDH with CLDH published through December 2015. There were no restrictions on publication date, type or language. Search terms included "donor hepatectomy" OR "liver transplantation" OR "donor liver resection" OR "donor sectionectomy" AND "open surgery" OR "right subcostal incision" OR "regular surgery" OR "conventional surgery" AND "laparoendoscopic" OR "laparoscopic". The reference lists of all selected articles were manually searched to determine if they should be included.

The literature search identified 937 articles, of which 288 from PubMed, 434 from EMBASE, 213 from Web of Science, and two from the Cochrane Library

(Figure 1). Two reviewers browsed the titles and abstracts independently. Articles were included if they: (1) compared the outcomes of MILDH and CLDH; and (2) reported at least some of the above outcomes. Articles were excluded if were submitted by the same authors or the same institutions to avoid duplication of patient populations.

Of the 937 identified articles, 199 were duplications; 665 did not focus on donor liver resection; nine were in animals; 11 did not compare MILDH with CLDH; two were editorials; and 22 were case reports. The full texts of the remaining 29 articles were carefully reviewed. Of these, three did not compare MILDH with CLDH; nine did not include outcomes of interest; one was a review article; and three were conference abstracts. Finally, 13 articles<sup>[12-24]</sup> were included in this meta-analysis.

### Data management

Data were analyzed by three authors (Li H, Zhang JB and Chen XL) independently. These reviewers were

**Table 1** Quality of cohort studies evaluated with modified Newcastle-Ottawa scale

Ref.	Case definition	Selection		Definition of controls	Comparability		Outcomes		Quality score
		Representativeness	Selection of controls		Comparable for 1, 2, 3	Comparable for 4, 5	Assessment of outcomes	Integrity of follow-up	
Choi <i>et al</i> <sup>[16]</sup> , 2012	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	8
Choi <i>et al</i> <sup>[17]</sup> , 2014	Yes	No	Yes	Yes	No	No	Yes	Yes	5
Makk <i>et al</i> <sup>[19]</sup> , 2014	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	8
Marubashi <i>et al</i> <sup>[20]</sup> , 2013	Yes	No	Yes	Yes	2, 3	4	Yes	Yes	7
Nagai <i>et al</i> <sup>[21]</sup> , 2012	Yes	No	Yes	Yes	1, 3	4	Yes	Yes	7
Samstein <i>et al</i> <sup>[22]</sup> , 2015	Yes	No	Yes	Yes	Yes	No	Yes	Yes	7
Soubrane <i>et al</i> <sup>[12]</sup> , 2006	Yes	No	Yes	Yes	1,3	4	Yes	Yes	7
Suh <i>et al</i> <sup>[23]</sup> , 2015	Yes	No	Yes	Yes	No	No	Yes	Yes	5

1 = gender; 2 = body mass index; 3 = graft generation; 4 = age; 5 = haemoglobin.

**Table 2** Quality of case-controlled studies evaluated with modified Newcastle-Ottawa scale

Ref.	Case definition	Selection		Definition of controls	Comparability		Outcomes		Quality score
		Representativeness	Selection of controls		Comparable for 1, 2, 3	Comparable for 4, 5	Ascertainment of exposure	Non-response	
Baker <i>et al</i> <sup>[13]</sup> , 2009	Yes	No	Yes	Yes	Yes	4	Yes	Yes	7
Kim <i>et al</i> <sup>[14]</sup> , 2009	Yes	No	Yes	Yes	Yes	4	Yes	Yes	7
Kim <i>et al</i> <sup>[18]</sup> , 2011	Yes	No	Yes	Yes	1, 3	Yes	Yes	Yes	6
Thenappan <i>et al</i> <sup>[15]</sup> , 2011	Yes	No	Yes	Yes	No	No	Yes	Yes	7
Zhang <i>et al</i> <sup>[24]</sup> , 2014	Yes	No	Yes	Yes	Yes	4	Yes	Yes	7

1 = gender; 2 = body mass index; 3 = graft generation; 4 = age; 5 = haemoglobin.

blinded to the authors, institutions, and journals of publication of all selected articles. Any disagreements between the reviewers were settled by the senior author (Wang GS).

Donor outcomes of interest included operative time, EBL, hospital costs, length of hospital stay, postoperative complications, analgesic use, graft weight, and postoperative liver function. Liver function was evaluated based on peak serum levels of aspartate transaminase (AST), alanine aminotransferase (ALT), and total bilirubin (TB). Recipient outcomes of interest included postoperative complications, postoperative liver function, and survival rate. If survival rate did not appear directly in an article, it was determined using Engauge software.

### Quality assessment

The methodological quality of retrospective studies was assessed using the modified Newcastle-Ottawa scale, which consists of three factors: patient selection, comparability of the study groups, and assessment of outcome<sup>[25,26]</sup>. As the maximum total score on this scale is 9, studies with scores  $\geq 7$  were defined as high-quality studies (Tables 1 and 2).

Data were pooled with the Cochrane Collaboration's

Review Manager 5.3 (Cochrane Collaboration, Oxford, United Kingdom). Mean differences and 95% CIs were calculated to pool functional outcomes. Statistical heterogeneity among studies was assessed using the  $\chi^2$  test with significance set at  $P < 0.1$ , and heterogeneity was quantified using the  $I^2$  statistic. A random-effects model was used if there was heterogeneity among studies; otherwise, a fixed-effects model was used<sup>[27]</sup>.

### Subgroups and publication bias

Grafts harvested from the left and right sides of the liver differ in weight, vascularity, and bile duct distribution, affecting outcomes in both donors and recipients. Therefore, subgroup analyses were performed on donors who underwent left hepatectomy (LH) and right hepatectomy (RH). Operative time, postoperative complications, and EBL were analyzed in these subgroups.

The publication bias of selected articles was analyzed by funnel plots, which were produced by Review Manager 5.3. If outcomes were associated with significant heterogeneity among studies, a random-effects model was used to minimize bias resulting from this heterogeneity.



**Table 3** Characteristics of included studies

Ref.	Level of evidence	Patient no.		Left/right	Recipients	TMI	TCI	Matching	Quality score
		MILDH	CLDH						
Baker <i>et al</i> <sup>[13]</sup> , 2009	3b	33	33	Right	W	LA	Midline epigastric	1, 2, 3, 4	7
Choi <i>et al</i> <sup>[16]</sup> , 2012	2b	60	90	Right	W/O	LA	Right subcostal	1, 2, 3, 4, 5	8
Choi <i>et al</i> <sup>[17]</sup> , 2014	4	25	484	Right	W/O	HAL or LA	Mercedes-Benz or L-shaped	NA	5
Kim <i>et al</i> <sup>[14]</sup> , 2009	3b	23	23	Right	W	Upper midline	J-shaped	1, 2, 3, 4	7
Kim <i>et al</i> <sup>[18]</sup> , 2011	3b	11	11	Left	W	L	J-shaped or midline	1, 3, 4, 5	7
Makk <i>et al</i> <sup>[19]</sup> , 2014	2b	26	24	Right	W	LA	Right subcostal with midline extension	1, 2, 3, 4, 5	8
Marubashi <i>et al</i> <sup>[20]</sup> , 2013	2b	31	79	Left	W	LA	Mercedes	2, 3, 4	7
Nagai <i>et al</i> <sup>[21]</sup> , 2012	2b	28	30	Right	W	Hal or upper midline	Mercedes	1, 3, 4	7
Samstein <i>et al</i> <sup>[22]</sup> , 2015	2b	22	20	Left	W	L	Midline	1, 2, 3	7
Soubrane <i>et al</i> <sup>[12]</sup> , 2006	2b	16	14	Left	W	L	Subcostal	1, 3, 4	7
Suh <i>et al</i> <sup>[23]</sup> , 2015	4	161	268	Un	W	LA or Upper midline	L-shaped	NA	5
Thenappan <i>et al</i> <sup>[15]</sup> , 2011	3b	15	15	Un	W	LA or Minimally-access	Midline epigastric with subcostal	NA	6
Zhang <i>et al</i> <sup>[24]</sup> , 2014	3b	25	25	Right	W	LA	Right subcostal	1, 2, 3, 4	7

MILDH: Minimally invasive living donor hepatectomy; CLDH: Conventional living donor hepatectomy; Left/right: Graft from left or right liver lobe of donors; Recipients: With or without analyzing recipients; TMI: Type of minimally incisions; TCI: Type of conventional incisions; W: With; W/O: Without; Un: Unclear or not only one kind; L: Laparoscopic approach; LA: Laparoscopy-assisted; HAL: Hand-assisted laparoscopic; Matching: 1 = gender; 2 = body max index; 3 = graft generation; 4 = age; 5 = haemoglobin; NA: No data available.

### Characteristics of selected articles

The meta-analysis included 13 articles<sup>[12-24]</sup> involving 1592 patients; the characteristics of the selected articles are shown in Table 3. Primary outcomes of interest included donor safety, as determined by perioperative complications and EBL; donor requirement for analgesics after hepatectomy; and recipient survival rate. Secondary outcomes included postoperative liver function, length of hospital stay, and total hospital cost. The level of evidence of these articles was estimated using the United Kingdom Cochrane Centre of Evidence (2001)<sup>[28]</sup>. Six articles described cohort studies comparing contemporary series of patients (level of evidence: 2b)<sup>[12,16,19-22]</sup>, five articles were retrospective case-control studies (level of evidence: 3b)<sup>[13-15,18,24]</sup>, and two articles were retrospective studies using historical series as controls (level of evidence: 4)<sup>[17,23]</sup>.

## RESULTS

### Donor outcomes

Of the 1592 donors included in the 13 articles, 476 underwent MILDH and 1116 underwent CLDH (Table 4)<sup>[12-24]</sup>. Operative times were similar in the two groups [weighted mean difference (WMD) = 20.68, 95%CI: -6.25-47.60,  $P = 0.13$ ] (Figure 2). Twelve studies<sup>[12-18,20-24]</sup> analyzed EBL among 1542 donors, finding no significant difference between those who underwent MILDH and CLDH (WMD = -32.61, 95%CI: -80.44-5.21,  $P = 0.18$ ). Hospital costs were reported by only two articles<sup>[18,24]</sup>, finding no significant difference between the two donor groups

(WMD = 0.56, 95%CI: -0.62-0.74,  $P = 0.35$ ). Ten studies<sup>[12,14-16,18,20-24]</sup>, including 967 patients, evaluated the length of hospital stay, finding that donors who underwent MILDH group had a significantly shorter hospital stay than those who underwent CLDH (WMD: -1.25, 95%CI: -2.35-0.14,  $P = 0.03$ ). Twelve articles<sup>[12-16,18-24]</sup> analyzed postoperative complications, finding that the rate of postoperative complications was significantly lower in the MILDH than in the CLDH group (OR = 0.62, 95%CI: 0.44-0.89,  $P = 0.009$ ). Ten articles<sup>[12-16,18-24]</sup> compared postoperative complications of donors between the two groups, finding no statistical difference (WMD = 0.56, 95%CI: 0.27-1.18,  $P = 0.13$ ) (Figure 3). Five articles<sup>[12,14-16,24]</sup> reported analgesic use, finding that the total analgesic use among donors was significantly lower in the MILDH than in the CLDH group (WMD = -7.79, 95%CI: -14.06-1.87,  $P = 0.01$ ) (Figure 4). Five studies<sup>[12-14,19,24]</sup> reported graft weight, finding no significant difference between the two groups (WMD = -3.32, 95%CI: -22.25-15.61,  $P = 0.73$ ). Seven articles<sup>[14,16,18,19,21,23,24]</sup> compared postoperative liver function, finding no significant difference between the two groups in peak AST (WMD = 6.41, 95%CI: -3.79-16.60,  $P = 0.50$ ), ALT (WMD = 11.86, 95%CI: -10.84-34.56,  $P = 0.031$ ), and TB (WMD = -0.10, 95%CI: -0.26-0.06,  $P = 0.21$ ) concentrations (Figure 5).

### Recipient outcomes

Six studies<sup>[12,13,18,19,23,24]</sup> compared postoperative complications in recipients, finding no significant difference in postoperative complication rates between the two groups (OR = 0.93, 95%CI: 0.66-1.31,  $P = 0.68$ ).

**Table 4 Results of meta-analysis comparison of minimally invasive living donor hepatectomy and conventional living donor hepatectomy**

Outcome of interest	Study (n)	MILDH (n)	CLDH (n)	WMD/OR (95%CI)	P value	Study heterogeneity			P value
						I <sup>2</sup>	df	I <sup>2</sup> , %	
Graft weight (g)	5	123	119	-3.32 (-22.25,15.61)	0.73	6.56	4	39	0.16
Donor outcomes									
Operative time (min)	13	476	1116	20.68 (-6.25,47.60)	0.13	147.62	12	92	< 0.01
Estimated blood loss (mL)	12	450	1092	-32.61 (-80.44,15.21)	0.18	61.26	11	82	< 0.01
Hospital cost (dollar)	2	36	36	0.56 (-0.62,1.74)	0.35	4.24	1	76	0.04
Length of hospital stay (d)	10	392	575	-1.25 (-2.35,-0.14)	0.03	99.31	9	91	< 0.01
Post complications	12	451	632	0.62 (0.44,0.89)	0.009	4.40	11	0	0.96
Analgesic use (h)	5	139	167	-7.97 (-14.06,-1.87)	0.01	7.50	4	47	0.11
Liver function									
Post AST peak (IU/L)	7	334	471	6.41 (-3.79,16.60)	0.22	13.60	6	56	0.03
Post ALT peak (IU/L)	8	350	485	11.86 (-10.84,34.57)	0.31	15.39	7	55	0.03
Post TB peak (mg/dL)	7	324	461	-0.10 (-0.26,0.06)	0.21	2.10	6	0	0.91
Recipient outcomes									
Liver function									
Post AST peak (IU/L)	3	59	59	-28.73 (-86.76,29.31)	0.33	0.90	2	0	0.64
Post ALT peak (IU/L)	3	59	59	-29.98 (-87.65,27.7)	0.31	0.31	2	0	0.86
Post TB peak (mg/dL)	3	59	59	-0.96 (-2.57,0.65)	0.24	1.26	2	0	0.53
Surviving	3			0.96 (0.27,3.47)	0.95	0.11	2	0	0.95
Post complications	6	272	375	0.93 (0.66,1.31)	0.68	3.28	5	0	0.66

MILDH: Minimally invasive living donor hepatectomy; CLDH: Conventional living donor hepatectomy; WMD/OR: Weight mean difference/odds ratio; df: Degree of freedom; Post: Postoperative.

