

# World Journal of *Gastroenterology*

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## Liver disease in menopause

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### Abstract

There are numerous physiologic and biochemical changes in menopause that can affect the function of the liver and mediate the development of liver disease. Menopause represents a state of growing estrogen deficiency, and this loss of estrogen in the setting of physiologic aging increases the likelihood of mitochondrial dysfunction, cellular senescence, declining immune responses to injury, and disarray in the balance between antioxidant formation and

oxidative stress. The sum effect of these changes can contribute to increased susceptibility to development of significant liver pathology, particularly nonalcoholic fatty liver disease and hepatocellular carcinoma, as well as accelerated progression of fibrosis in liver diseases, as has been particularly demonstrated in hepatitis C virus liver disease. Recognition of the unique nature of these mediating factors should raise suspicion for liver disease in perimenopausal and menopausal women and offer an opportunity for implementation of aggressive treatment measures so as to avoid progression of liver disease to cirrhosis, liver cancer and liver failure.

**Key words:** Liver disease; Menopause; Aging

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**Core tip:** There is an interplay of hormonal issues and aging that create a unique path for development of liver disease in menopausal women. Reviewed in this article are the expected liver-related physiologic and biochemical features of menopause and the impact of menopause on the natural history of liver disease. The impact of an understanding of how menopause mediates liver disease is important as there are growing numbers of menopausal women worldwide.

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### INTRODUCTION

Chronic liver disease and cirrhosis impose a significant burden on worldwide health. Representing the 12<sup>th</sup> leading cause of death worldwide, cirrhosis resulted in the death of one million people in 2010, which was 33% more than the number of persons worldwide

**Table 1** Benefits of estrogen in liver disease

Inhibition of fibrogenesis
Protection of mitochondrial structure and function
Inhibition of cellular senescence
Increase in innate immunity
Promotion of antioxidant effects

who died of cirrhosis in 1990<sup>[1]</sup>. Similarly, in the United States, chronic liver disease and cirrhosis represent the 12<sup>th</sup> leading cause of death with approximately 30000 lives lost each year<sup>[2]</sup>. More notably, the rate of liver-related death increases among those of older age, with chronic liver disease and cirrhosis representing the fourth leading cause of death in persons aged 45 to 54 years and representing the seventh leading cause of death in persons aged 55 to 64 years. Thus, with increasing age there appears to be an increasing burden of liver disease, and this has been demonstrated in both men and women<sup>[3]</sup>.

For women, issues regarding liver disease are unique in that both age and hormonal factors influence the development and progression of various liver diseases, and it has been demonstrated that this interplay of factors negatively affects the course of liver-related health in postmenopausal women. The average age of natural menopause in the western world is 51 years, and increasingly larger numbers of women are approaching menopause. About 6000 women in the United States reach menopause each day, and it has been estimated that by 2020, more than 50 million women in the United States will be older than age 51 years, the majority of whom will either have fully transitioned to or be in the midst of transitioning into menopause<sup>[4]</sup>. Worldwide estimates demonstrated that in 1998, more than 477 million women were postmenopausal, and it is estimated that by 2025, 1.1 billion women worldwide will be postmenopausal<sup>[5]</sup>. Thus, it is becoming increasingly important that the natural history and management of chronic liver disease are understood in the unique context of how they are mediated by menopause.

## THE LIVER IN MENOPAUSE

### Menopause physiology

Menopause is a gradual process of reproductive aging involving a sequence of hormonal changes over several years that culminates in the cessation of ovarian follicular activity and menstrual cycles. The changes that ensue involve an early follicular phase in the late reproductive years with increased levels of follicle stimulating hormone (FSH) and a decline in levels of inhibin B, a glycoprotein that suppresses FSH and occurs as a result of a decrease in the number of ovarian follicles<sup>[6,7]</sup>. During this time, estrogen levels are preserved. However, as the menopausal transition

unfolds, FSH levels remain high, but estrogen levels begin to fluctuate and eventually decline as the permanent cessation of menses that defines menopause ensues. In the premenopausal state, 17 $\beta$ -estradiol is the predominant and most potent form of estrogen<sup>[8]</sup>. In the postmenopausal state, estrone, a much weaker form of estrogen, predominates and is produced *via* conversion of androstenedione in adipose tissue and in the liver. In menopause, the interplay of reductions in estrogen levels along with biochemical effects of the aging process foster an environment that increases the propensity for damage within the liver.

### Physiologic significance of estrogen

Estrogen exhibits a number of beneficial roles in the body, as it has been shown to promote coagulation, aid in maintaining proper fluid balance, and foster increases in high density lipoproteins and decreases in low density lipoproteins that lead to favorable lipid profiles. Likewise, estrogen exerts a number of liver-related benefits (Table 1). Within the liver, estrogen inhibits proliferation of stellate cells and fibrogenesis. Important steps in the development of fibrosis in the liver include activation of stellate cells, which transform into myofibroblast-like cells, and these cells then proliferate and express  $\alpha$  smooth muscle actin ( $\alpha$ -SMA)<sup>[9]</sup>. They synthesize large amounts of extracellular matrix components such as type I collagen, type III collagen, type IV collagen, laminin, fibronectin and proteoglycans. In rat models in which hepatic fibrosis was induced with dimethylnitrosamine, treatment with estradiol led to reduced expression of type I procollagen as well as reduced deposition of type I and type III collagens, reduced expression of  $\alpha$ -SMA and reduced stellate cell proliferation<sup>[10,11]</sup>.

Estrogen has been shown to regulate the structure and function of mitochondria, particularly in tissues that have a high energy demand. Mitochondria have an important role in ATP production, cell metabolism and apoptosis, and homeostasis of reactive oxygen species (ROS) as well as hepatocyte metabolism of glucose, lipids, and proteins<sup>[12]</sup>. Dysfunction of mitochondria can lead to disruption of normal cell function and cell membrane permeability and cell senescence, the permanent arrest of cell growth. This process contributes to aging of tissues. Data have shown that estrogen receptors (ERs) and estrogen binding proteins are located within the mitochondria of many cell types, including the liver<sup>[13]</sup>. Estrogen mediates the structure and function of mitochondria *via* transcription of mitochondrial DNA and induction of nuclear DNA-encoded proteins within the mitochondrial respiratory chain (MRC), leading to enhanced MRC activity with subsequent increased ATP and superoxide<sup>[14]</sup>. Further, there are data suggesting that knockout of one ER isoform (ER $\beta$ 1) prevents estrogen-derived protection against mitochondrial membrane depolarization<sup>[15]</sup>.



Demonstration of the ability of estrogen to protect and enhance the function of mitochondria suggests that estrogen may be able to inhibit cellular senescence induced by mitochondrial damage and thus, in turn, slow down the aging process in tissues. This further suggests that the reduction of estrogen as seen in menopause may promote mitochondrial dysfunction, cellular senescence, and tissue aging.

#### **Expected morphologic features of the menopausal liver**

There are a number of morphologic changes that occur within the liver as it ages, and thus, such features would be expected to evolve within the liver in menopause. Such changes include reductions in liver blood flow and volume as well as changes in the capacity for liver regeneration. Data have shown that liver volume, blood flow and function decrease approximately 1% per year after age 40 to 50 years<sup>[16]</sup>. Overall, liver volume decreases by 20% to 40% by the time persons reach elderly age, and this reduction is noted to be more marked in women<sup>[17]</sup>. Blood flow is reduced by 35% to 50% in the elderly and may contribute to the reduced liver volume that is seen with increasing age<sup>[18]</sup>.

#### **Immunosenescence**

Liver injury is mediated in part by the host immune response, and gender and aging influence the role of innate and adoptive immunity in liver disease. Data have shown that female cells show a 10-fold greater expression of the induction of genes associated with toll-like receptor pathways and antiviral type I interferon responses, leading to an increase in the host detection of viral and bacterial nucleic acids<sup>[19]</sup>. The number and activity of cells of innate immunity, including dendritic cells, monocytes and macrophages are higher in females compared to males<sup>[20]</sup>. Likewise, the humoral and cellular immune responses to antigens are stronger in females than in males. Women have higher CD3<sup>+</sup> and CD4<sup>+</sup> cell counts and higher inflammatory helper T-cell type 1 responses than men<sup>[21,22]</sup>. They also have greater cytotoxic T cell activity and greater upregulation of antiviral and proinflammatory genes than men. It has been observed that these gender differences in components of the immune response are related to the binding of sex steroids, including estradiol, to specific receptors and the subsequent alteration of cell signaling pathways and production of cytokines and chemokines<sup>[23]</sup>. As the body ages, monocytes, macrophages and dendritic cells have decreased function, and the number and function of T cells also declines<sup>[17]</sup>. Thus, despite the hormonally-mediated immune advantage that women may have over men in adaptation to injury, this advantage may decline in menopause. As an example, data have shown decreasing CD4<sup>+</sup> cells and B lymphocytes as well as declining cytotoxic activity of natural killer cells in menopause<sup>[24]</sup>. Levels of inflammatory cytokines and

interleukins increases, thus creating the inflammatory state associated with menopause<sup>[25]</sup>.

#### **Antioxidants and oxidative stress**

Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione S-transferases are important in eliminating ROS and protecting cells against insults from toxic substances and oxidative stress<sup>[26,27]</sup>. These antioxidants decrease in the liver with aging, thus raising the susceptibility to liver damage. There are decreases in Na<sup>+</sup>K<sup>+</sup>ATPase and Ca<sup>2+</sup>ATPase functions in the aging liver as well, and this can negatively alter signal transduction pathways and cell functions, leading to increased lipid peroxidation and fostering a predisposition to liver disorders<sup>[28]</sup>. Aging livers have been shown to have increases in lipofuscin, which is considered as an end product of increases in lipid peroxidation, thus providing further proof of the aberrant balance of antioxidants and oxidative stress seen in the aging liver<sup>[29]</sup>. Rat models have shown that the administration of estradiol to aged rats can restore to normal function the otherwise age-related decline in the activity of antioxidant enzymes and membrane-linked ATPases and can lower lipid peroxidation and lipofuscin content in the aged liver<sup>[30]</sup>. At higher concentrations, such as in the premenopausal state, estrogen exerts antioxidant effects, but at lower concentrations and particularly when containing a catechol, estrogen exhibits pro-oxidant effects, contributing to breaks in genetic material and oxidation of bases, and increasing oxidative stress in the body<sup>[8]</sup>.

## **LIVER DISEASES IN MENOPAUSE**

### **Hepatitis C**

Natural history studies on hepatitis C virus (HCV) disease have demonstrated slower fibrosis progression in women compared to men. Fibrosis progression does not appear to be linear with advancing age, and hormonal differences may mediate the effect of gender on fibrosis progression. Di Martino *et al*<sup>[31]</sup> demonstrated that among HCV-infected women, higher rates of fibrosis progression occur in postmenopausal women compared to premenopausal women, and this rate of fibrosis progression is higher in postmenopausal women who are not on hormone therapy (HT) compared to postmenopausal women who are on HT<sup>[31]</sup>. To further underscore the potential beneficial effect of estrogen, it was also shown that fibrosis progression toward advanced liver disease occurs at a higher rate among nulliparous women compared to women who have had at least one pregnancy. Additional data underscore that menopause is associated with higher rates of advanced fibrosis, and the presence of HT in menopause appears to be associated with a lower level of fibrosis<sup>[32]</sup>. Concurrent steatosis is also seen more frequently in postmenopausal women infected with HCV compared to premenopausal women with

HCV, and the concurrent presence of steatosis is also associated with higher stages of fibrosis.

Menopause has also been demonstrated to have a negative effect on outcomes in the management of HCV liver disease. Recent data have shown that HCV-infected postmenopausal women, particularly nulliparous postmenopausal women, have been less likely to achieve sustained virologic response (SVR) following treatment with pegylated interferon and ribavirin<sup>[33]</sup>. In these data, a longer duration of menopause (greater than five years) was associated with more severe fibrosis, whereas past pregnancies and HT were not associated with fibrosis severity. Menopause was also associated with higher necroinflammatory activity and higher rates of steatosis compared to that seen in premenopausal women. Potentially identifiable reasons for this were demonstrated through differences in cytokine levels seen in postmenopausal women compared to premenopausal women. Higher levels of TNF- $\alpha$  and IL-6 are seen in postmenopausal women compared to premenopausal women, and higher levels of IL-6 are associated with higher levels of necroinflammatory activity and more severe fibrosis<sup>[33,34]</sup>. Levels of these cytokines are upregulated in HCV infection and may negatively influence the response to HCV treatment with pegylated interferon and ribavirin. Presently, newer, non-interferon based therapies are available for HCV liver disease. Although data do not exist currently on whether there is any impact of menopause on these rates of response to such treatment, the overall impact may not be as significant as with interferon-based therapies given the overall markedly improved SVRs with these newer therapies.