Seven studies<sup>[12,13,18,19,23,24]</sup> analyzed postoperative biliary complications for recipients, showing no significant difference between the two groups (WMD = 1.10, 95%CI: 0.73-1.66,  $P = 0.65$ ) (Figure 6). Three studies<sup>[14,18,24]</sup> compared postoperative liver function in recipients, finding no significant differences between the two groups in peak AST (WMD = -28.73, 95%CI: -86.76-29.31,  $P = 0.33$ ), ALT (WMD = -29.98, 95%CI: -87.65-27.7,  $P = 0.31$ ), and TB (WMD = -0.96, 95%CI: -2.57-0.65,  $P = 0.24$ ) concentrations. Three articles<sup>[13,20,22]</sup> compared overall recipient survival, finding no significant difference between the two groups in recipient survival rate (HR = 0.96, 95%CI: 0.27-3.47,  $P = 0.95$ ) (Figure 7).

### Subgroup analysis

Postoperative complications, operative time, and EBL were analyzed in donors who underwent RH and LH. In assessing postoperative complications, three studies<sup>[15,17,23]</sup> were excluded, as data were unmatched. Pooled data of six studies<sup>[13,14,16,19,21,24]</sup> showed no significant difference in postoperative complication rates in donors who underwent RH by MILDH and CLDH, but did favor MILDH (OR = 0.73, 95%CI: 0.45-1.19,  $P = 0.21$ ). In contrast, pooled data of four studies<sup>[12,18,20,22]</sup> that evaluated donors who underwent LH showed that the postoperative complication rate was significantly lower in patients who underwent MILDH (OR = 0.37, 95%CI: 0.16-0.87,  $P = 0.02$ ) (Figure 8).

Seven studies<sup>[13,14,16,17,19,21,24]</sup> compared operative time for RH, finding no significant difference between MILDH and CLDH (WMD = 14.99, 95%CI: -22.52-52.50,  $P = 0.43$ ). In contrast, four studies<sup>[12,18,20,22]</sup> that

compared operative time for LH found that this time was significantly shorter for CLDH (WMD = 62.04, 95%CI: 37.04-87.03,  $P < 0.0001$ ). The remaining two studies<sup>[15,23]</sup> were excluded (Figure 9).

Six studies<sup>[13,14,16,17,21,24]</sup> reported EBL in donors who underwent RH, finding no significant difference between the MILDH and CLDH groups (WMD = -1.67, 95%CI: -66.05-62.72,  $P = 0.96$ ). Similarly, four studies<sup>[12,18,20,22]</sup> compared EBL in donors who underwent LH, finding no significant difference between the two groups (WMD = -93.04, 95%CI: -215.56-29.48,  $P = 0.14$ ) (Figure 10).

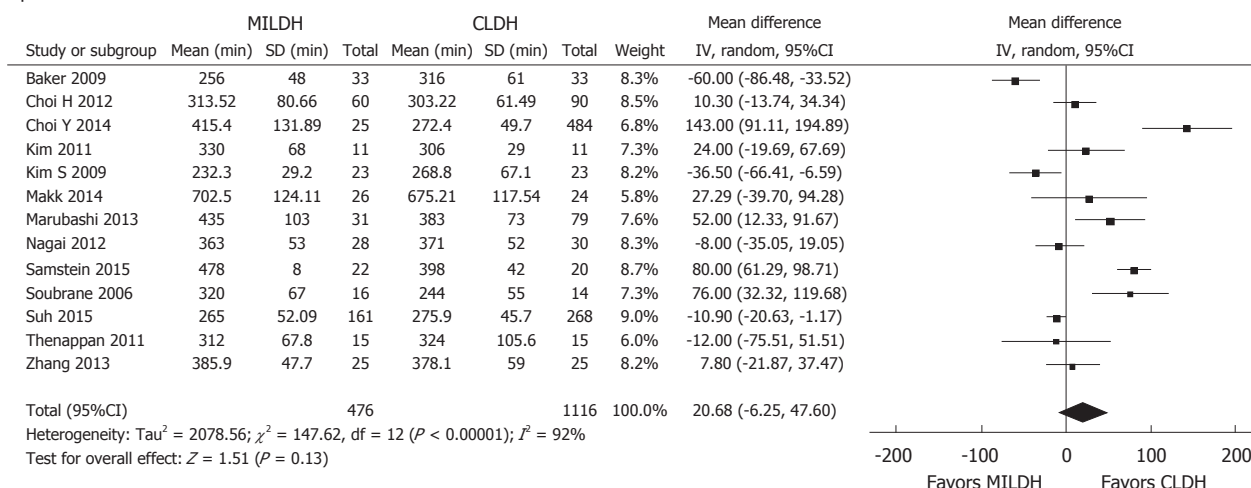
### Publication bias

The funnel plot of postoperative complications showed that all articles included in this meta-analysis were symmetrically distributed around the center line, indicating a lack of obvious publication bias (Figure 11).

## DISCUSSION

Primary laparoscopic living donor hepatectomy was introduced in 2002 to reduce the impact of open hepatectomy on donors<sup>[29]</sup>. Since then, minimally invasive approaches have been considered safe and effective, reducing postoperative pain and surgical morbidity and providing a faster recovery time<sup>[9,11,30,31]</sup>. Despite these findings, minimally invasive approaches to living donor hepatectomy have not been accepted by consensus guidelines. A hybrid technique for donor hepatectomy was introduced in 2006<sup>[32]</sup>, and subsequent studies have compared minimally invasive and conventional donor liver resection<sup>[33]</sup>. To date, however, these two methods have not been

## Operative time



## Estimated blood loss

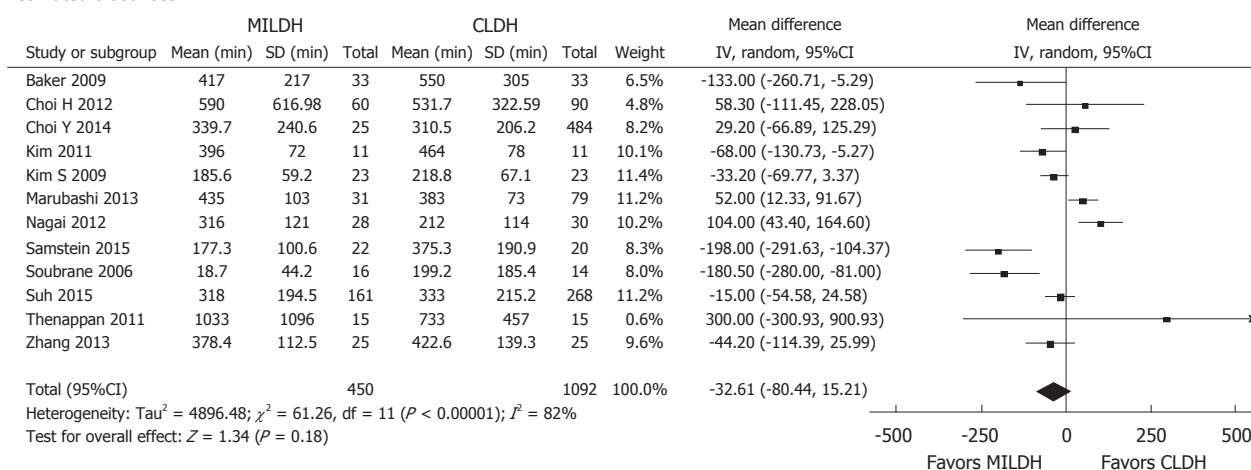


Figure 2 Forest plots and meta-analysis of intraoperative outcomes of donors.

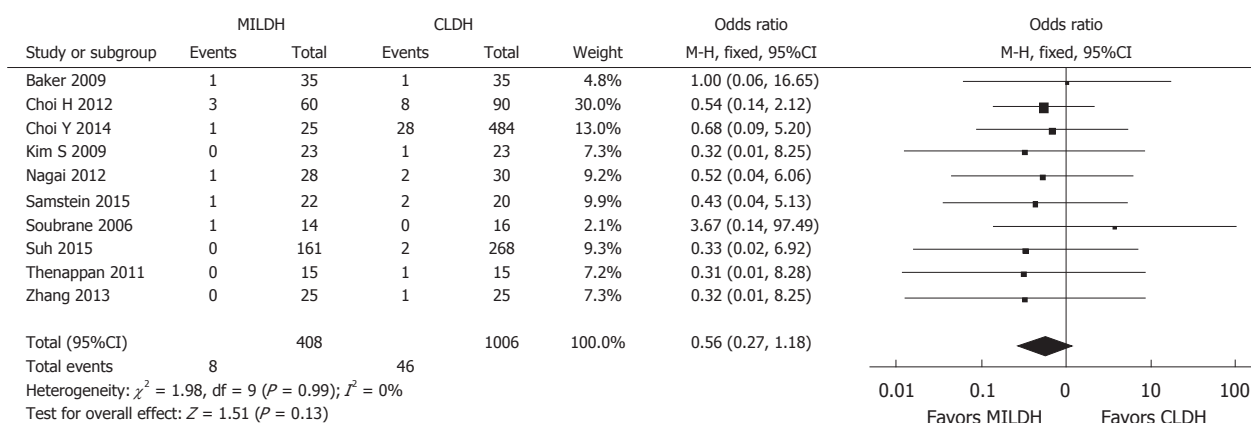


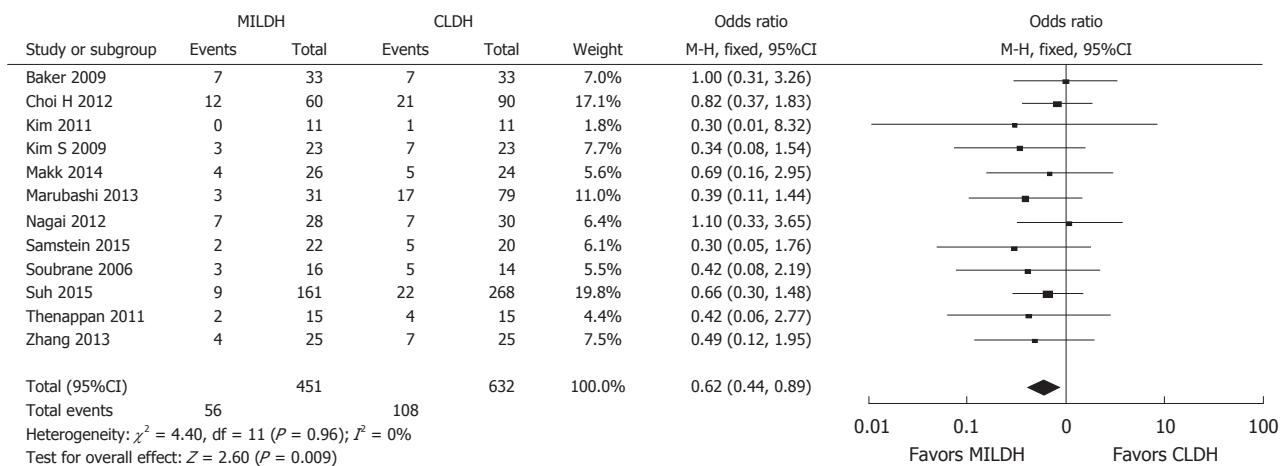
Figure 3 Forest plot and meta-analysis of postoperative biliary complications for donors.

systematically analyzed in large donor populations.