Gender-specific data on post-transplant outcomes in patients transplanted for HCV liver disease suggest potential concerns regarding the possible effect of menopause and its interplay with age on graft and patient survival following liver transplantation. Belli *et al*<sup>[35]</sup> were able to demonstrate in a multicenter study that female sex is an independent factor in progression to severe fibrosis in recurrent HCV disease post transplant. Furthermore, in approximately 90% of cases, severe fibrosis progression occurred within five years post transplant in female patients who were allocated older donor (greater than age 60 years) livers, whereas similarly older donor livers allocated to men led to severe fibrosis progression in less than 50% of those male recipients. Whereas this study did not specifically assess the impact of menopausal status on such outcomes, most women in this study were postmenopausal. Lai *et al*<sup>[36]</sup> provided later data that similarly demonstrated female sex as an independent predictor of severe fibrosis in liver allografts of and increased mortality of patients with recurrent HCV liver disease post-transplantation. Compared to male recipients, women had a 33% increased risk of developing graft failure.

### **Nonalcoholic fatty liver disease**

Recognized as the most common chronic liver disease in the Western world, the median prevalence of nonalcoholic fatty liver disease (NAFLD) in the general population is about 20%, and various factors have been associated with an increased likelihood of developing NAFLD, including obesity, diabetes mellitus, and dyslipidemia<sup>[37]</sup>. Emerging data suggest that postmenopausal women may be uniquely at risk for NAFLD. Although data have reported a higher incidence of NAFLD in men compared to women, there are observations of persistent increases in the incidence of NAFLD in women beyond middle age, whereas such continued increases in NAFLD incidence are not demonstrated in men<sup>[38-40]</sup>. Postmenopausal women are clearly at increased risk for the development of the metabolic syndrome compared to premenopausal women, and this is due to multiple changes occurring in menopause, including decreased energy expenditures with development of increased visceral fat, increased weight gain, and increases in triglycerides and cholesterol<sup>[41]</sup>. Newer data have demonstrated that whereas men with NAFLD have greater severity of hepatic fibrosis than premenopausal women, the severity of hepatic fibrosis in postmenopausal women is similar to that of men, and this effect has been observed even in nonobese postmenopausal women<sup>[42,43]</sup>. Similar to what has been demonstrated with HCV liver disease, it is believed that such increase in the likelihood of worsening hepatic fibrosis is related to estrogen loss in the postmenopausal state.

Yet, there are still important age-related factors that influence the progression of NAFLD, factors that postmenopausal women would be exposed to given their older age. Features associated with hepatocyte senescence, including telomere shortening and increased expression of p21, are seen in NAFLD, and the increased expression of p21 has been associated with increased levels of fibrosis in NAFLD patients<sup>[44]</sup>. Murine models demonstrated increased levels of pro-inflammatory cytokines, TNF and monocyte chemoattractant protein 1 in older mice fed high fat diets compared to younger mice given high fat diets, and significant steatohepatitis was observed only in these older mice<sup>[45]</sup>. Additionally, with aging, increased accumulation of fat is seen in many non-adipose tissues, including the liver<sup>[46]</sup>. This accumulation of fat in the liver is linked to insulin resistance and increased levels of cytokines, including TNF- $\alpha$  and IL-1, which contribute to oxidative stress and mediate the inflammatory response seen with obesity<sup>[47]</sup>.

### **Hepatocellular carcinoma**

The incidence of hepatocellular carcinoma (HCC) increases with older age, and it occurs in men two to three times more frequently than in women. Recent data from the Surveillance, Epidemiology, and End

Results database have demonstrated that women with HCC have a higher rate of overall survival than men regardless of age, race, stage of HCC, or treatment<sup>[48]</sup>. Women between the ages of 18 years and 64 years were noted to have longer survival than men of the same age, and the largest difference in survival was noted in women aged 18 years to 44 years. Furthermore, this gender-based disparity in survival in HCC patients disappeared in patients older than 65 years of age. Such gender disparity seen in the development of and survival in HCC is thought to be mediated by estrogen, and these epidemiologic data further suggest that menopausal status may mediate outcomes in HCC. Shimizu *et al*<sup>[49]</sup> demonstrated that decreased levels of hepatic ERs are associated with development of HCC and that ER levels were correlated with levels of the antioxidant, copper zinc superoxide dismutase (CuZn-SOD), and inversely proportional to the lipid peroxidation product, malondialdehyde. In addition, it was demonstrated that hepatic ER levels were significantly higher in normal livers of premenopausal women compared to hepatic ER levels in postmenopausal women and in cirrhotic patients that either had or did not have HCC. Furthermore, hepatic ER levels were higher in women with cirrhosis than in men with cirrhosis. Other data have shown in murine models that estrogen can suppress production of the inflammatory cytokine interleukin-6 (IL-6) and thus inhibit formation of diethylnitrosamine (DEN)-induced hepatocarcinogenesis<sup>[50]</sup>. Additional data have also demonstrated that the ER- $\alpha$  protein is downregulated in 60% of cases of HCC in women, and it is believed that activation of the p53/microRNA-18a pathway may promote the upregulation of microRNA-18a that leads to ER- $\alpha$  downregulation and cell proliferation in HCC in women<sup>[51,52]</sup>. Whereas this mechanism is purported as an explanation for why women develop HCC, further studies will be needed to determine if age mediates this mechanism.

## LIVER DISEASE MANAGEMENT IN MENOPAUSE

Data have clearly shown unique issues in the development and natural history of liver disease in menopause. As there is ongoing concern about the progression of liver injury with liver disease in the menopausal state, this also raises concern about the possible need for specialized approaches to liver disease management among women with chronic liver disease who are in menopause or approaching menopause. Previous therapy for HCV liver disease, namely pegylated interferon, carried a substantial side effect profile, and thus, recommendations often suggested consideration for use of pegylated interferon in the setting of patients with more advanced levels of fibrosis<sup>[53]</sup>. Historically, many HCV-infected patients

waited until achieving this level of fibrosis before consideration for treatment. Concern regarding a more accelerated progression of fibrosis in postmenopausal women with HCV liver disease suggests a need for consideration for a more aggressive treatment approach in women, particularly among those who are perimenopausal or in their younger years of menopause, so as to avoid accelerated progression toward advanced HCV liver disease. Presently, newer HCV treatments, including sofosbuvir, simeprevir, sofosbuvir/ledipasvir, and paritaprevir/ritonavir/ombitasvir/dasabuvir, have minimal side effect profiles, and thus, issues regarding timing of HCV treatment are no longer influenced by concerns about side effects. As these drugs are relatively new and markedly superior in the ability to eradicate HCV infection, there are no data regarding whether there is a reduced likelihood of response to treatment in menopause as has previously been demonstrated in interferon-based treatment. However, there should continue to be concern about ensuring that HCV infection is aggressively managed in all populations and particularly in women who are at risk for acceleration of the severity of HCV liver disease.

Therapy for NAFLD centers on control of the underlying metabolic features associated with NAFLD, including obesity, diabetes mellitus, hypertension and dyslipidemia. As the incidence and severity of NAFLD is increased among older women, particularly in those who have achieved menopause, these observations raise concern about the potential need for a heightened emphasis on weight loss and control of other associated metabolic factors in women who are peri-menopausal and in their early years of menopause so as to hopefully avoid development of advanced fatty liver disease in their older years of life.

More broadly, the influence of estrogen on the progression of liver disease raises query about whether HT may be beneficial in liver disease. HT has been shown to improve a number of clinical conditions associated with menopause, including osteoporosis, vasomotor symptoms, and atherosclerosis. Concern about the negative effects of HT on cardiovascular health were raised in the 2002 Women's Health Initiative trial, which demonstrated an increased risk in the development of heart disease<sup>[54]</sup>. Secondary analysis of data from this trial led to discovery that there was no increased incidence of heart disease in women on HT between ages 50 and 59 years and among those who were within 10 years of menopause<sup>[55]</sup>. As menopause ensues and estrogen becomes more deficient, there is decreased activity of estrogen receptors<sup>[8]</sup>. Further, with aging, there is accumulation of mitochondrial DNA mutations and subsequent mitochondrial dysfunction<sup>[56]</sup>. Such mitochondrial dysfunction can promote cellular senescence, and senescent cells may produce inflammatory cytokines that oppose the response of estrogen to cytokine formation and may alter expression of certain estrogen-regulated genes<sup>[57]</sup>. These factors

may contribute to the age-dependent function of estrogen that can be beneficial at younger ages but deleterious in older age. Whereas studies have shown an association of HT with slower fibrosis progression in HCV liver disease and in fatty liver disease, further studies are needed to determine the extent of benefits and overall safety of HT in liver disease.

## CONCLUSION

The combination of age and hormonal factors uniquely influence the development and progression of liver disease in postmenopausal women. With recognition of the various physiologic and biochemical changes that occur in menopause, there should be a heightened suspicion for possible liver disease and early implementation of therapies to minimize the likelihood of progression to advanced liver disease, liver cancer, and liver-related death.

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## Recent developments in the pathophysiology of irritable bowel syndrome

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### Abstract

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder, the pathophysiology of which is not completely known, although it has been shown that genetic/social learning factors, diet, intestinal microbiota, intestinal low-grade inflammation, and abnormal gastrointestinal endocrine cells play a major role. Studies of familial aggregation and on twins have confirmed the heritability of IBS. However, the proposed IBS risk genes are thus far nonvalidated hits rather than true predisposing factors. There is no convincing evidence that IBS patients suffer from food allergy/intolerance, with the effect exerted by diet seemingly caused by intake of poorly absorbed carbohydrates and fiber. Obesity is a possible comorbidity of IBS. Differences in the microbiota between IBS patients and healthy controls have been reported, but the association between IBS symptoms and specific bacterial species is uncertain. Low-grade inflammation appears to play a role in the pathophysiology of a major subset of IBS, namely postinfectious IBS. The density of intestinal endocrine cells is reduced in patients with IBS, possibly as a result of genetic factors, diet, intestinal microbiota, and low-grade inflammation interfering with the regulatory signals controlling the intestinal stem-cell clonogenic and differentiation activities. Furthermore, there is speculation that this decreased number of endocrine cells is responsible for the visceral hypersensitivity, disturbed gastrointestinal motility, and abnormal gut secretion seen in IBS patients.

**Key words:** Diet; Endocrine cells; Genetic factors; Low-grade inflammation; Microbiota; Stem cells

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**Core tip:** There are several factors that play a major role in the pathophysiology of irritable bowel syndrome

(IBS). These factors are genetic disposition, diet, the intestinal microbiota, and mucosal low-grade inflammation. These factors are known to affect the gastrointestinal endocrine cells, with the densities of intestinal endocrine cells being reduced in IBS patients. The reduction in the gastrointestinal endocrine cells seems to be caused by abnormal clonogenic and differentiation activities of the intestinal stem cells. The abnormalities in the gastrointestinal endocrine cells can explain the visceral hypersensitivity, disturbed gastrointestinal motility, and abnormal gut secretion observed in IBS patients.

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## INTRODUCTION

Patients with irritable bowel syndrome (IBS) suffer from intermittent abdominal pain or discomfort in combination with altered bowel habits and abdominal distension/bloating<sup>[1-3]</sup>. These symptoms cause significant morbidity, with impaired quality of life and reduced work productivity<sup>[4,5]</sup>, and is an economic burden to both the patients and society<sup>[6-12]</sup>. IBS patients can be divided into three subtypes according to the predominant bowel pattern: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), and both diarrhea and constipation (IBS-M)<sup>[13]</sup>.

The pathophysiology of IBS is incompletely understood, and there is no diagnostic test or effective treatment for this condition<sup>[14-16]</sup>. Thus, IBS patients visit doctors more frequently, undergo more diagnostic tests and examinations, consume more medications, and are hospitalized more frequently than those without IBS<sup>[6-12]</sup>. Understanding the pathophysiology of IBS is necessary in order to develop better diagnostic methods and effective treatments, and consequently reduce the economical costs both for patients and society. New data on the pathophysiology of IBS have accumulated over the past few years, improving our understanding of this disorder<sup>[1,15-20]</sup>. The aim of this review was to account for these new data and integrate them into the current knowledge on the pathophysiology of IBS.

## PATHOPHYSIOLOGY OF IBS

There is evidence that several factors play a central role in the pathophysiology of IBS, such as genetic/social learning factors, diet, the intestinal microbiota, low-grade chronic intestinal inflammation, and abnormal gastrointestinal endocrine cells<sup>[1,14-20]</sup>.

### Heritability and social learning

**Familial aggregation:** Familial clustering of IBS has been noted in everyday clinical practice, with 37% of IBS patients reportedly having a family history of the disorder<sup>[21]</sup>. Moreover, it has been shown that IBS patients are more likely (33.9%) than controls (12.6%) to have a family history of IBS<sup>[22]</sup>. In a cohort of IBS patients from Olmsted County, USA, a significant association was found between IBS patients and having a first-degree family member with IBS, but not for their non-IBS spouses<sup>[23]</sup>. The prevalence of IBS was 17% among IBS patients' relatives, compared to 7% among their spouses' relatives<sup>[24]</sup>. Similarly, the prevalence rates of IBS were reported to be 21%-50% and 4%-27% among relatives of IBS patients and non-IBS controls, respectively<sup>[25,26]</sup>. In a recently published, nationwide survey of the Swedish population, the risk of IBS was found to be increased in the first-, second-, and third-degree relatives of patients with IBS compared with their non-IBS counterparts, with the risk tending to be higher in more closely related relatives<sup>[27]</sup>.

**Twin studies:** All twin studies confirm a substantial genetic component in IBS<sup>[28-31]</sup>, with one exception<sup>[32]</sup>. Among 343 Australian twin pairs, IBS was found to occur at rate of 33.3% in monozygotic twins compared to 13.3% in dizygotic twins, with 56.9% of the variance being due to additive genetic factors.<sup>[28]</sup> In two studies involving 6060 and 986 American twin pairs<sup>[29,33]</sup>, the first study showed that the concordance of IBS was significantly greater in monozygotic (17.2%) than in dizygotic (8.4) twins<sup>[29]</sup>, and in the second study the polychoric correlation of IBS for monozygotic twins with IBS was greater than that for dizygotic twins (47% and 17%, respectively)<sup>[33]</sup>. In Scandinavia, a study conducted involving 3286 Norwegian twin pairs found that the concordance for IBS was significantly higher among monozygotic (22.4%) than dizygotic (9.1%) twins, and that the concordance was higher (48.5%) in females<sup>[31]</sup>. However, in contrast to all other twin studies, a study of 1870 British twin pairs did not reveal any significant difference in the rates of IBS between monozygotic and dizygotic twins<sup>[32]</sup>.

**Genetic studies:** The aforementioned epidemiological and twin studies point to a potential involvement of specific genes in the pathogenesis of IBS. Most of the genetic research has concentrated on the serotonin signaling pathways, control of immune activation, bile acid synthesis, neuropeptide activity, and intestinal secretion<sup>[34-37]</sup>. More than 60 gene candidates have been proposed to play a role in the genetic predisposition to IBS, but these risk genes have yet to be validated<sup>[38]</sup>. The most important of these gene candidates are described in detail elsewhere<sup>[39]</sup>. Several studies have focused on the *HTTLPR* genotype, which controls the expression of the SLC6A4 (serotonin transporter



protein); however, the reported association with IBS is equivocal<sup>[40-43]</sup>.

The gene that is most likely to be associated with IBS, and with IBS-C in particular, is that encoding tumor necrosis factor superfamily 15 (*TNFSF15*). It was first described in Swedish and US patients, and was confirmed in patient cohorts in the UK and Canada<sup>[44-46]</sup>. However, in a genome-wide association study (GWAS) the association between *TNFSF15* and IBS was found to be nonsignificant<sup>[47]</sup>. It was suggested that this seemingly contradictory finding can be explained by the possibility that genetic effects are diluted and more difficult to detect at the general population level<sup>[47]</sup>.

In a general population GWAS, a locus at 7p22.1, which includes the genes *KDEL2* and *GRID2IP*, showed consistent IBS risk effects<sup>[47]</sup>. *KDEL2* encodes a family of receptors, the most well known of which is KDEL1 [KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1], which is an integral membrane protein that binds the Lys-Asp-Leu-Glu and Arg-Asp-Leu-Glu amino acid motifs of target proteins and mediates their retrograde transport to the endoplasmic reticulum<sup>[48-52]</sup>. *GRID2IP* encodes delphilin, which is expressed in nerve-fiber-Purkinje-cell synapses in the brain<sup>[53,54]</sup>.

The reasons underlying the conflicting results yielded by genetic association studies, and especially in IBS, are discussed elsewhere<sup>[38,55]</sup>. The IBS risk genes proposed so far are nonvalidated hits rather than true predisposing factors, and the studies conducted have been largely too underpowered to capture true association signals<sup>[38]</sup>. In the future, research in this field should apply the promising GWAS approach to research candidate mechanisms rather than symptom definition<sup>[38]</sup>.

### Environment and social learning

Parental modeling and reinforcement of illness behaviors may play a role in the pathophysiology of IBS<sup>[29,56-59]</sup>. Having a mother with IBS accounts for as much variance as having an identical twin with IBS<sup>[56]</sup>. Aggregation of IBS among spouses to IBS patients has been reported to indicate that nongenetic - and most probably environmental factors - are responsible for IBS clustering<sup>[27]</sup>. In a more recent comprehensive review, where a careful weighing of evidence was made, concluded that social learning may be one of the factors involved in the etiology of IBS<sup>[60]</sup>. Moreover, the pain caused by visceral hypersensitivity in IBS has been attributed to atypical attention to pain as a part of illness behavior<sup>[61,62]</sup>.

**Diet:** Most IBS patients believe that certain food items trigger their symptoms<sup>[63-71]</sup>. This has resulted in IBS patients making conscious choices to avoid foodstuffs such as milk and milk products, wheat products, caffeine, cabbage, onions, peas, beans, hot spices, and fried and smoked food<sup>[63,68,72,73]</sup>. The intake of energy,

carbohydrates, proteins, and fats in IBS patients does not differ from that of the general population<sup>[72-78]</sup>. However, IBS patients tend to avoid several food sources that are important to their health, especially those rich in vitamins and minerals<sup>[73]</sup>. Several factors have been discussed to explain the mechanisms by which diet plays its role in the pathophysiology of IBS, such as poorly absorbed carbohydrates and fiber, food allergy/intolerance, and the comorbidity of obesity and IBS<sup>[1,17,20,79-83]</sup>.

**Poorly absorbed carbohydrates and fiber:** Several food items contain indigestible and poorly absorbed short-chain carbohydrates, namely fermentable oligo-, di-, and monosaccharides, and polyols (FODMAPs)<sup>[1]</sup>. FODMAPs include fructose, lactose, sugar sources (sorbitol, maltitol, mannitol, xylitol, and isomalt), fructans, and galactans<sup>[1,84]</sup>, and occur in a wide range of foods such as wheat, rye, vegetables, fruits, and legumes<sup>[85-87]</sup>. These unabsorbed carbohydrates enter the distal small intestine and colon, where they increase the osmotic pressure in the luminal cavity and provide a substrate for bacterial fermentation<sup>[84,88,89]</sup>. This bacterial fermentation leads to gas production, which in turn causes abdominal distension and abdominal pain/discomfort. FODMAPs have been found to trigger gastrointestinal symptoms in IBS, and a low-FODMAPs diet reduces symptoms and improves the patient's quality of life<sup>[73,78,90-95]</sup>.

Recent studies have shown that the triggering of IBS symptoms by FODMAPs is much more complicated than was originally thought. Thus, a low FODMAPs intake induces favorable changes in the intestinal microbiota<sup>[96]</sup> and gastrointestinal endocrine cells<sup>[97-100]</sup>. The change from a diet of typical Australian food to a low-FODMAPs diet was found to change the intestinal microbiota; whereas a typical Australian diet increases the relative abundance of butyrate-producing *Clostridium* cluster XIVa and the mucus-associated *Akkermansia muciniphila*, and reduces *Ruminococcus torques*, a low-FODMAPs diet reduces the total bacterial abundance<sup>[96]</sup>. Several endocrine cell types in the gastrointestinal tract of IBS patients are abnormal<sup>[101-120]</sup>, and these abnormalities are considered to play a major role in the development of IBS symptoms and represent future targets for treatment<sup>[16,121]</sup>. Switching from a typical Norwegian diet to a low-FODMAPs diet has been shown to change the densities of endocrine cells in the stomach and large intestine toward normal levels<sup>[97-100]</sup>.

**Food allergy/intolerance:** There is no convincing evidence to support the idea that IBS patients suffer from food allergy/intolerance<sup>[64,67,122-128]</sup>. The prevalence of nonceliac gluten sensitivity (NCGS) in the United States has been reported to range from 0.55% to 6%<sup>[129,130]</sup>. NCGS is defined as patients with gastrointestinal and extragastrointestinal IBS-like symptoms without celiac disease or wheat allergy, and

with symptom relief on a gluten-free diet (GFD) and relapse on gluten challenge<sup>[130-137]</sup>.

NCGS was first described more than 30 years ago<sup>[138,139]</sup>, and has been the focus of several recent reports<sup>[140-144]</sup>. Contradictory results have been reported regarding whether or not NCGS patients have low-grade inflammation and abnormal intestinal permeability<sup>[141,144-151]</sup>. However, in double-blind, randomized, placebo-controlled studies<sup>[141,143,144]</sup>, the positive effects on symptoms in NCGS patients were actually found to be the result of wheat withdrawal rather than gluten withdrawal<sup>[152]</sup>. In a placebo-controlled, crossover study of patients with IBS-like symptoms with self-imposed GFD<sup>[153]</sup>, the gastrointestinal symptoms consistently and significantly improved when the FODMAPs intake was reduced, and these symptoms were not worsened by either a low- or high-dose challenge with gluten. It therefore seems that it is the carbohydrate content (fructans and galactans) in the wheat rather than gluten that is responsible for triggering NCGS symptoms. This conclusion is supported further by the findings that in those who believed that they had NCGS, 24% had uncontrolled symptoms despite consuming a GFD, 27% were not following a GFD alone, and 65% avoided other foods that contain high levels of FODMAPs<sup>[154]</sup>.

NCGS and IBS patients experience the same symptoms that are triggered by wheat intake, and both groups have a high frequency of antigliadin antibodies (AGAs) with negative tissue transglutaminase, or deamidated gliadin peptide antibodies<sup>[133,143,155-158]</sup>. AGAs have been reported to have a good sensitivity but a low specificity for celiac disease<sup>[159]</sup>, and 12%-15% of healthy subjects are reportedly positive for AGAs<sup>[155,159,160]</sup>. It is thus possible to conclude that NCGS patients are IBS patients who are self-diagnosed and have self-treated by adhering to a GFD.

**Obesity and IBS:** There has been some concern that the onset of symptoms upon ingesting food would result in low food intake with consequent malnutrition in patients with IBS<sup>[73,161]</sup>. However, while some studies have found an association between low body mass index (BMI) and IBS<sup>[162]</sup>, others have found the predominance of normal-weight or overweight IBS patients<sup>[63]</sup>. The association between IBS and obesity was found to be controversial in a comprehensive review, and the author concluded that obesity and IBS might be linked<sup>[163]</sup>.

Appetite is regulated by a large number of hormones, including those secreted by the gastrointestinal endocrine cells<sup>[164]</sup>. The densities of the following five gastrointestinal endocrine cell types that secrete hormones known to regulate appetite are abnormal in patients with IBS: ghrelin, cholecystokinin, peptide YY, enteroglucagon (oxyntomodulin), and serotonin<sup>[101,103,104,107,165-171]</sup>. Ghrelin stimulates food intake and body weight gain<sup>[172,173]</sup>. The density of this endocrine cell type is increased in IBS-D patients. The densities of endocrine cells that secrete

the other four hormones, which have an anorexigenic action<sup>[174-189]</sup>, are reduced in patients with IBS. This would be predictive of an increased appetite and food intake in IBS patients. BMI and appetite in IBS patients have not been fully studied, and the currently available data are controversial. It is not clear whether IBS patients have an increased appetite, which is opposed by the avoidance of eating because of worsening of symptoms upon eating, or a high BMI.