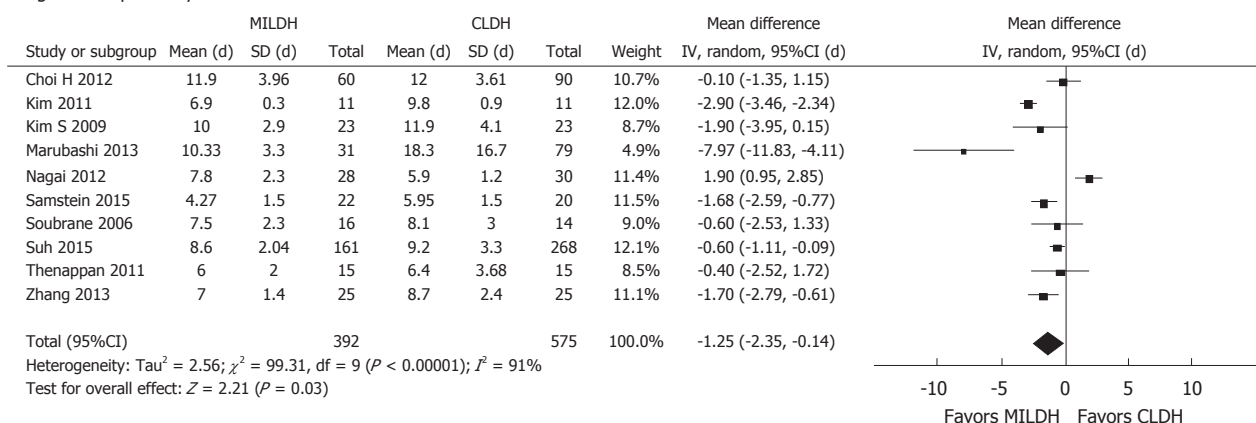
This systematic review and meta-analysis of 13 studies, involving 1592 patients, compared minimally invasive with conventional methods for living donor hepatectomy, finding that MILDH was not less safe than CLDH. MILDH was associated with a significantly

lower postoperative complication rate, a significantly lower analgesic requirement, and a significantly shorter hospital stay for donors than CLDH. However, operative time, EBL, graft weight, hospital costs, and postoperative liver function for donors were similar in the two groups. Moreover, comparisons of

## Postoperative complications



## Length of hospital stay



## Analgesics use

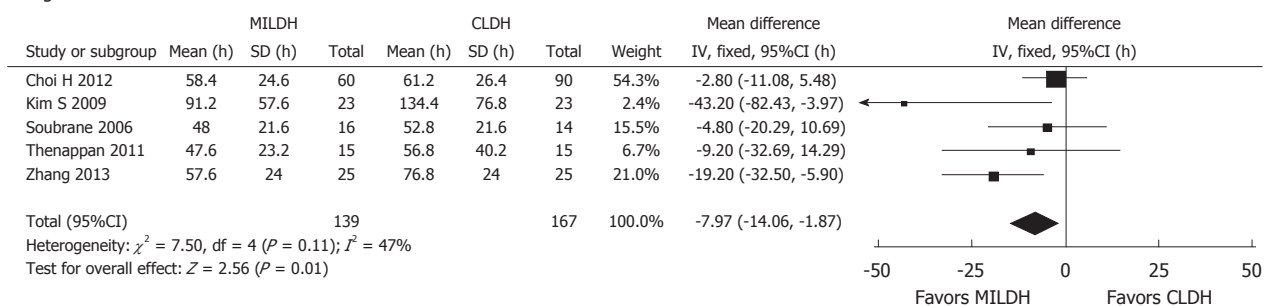


Figure 4 Forest plots and meta-analysis of postoperative outcomes of donors.

postoperative recipient liver function, complication rate, and survival rate showed no differences between these two groups.

Donor safety is of paramount importance during LDLT, regardless of the technique used. Our pooled data on perioperative outcomes indicated that MILDH was as safe and effective for LDLT as CLDH.

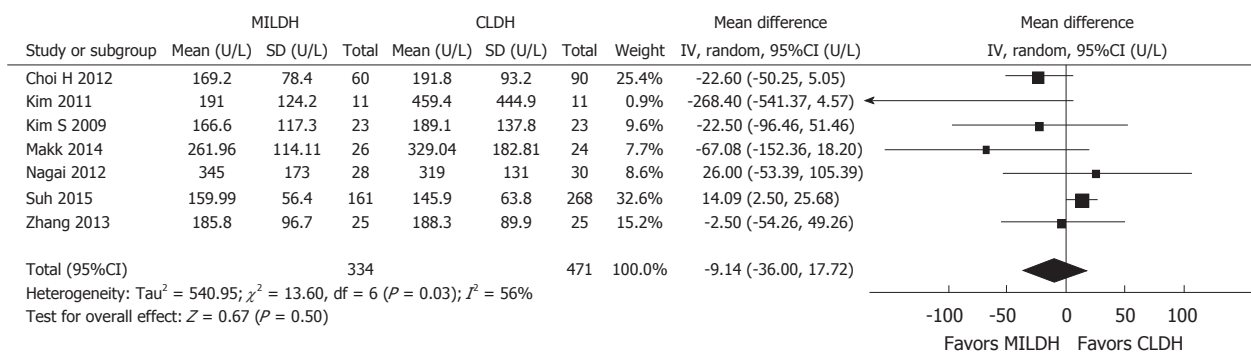
Our finding that operative times were comparable in the two groups is inconsistent with several studies suggesting that MILDH was associated with a shorter average operative time<sup>[13,14]</sup>. This may have been owing to the dissimilarity of operative procedures in different institutions. Nevertheless, the operative time for making an upper midline incision was

generally shorter in the MILDH group, as the incision was shorter. Although the small incision reduced the time spent in opening and closing the abdomen, it was apparently balanced by the additional time required to mobilize grafts laparoscopically, as this approach required frequent installation and removal of laparoscopic devices, application of the hanging maneuver, and dissection of the deep parenchyma.

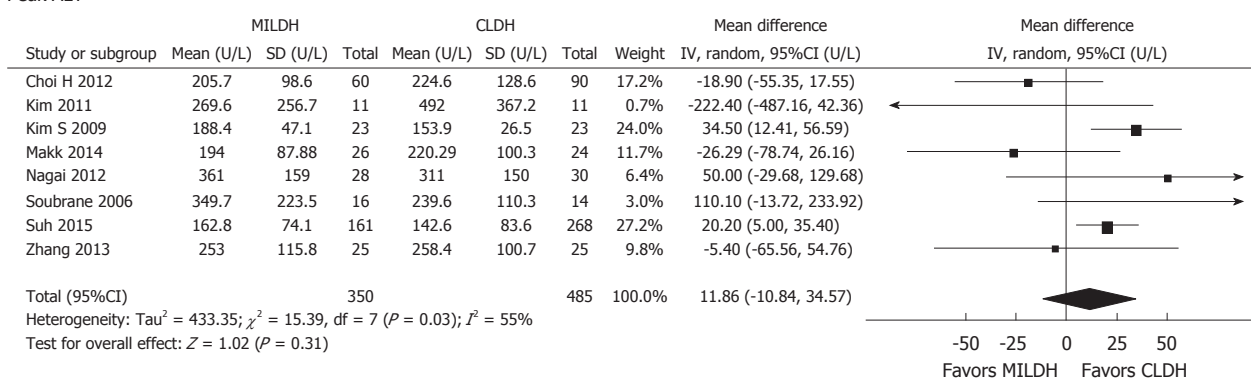
EBL did not differ significantly between the MILDH and CLDH groups, although it was lower in the MILDH group. Laparoscopic parenchymal dissection and the high intra-abdominal pressure attained by pneumoperitoneum use apparently resulted in lower blood loss in the MILDH group<sup>[34]</sup>. Furthermore,



## Peak AST



## Peak ALT



## Peak TB

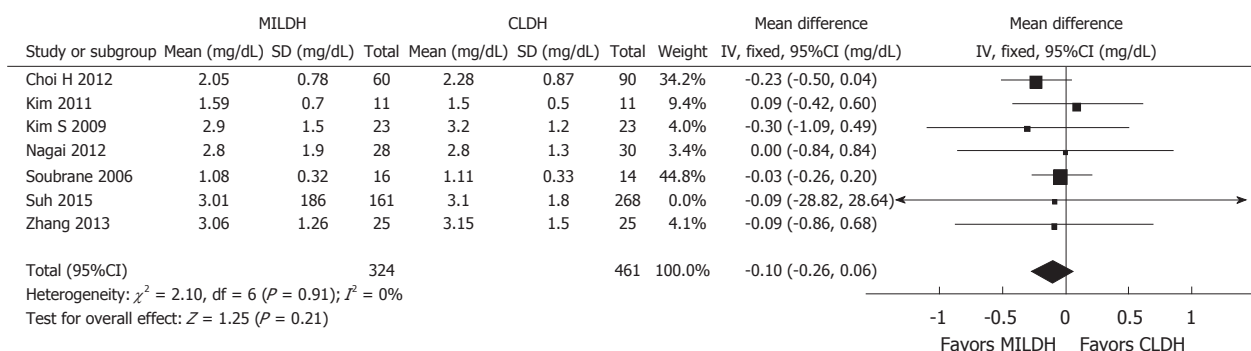


Figure 5 Forest plots and meta-analysis of postoperative liver function of donors.

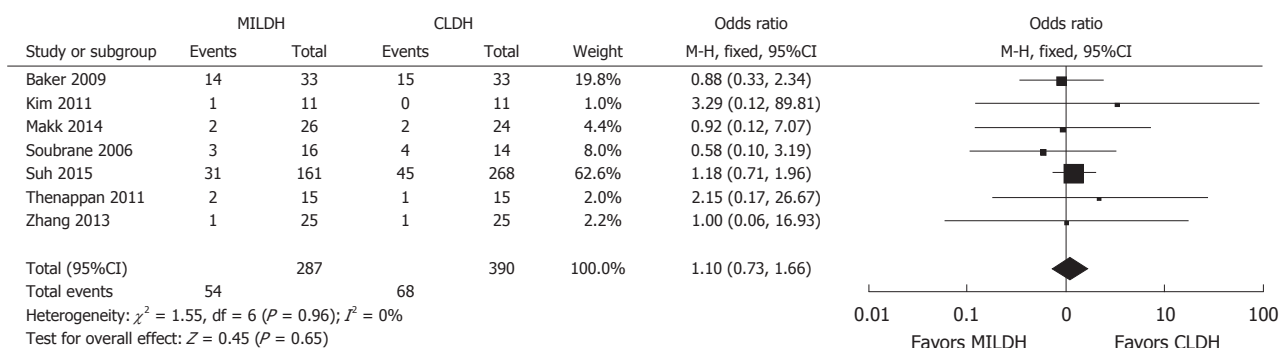
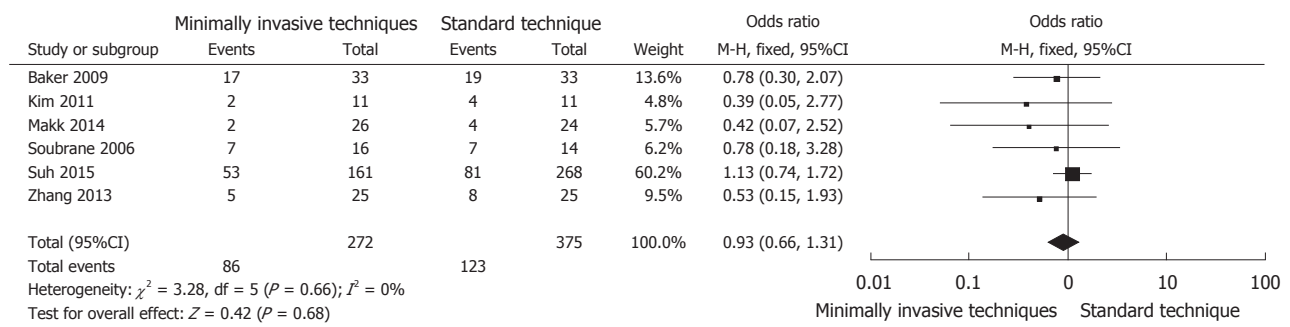


Figure 6 Forest plot and meta-analysis of postoperative biliary complications for recipients.

## Postoperative complications



## Surviving

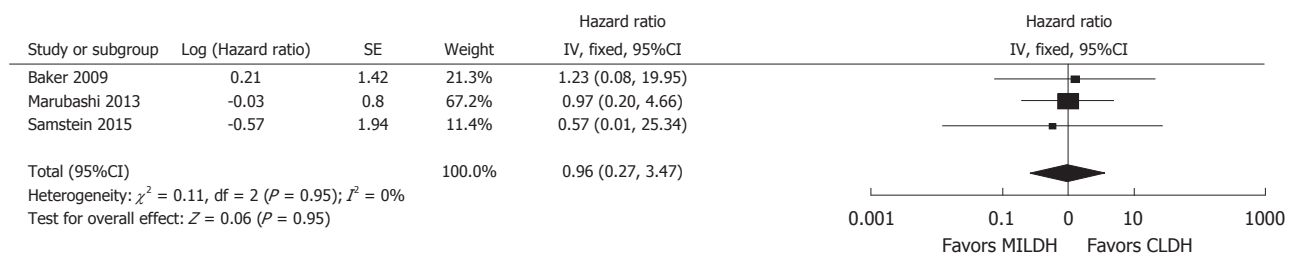


Figure 7 Forest plots and meta-analysis of postoperative outcomes of recipients.