**Intestinal microbiota:** The role of the intestinal microbiota in the pathophysiology of IBS has been discussed in detail elsewhere<sup>[190-194]</sup>. The human intestine contains about  $10^{14}$  bacteria belonging to over 1000 species<sup>[190,195,196]</sup>. These bacteria can be present in the lumen or attached to or embedded in the mucus layer of the gastrointestinal tract<sup>[197]</sup>. The number of bacteria is lower in the small intestine than in the colon, and decreases gradually toward the upper parts of the gastrointestinal tract<sup>[198-200]</sup>. The gastrointestinal microbial composition is determined by host genetic factors and environmental factors<sup>[193]</sup>. The environmental factors include mode of delivery at birth, aging, treatment with antibiotics, and sanitation status<sup>[201]</sup>. The gastrointestinal microbiota plays a role in gastrointestinal motility, gut immune defense, digestion and metabolism, inflammation, and cell proliferation<sup>[193,202]</sup>.

Several studies using culture-based and culture-independent methods have shown that the microbiota - as detected in feces samples - differs between in IBS patients and healthy controls<sup>[203-229]</sup>. However, the association between IBS symptoms and specific bacterial species is uncertain<sup>[191]</sup>. Although contradictory results have been reported, decreased levels of lactobacilli and bifidobacteria, and increased levels of anaerobic bacteria such as streptococci and *Escherichia coli*, as well as increased ratios of *Firmicutes*, *Bacteroidetes*, and *Clostridium* species have been confirmed in several studies<sup>[206,226]</sup>.

**Low-grade inflammation:** It has been suggested that the presence of colonic mucosal low-grade inflammation plays a role in the pathophysiology of IBS<sup>[18,230]</sup>. However, studies of mucosal low-grade inflammation in the colon have yielded contradictory results<sup>[231]</sup>. There are reports of increased numbers of intraepithelial immune cells, and elevated numbers of immune cells and mast cells in lamina propria of rectal biopsies taken from patients with postinfectious IBS (PI-IBS)<sup>[116,232,233]</sup>. The densities of immune and mast cells in the mucosa of patients with sporadic (nonspecific) IBS (non-PI-IBS) did not differ from those in healthy controls<sup>[234]</sup>. An increased number of intraepithelial lymphocytes has been found in studies in which no attention was paid to the previous history of gastrointestinal infection<sup>[235-237]</sup>. However, an unchanged density of mast cells was found in studies in which no distinction was made between PI-IBS and non-PI-IBS<sup>[235,238,239]</sup>. Moreover, the mast cell density was elevated in PI-IBS but not in non-PI-

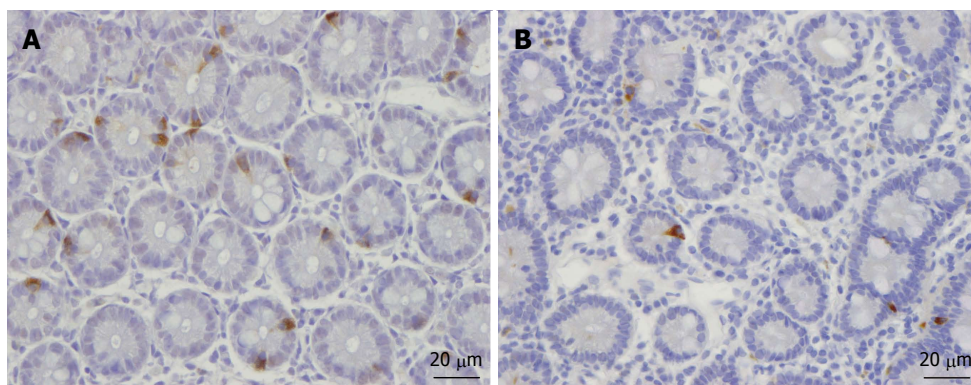


Figure 1 Chromogranin A-immunoreactive cells in (A) a healthy subject and (B) an irritable bowel syndrome patient.

IBS<sup>[118,235]</sup>. Similar to the immune cells and mast cells, inconsistent findings have been reported regarding cytokines in patients with IBS<sup>[240]</sup>, whereby changes in cytokines were reported in IBS patients<sup>[240-242]</sup>, but not in those with non-PI-IBS<sup>[243]</sup>.

The research performed to date provides compelling evidence that low-grade inflammation occurs in a subset of IBS patients, namely those with PI-IBS, but not in those with non-PI-IBS. PI-IBS represents a considerable proportion of IBS patients, with an incidence of 7%-31% among IBS patients<sup>[244-246]</sup>. Thus, low-grade inflammation plays a significant role in the pathophysiology in a subset of IBS patients.

#### Abnormal gastrointestinal endocrine cells

**Gastrointestinal endocrine cells:** The gastrointestinal tract contains at least 15 different types of endocrine cells that are spread among the epithelial cells of the mucosa<sup>[14,78,170,247-250]</sup>. These cells, which constitute about 1% of all epithelial cells in the gastrointestinal tract<sup>[247,248,251-253]</sup>, have specialized sensors in the form of microvilli that project into the lumen and respond to luminal stimuli by releasing hormones<sup>[101,254-265]</sup>. The distribution, functions, and modes of action of the most important endocrine/paracrine cells are described in detail elsewhere<sup>[15,16,170]</sup>. In brief, they secrete one or more signaling substances into the lamina propria, where these substances act directly on nearby structures (autocrine/paracrine mode) and/or indirectly *via* an endocrine mode of action (by circulating in the blood to reach distant targets)<sup>[266]</sup>. They regulate several functions of the gastrointestinal tract such as sensation, motility, secretion, absorption, local immune defense, and food intake<sup>[1,166,170,247,248]</sup>. These cells interact and integrate with each other and with the enteric nervous system and the afferent and efferent nerve fibers of the central nervous system<sup>[1,166,170,267]</sup>.

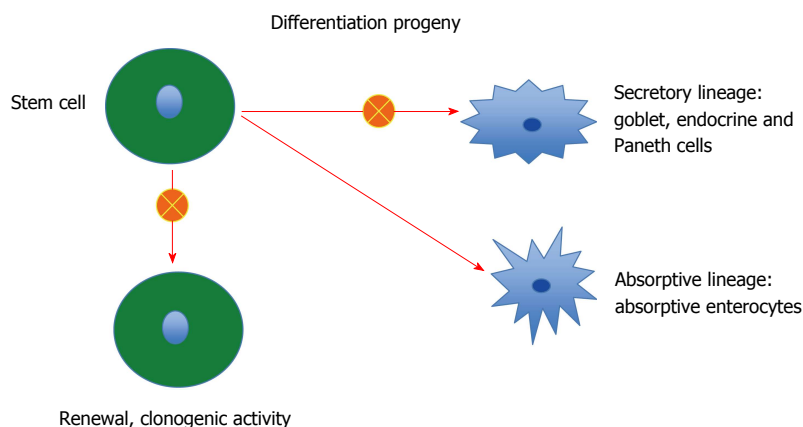
Abnormal endocrine cells have been found in both sporadic IBS and PI-IBS patients. In sporadic IBS, abnormal endocrine cells were found in the stomach, proximal small intestine (duodenum), distal small intestine (ileum), colon, and rectum<sup>[111-113,167,171,268-276]</sup>. Although the densities of endocrine cell types can vary (*i.e.*, decrease or increase), the general trend of the

entire intestinal endocrine cell population is toward a decrease in IBS. This becomes evident when intestinal biopsy samples are stained with chromogranin A, which is a common marker for endocrine cells. Thus, the densities of the total endocrine cells in the duodenum, ileum, and colon are reportedly decreased, while those of the stomach and rectum are unchanged (Figure 1)<sup>[102,269,271,272]</sup>. In contrast to sporadic IBS, the densities of intestinal endocrine cells in patients with PI-IBS tend to increase<sup>[109,113,114,116-120,277]</sup>.

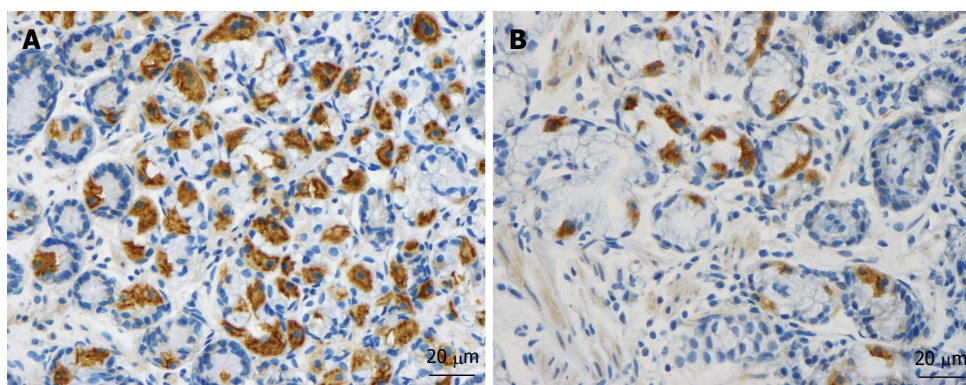
**Stem cells:** Each intestinal crypt contains four to six stem cells, which originate from pluripotent stem cells of endodermal origin<sup>[247,248,278]</sup>. These cells divide into new stem cells (self-renewal; clonogeny) and into cells that differentiate into all epithelial cell types including enterocytes, goblet cells, Paneth cells, and endocrine cells (differentiation progeny)<sup>[279-293]</sup>. The differentiation progeny includes two lineages: secretory and absorptive. The secretory lineage gives rise to goblet, endocrine, and Paneth cells, and the absorptive lineage to absorptive enterocytes (Figure 2)<sup>[279-293]</sup>.

As mentioned above, the total density of intestinal endocrine cells is reduced in sporadic IBS. A similar reduction in the density of intestinal endocrine cells has been observed in congenital malabsorptive diarrhea, and following small-intestine allograft rejection<sup>[294,295]</sup>. The decrease in the density of endocrine cells in both conditions has been found to be caused by a mutation in the gene encoding the protein neurogenin 3 (NEUROG3), which is expressed in the endocrine progenitor cells required for intestinal endocrine development, and a reduction in the progenitors of intestinal endocrine cells that express NEUROG3 and NeuroD<sup>[294,295]</sup>. It has recently been reported that the densities of cells expressing Musashi 1 (Msi-1, expressed in both stem cells and in their early progeny; Figure 3) and NEUROG3 (expressed in early endocrine cell progenitors; Figure 4)<sup>[296]</sup> were reduced in the duodenum of sporadic IBS patients<sup>[299]</sup>. It was concluded that disturbance of the clonogenic and differentiation activities of intestinal stem cells could be responsible for the reduction of intestinal endocrine cells observed in IBS patients<sup>[296]</sup>.

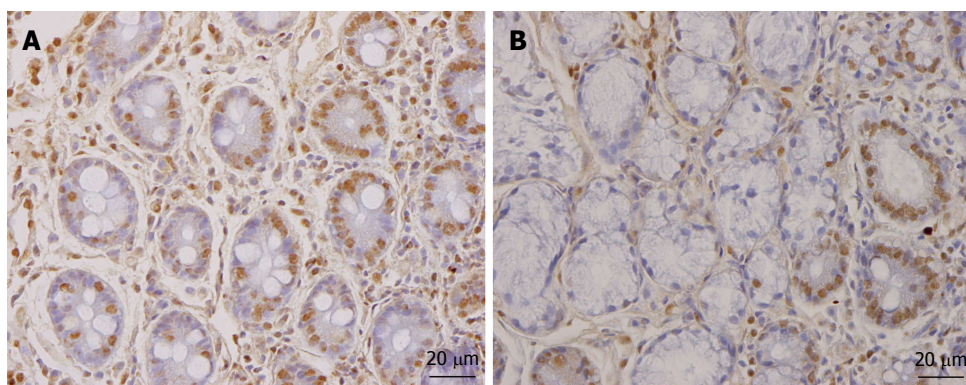




**Figure 2** The intestinal stem cell exerts 2 activities: clonogenic activity, where it produce a copy of itself to maintain the number of stem cells constant in the crypts, and differentiation activity. The differentiation consists of 2 lineages: secretory lineage and absorptive lineage. Through a cascade of progenitors the secretory lineage give rise to goblet, endocrine and Paneth cells and the absorptive lineage to absorptive enterocytes. In IBS patients, both clonogenic and differentiation activities are abnormal.