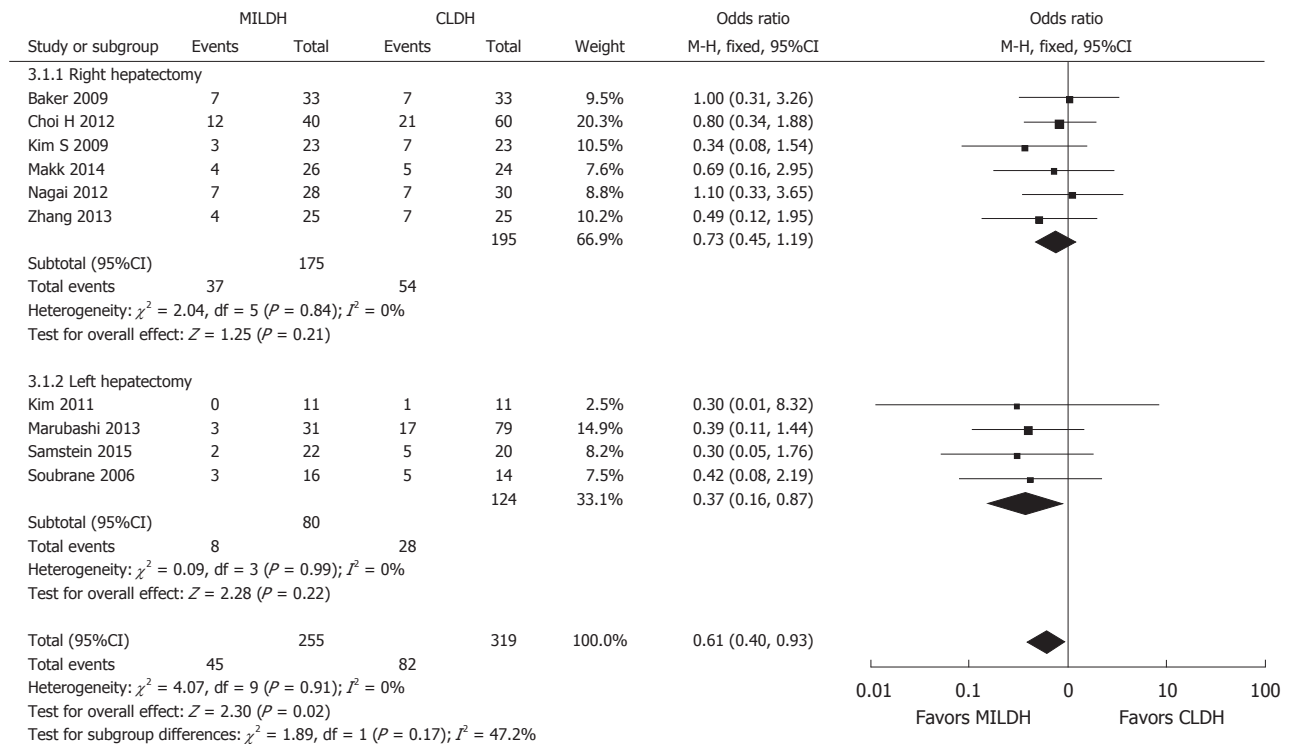


Figure 8 Forest plot and meta-analysis of postoperative complication rates for donors.

laparoscopy provided a magnified view of the liver, which was good for bleeding control.

Unlike intraoperative indices, postoperative outcomes favored MILDH. Our meta-analysis showed that postoperative complications (including wound-related, biliary, and vascular complications) occurred in 164 of 1093 patients in 12 studies, with a significantly lower postoperative donor complication rate in the

MILDH group (12.4%) than in the CLDH group (17.1%). Few patients in either group experienced severe complications, including death or need for retransplantation. Our study also showed no statistical difference in the donor biliary complication rate between the MILDH group (1.96%) and CLDH group (4.57%), but favored the MILDH group. The incidence of postoperative biliary complications of donors was

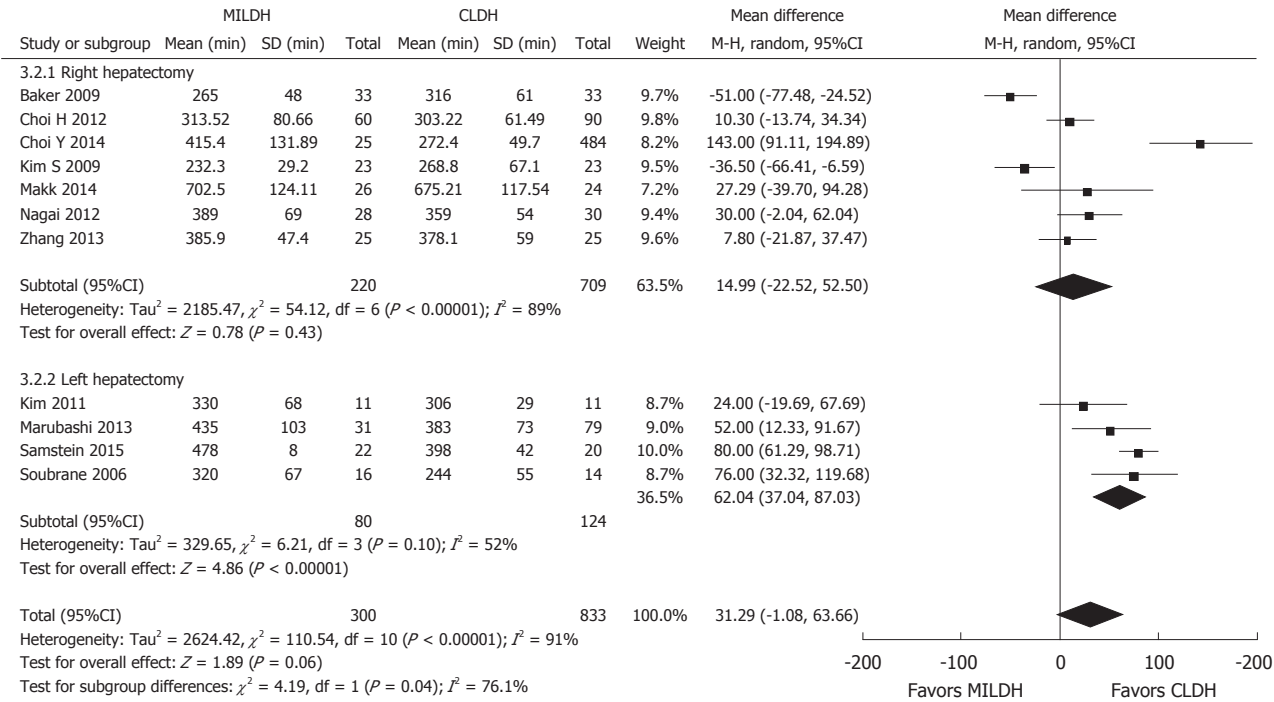


Figure 9 Forest plot and meta-analysis of operative time for donors.

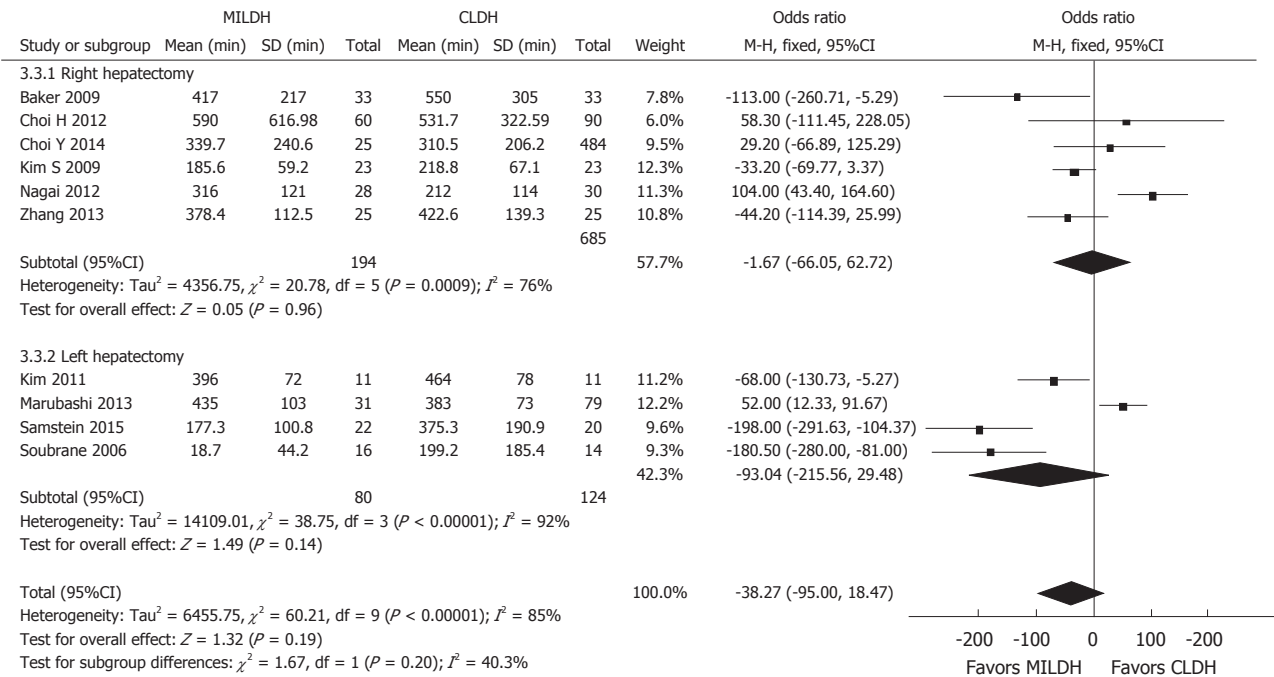


Figure 10 Forest plot and meta-analysis of estimated blood loss for donors.

closely related with the preoperative assessment of the biliary system and intraoperative anatomical techniques<sup>[35]</sup>. In laparoscopic liver resection, hepatic bile duct and artery could be identified more precisely with the amplification effect of laparoscopy, and the probability of bile duct injury would be reduced. We considered them as the main reasons for lower donor biliary complication rate in the MIDH

group. In addition, preoperative magnetic resonance cholangiopancreatography (MRCP), intraoperative cholangiography and marking bile duct cut line would help to reduce postoperative biliary complications in donors<sup>[16]</sup>.

Vascular complications (including postoperative bleeding and vascular embolization) of donors were related to the preoperative assessment of hepatic

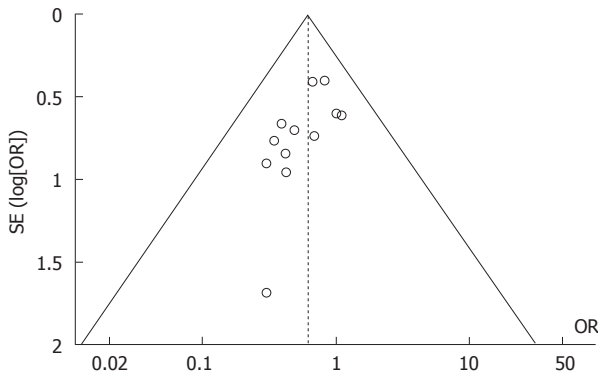


Figure 11 Funnel plot of postoperative complication rates.

vascular system and intraoperative anatomical techniques. Preoperative accurate assessment of hepatic vascular structures and careful intraoperative dissection techniques could reduce postoperative vascular complications effectively. Dissecting the liver precisely by minimally invasive approaches would also help to reduce donor vascular complications.

The rate of incision complications of the CLDH group was obviously higher than that of the MILDH group. The CLDH group adopted the "J" and "L" shape and "Mercedes" incision, which were larger compared to the other group. Large incision might cut off more abdominal nerves and was a high risk factor for incision complications. The smaller incision employed for MILDH, especially during laparoscopic surgery, could minimize surgical tissue trauma and abdominal nerve injury, thus reducing the rate of wound-related complications (infections, hematomas, and incisional hernias) and abdominal injuries. This finding is in good agreement with previous results<sup>[30]</sup>. Laparoscopic graft harvesting, in particular, could reduce incision hernia effectively. Smaller incision also contributes to reducing postoperative analgesia drug dose. Furthermore, minimally invasive approaches could result in earlier postoperative recovery of donors, thus minimizing other complications such as pleural effusion and intestinal obstruction.

Our study also found that the duration of continuous intravenous analgesic use was shorter in donors who underwent MILDH than in those who underwent CLDH. Minimally invasive approaches can reduce postoperative pain, as these approaches avoid cutting the subcostal muscle that is cut by conventional incisions, as well as minimizing surgical tissue trauma. Length of hospital stay was also significantly shorter for donors who underwent MILDH than in those who underwent CLDH, enabling the former to return to their normal lives earlier after surgery. A meta-analysis of 112 studies evaluating six laparoscopic surgical procedures showed a more rapid return to work after minimally invasive surgery<sup>[36]</sup>. Shorter hospital stay also contributed to lower hospital costs, increasing donor satisfaction with operative procedures. These outcomes were consistent with several studies of laparoscopic-assisted living

donor hepatectomy, which found less pain, improved postoperative symptoms, and faster recovery compared with conventional open surgery<sup>[33,37]</sup>.

Postoperative donor liver function was evaluated by measuring peak levels of serum AST, ALT, and TB. The pooled data showed no significant difference between the two groups. Reduced liver volume after donor liver resection may result in immediate, but transient, increases in peak AST, ALT, and TB. As the liver regenerates, all three indices would decline gradually. MILDH was a more difficult surgical procedure, but did not worsen liver function. Recovery time would be similar in donors who underwent MILDH and CLDH.

In subgroup analysis, we separately analyzed outcomes, including operative time, EBL, and postoperative complication rates, in patients who underwent LH or RH, to minimize any bias resulting from the side of liver resection. Donors who underwent minimally invasive LH had a lower rate of postoperative complications than those who underwent conventional LH; however, there was no between-group difference in donors who underwent RH. EBL and operative time were similar in donors who underwent minimally invasive and conventional LH and RH.