**Figure 3** Msi-1-immunoreactive cells in duodenum of subjects from the (A) control, and (B) irritable bowel syndrome patient.



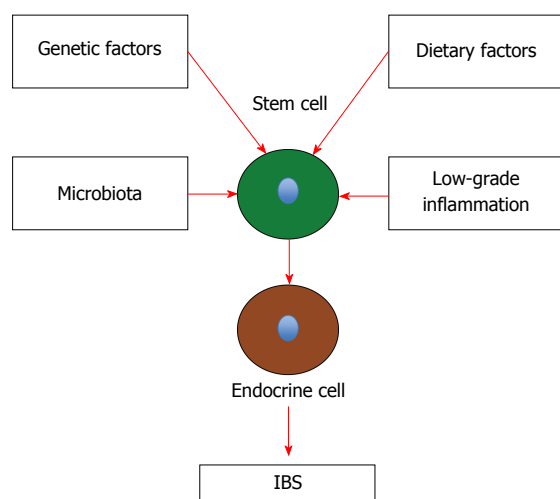
**Figure 4** NEUROG3-immunoreactive cells in (A) a healthy subject and (B) an irritable bowel syndrome patient.

## HYPOTHESIS

IBS patients exhibit visceral hypersensitivity, disturbed gastrointestinal motility, and abnormal gut secretion<sup>[297-301]</sup>. The gastrointestinal endocrine cells, as mentioned above, regulate several functions of the gut including sensation, motility, and secretion. The density of the intestinal endocrine cells is generally reduced in sporadic IBS. This reduction appears to

be caused by a reduction in intestinal stem-cell self-renewal and proliferation. Intestinal stem-cell self-renewal (clonogeny) and proliferation are regulated by several signaling pathways<sup>[287]</sup>. As demonstrated in this review, heredity, diet, the intestinal microbiota, and low-grade inflammation play a major role in the pathophysiology of IBS. Changes in diet, intestinal bacterial flora, and inflammation have been reported to affect the density of endocrine cells in the gut<sup>[1,97,302]</sup>.





**Figure 5** Schematic drawing to illustrate the possible pathophysiology of irritable bowel syndrome. Details are described in the text. IBS: Irritable bowel syndrome.

It can be speculated that the factors that have been shown to play a major role in the pathophysiology of IBS will affect the signaling pathways for stem-cell clonogenic renewal and proliferation, resulting in abnormalities in the gastrointestinal endocrine cells with the development of IBS symptoms (Figure 5).

## CONCLUSION

There is compelling evidence that genetic factors, diet, the intestinal microbiota, and mucosal low-grade inflammation play a major role in the pathophysiology of IBS. These factors are known to affect the gastrointestinal endocrine cells, with the densities of intestinal endocrine cells being reduced in IBS patients. The abnormalities in the gastrointestinal endocrine cells can explain the visceral hypersensitivity, disturbed gastrointestinal motility, and abnormal gut secretion observed in IBS patients.

The reduction in intestinal endocrine cells appears to be caused by disturbance of the clonogenic and differentiation activities of the intestinal stem cells. The clonogeny and proliferation of intestinal stem cells are regulated by several signaling pathways. It is possible that genetic factors, diet, the intestinal microbiota, and mucosal low-grade inflammation interfere with the signals regulating the clonogenic and proliferation activities of stem cells, resulting in a reduction in the density of intestinal endocrine cells. This reduction of intestinal endocrine cells can in turn result in the development of IBS symptoms.

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## Sarcopenia in the prognosis of cirrhosis: Going beyond the MELD score

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### Abstract

Estimating the prognosis of patients with cirrhosis remains challenging, because the natural history of cirrhosis varies according to the cause, presence of portal hypertension, liver synthetic function, and the reversibility of underlying

disease. Conventional prognostic scoring systems, including the Child-Turcotte-Pugh score or model for end-stage liver diseases are widely used; however, revised models have been introduced to improve prognostic performance. Although sarcopenia is one of the most common complications related to survival of patients with cirrhosis, the newly proposed prognostic models lack a nutritional status evaluation of patients. This is reflected by the lack of an optimal index for sarcopenia in terms of objectivity, reproducibility, practicality, and prognostic performance, and of a consensus definition for sarcopenia in patients with cirrhosis in whom ascites and edema may interfere with body composition analysis. Quantifying skeletal muscle mass using cross-sectional abdominal imaging is a promising tool for assessing sarcopenia. As radiological imaging provides direct visualization of body composition, it is useful to evaluate sarcopenia in patients with cirrhosis whose body mass index, anthropometric measurements, or biochemical markers are inaccurate on a nutritional assessment. Sarcopenia defined by cross-sectional imaging-based muscular assessment is prevalent and predicts mortality in patients with cirrhosis. Sarcopenia alone or in combination with conventional prognostic systems shows promise for a cirrhosis prognosis. Including an objective assessment of sarcopenia with conventional scores to optimize the outcome prediction for patients with cirrhosis needs further research.

**Key words:** Liver cirrhosis; Model for end-stage liver diseases score; Mortality; Prognosis; Sarcopenia

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**Core tip:** Sarcopenia is one of the most common complications associated with survival in cirrhotic patients. However, the lack of an objective and reliable method to quantify muscle mass has limited the general incorporation of sarcopenia into cirrhosis prognostic scores. In this article, we highlight cross-sectional imaging-based estimation of skeletal muscle mass for

diagnosing sarcopenia and assessing the prognosis of cirrhosis patients. In addition, we explore the possibility of incorporating sarcopenia into conventional prognostic scoring systems for better prognostication in cirrhosis patients.

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## INTRODUCTION

Cirrhosis is a consequence of chronic liver injury that leads to necroinflammation, fibrosis, hepatocellular dysfunction, and vascular remodeling. Although liver transplantation is the only curative treatment for cirrhosis, this option is not available for most patients. Therefore, management is generally focused on preventing and controlling complications. Complications including ascites, variceal bleeding, hepatic encephalopathy, hepatorenal syndrome, or hepatocellular carcinoma (HCC) are the most widely recognized<sup>[1]</sup>. Malnutrition is one of the most frequent complications in patients with cirrhosis, and it adversely affects other complications, quality of life, survival, and outcome after liver transplantation<sup>[2]</sup>. Despite its high prevalence and important prognostic role, muscle wasting or sarcopenia, which is a major feature of malnutrition, has not been highlighted until recently.

Conventional prognostic scores for patients with cirrhosis, such as the Child-Turcotte-Pugh (CTP) score or the model for end-stage liver diseases (MELD) score, have limitations, including the lack of a nutritional status evaluation. This may be caused by the lack of a clear definition and the complexity of a nutritional assessment in patients with cirrhosis and fluid overload<sup>[3-5]</sup>. Several tools have been introduced to measure the nutritional status of patients with cirrhosis; however, a lack of objectivity, reproducibility, and prognostic performance limits their wide application<sup>[3]</sup>. Currently, muscular assessments using cross-sectional imaging obtained by computed tomography (CT) or magnetic resonance imaging (MRI) constitute objective and reproducible methods for nutritional assessment and detection of sarcopenia. Quantifying skeletal muscle mass is not biased by edema or ascites, which frequently presents in decompensated patients with cirrhosis, and reflects a chronic decrease in overall health rather than the acute severity of liver disease<sup>[6]</sup>.

Several investigators have reported that sarcopenia is highly prevalent and an independent prognostic factor for mortality in patients with cirrhosis<sup>[7-11]</sup>. Adding muscle wasting to the currently accepted prognostic scores has shown promising results<sup>[12]</sup>. Therefore, sarcopenia quantified by an objective

method combined with commonly used prognostic systems has the potential to improve prognostication of patients with cirrhosis; however, prospective validation in large cohorts remains elusive. We discuss the current prognostic models and investigate the prevalence and prognostic value of sarcopenia in patients with cirrhosis.

## CONVENTIONAL PROGNOSTIC SYSTEMS FOR LIVER CIRRHOSIS

Predicting prognosis is crucial in the management of patients with cirrhosis. A number of prognostic models have been derived and validated. The CTP and MELD scores are the most widely used systems to predict mortality in patients with cirrhosis. The CTP score was originally designed to predict the outcome of patients with cirrhosis during surgery<sup>[13]</sup> and was extended for determining prognosis, treatment response, and prioritizing patients for liver transplantation (LT) who were on the waiting list<sup>[14]</sup>. The MELD scoring system was initially developed to predict early mortality in patients with cirrhosis undergoing a transjugular intrahepatic portosystemic shunt<sup>[15]</sup> and is composed of three objective parameters, including serum bilirubin, the international normalized ratio of prothrombin time, and serum creatinine. Subsequently, the MELD score has been shown to be useful for predicting short-term mortality in various patients with cirrhosis<sup>[16,17]</sup>. Since 2002, the MELD score has replaced the CTP score for organ allocation in patients waiting for LT in the United States, due to the advantage of including only objective laboratory variables and its superior ability to predict short-term outcomes compared to the CTP score<sup>[18]</sup>.

However, the MELD score also has some drawbacks, including variability in laboratory parameters, misclassification of some patients with a low MELD score, and the lack of a nutritional status assessment<sup>[19]</sup>. Many researchers have tried to improve the prognostic performance of the MELD score. Hyponatremia accurately predicts short-term mortality independently of the MELD score and is often associated with ascites, hepatorenal syndrome, and liver-related mortality<sup>[20-22]</sup>. Incorporating serum sodium into the MELD score, known as MELD-Na, improves its predictive ability, particularly for patients with a low MELD score<sup>[22]</sup>. The MELD-to-sodium ratio (MESO) index and the ReFit MELD-Na have been proposed to optimize prognostic scoring systems further. The MESO index provides better predictive ability compared to the original MELD score<sup>[23]</sup>, and the ReFit MELD-Na shows better performance for predicting short-term mortality in patients waiting for LT compared to the original MELD score and MELD-Na<sup>[24]</sup>. In addition, hypoalbuminemia negatively impacts waiting-list mortality after adjusting for the MELD score, serum sodium, and other covariates. A novel model including the MELD

score and serum sodium and albumin, called the five-variable MELD, improves the predictive performance of short-term mortality among patients on the LT waiting list<sup>[25]</sup>.

Despite these efforts to modify the original MELD scoring systems, little has been done to incorporate nutritional status into conventional prognostic models. This may be caused by the heterogeneity in the definition of malnutrition and the complexity of a nutritional assessment in patients with cirrhosis and water retention or ascites<sup>[3-5]</sup>. Adding an objective and readily available marker of nutritional status to the conventional prognostic scoring systems is a promising target to further improve prognostication in patients with cirrhosis.

## NUTRITIONAL ASSESSMENT IN PATIENTS WITH CIRRHOSIS

Numerous tools for nutritional assessment, for example, body mass index (BMI), anthropometric measures, and subjective global assessment (SGA), have been introduced<sup>[26,27]</sup>. However, the usefulness of these methods is limited due to their subjectiveness and the impact of body composition changes in patients with cirrhosis and edema or ascites<sup>[28]</sup>. Standard laboratory tests have been used to estimate nutritional status, including prothrombin time, albumin, prealbumin, the creatinine height index, and transferrin. Because these common nutritional status parameters are confounded by cirrhosis, their utility in patients with cirrhosis is limited. Serum albumin, prealbumin, and transferrin levels decrease, and prothrombin time is prolonged due to impaired hepatic synthetic function, which results in an underestimation of nutritional status in patients with cirrhosis<sup>[29]</sup>. In addition, the creatinine height index is not an accurate marker of malnutrition due to frequently impaired kidney function in patients with cirrhosis<sup>[30]</sup>.

The interpretation of anthropometric measures is also confusing, because they are influenced by ascites, edema, and salt or diuretic intake in patients with cirrhosis<sup>[31]</sup>. The SGA scale assesses weight changes, dietary intake, gastrointestinal symptoms, medical diagnoses, and a physical examination. However, the SGA underestimates nutritional status in patients with cirrhosis<sup>[26]</sup>.