Evaluation of recipients showed no statistically significant differences in postoperative complication rate, postoperative liver function, or survival rate, although the complication rate was lower in the MILDH than in the CLDH group. There were also no significant differences in recipient postoperative liver function, as determined by peak serum AST, ALT, and TB levels. These findings indicate that the method of procuring liver grafts would have little effect on postoperative recipient liver function. Recovery times are similar in recipients who received grafts procured through MILDH and CLDH. The three studies<sup>[13,20,22]</sup> that evaluated recipient survival rate found no significant between-group difference. Other reports<sup>[38]</sup> evaluated several of the studies included in our meta-analysis, reporting survival rate but not postoperative liver function.

The biliary tree manipulation and identification have key impacts on the functions of graft. Our results showed no significant difference in the rate of recipient biliary complications between the MILDH group (18.8%) and CLDH group (17.4%). The recipient biliary complications include bile leakage and biliary stenosis and are closely related to the quality of liver graft<sup>[39]</sup>. Dissecting the liver precisely by minimally invasive approaches would help to harvest high-quality liver grafts and reduce the recipient biliary complications. In addition, comprehensive preoperative assessment of the biliary tract for donors (clear whether there are anomalies), familiarity with the hepatic biliary anatomy, cutting off donor bile duct precisely and feasible measures such as intraoperative cholangiography could minimize the risk of postoperative biliary complications<sup>[15]</sup>.

This meta-analysis had several limitations, including the quality of the included studies. No randomized



controlled trials were included, increasing the risk of bias owing to inadequate random sequence generation and blinding. In addition, all included articles were single-center studies, but differences in surgeons' experiences with the two techniques may have influenced patient outcomes. Moreover, within each study comparing MILDH with CLDH, not all operations were performed by a single surgeon, which may have introduced selection bias. Third, the follow-up period was generally short; therefore, long-term donor outcomes could not be evaluated. Finally, only three of the 13 included studies evaluated recipient survival rate. Because these recipients underwent LDLT with curative intent, their survival rate would be an important indicator of the safety and efficacy of these surgical procedures.

Nevertheless, the results of this meta-analysis are encouraging, as MILDH, which is more challenging to perform than CLDH, was always performed by experienced liver surgeons with a commitment to minimally invasive surgery<sup>[1]</sup>. Moreover, sufficient data on a large patient cohort that had undergone MILDH had accumulated, allowing evaluation by meta-analytical methods. Multiple strategies were used to identify applicable studies, with strict criteria used for study inclusion and evaluation. Subgroup analysis was performed to minimize heterogeneity. Future studies comparing MILDH and CLDH should include larger numbers of patients, with more data about recipients and a longer follow-up period.

In conclusion, the results of this meta-analysis comparing MILDH to CLDH show that MILDH could result in lower postoperative complication rate and analgesics requirement and shorter hospital stay with similar recipient outcomes. MILDH is safe, effective, and feasible for living donor liver resection. Nevertheless, MILDH, especially fully laparoscopic approach for the right lobe harvesting, is still an immature procedure with uncertain risk and effect, and should be performed cautiously.

## COMMENTS

### Background

Living donor liver transplantation has become an established treatment modality for patients with end-stage liver disease. With the wide use of minimally invasive techniques in hepatic surgery in recent years, more importance has been attached to minimally invasive living donor hepatectomy. Several centers considered minimally invasive approaches as safe and efficient techniques for graft harvested compared to conventional techniques. Despite this, no consensus is available in the literature about which of these two approaches is more beneficial to the patient.

### Research frontiers

Nowadays living donor liver harvesting is performed with minimally invasive approaches in a growing number of centers. The worldwide research is directed towards a type of technique to guarantee the safety of donors.

### Innovations and breakthroughs

In the present study, the authors investigated the outcomes of minimally invasive living donor liver resection and conventional approaches by pooling

results from different centers. This is the first report of a meta-analysis comparing these two kinds of surgical approaches with concluding satisfactory results.

### Applications

This report allows understanding the role of two surgical techniques for living donor hepatectomy.

### Peer-review

This systematic review and meta-analysis of retrospective studies adds useful information for practice and research, and probably for policy.

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## Successful treatment of a pancreatic schwannoma by spleen-preserving distal pancreatectomy

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## Abstract

Schwannomas are neurogenic tumors that arise from the neural sheaths of peripheral nerves. These tumors can be located in any area of the human body; the most common locations are the head, neck, trunk and extremities. Pancreatic schwannomas are very rare. Over the past 40 years, only 67 cases of pancreatic schwannomas have been reported in the English literature. Here we present a case of pancreatic schwannoma in a 62-year-old male. The tumor was revealed by ultrasound and computed tomography in the neck and body of the pancreas. An accurate diagnosis was difficult to obtain preoperatively. The patient consented to the performance of a laparotomy, and the mass was found in the neck and body of the pancreas and successfully treated using a spleen-preserving distal pancreatectomy with splenic artery and vein preservation. The procedure has only been reported in one other case of pancreatic schwannoma; here we present the second reported case. Macroscopically, the tumor was well circumscribed, gray-white in color and 3.3 cm × 2.8 cm in size. Microscopically, the tumor cells were spindle-shaped and had a palisading arrangement with no atypia, which are results compatible with a benign tumor. Both hypercellular and hypocellular areas were visible. Immunohistochemically, the tumor cells were strongly positive for S-100 protein. The tumor was definitively diagnosed as a schwannoma of the pancreatic neck and body. The patient was followed for 72 mo and has been doing well without any complications.

**Key words:** Schwannoma; Pancreas; Spleen-preserving distal pancreatectomy; S-100; Mesenchymal tumor

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**Core tip:** Over the past 40 years, only 67 cases of pancreatic schwannoma have been reported in the English literature. An accurate preoperative diagnosis is difficult to obtain. Here, we present the case of a patient with a pancreatic schwannoma who underwent spleen-preserving distal pancreatectomy. This surgical intervention has only been previously reported in one case of pancreatic schwannoma. After surgery, the patient recovered quickly and had a good prognosis. In this case report, we share our experience in the diagnosis and treatment of a rare pancreatic schwannoma and performed a literature review to deepen the understanding of the subject.

Xu SY, Wu YS, Li JH, Sun K, Hu ZH, Zheng SS, Wang WL. Successful treatment of a pancreatic schwannoma by spleen-preserving distal pancreatectomy. *World J Gastroenterol* 2017; 23(20): 3744-3751 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3744.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3744>

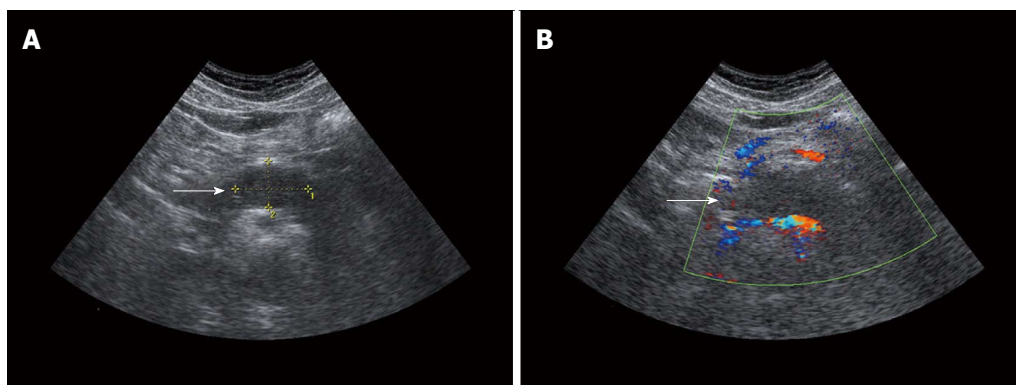
## INTRODUCTION

Schwannomas are mesenchymal tumors that originate from the Schwann cells of peripheral nerves<sup>[1]</sup>. Schwannomas are generally encapsulated, and over 90% are benign<sup>[2]</sup>. These tumors can occur in patients of all ages, with an equal frequency in males and females, and are most often reported in patients between 20 and 50 years of age<sup>[3]</sup>. A considerable number of these patients are asymptomatic, and the tumors are found incidentally<sup>[4]</sup>. Schwannomas can show either monosomy 22 or loss of 22q material; the definitive pathogenesis of the tumor remains uncertain<sup>[1]</sup>. Occasionally, the tumor can become cystic, hemorrhagic, calcified or even ossified<sup>[5]</sup>. Surgery may be the optimal treatment for schwannomas, after which patients generally have a good prognosis<sup>[6]</sup>. Although schwannomas can develop in any part of the body, the most common locations include the head, neck, trunk and extremities<sup>[7]</sup>. Schwannomas in the pancreas are extremely rare. To our knowledge, over the past 40 years, only 67 cases have been reported in the English literature<sup>[8-68]</sup>. In the present study, we present a case of a pancreatic schwannoma that was successfully treated using a spleen-preserving distal pancreatectomy technique and performed a review of the available literature.

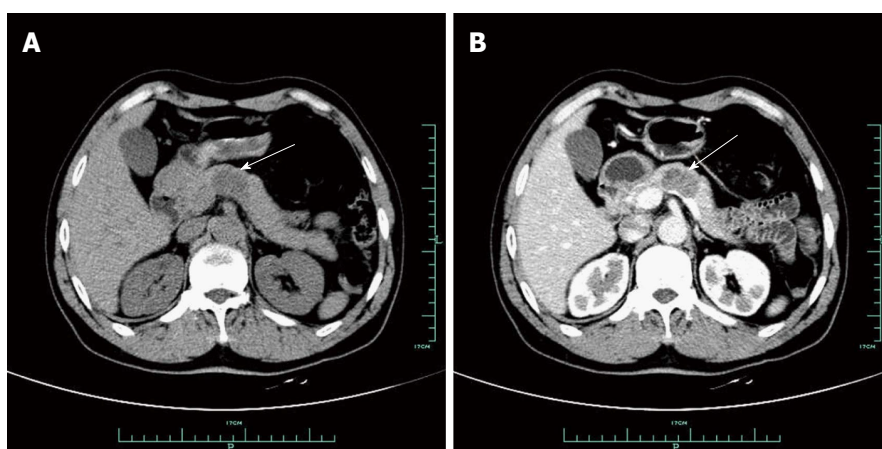
## CASE REPORT

On January 6, 2011, a 62-year-old male was referred to our hospital because of a pancreatic mass that was found on ultrasound during a routine health examination at the local hospital. His abdomen was soft, non-tender, and non-distended, with no evidence of a palpable or pulsatile mass. There was no history of weight loss or trauma and no family history of significant disease. Abnormal laboratory results included an international normalized ratio of 1.16 (normal range: 0.85-1.15) and a thrombin time of 21.7 s (normal range: 14.5-21.5). Other laboratory results, which included tumor markers, were normal. The ultrasound showed a well-defined, low-density lesion measuring 3.7 cm × 2.3 cm in the neck and body of the pancreas (Figure 1A). No blood flow signal was detected within the lesion on color Doppler ultrasound (Figure 1B). An unenhanced computed tomography (CT) scan revealed a well-marginated and hypodense mass measuring 2.8 cm × 1.9 cm in the pancreatic neck and body (Figure 2A). On contrast-enhanced CT, the mass was slightly and heterogeneously enhanced (Figure 2B). Endoscopic ultrasound-fine needle aspiration (EUS-FNA) was also performed. However, we failed to acquire a tumor sample. Although imaging results were obtained, the mass in the pancreatic neck and body was still unable





**Figure 1** Ultrasound findings. A: Ultrasound showed a well-defined, low-density lesion (arrow) measuring 3.7 cm × 2.3 cm in the pancreatic neck and body; B: No blood flow signal within the lesion was detected by color Doppler ultrasound.



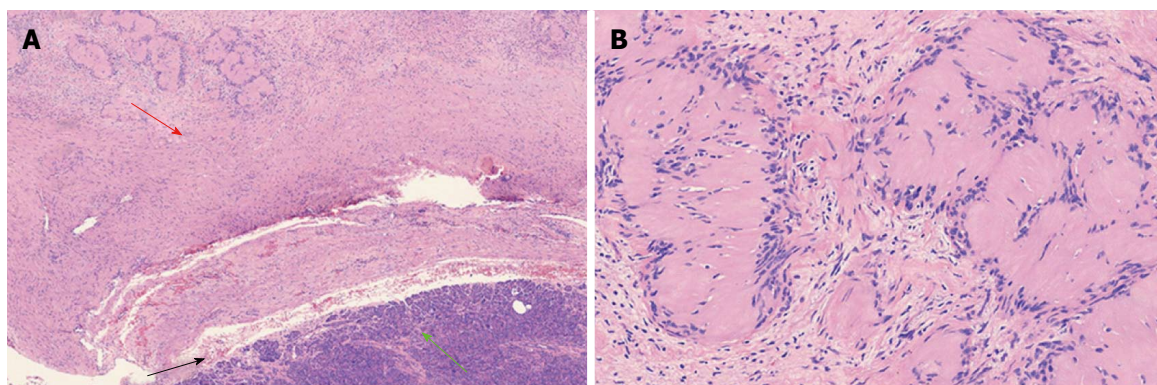
**Figure 2** Computed tomography findings. A: An unenhanced CT scan revealed a well-margined and hypodense mass (arrow) measuring 2.8 cm × 1.9 cm in the pancreatic neck and body; B: On contrast-enhanced CT, the mass was slightly and heterogeneously enhanced.

to be accurately diagnosed.

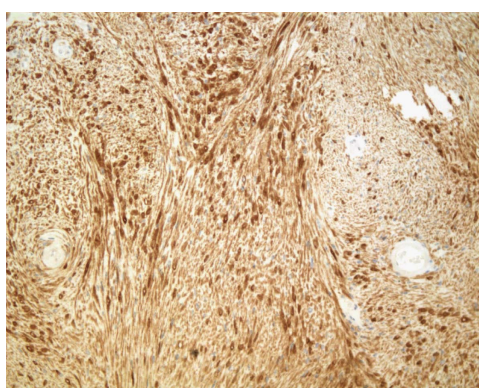
The patient consented to the performance of a laparotomy, and the mass was observed to originate from the pancreatic neck and body. We performed a spleen-preserving distal pancreatectomy with preservation of the splenic artery and vein. Intraoperative frozen pathology revealed a pancreatic schwannoma. Macroscopically, the mass was observed in the pancreatic neck and body, gray-white in color and 3.3 cm × 2.8 cm in size. Microscopically, the tumor cells were spindle-shaped and had a palisading arrangement with no atypia, which are results that are compatible with a benign tumor. Both hypercellular and hypocellular areas were visible (Figure 3). Immunohistochemically, the tumor cells were strongly positive for S-100 protein (Figure 4), but negative for SMA, CD34 and CD117. The tumor was definitively diagnosed as a schwannoma of the pancreatic neck and body. After surgery, the patient recovered smoothly and was discharged from the hospital 10 d later. The patient was followed for 72 mo and has been doing well without any further complications.

## DISCUSSION

Schwannomas are neoplasms that originate from Schwann cells in nerve sheaths<sup>[69]</sup>. More than 90% of schwannomas are benign, and they account for approximately 5% of benign soft-tissue neoplasms<sup>[3]</sup>. Malignant schwannomas are rare and are usually associated with von Recklinghausen's disease<sup>[70]</sup>. Schwannomas can occur in patients of all ages, with equal frequencies in males and females, and cases are most often reported in patients between 20 and 50 years of age<sup>[3]</sup>. Almost any site in the human body can be involved, although the head, neck, and extremities are the most reported areas of tumor development<sup>[71]</sup>. Schwannomas that originate from the nerve sheaths of the pancreas are extremely rare. Intra-pancreatic innervation includes the perivascular plexus, periacinous plexus and peri-insular plexus. These three plexuses connect with each other to form a net-like structure. However, the type of nerve fibers that produce the origination of pancreatic schwannomas has not yet been described. To our knowledge,



**Figure 3 Microscopic examination.** A: A thin capsule (black arrow) was found between the tumor (red arrow) and the normal pancreatic tissues (green arrow) (HE,  $\times 40$ ); B: The tumor cells were spindle-shaped and had a palisading arrangement with no atypia, which is compatible with a benign tumor. Both hypercellular and hypocellular areas were visible (HE staining,  $\times 200$ ). HE: Hematoxylin and eosin.



**Figure 4 Immunohistochemical staining.** The tumor cells were strongly positive for S-100 protein (HE staining,  $\times 200$ ). HE: Hematoxylin and eosin.

over the past 40 years, only 67 cases of pancreatic schwannoma have been reported in the English literature<sup>[8-68]</sup>. Table 1 summarizes the important available clinicopathological characteristics of these 68 cases, which include the present case. Continuous variables are summarized as the mean  $\pm$  SD and the range. Statistical analyses were conducted using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL).

Because the clinical symptoms and imaging characteristics of schwannomas are nonspecific, the accurate preoperative diagnosis of a pancreatic schwannoma is nearly impossible. A definitive diagnosis relies on the combined findings of the histopathological and immunohistochemical examination of surgical specimens. Microscopically, pancreatic schwannomas are usually encapsulated with varying relative amounts of the two histologic components of hypercellular Antoni A areas and hypocellular Antoni B areas<sup>[22]</sup>. The former is composed of closely packed spindle cells with occasional nuclear palisading. The latter consists of loosely arranged tumor cells and abundant myxoid stroma<sup>[13]</sup>. Over 90% of pancreatic schwannomas are benign. Thus far, only 5 (7.35%) malignant pancreatic schwannomas have been reported in the English

literature<sup>[29,64,66-68]</sup>. Immunohistochemical staining showed that schwannomas are positive for S-100 protein, but negative for desmin, smooth muscle myosin, SMA, CD34 and CD117<sup>[23,72]</sup>.

Imaging modalities including ultrasound, CT and magnetic resonance imaging (MRI) have a certain diagnostic value but lack specificity. On ultrasound, a pancreatic schwannoma is usually shown as a well-margined hypodense lesion. On unenhanced CT scans, schwannomas are usually well-defined hypodense lesions with an associated capsule. Schwannomas with high Antoni A areas show a high density and appear inhomogeneous. Schwannomas with high Antoni B areas appear cystic and show a low density<sup>[17]</sup>. On contrast-enhanced CT scans, Antoni A areas are enhanced, while Antoni B areas are unenhanced<sup>[14]</sup>. On MRI, schwannomas usually appear hypointense in T1-weighted images and inhomogeneous and hyperintense in T2-weighted images<sup>[23]</sup>. EUS-FNA may also be valuable for the preoperative diagnosis of schwannomas. Li *et al.*<sup>[35]</sup> reported a pancreatic schwannoma that was definitively diagnosed by EUS-FNA. In the present study, EUS-FNA was also performed; however, we failed to acquire a sufficient sample of the tumor.

Surgery is the curative treatment for pancreatic schwannomas, and most cases are treated by laparotomy. Only one case of pancreatic schwannoma was treated using minimally invasive laparoscopic surgery (1.47%)<sup>[21]</sup>. Enucleation has been reported in 10 (14.71%) cases. Patients treated by minimally invasive surgery might have less pain and faster recovery. Since the tumor can be located in different sections of the pancreas, surgical approaches may vary. In the present case, we performed a spleen-preserving distal pancreatectomy for the mass that was found in the neck and body of the pancreas. To date, spleen-preserving distal pancreatectomy has only been reported in one other case of pancreatic schwannoma<sup>[38]</sup>; here we describe the second

**Table 1 Summary of clinicopathological data from all 68 cases of pancreatic schwannoma**

	<i>n</i> (%) or mean $\pm$ SD (range)
Age (yr) ( <i>n</i> = 67)	
Mean	55.67 $\pm$ 15.13 (20-87)
Sex (male/female), (male %) ( <i>n</i> = 67)	30/37 (44.78)
Symptoms <sup>1</sup> ( <i>n</i> = 67)	
Asymptomatic	25 (37.31)
Symptomatic	
Abdominal pain	29 (43.28)
Weight loss	9 (13.43)
Back pain	4 (5.97)
Nausea/vomiting	3 (4.48)
Anorexia	2 (2.99)
Anemia	2 (2.99)
Melena	2 (2.99)
Jaundice	2 (2.99)
Dyspepsia	1 (1.49)
Abdominal discomfort	1 (1.49)
Abdominal mass	1 (1.49)
Location ( <i>n</i> = 68)	
Head	26 (38.24)
Head/body	3 (4.41)
Neck/body	1 (1.47)
Body	14 (20.59)
Body/tail	7 (10.29)
Tail	8 (11.76)
Uncinate process	8 (11.76)
Mean size (cm) ( <i>n</i> = 63)	5.75 $\pm$ 4.52 (1-20)
Benign	59 (5.21 $\pm$ 3.89) (1-20)
Malignant	4 (13.75 $\pm$ 6.24) (7-20)
Operation ( <i>n</i> = 68)	
PD <sup>2</sup>	20 (29.41)
PPPD	2 (2.94)
DP + splenectomy <sup>3</sup>	16 (23.53)
DP + splenic preservation	2 (2.94)
Enucleation	10 (14.71)
Central pancreatectomy	1 (1.47)
Unresectable	2 (2.94)
Refused	1 (1.47)
Not specified	13 (19.12)
Histology ( <i>n</i> = 68)	
Malignant	5 (7.35)
Benign	62 (91.18)
Not specified	1 (1.47)
Nature of tumor ( <i>n</i> = 68)	
Solid	21 (30.88)
Cystic	27 (39.71)
Solid and cystic	14 (20.59)
Not specified	6 (8.82)
Mean follow-up months ( <i>n</i> = 30)	22.23 $\pm$ 19.56 (3-67)
No. of deaths	0

<sup>1</sup>Some patients had two or several symptoms; <sup>2</sup>One patient underwent resection of the portal vein; <sup>3</sup>One patient underwent resection of the transverse colon. PD: Pancreaticoduodenectomy; PPPD: Pylorus preserving pancreaticoduodenectomy; DP: Distal pancreatectomy.

reported case. Compared with the traditional distal pancreatectomy and splenectomy for tumors in the body or tail of the pancreas, spleen-preserving distal pancreatectomy can not only offer complete resection of the tumor but also preserve the spleen. After complete removal of the tumor, patients usually have a good prognosis.

In conclusion, the finding of a schwannoma in the pancreas is extremely rare. Over the past 40 years, only 67 cases of pancreatic schwannoma have been reported in the English literature. Although multiple imaging modalities are currently available, it is challenging to obtain an accurate diagnosis prior to the performance of surgery, which is the optimal treatment for pancreatic schwannomas. Spleen-preserving distal pancreatectomy has only been reported in one previous case. After complete resection of the tumor, patients with pancreatic schwannoma usually have a good prognosis.

## COMMENTS

### Case characteristics

On January 6, 2011, a 62-year-old male was referred to the authors' hospital because of a pancreatic mass found during a routine health examination at the local hospital.

### Clinical diagnosis

The patient's abdomen was soft, non-tender, and non-distended, with no evidence of a palpable or a pulsatile mass.

### Differential diagnosis

Intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, solid pseudopapillary tumor, pancreatic endocrine tumor or pancreatic ductal adenocarcinoma.

### Laboratory diagnosis

Abnormal laboratory results included an international normalized ratio of 1.16 (normal range: 0.85-1.15) and a thrombin time of 21.7 s (normal range: 14.5-21.5). Other laboratory results, including tumor markers, were normal.

### Imaging diagnosis

Ultrasound showed a well-defined, low-density lesion measuring 3.7 cm  $\times$  2.3 cm in the pancreatic neck and body. No blood flow signal was detected by color Doppler ultrasound within the lesion. An unenhanced CT scan revealed a well-marginated and hypodense mass measuring 2.8 cm  $\times$  1.9 cm in the pancreatic neck and body. On contrast-enhanced CT, the mass was slightly and heterogeneously enhanced. Although they obtained these imaging results, the mass in the pancreatic neck and body was still unable to be definitively diagnosed.

### Pathological diagnosis

Macroscopically, the mass was shown in the pancreatic neck and body, gray-white in color and 3.3 cm  $\times$  2.8 cm in size. Microscopically, the tumor cells were spindle-shaped and had a palisading arrangement with no atypia, which are results that are compatible with a benign tumor. Both hypercellular and hypocellular areas were visible. Immunohistochemically, the tumor cells were strongly positive for S-100 protein, but negative for SMA, CD34 and CD117. The tumor was definitively diagnosed as a schwannoma of the pancreatic neck and body.

### Treatment

The patient underwent a spleen-preserving distal pancreatectomy.

### Related reports

Schwannoma in the pancreas is extremely rare. Over the past 40 years, only 67 cases of pancreatic schwannomas have been reported in the English literature. To date, the use of a spleen-preserving distal pancreatectomy has only been



reported in one other case.

## Experiences and lessons

Imaging modalities, including ultrasound, CT and magnetic resonance imaging, have a certain diagnostic value but lack specificity in the diagnosis of pancreatic schwannoma. Surgery is the curative treatment for the tumor. Since the tumor can be located in different sections of the pancreas, surgical approaches may vary. After complete resection of the tumor, patients with pancreatic schwannomas usually have a good prognosis.

## Peer-review

This study shares the experience in the diagnosis and the treatment of a rare pancreatic schwannoma with an accompanying literature review to deepen the understanding of the subject. The information in this paper is useful for readers.

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## Preoperative detection and localization of small bowel hemangioma: Two case reports

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### Abstract

Among the various diagnostic modalities for small bowel hemangioma, video capsule endoscopy (VCE) and double-balloon enteroscopy (DBE) can be recommended as part of the work-up in patients with obscure gastrointestinal bleeding (OGIB). DBE is superior to VCE in the accuracy of diagnosis and therapeutic potential, while in most cases total enteroscopy cannot be achieved through only the antegrade or retrograde DBE procedures. As treatment for small bowel bleeding, especially spout bleeding, localization of the lesion for the decision of DBE insertion facilitates early treatment, such as endoscopic hemostatic clipping, allowing patients to avoid useless transfusion and the worsening of their disease into life-threatening status. Applying endoscopic India ink marking prior to laparoscopic surgical resection is a particularly useful technique for more minimally invasive treatment. We report two cases of small bowel hemangioma found in examinations for OGIB that were treated with combination of laparoscopic and endoscopic modalities.

**Key words:** Laparoscopic surgery; India ink marking; Small bowel hemangioma; Obscure gastrointestinal bleeding; Minimally invasive

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**Core tip:** Small bowel hemangioma is a relatively rare small bowel tumor and can cause gastrointestinal bleeding, which often results in a diagnostic dilemma. video capsule endoscopy (VCE) and double-balloon enteroscopy are both useful modalities for the diagnosis of small bowel disease with obscure gastrointestinal bleeding including hemangioma, and preceding observation by VCE can result in a synergistic treatment effect. Furthermore, applying endoscopic India ink marking prior to laparoscopic surgical resection is a useful technique for achieving minimally invasive treatment.