Body composition (*i.e.*, body fat mass and lean mass) is essential to estimate nutritional status. Several indirect methods have been used to measure body composition in patients with cirrhosis, including total-body electrical conductivity, bioelectrical impedance, dual energy X-ray absorptiometry, air displacement plethysmography, and magnetic resonance spectroscopy<sup>[32-34]</sup>. These tools work on the basis of the two-compartment model composed of body fat mass and fat-free mass. Nonfat or lean mass is estimated from the weight remaining after

determining whole body weight and fat mass. Because skeletal muscle mass accounts for about 50% of lean body mass, measures of lean body or fat-free mass indirectly estimate whole-body skeletal muscle mass<sup>[35]</sup>. A bioelectrical impedance analysis measures the body's resistance to the flow of alternating current, and dual energy X-ray absorptiometry estimates body composition using low-dose X-rays. Yet, there is a lack of accuracy in these methods in the presence of fluid retention, which is frequently encountered in patients with cirrhosis<sup>[34,36]</sup>.

CT or MRI is the gold standard method to quantify skeletal muscle mass. Muscle area determined from a single-slice abdominal scan obtained by CT or MRI is highly correlated with total-body skeletal muscle quantified by whole-body multislice analysis<sup>[37]</sup>. Single abdominal CT or MRI cross-sectional images have emerged as a novel way to objectively and reproducibly assess nutritional status and detect muscle wasting in patients with cirrhosis. Skeletal muscle area is quantified using tissue-specific Hounsfield unit thresholds of -29 to +150<sup>[38]</sup>. Quantifying psoas muscle or total abdominal muscle areas on a single abdominal CT section at the L3 or L4 level is linearly associated with whole body muscle mass<sup>[39]</sup> and is a reliable, noninvasive marker of muscle wasting in patients with cirrhosis<sup>[8,11,40-45]</sup>. Psoas muscle thickness rather than cross-sectional area has also been investigated to improve simplicity and applicability in daily practice<sup>[7,9]</sup>.

A radiological assessment of skeletal muscle mass has several advantages over traditional methods for patients with cirrhosis. First, it provides direct visualization and measurements of tissue compartments and is not biased by fluid retention that commonly presents in patients with cirrhosis. Second, additional scanning is not required to quantify body tissues, because abdominal CT scans are routinely performed to screen for HCC in patients with cirrhosis. Third, it provides an accurate, objective, and reproducible measure of skeletal muscle mass.

## DEFINITION OF SARCOPENIA

Sarcopenia is generally defined as a reduction in muscle mass two standard deviations below the healthy young adult mean<sup>[46]</sup>. Sarcopenia is traditionally associated with aging; however, it can occur earlier in patients with malignancy and chronic disease<sup>[47]</sup>. Despite the recent consensus statement of the European Working Group on Sarcopenia in Older People that recommends taking into account both low muscle mass and low muscle function (strength or performance) for the diagnosis of sarcopenia<sup>[48]</sup>, the use of muscle mass vs function to define sarcopenia remains controversial. Moreover, muscle mass alone has been widely used to define sarcopenia and is associated with prognosis in patients with various conditions<sup>[49]</sup>. As CT or MRI imaging is the gold standard tool to quantify skeletal muscle



**Table 1** Definition and prevalence of sarcopenia in cirrhosis

Ref.	n	Men, n(%)	Unit of measure	Cutoffs for sarcopenia	Prevalence	Predictors of sarcopenia
Cruz <i>et al</i> <sup>[60]</sup>	234	157 (67)	L3-4 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men: $\leq 52.4$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 38.5$ cm <sup>2</sup> /m <sup>2</sup>	70% (men 76%)	
DiMartini <i>et al</i> <sup>[40]</sup>	338	223 (66)	L3-4 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men: $\leq 52.4$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 38.5$ cm <sup>2</sup> /m <sup>2</sup>	68% (men 76%, women 51%)	80% prevalence in alcoholic liver disease <i>vs</i> 31%-71% in other diseases 80% prevalence in normal-weight <i>vs</i> 62% in obese
Hanai <i>et al</i> <sup>[8]</sup>	130	76 (58)	L3 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men: $\leq 52.4$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 38.5$ cm <sup>2</sup> /m <sup>2</sup>	68% (men 82%, women 50%)	In the multivariate analysis, only the male gender [OR (95%CI) = 5.65 (1.43-24.23), $P = 0.01$ ] and BMI [0.77 (0.66-0.87), $P < 0.0001$ ] were independent predictors of sarcopenia
Meza-Junco <i>et al</i> <sup>[43]</sup>	116	98 (84)	L3 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men BMI $\geq 25$ kg/m <sup>2</sup> : $\leq 53$ cm <sup>2</sup> /m <sup>2</sup> BMI $< 25$ kg/m <sup>2</sup> : $\leq 43$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 41$ cm <sup>2</sup> /m <sup>2</sup>	30% (men 31%, women 28%)	Age was older ( $61 \pm 1$ yr <i>vs</i> $57 \pm 1$ yr, $P = 0.001$ ), and the INR was higher ( $1.4 \pm 0.08$ <i>vs</i> $1.2 \pm 0.03$ , $P = 0.01$ ) in sarcopenic patients than nonsarcopenic patients
Montano-loza <i>et al</i> <sup>[10]</sup>	112	78 (70)	L3 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men: $\leq 52.4$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 38.5$ cm <sup>2</sup> /m <sup>2</sup>	40% (men 50%, women 18%)	Sarcopenia was more frequent in men (50% <i>vs</i> 18%, $P < 0.001$ ) and patients with a low BMI ( $26 \pm 0.7$ kg/m <sup>2</sup> <i>vs</i> $29 \pm 0.8$ kg/m <sup>2</sup> , $P = 0.003$ )
Montano-loza <i>et al</i> <sup>[45]</sup>	248	169 (68)	L3 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men BMI $\geq 25$ kg/m <sup>2</sup> : $\leq 53$ cm <sup>2</sup> /m <sup>2</sup> BMI $< 25$ kg/m <sup>2</sup> : $\leq 43$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 41$ cm <sup>2</sup> /m <sup>2</sup>	45% (men 52%, women 30%)	Sarcopenia was more common in men ( $P = 0.002$ ), patients with ascites ( $P = 0.02$ ), patients with low BMI ( $P < 0.001$ ), and patients with higher bilirubin levels ( $P = 0.05$ ), creatinine levels ( $P = 0.02$ ), INR ( $P = 0.04$ ), CTP scores ( $P = 0.002$ ), and MELD scores ( $P = 0.002$ )
Tandon <i>et al</i> <sup>[11]</sup>	142	85 (60)	L3 SMI (cm <sup>2</sup> /m <sup>2</sup> )	Men: $\leq 52.4$ cm <sup>2</sup> /m <sup>2</sup> Women: $\leq 38.5$ cm <sup>2</sup> /m <sup>2</sup>	41% (men 54%, women 21%)	In a multivariate logistic regression analysis, male sex [OR (95%CI) = 5.91 (2.38-14.6)], CTP class C [ <i>vs</i> CTP class A: 15.4 (1.44-165.7)], and a BMI [0.82 (0.74-0.90)] were independent predictors of sarcopenia

mass, skeletal muscle mass calculated from abdominal cross-sectional images is a great resource to define sarcopenia.

Recent studies investigating sarcopenia in patients with cirrhosis utilized cross-sectional muscle area normalized for stature (cm<sup>2</sup>/m<sup>2</sup>), called the L3 skeletal muscle index (SMI). In most studies<sup>[8,10,11,40]</sup>, the L3 SMI cutoffs for defining sarcopenia were chosen based on a sarcopenia study of patients with cancer<sup>[50]</sup> (L3 SMI:  $\leq 38.5$  cm<sup>2</sup>/m<sup>2</sup> for women and  $\leq 52.4$  cm<sup>2</sup>/m<sup>2</sup> for men). More recent studies<sup>[43,45]</sup> have adopted sarcopenia cutoffs based on a study that optimally stratified patients with solid tumors<sup>[51]</sup> (L3 SMI:  $\leq 41$  cm<sup>2</sup>/m<sup>2</sup> for women and  $\leq 53$  cm<sup>2</sup>/m<sup>2</sup> for men with a BMI  $\geq 25$  kg/m<sup>2</sup> and  $\leq 43$  cm<sup>2</sup>/m<sup>2</sup> for patients with BMI  $< 25$  kg/m<sup>2</sup>) (Table 1). In addition, new sarcopenia cutoff values for patients with cirrhosis have been reported (L3 SMI:  $\leq 42$  cm<sup>2</sup>/m<sup>2</sup> for women and  $\leq 50$  cm<sup>2</sup>/m<sup>2</sup> for men)<sup>[52]</sup> and are similar to those of cancer patients.

## PATHOGENESIS OF SARCOPENIA IN CIRRHOSIS

The pathogenesis of sarcopenia in cirrhosis is multifactorial and not fully understood. The mechanisms that contribute to sarcopenia include inadequate dietary intake, metabolic disturbances, and malabsorption

(Figure 1).

Inadequate dietary intake is common in patients with cirrhosis. Nausea and early satiety secondary to ascites, delayed gastric emptying, impaired gut motility, and small intestinal bacterial overgrowth contribute to poor intake<sup>[53]</sup>. Loss of appetite related to upregulation of tumor necrosis factor- $\alpha$  and leptin<sup>[54,55]</sup> and altered taste sensation associated with zinc deficiency<sup>[56]</sup> also contribute to decreased dietary intake. Dietary restriction, such as sodium restriction, decreased protein intake, and iatrogenic fasting during hospitalization can aggravate poor oral intake. Additionally, poor and irregular feeding is common in cirrhotic patients with active alcoholism, and might be aggravated by low socioeconomic status<sup>[57]</sup>.

Because cirrhotic liver tissue exhibits impaired synthesis and storage of glycogen, relatively short periods of fasting in patients with cirrhosis result in the breakdown of fat and muscle and promote gluconeogenesis from non-carbohydrate sources<sup>[58]</sup>. Unless dietary protein intake is sufficient, this can lead to muscle wasting. About 15%-30% of cirrhotic patients are hypermetabolic. The cause of hypermetabolism is unclear; activation of the sympathetic nervous system through hyperdynamic circulation, intestinal bacterial translocation, or systemic inflammation may partially explain the underlying mechanism of hypermetabolism in cirrhosis. Increased energy expenditure in cirrhotic

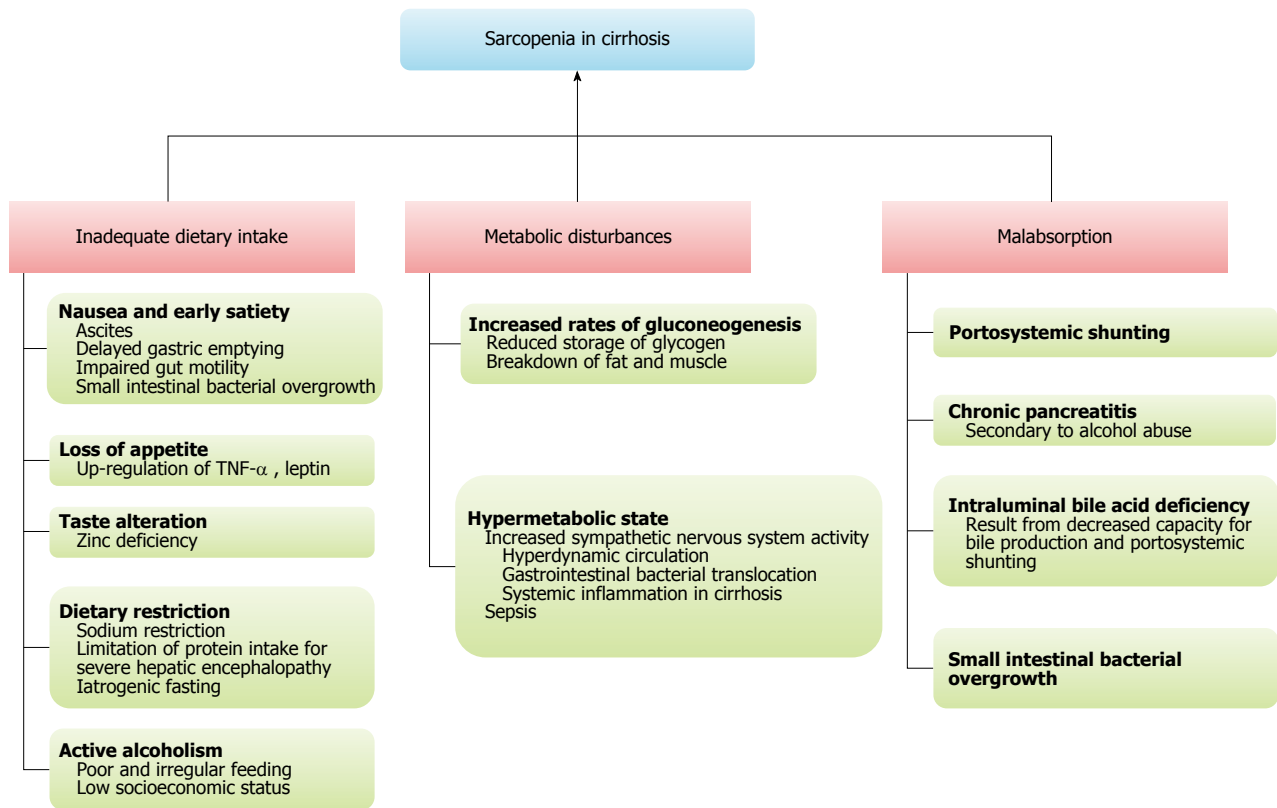


Figure 1 Pathogenesis of sarcopenia in cirrhosis.

patients accelerates the degradation of protein, which may be aggravated by sepsis<sup>[5]</sup>.