Takase N, Fukui K, Tani T, Nishimura T, Tanaka T, Harada N, Ueno K, Takamatsu M, Nishizawa A, Okamura A, Kaneda K. Preoperative detection and localization of small bowel hemangioma: Two case reports. *World J Gastroenterol* 2017; 23(20): 3752-3757 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i20/3752.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i20.3752>

## INTRODUCTION

The detection of small bowel bleeding using conventional endoscopy is difficult because of the anatomical features of the small intestine. The current clinical guideline including diagnosis and management for patients with suspected small bowel bleeding is provided by American College of Gastroenterology<sup>[1]</sup>. Among the diagnostic modalities, such as video capsule endoscopy (VCE), double-balloon enteroscopy (DBE), computed tomography (CT), magnetic resonance, enterography, angiography and scintigraphy, VCE and DBE show the greatest impact on the diagnosis and treatment of small-bowel disease<sup>[2-4]</sup>. We herein report two cases of small bowel hemangioma found in examinations for obscure gastrointestinal bleeding.

## CASE REPORT

### Case 1

A 62-year-old Japanese female who had been admitted to another facility presented with black (tar) colored stools and general malaise as her chief complaints. There was no past medical problem. Though she had a severe iron-deficiency anemia, general gastrointestinal examinations including upper endoscopy and total colonoscopy to the terminal ileum were normal. She was referred to our hospital for a detailed examination of obscure gastrointestinal bleeding (OGIB). Although she had received repeated transfusions of red blood cells with one as recent as one week prior, her initial hemoglobin level was 3.8 g/dL. Her coagulating system and tumor markers including carcinoembryonic

antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were normal. VCE revealed the exact location of the lesion (Figure 1A), and DBE revealed spout bleeding in the upper jejunum (Figure 1B). The DBE showed a raised lesion (15 mm) with smooth surface in the upper jejunum site of approximately 60 cm (Figure 1B and C). We suspected the lesion as small intestinal hemangioma or arteriovenous malformation. We performed endoscopic hemostatic clipping of the lesion (Figure 1D), and during the endoscopic procedure, applied a visualization technique for tumor localization using India ink tattooing. Finally, we performed a video-assisted single-port laparoscopic enterectomy (Figure 2A). The resected specimen contained a depressed lesion with necrosis-related slough of endoscopic clipping lesion (Figure 2B). Histological findings revealed a cavernous hemangioma without atypia mainly composed of submucosa (Figure 2C). She was dismissed from the hospital 8 d after undergoing surgery.

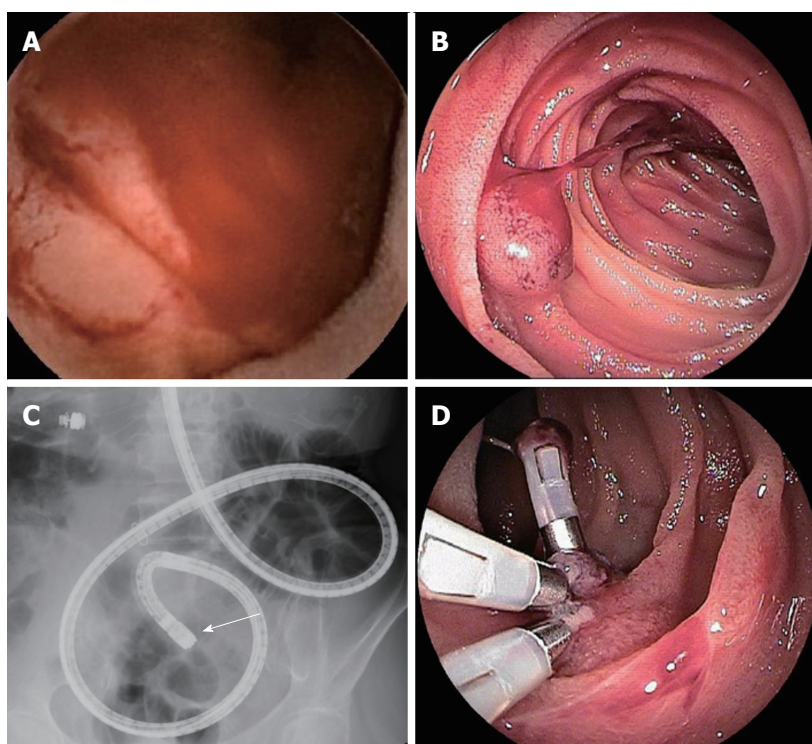
### Case 2

A 52-year-old Japanese male visiting his family doctor presented with black (tar) colored stools for 3 mo as his chief complaint. He had hypertension, hyperlipidemia and type 2 diabetes mellitus without complications. Though he was diagnosed with only mild iron-deficiency anemia, his hemoglobin level had decreased by approximately 35% in 6 mo. Therefore, a general gastrointestinal examination was performed. When upper endoscopy and total colonoscopy showed almost normal results, he was referred to our hospital for a detailed examination of OGIB. His initial hemoglobin level was 11.3 g/dL. Early-phase contrast-enhanced computed tomography (CECT) showed a small nodule enhancement in the ileum (Figure 3A). VCE revealed the precise location of the lesion (Figure 3B), and DBE revealed submucosal tumor-like (SMT) raised lesion (10 mm) with central erosion in the lower ileum (Figure 3C); still, histological findings from the endoscopic biopsy showed nonspecific changes. We preoperatively diagnosed the lesion as hemangioma, and performed a video-assisted laparoscopic enterectomy using preoperative endoscopic India ink tattooing (Figure 3D). Histological findings revealed a capillary hemangioma without atypia from the muscle layer to the mucosal layer (Figure 3E). The patient was dismissed from the hospital 8 days after surgery.

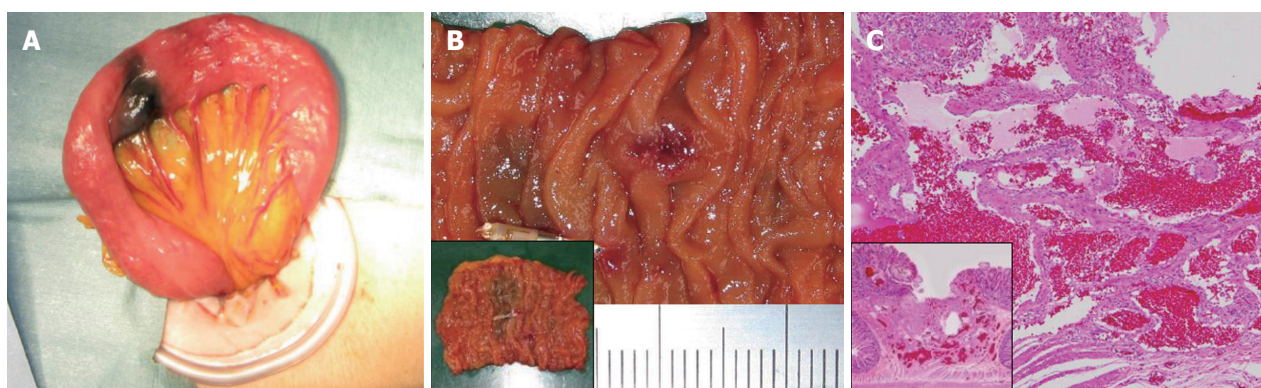
## DISCUSSION

Intraluminal gastrointestinal bleeding can be a life-threatening condition. In case of OGIB this is rarely the case when esophagogastroduodenoscopy and colonoscopy with adequate preparation have been performed<sup>[5,6]</sup>, which often results in a diagnostic dilemma. In 75% of OGIB patients, the lesion is ultimately found in the small intestine, and occasionally





**Figure 1 Evaluation of endoscopic findings (case 1).** Video capsule endoscopy (A) and double-enteroscopy (B and C) show a raised lesion with smooth surface in the upper jejunum, and double-balloon enteroscopy showed spout bleeding of the lesion. The lesion in the jejunum was disclosed 29 min after capsule ingestion (pylorus passage at 16 min) (A). Detailed localization of the target lesion using fluoroscopy is shown by the end of endoscopic insertion (arrow) (C). The lesion underwent endoscopic hemostatic clipping (D).



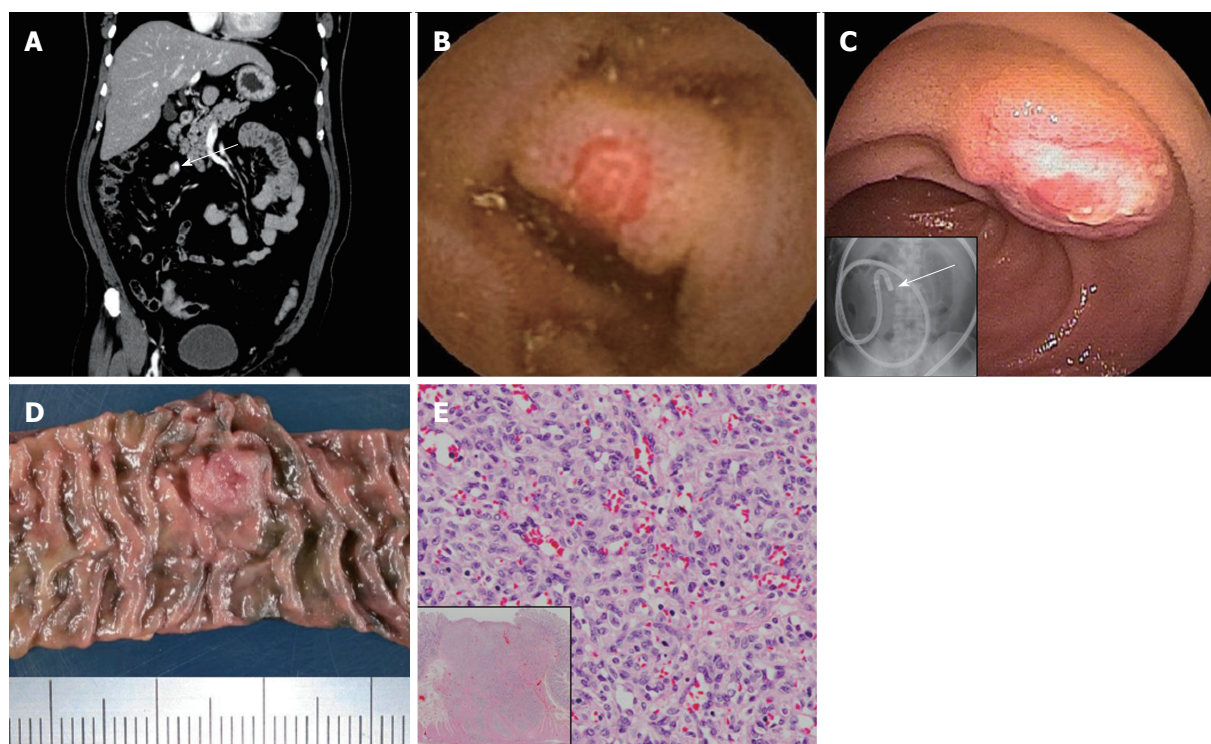
**Figure 2 Surgical and pathological finding (case 1).** The surgical finding on single port laparoscopic survey shows an objective site with India link tattooing (A). Surgical specimen from the small intestine. Including the lesion indicated with India link shows a whole view of the resected lesion (B). Histological (h-e stain) in the resected specimen show different-sized blood vessels circumferentially proliferated from the mucosa to submucosa. Inset shows different-sized distended blood vessels circumferentially proliferated from the mucosa to submucosa. Inset shows a low-power field view (C).

causes recurrent or persistent bleeding<sup>[6]</sup>.

Small bowel bleeding is relatively rare, comprising only about 5% of gastrointestinal bleeding<sup>[1,7]</sup>. Concerning the common causes of small bowel bleeding, the causative disease varies by age. In patients under 40 years of age, inflammatory bowel disease, the most common cause, is followed by Dieulafoy's lesions, neoplasms, Meckel's disease and Polyps. In those over 40, angioectasia including arteriovenous malformation and hemangioma is followed by Dieulafoy's lesions, neoplasms and NSAIDs ulcers<sup>[1]</sup>. In addition, Yamamoto *et al.*<sup>[8]</sup>

reported that the frequency of angioectasia-related bleeding among small bowel bleeding cases was 20%.

Of all primary small bowel tumor cases, 47% are benign<sup>[9]</sup>. Among them, small bowel hemangioma is a relatively rare primary small bowel tumor, histologically defined as a benign tumor. Wilson *et al.*<sup>[10]</sup> reported that hemangioma constitutes approximately 10% of benign small bowel tumors and that most reported cases have occurred in the jejunum and ileum. Concerning the initial common symptoms of small bowel hemangioma, the occurrence frequency of iron-deficiency anemia, pain and intussusception are 41%, 31% and 13%,



**Figure 3 Evaluation of clinical finding (case 2).** Early-phase contrast-enhanced computed tomography reveals small nodule enhancement in the ileum (arrow) (A). Video capsule endoscopy (B) and double-balloon enteroscopy (C) show a submucosal tumor-like raised lesion with central erosion in the lower ileum. The lesion in the jejunum was disclosed 145 min after capsule ingestion (pylorus passage at 140 min) (B), inset indicates fluoroscopic localization of target at the end of the endoscope insertion (arrow) (C). Surgical specimen from the small intestine, including the indicated lesion with India ink tattooing (D). Histological finding (H-E stain) in the resected specimen show circumferential capillary growth without atypia from the mucosa to the muscle. Inset shows a low-power field view (E).

respectively<sup>[10]</sup>. Macroscopically, Chen *et al.*<sup>[11]</sup> summarized previous endoscopy reports of intestinal hemangiomas describing the lesions as typically submucosal, purple to red, soft and pedunculated. Histologically, hemangiomas can be broadly classified in capillary, cavernous and mixed type, with the cavernous type being the most common<sup>[12]</sup>. They generally consist of numerous dilated, irregular blood-filled spaces or sinuses lined by layers of endothelial cells<sup>[13]</sup>.

Recent advances in endoscopic technique including VCE and DBE have allowed preoperative diagnosis of small bowel hemangioma<sup>[11]</sup>. VCE can be recommended as part of the routine work-up in patients with obscure bleeding<sup>[14]</sup>, and it is not contraindicated except in patients with stenosis of the intestine. Compared with VCE, DBE has the advantage of biopsy and therapeutic potential, such as preoperative localization, coagulation and hemostasis by clipping<sup>[15]</sup>, whereas Xin indicated that successful total enteroscopy is achieved in only 1.6% of patients through the antegrade procedure<sup>[16]</sup>.

In recent years, the therapeutic options including minimally invasive laparoscopic surgery represented by our cases and even non-surgical endoscopic approaches for small bowel hemangioma have been proposed. The laparoscopic approach to gastrointestinal diseases is now widely accepted, but it is generally difficult to locate the lesion by palpation, especially in the jejunum and ileum. A previous

study reported on colonic tattooing in animal model with various agents, such as methylene blue, indigo carmine, toluidine blue, lymphazurine, hematoxylin, eosin, indocyanine green (ICG) and India ink<sup>[17]</sup>. Only ICG and India ink tattoos persisted for more than 48 h, while ICG was associated with allergic reactions and systemic toxicity<sup>[17]</sup>. Therefore, endoscopic marking with India ink is used widely as a visualization technique for colorectal cancer, to define the operative location. India ink tattooing has a low incidence of complications (0.22%), and remains for a prolonged duration<sup>[17,18]</sup>. In the present case, we selected this visualization technique for video-assisted single-port laparoscopic enterectomy, expecting a minimally invasive effect. Concerning non-surgical endoscopic approaches, several clinical studies have reported that endoscopic mucosal resection can be a useful tool for small bowel hemangioma<sup>[19-21]</sup>. However, in addition to the common endoscopic complications including intestinal perforation and lesion persistence, the endoscopic approach for small bowel hemangioma with rich vascularity has the potential for flooding, given the potential for misconception of the lesion depth. Chen *et al.*<sup>[11]</sup> also recommended careful consideration of the indications for endoscopic surgery. As one of the methods to overcome these potential risks, laparoscopic and endoscopic cooperative surgery (LECS) has been suggested as a new concept for tumor dissection<sup>[22]</sup>. In recent years, Kanaji *et al.*<sup>[23]</sup>



demonstrated safe and total laparoscopic resection of hemangiomas in the third portion of the duodenum using the LECS technique. Therefore, LECS may become a useful therapeutic option for small bowel hemangioma.

In conclusion, we have documented two cases of small bowel hemangioma found in examinations for OGIB. The preceding implementation of VCE made the selective decision of DBE insertion easy, and the endoscopic process facilitated early treatment, resulting in avoidance of progression to life-threatening status. In the present case report (Case 2), CECT showed small nodule enhancement in the lesion. Therefore, the preceding CECT with a characteristic of rapid and minimally invasive technique may omit VCE, making it a useful algorithm for further early treatment. These findings imply that various preoperative endoscopic contrivances may result in safer, more minimally invasive treatment.

## COMMENTS

### Case characteristics

Two cases of small bowel hemangioma found in examinations for obscure gastrointestinal bleeding (OGIB) were treated with combination of laparoscopic and endoscopic modalities.

### Clinical diagnosis

Preoperative enteroscopy and imaging tests in each case suggested that the lesion was a small intestinal hemangioma or arteriovenous malformation.

### Differential diagnosis

Arteriovenous malformation.

### Laboratory diagnosis

Iron-deficiency anemia was diagnosed, with one case showing severe anemia.

### Imaging diagnosis

Video capsule endoscopy (VCE) showed the precise location of the lesion, and double-balloon enteroscopy (DBE) revealed raised lesions with or without spout bleeding in the small intestine.

### Pathological diagnosis

They diagnosed Case 1 and Case 2 as cavernous hemangioma and capillary hemangioma, respectively.

### Treatment

The patients were treated with combination of laparoscopic and endoscopic modalities.

### Related reports

There are few reports on treatments combining VCE, DBE on fluoroscopy, preoperative endoscopic marking, and single-port laparoscopic surgery.

### Term explanation

There are no uncommon terms used in this manuscript.

### Experiences and lessons

The authors share this case as important knowledge for the appropriate treatment process for small bowel hemangioma.

## Peer-review

These two case reports demonstrate that small bowel hemangioma found as an examination for OGIB were successfully treated with combination of laparoscopic and endoscopic modalities.

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## Non-invasive stimulation techniques to relieve abdominal/pelvic pain: Is more always better?

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### Abstract

Chronic abdominal and pelvic pain is a common condition that has significant impact on quality of life, and causes billions of dollars in direct and indirect costs. Emerging data suggest that transcranial direct current stimulation (tDCS), alone or in combination with transcutaneous electrical nerve stimulation (TENS), could be a promising therapeutic avenue to reduce chronic pain. The encouraging results coming from these studies prompted us to try combining TENS and tDCS in 4 of our patients who suffered from chronic abdominal/pelvic pain and to compare the effect with 5 other patients who received TENS alone. Pain intensity was assessed with a visual analog scale before, during and after the stimulation. We observed that there was a slight decrease in pain which was similar in both patient groups (TENS alone and TENS combined with tDCS). These observations suggest that combining TENS and tDCS in patients suffering from chronic pelvic and/or abdominal pain produces no additional benefit, compared to TENS alone. Future studies, looking at the effect of several/consecutive TENS and tDCS sessions should be conducted.

**Key words:** Transcranial direct current stimulation; Transcutaneous electrical nerve stimulation; Pelvic pain; Abdominal pain; Chronic pain

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**Core tip:** Past studies have showed that combining transcutaneous electrical nerve stimulation (TENS) and transcranial direct current stimulation (tDCS) can be an effective strategy to relieve chronic pain. In this letter, we describe the observations made on nine patients suffering from chronic pelvic and/or abdominal pain. Combining TENS and tDCS produced negligible effect on pain. The reduction in pain noted after the application of TENS and tDCS was comparable to the reduction noted after the application of TENS only. These clinical observations question the added value of tDCS in patients suffering from chronic pelvic and abdominal pain.

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## TO THE EDITOR

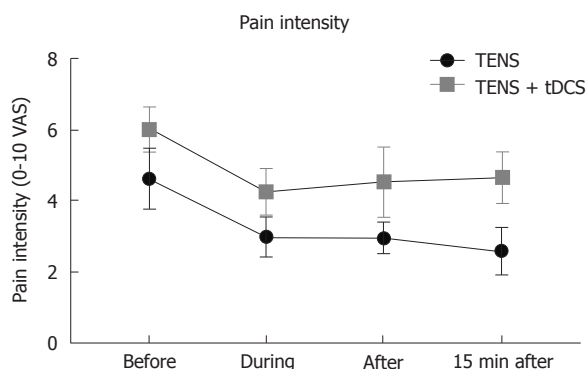
Chronic pelvic pain syndrome is quite prevalent and disabling, and should definitely receive more attention<sup>[1]</sup>. Abnormalities in the brain-gut axis play an important role in functional gastro-intestinal disorders, suggesting that brain modulation can be a part of the solution to relieve visceral pain, such as pelvic and abdominal pain<sup>[2]</sup>. Over the last few years, two studies looking into the usefulness of brain stimulation techniques to reduce chronic abdominal and pelvic pain syndromes were published. The first article, published by Fenton *et al.*<sup>[3]</sup>, looked into the safety and efficacy of transcranial direct current stimulation (tDCS) in patients suffering from refractory chronic pelvic pain. Then, a few years later, Schabrun *et al.*<sup>[4]</sup> published a study in which they suggested that combining tDCS to transcutaneous electrical nerve stimulation (TENS) could be more effective to reduce pain than tDCS or TENS alone. TENS is a modality that is frequently used with our patients. Although the outcomes are generally good, some patients report no significant benefit following TENS application. The results reported by Schabrun *et al.*<sup>[4]</sup> prompted us to try combining TENS and tDCS in our patients who suffered from chronic abdominal and/or pelvic pain and who failed standard pharmacological/surgical therapies.

Patients were randomly allocated to TENS alone ( $n = 5$ ) or TENS combined with tDCS ( $n = 4$ ) using a random numbers table with a ratio of 1: 1, based on their order of entry in the trial. All patients (mean age  $43 \pm 10$  years old) were medicated for their pain (8 with opioids, 5 with cannabinoids, 5 with

anticonvulsants, and 1 with tricyclic antidepressants; note that every patient had at least two medications). They were asked to keep their medication stable at least 1 mo before receiving the neurostimulation treatments. There was no difference between the 2 treatment groups regarding the age and medical diagnosis, although the proportion of women tended to be higher in the TENS-only group. For both groups, TENS was delivered using 2 pairs of rubber silicone electrodes connected to a digital Eclipse Plus apparatus (Empi, St. Paul, Minnesota). Two electrodes were placed on the lower lumbar or abdominal region and two other electrodes were placed over the right tibial nerve, in order to target the painful area (directly or via the associated dermatome)<sup>[5]</sup>. TENS frequency was set at 3 Hz and the pulse duration at 400 ms, and the intensity was adjusted to produce strong and painful sensations<sup>[5,6]</sup>. For the TENS + tDCS group, a 2 mA direct current was transferred to the patients, through the scalp, by a saline-soaked pair of surface sponge electrodes (5 cm × 7 cm) and delivered by a constant current stimulator, battery-driven, 1 × 1 tDCS device (Model 1300-A; Soterix Medical Inc, New York). Patients received anodal stimulation of the primary motor cortex (M1), as suggested by Fenton *et al.*<sup>[3]</sup> and by Schabrun *et al.*<sup>[4]</sup>. The anodal electrode was placed over M1, contralateral to the most painful site (C3 or C4 according to the electroencephalogram 10/20 system), and the cathodal electrode was placed on the supraorbital area contralateral to the anode<sup>[3,7]</sup>. Both TENS and tDCS were applied for 30 min. Patients who received TENS + tDCS received both stimulations simultaneously. Pain intensity was assessed 4 times during the patients' visit (before, during, after and 15 min following the treatment) using a visual analog scale (VAS) of 10 cm ranges from "no pain" (0 cm) to "the worst imaginable pain" (10 cm). The study was approved by the local institutional ethics committee and written informed consent was obtained from all patients.

As can be seen in Figure 1, there was a slight decrease in pain during treatment; however the pain reduction was not clinically significant and was similar between both groups (average reduction of 1.6 in the TENS group and 1.8 in the TENS+tDCS group)<sup>[8]</sup>. Pain intensity continued to slightly decrease and barely reached clinical significance (2 points on VAS) 15 min after stimulation in the TENS group<sup>[8]</sup>.

These results somewhat contrast with those of Schabrun *et al.*<sup>[4]</sup> who observed a decrease 2.5 in the pain severity score after TENS alone and a decrease of 2.8 after combined TENS and tDCS, a change that was both statistically and clinically significant<sup>[8]</sup>. The inconsistencies between our observations and those of Schabrun *et al.*<sup>[4]</sup> could be explained by the different populations studied (chronic low back pain vs chronic abdominal/pelvic pain). It is also important to mention that the beneficial effect of TENS+tDCS noted by



**Figure 1** Pain intensity assessed with a 10 cm visual analog scale ranges from 0 to 10. Each point represents mean  $\pm$  SEM (standard error of mean). There were 5 patients in the transcutaneous electrical nerve stimulation (TENS) group and 4 patients in the TENS + transcranial direct current stimulation (tDCS) group.

Schabrun *et al.*<sup>[4]</sup> was observed in a subsample of patients only (*i.e.*, in individuals with more pronounced pain sensitization). Different results could have perhaps been obtained if we had included solely chronic abdominal/pelvic pain patients with increased pain sensitization. Furthermore, it should be noted that the effects noted in our patients were observed after one single session of neurostimulation. Providing chronic pain patients with only one tDCS session is perhaps not sufficient to drive important and long-lasting changes in symptoms. Finally, it should be pointed out that 8 of our 9 patients took opioids on a regular basis, a medication that is known to have a negative effect on the reaction to low frequency TENS<sup>[9]</sup>.

In conclusion, our observations suggest that one session of TENS, alone or in combination with tDCS, can slightly reduce pain in patients suffering from chronic abdominal or pelvic pain. However, combining TENS with tDCS does not seem to provide any additional benefit. Contrary to TENS, which can

be self-administered by patients at home during their everyday activities, tDCS must be administered by a healthcare professional. Future studies, looking at the effect of several/consecutive TENS and tDCS sessions should be conducted.

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