Malabsorption of nutrients in cirrhotic patients is caused by portosystemic shunting, chronic pancreatitis secondary to alcohol abuse, intraluminal bile salt deficiency in cholestasis, and overgrowth of bacteria in the small intestine<sup>[59]</sup>.

## PREVALENCE AND PREDICTORS OF SARCOPENIA IN CIRRHOSIS

Cross-sectional imaging studies have reported that the prevalence of sarcopenia is 30%-70% among patients with cirrhosis (Table 1). This wide range is partly explained by the lack of an operational definition for sarcopenia in patients with cirrhosis, patient baseline characteristics, and diversity in the cause and severity of liver disease among studies<sup>[8,10,11,40,43,45,60]</sup>.

Sarcopenia is more frequent in men than in women<sup>[8,10,11,45]</sup> and in patients with a low BMI<sup>[8,10,11,40,45]</sup>. The proportion of patients with sarcopenia is higher in those with alcoholic liver disease (80%) compared to other diseases (31%-71%)<sup>[40]</sup>. In some reports, CTP or MELD scores were predictors of sarcopenia<sup>[11,45]</sup>, whereas others found that sarcopenia was not correlated with the degree of liver dysfunction assessed by conventional scoring systems (CTP or MELD score)<sup>[8,10,43]</sup>.

## CLINICAL IMPACT OF SARCOPENIA

### *Effect of sarcopenia on survival in patients with cirrhosis*

The survival rates of patients with cirrhosis are significantly lower in those with sarcopenia than in those without (Table 2). The median survival is  $19 \pm 6$  mo in patients with sarcopenia, compared to  $34 \pm 11$  mo in patients without sarcopenia (log-rank,  $P = 0.005$ )<sup>[10]</sup>. Another study evaluating patients with concurrent cirrhosis and HCC reported a median survival of  $16 \pm 6$  mo for patients with sarcopenia compared to  $28 \pm 3$  mo for those without sarcopenia (log-rank,  $P = 0.003$ )<sup>[43]</sup>. The 1-year probability of survival in patients with sarcopenia is significantly lower than that in patients without sarcopenia (85% vs 97%,  $P = 0.01$ <sup>[8]</sup>; 52% vs 82%,  $P = 0.003$ <sup>[43]</sup>; 53% vs 83%,  $P = 0.005$ <sup>[10]</sup>; 63% vs 79%,  $P = 0.04$ <sup>[11]</sup>).

### *Causes of mortality in patients with sarcopenia and cirrhosis*

The lower survival rate in cirrhotic patients with sarcopenia is thought to be related to a higher proportion of sepsis-related deaths. The sepsis-related mortality rates in patients with and without sarcopenia patients are 22% and 8%, respectively ( $P = 0.02$ )<sup>[10]</sup>. As previously reported, the risk of infection is higher in elderly patients with sarcopenia than in those without<sup>[61]</sup>; therefore,

**Table 2** Clinical impact of sarcopenia on mortality in cirrhosis patients

Ref.	n	Unit of measure	Level of measure	Factors associated with survival (HR, 95%CI)	Survival among sarcopenic and nonsarcopenic patients	Cause of death
Durand <i>et al</i> <sup>[7]</sup>	562	TPMT/height, mm/m	umbilicus	MELD score (1.2, 1.14-1.27) TPMT/height (0.86, 0.78-0.94) in MELD-era cohort		
Hanai <i>et al</i> <sup>[8]</sup>	130	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3 vertebrae	CTP class B (2.39, 1.07-5.95) CTP class C (5.49, 2.11-15.12) BCAA (0.38, 0.19-0.79) Sarcopenia (3.03, 1.42-6.94)	The 1-, 3-, and 5-yr survival rates in patients with sarcopenia and nonsarcopenia were 85% and 97%, 63% and 79%, and 53% and 79%, respectively ( <i>P</i> = 0.01) The median survival was 16 (95%CI: 7-26) mo in patients with PMTH ≤ 14 mm/m The 1- and 2-yr mortality rates in patients with PMTH ≤ 14 mm/m and PMTH > 14 mm/m were 41.6% and 2.6%, and 66.8% and 15.2%, respectively ( <i>P</i> < 0.001)	No significant difference was seen in cause of death between patients with and without sarcopenia
Kim <i>et al</i> <sup>[9]</sup>	65	PMTH, mm/m	L4 vertebrae	PMTH (0.81, 0.68-0.97)		
Meza-Junco <i>et al</i> <sup>[43]</sup>	116	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3 vertebrae	Serum Na (0.89, 0.81-0.98) MELD (1.06, 1.01-1.12) CTP (2.39, 1.43-4.01) TNM stage (2.03, 1.45-2.84) Sarcopenia (2.20, 1.21-4.02)	The median survival was 16 ± 6 mo vs 28 ± 3 mo in sarcopenic patients compared to nonsarcopenic ( <i>P</i> = 0.003) The 6-mo, and 1-yr survival rates in patients with sarcopenia and nonsarcopenia were 67% and 90%, and 52% and 82%, respectively Median survival was 19 ± 6 mo vs 34 ± 11 mo in sarcopenia patients compared to nonsarcopenic patients ( <i>P</i> = 0.005) The 6-mo and 1-yr survival rates in patients with sarcopenia and nonsarcopenia were 71% and 90%, and 53% and 83%, respectively	No significant difference was seen in the frequency of sepsis-related death between patients with and without sarcopenia (12% vs 4%, <i>P</i> = 0.2)
Montano-Loza <i>et al</i> <sup>[10]</sup>	112	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3 vertebrae	CTP (1.85, 1.02-3.36) MELD (1.08, 1.03-1.14) Sarcopenia (2.21, 1.23-3.95)	The 6-mo and 1-yr survival rates in patients with sarcopenia and nonsarcopenia were 71% and 90%, and 53% and 83%, respectively	The rate of sepsis-related death was significantly higher in sarcopenic patients than nonsarcopenic patients (22% vs 8%, <i>P</i> = 0.02)
Tandon <i>et al</i> <sup>[11]</sup>	142	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3 vertebrae	Age (1.06, 1.01-1.10) MELD (1.13, 1.09-1.19) Sarcopenia (2.36, 1.23-4.53)	The 1-, 2-, and 3-yr survival rates in patients with sarcopenia and nonsarcopenia were 63% and 79%, 51% and 74%, and 51% and 70%, respectively ( <i>P</i> = 0.04)	Rates of sepsis-related death: 47% in sarcopenic patients vs 31% in nonsarcopenic patients ( <i>P</i> = 0.48)

BCAA: Branched chain amino acid; PMTH: Psoas muscle thickness by height.

sarcopenia, which reflects impaired immunity, may increase the risk for severe infections in patients with cirrhosis<sup>[62]</sup>. However, other studies have reported no difference in the frequency of sepsis-related death between patients with and without sarcopenia<sup>[8,11,43]</sup> (Table 2). Because sarcopenia affects immunity and physiological function<sup>[63]</sup>, sepsis is considered one of the leading causes of death in sarcopenic cirrhosis patients. However, the pathophysiological mechanism linking sarcopenia and mortality in cirrhosis is unproven. Conflicting results on causes of death call for further research regarding the pathogenic mechanism of sarcopenia in the prognosis of cirrhosis.

### Post-transplantation survival

Several investigators have reported that muscle mass is significantly associated with post-transplantation mortality (Table 3). In an exploratory analysis, the SMI

was significantly associated with post-transplantation survival (HR = 0.97, *P* = 0.014)<sup>[60]</sup>. DiMartini *et al*<sup>[40]</sup> demonstrated that muscle mass is a significant predictor of survival in men (HR = 0.95, *P* = 0.01), but not in women (HR = 0.98, *P* = 0.55). Englesbe *et al*<sup>[41]</sup> showed that the risk of post-transplantation mortality increases as the psoas muscle cross-sectional area decreases (HR = 3.7/1000 mm<sup>2</sup> decrease in psoas area; *P* < 0.0001). It has also been reported that sarcopenia is an independent prognostic factor for post-transplant mortality (HR = 2.06, *P* = 0.047)<sup>[64]</sup>. However, other studies have reported that sarcopenia is not associated with increased mortality after LT<sup>[7,45]</sup>. Some differences in the units of measure and definitions of sarcopenia used may partly explain dissimilarities between the results of these studies. Further prospective studies are needed to identify the association between sarcopenia and post-transplantation survival.

**Table 3** Impact of pretransplant sarcopenia on outcomes after liver transplantation

Ref.	n	Unit of measure	Level of measure	Impact on the post-transplant survival	Impact on the post-transplant infection	Impact on the length of post-transplant hospitalization
Cruz <i>et al</i> <sup>[60]</sup>	234	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3-4	SMI was significantly associated with survival post-transplantation (HR, 95%CI: 0.97, 0.94-0.99); <i>P</i> = 0.014)		
DiMartini <i>et al</i> <sup>[40]</sup>	338	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3-4	Muscle mass was a significant predictor of survival only in men (HR = 0.95, <i>P</i> = 0.01)		Muscle mass predicted ICU stay, total length of stay, and days of intubation
Durand <i>et al</i> <sup>[7]</sup>	562	TPMT/height, mm/m	umbilicus	MELD-psoas score was not an independent prognostic factor for post-transplant mortality in pre-MELD and MELD-era cohorts		
Englesbe <i>et al</i> <sup>[41]</sup>	163	TPA, mm <sup>2</sup>	L4	The risk of post-transplantation mortality increased as psoas area decreased (HR = 3.7/1000 mm <sup>2</sup> decrease in psoas area; <i>P</i> < 0.0001)		
Krell <i>et al</i> <sup>[42]</sup>	207	TPA, mm <sup>2</sup>	L4		Pretransplant TPA (HR = 0.38, <i>P</i> < 0.01) was an independent risk factor for developing a serious posttransplant infection	
Masuda <i>et al</i> <sup>[64]</sup>	204	Area of the psoas muscle, cm <sup>2</sup>	L3	Sarcopenia was an independent prognostic factor for posttransplant mortality (HR = 2.06, <i>P</i> = 0.047)	The rate of postoperative sepsis was higher in sarcopenic patients than in nonsarcopenic patients (17.7% vs 7.4%, <i>P</i> = 0.03)	
Montano-Loza <i>et al</i> <sup>[45]</sup>	248	SMI, cm <sup>2</sup> /m <sup>2</sup>	L3	L3 SMI and the presence of sarcopenia were not associated with increased mortality after liver transplantation	Bacterial infections within the first 90 d after liver transplantation were more common in sarcopenic patients than in nonsarcopenic patients (26% vs 15%, <i>P</i> = 0.04)	Sarcopenic patients had longer hospital stays (40 ± 4 d vs 25 ± 3 d, <i>P</i> = 0.005) and longer ICU stays (12 ± 2 d vs 6 ± 1 d, <i>P</i> = 0.001) after liver transplantation than nonsarcopenic patients

ICU: Intensive care unit.

**Other post-transplantation outcomes**

The frequency of post-transplantation infection is higher in patients with sarcopenia than in those without (17.7% vs 7.4%, *P* = 0.03<sup>[64]</sup>; 26% vs 15%, *P* = 0.04<sup>[45]</sup>). Krell *et al*<sup>[42]</sup> also showed that as the total psoas area (TPA) decreases, the risk of developing infection increases [odds ratio for tertile 1 vs tertile 3, 4.6; 95%CI: 2.25-9.53]. Moreover, patients with sarcopenia have longer hospital and intensive care unit stays after LT compared to those of patients without sarcopenia<sup>[40,45]</sup> (Table 3).

## PROGNOSTIC IMPLICATIONS FOR PATIENTS WITH SARCOPENIA AND CIRRHOSIS

As described previously, a growing body of literature has emphasized the negative impact of sarcopenia assessed by imaging on the outcome of patients with cirrhosis. Sarcopenia or a measure of muscle mass is an independent predictor of survival for patients with

cirrhosis<sup>[7-11,43]</sup>.

The c-statistics for the L3 SMI for predicting 3- and 6-mo mortality are 0.64 (0.46-0.83; *P* = 0.1) and 0.67 (0.54-0.81; *P* = 0.02), respectively<sup>[43]</sup>. The c-statistics for the L3 SMI was also significant for predicting 6-mo mortality (0.67, 0.55-0.79; *P* = 0.02) but not 3-mo mortality (0.61, 0.47-0.75; *P* = 0.2)<sup>[10]</sup>. The predictive ability of sarcopenia alone was inferior to that of the MELD or CTP score<sup>[10,43]</sup>.

Considering that the MELD lacks a nutritional assessment and the inferior predictive performance of sarcopenia alone, recent studies have investigated whether modifying the MELD score to include sarcopenia could improve mortality prediction in patients with cirrhosis. The discriminating ability of transverse psoas muscle thickness (TPMT)/height is inferior to that of the MELD score [overall C index (95%CI): 0.67 (0.47-0.82) for TPMT/height, 0.80 (0.60-0.91) for MELD score in a MELD-era cohort]. However, the overall C index (0.82; 95%CI: 0.64-0.93) of the MELD-psoas score, which combines MELD and TPMT/height, is superior to that of the MELD score



(0.80; 95%CI: 0.60-0.91) and was similar to that of the MELD-Na score (0.82; 95%CI: 0.63-0.93) in the MELD-era cohort<sup>[7]</sup>. Another study showed that a novel MELD-sarcopenia score, derived from estimated values given by a Cox model including the MELD score and L3 SMI, is associated with a modest improvement for predicting mortality in patients with cirrhosis [c-statistic (95%CI) for 3-mo mortality was 0.68 (0.60-0.76) for MELD and 0.72 (0.65-0.79) for MELD-sarcopenia]<sup>[65]</sup>.

The presence of sarcopenia was an independent predictor of mortality in patients with low MELD scores ( $< 15$ ; log-rank,  $P = 0.02$ ) but not in patients with higher MELD scores ( $\geq 15$ ,  $P = 0.59$ )<sup>[11]</sup>. Another study also demonstrated that low TPMT/height is associated with increasing mortality among patients with refractory ascites and a MELD score  $\leq 25$ , but not in patients without refractory ascites<sup>[7]</sup>. Therefore, sarcopenia may be useful for risk stratifying in patients with low MELD scores.

Sarcopenia is an attractive prognostic factor to reduce waiting-list mortality and improve organ allocation in addition to conventional scores, because the CTP and MELD scores mainly reflect liver function but not nutritional status. However, prospective studies that include a large number of patients with cirrhosis are needed prior to the widespread use of sarcopenia alone or in combination with the MELD score as a prognostic factor.

## CHALLENGES IN CLINICAL APPLICATIONS

### Standardizing muscularity assessment

Many studies that investigated the prevalence and impact of sarcopenia on waiting-list mortality or post-transplantation outcomes used muscle cross-sectional area on a single abdominal CT scan as the assessment of muscularity in patients with cirrhosis. Cross-sectional areas of surrounding muscles (*i.e.*, psoas, erector spinae, quadrates lumborum, transverses abdominis, external and internal obliques, and rectus abdominis) in the L3 or L3-4 regions have been quantified using specific computer software and tissue-specific Hounsfield unit thresholds<sup>[8,10,11,40,43,45,60]</sup>. Other investigators have used TPA measured by outlining the borders of both psoas muscles and computed the cross-sectional area of the psoas muscles<sup>[41,42,64]</sup>. Measuring psoas muscle mass on a CT scan is easy and accessible. However, total psoas muscle area is only part of the total skeletal muscle mass, and TPA has not been validated as a predictor of total body mass. In contrast, L3 SMI has been shown to be correlated with whole-body muscle mass<sup>[37]</sup>. Because muscularity assessment based on the muscle cross-sectional area is complex and requires specific software, evaluations of the psoas muscle thickness were introduced and have been found to be associated with waiting-list and post-transplant mortality<sup>[7,9]</sup>.

The L3 vertebra level has been commonly used to calculate the cross-sectional area or psoas muscle thickness on CT scans<sup>[8,10,11,43,45,64]</sup> based on the finding that cross-sectional muscle area measured at the L3 level best correlates with whole-body muscle mass in patients with or without malignancy<sup>[37]</sup>. However, others have measured cross-sectional muscle area or psoas muscle thickness at the level of L4<sup>[9,41,42]</sup>, L3-4<sup>[40,60]</sup>, or the umbilicus<sup>[7]</sup>. Although the umbilicus level is easily recognized on an abdominal CT scan, it may vary in patients with massive ascites. In contrast, the sacralization of the L4 vertebrae, lumbarization of the S1 vertebrae, and prominent lordosis in patients with refractory ascites may cause errors when identifying the vertebral level<sup>[7]</sup>. Thus, the best muscle measurement method that readily reflects whole-body skeletal muscle needs to be determined.

### Cutoff values for sarcopenia measured by cross-sectional imaging

As predefined sarcopenia cutoff values are lacking for patients with cirrhosis, most studies<sup>[8,10,11,40]</sup> defined sarcopenia using the L3 SMI sex-specific cutoff values from a previous study<sup>[50]</sup>. These values (L3 SMI:  $\leq 38.5$  cm<sup>2</sup>/m<sup>2</sup> for women and  $\leq 52.4$  cm<sup>2</sup>/m<sup>2</sup> for men) are derived from a sarcopenia study that stratified mortality in cancer patients; therefore, it may not be optimal for prognostication of patients with cirrhosis. More recent studies<sup>[43,45]</sup> adopted sex- and BMI-specific cutoff values for sarcopenia (L3 SMI:  $\leq 41$  cm<sup>2</sup>/m<sup>2</sup> for women and  $\leq 53$  cm<sup>2</sup>/m<sup>2</sup> for men with a BMI  $\geq 25$  kg/m<sup>2</sup> and  $\leq 43$  cm<sup>2</sup>/m<sup>2</sup> for patients with a BMI  $< 25$  kg/m<sup>2</sup>)<sup>[51]</sup>. A preliminary report that included 350 patients with cirrhosis established new sarcopenia cutoff values for patients with cirrhosis (L3 SMI:  $\leq 42$  cm<sup>2</sup>/m<sup>2</sup> for women and  $\leq 50$  cm<sup>2</sup>/m<sup>2</sup> for men)<sup>[52]</sup>.

### Muscle function

It may be insufficient to define sarcopenia based only on skeletal muscle mass. Although using muscle function together with muscle mass is controversial<sup>[48]</sup>, the nonlinear relationship between muscle strength and mass provides a basis for adopting both criteria to define sarcopenia<sup>[66]</sup>.

### Sex-specific sarcopenia differences

The prevalence of sarcopenia is higher in men than in women<sup>[8,10,11,40,45,60]</sup>. In addition, results regarding the impact of muscle mass on survival or other clinical outcomes differ between men and women<sup>[40]</sup>. Similarly, skeletal muscle mass predicts 3- and 6-mo survival in men with cirrhosis waiting for LT but not in women<sup>[10]</sup>.

Women have more abundant fat stores and more preferentially utilize fat stores compared to skeletal muscle stores<sup>[67]</sup>. Therefore, fat reserves are more depleted in women, whereas men have a more depleted skeletal muscle mass<sup>[68]</sup>. Moreover, sex hormone differences may play a role in the way

skeletal muscle is turned over<sup>[69]</sup>. These factors may explain the sex-specific differences in the prevalence and pathophysiology of sarcopenia in patients with cirrhosis. These differences may influence the use of sarcopenia to assess nutritional status and on the utility of a sarcopenia-based prognostic score.

## CONCLUSION

In view of emerging findings linking sarcopenia with a poor outcome in cirrhotic patients, adopting sarcopenia as a surrogate marker appears to be an appealing approach to prognostication in cirrhosis. Furthermore, sarcopenia determined by cross-sectional imaging-based muscular assessment is objective and reproducible and reflects nutritional and functional status, which is not included in current cirrhosis prognostic models. Accumulating evidence suggests a compelling rationale for the review of current prognostic scoring systems as well as the incorporation of sarcopenia into prognostic models for patients with cirrhosis. Although awareness of the effects of sarcopenia on the outcome of cirrhotic patients is increasing, there are many practical challenges to the application of these findings. Further studies are required to validate the methodology of quantifying muscle mass using cross-sectional imaging and to derive optimal gender-specific cutoffs of the muscle mass index as a determinant of mortality in cirrhotic patients.

In conclusion, optimizing a prognostic scoring system is a crucial topic when managing patients with cirrhosis. Despite the high prevalence of sarcopenia and its potential to influence morbidity and mortality in patients with cirrhosis, sarcopenia is not included in the conventional prognostic scores for cirrhosis, such as the MELD and CTP scores. The lack of an objective, available, and reproducible muscle wasting index has limited the inclusion of sarcopenia into prognostic scoring systems for cirrhosis. Quantifying skeletal muscle mass in patients with liver cirrhosis is challenging; however, a muscularity assessment using single-slice cross-sectional imaging provides a possible application for sarcopenia in the prognostication of patients with cirrhosis. Several novel attempts have been made to combine measurements of sarcopenia with current prognostic models to assess the severity of liver disease. To date, the proposed composite models have been associated with only modest improvement in the prognostication of cirrhosis. While there is still much to be defined, quantification of skeletal muscle mass sheds light on the prognostic role of sarcopenia and might hold promise for further development of prognostic models utilizing sarcopenia. Large prospective studies are required to validate the prognostic implication of sarcopenia in addition to conventional prognostic systems.

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## Personalized targeted therapy for esophageal squamous cell carcinoma

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### Abstract

Esophageal squamous cell carcinoma continues to heavily burden clinicians worldwide. Researchers have discovered the genomic landscape of esophageal squamous cell carcinoma, which holds promise for an era of personalized oncology care. One of the most pressing problems facing this issue is to improve the understanding of the newly available genomic data, and identify the driver-gene mutations, pathways, and networks. The emergence of a legion of novel targeted agents has generated much hope and hype regarding more potent treatment regimens, but the accuracy of drug selection is still arguable. Other problems, such as cancer heterogeneity, drug resistance, exceptional responders, and side effects, have to be surmounted. Evolving topics in personalized oncology, such as interpretation of genomics data, issues in targeted

therapy, research approaches for targeted therapy, and future perspectives, will be discussed in this editorial.

**Key words:** Cancer heterogeneity; Cultured tumor cells; Driver mutation; Drug side effects; Esophageal squamous cell carcinoma; Exceptional responder; High-throughput nucleotide sequencing; Neoplasm drug resistance; Personalized medicine; Xenograft model

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**Core tip:** Esophageal squamous cell carcinoma represents a heavy burden on clinicians worldwide. Recently, researchers have discovered the genomic landscape of this cancer, which holds promise for an era of personalized oncology care. Evolving topics in personalized oncology, such as interpretation of genomics data, critical issues in targeted therapy, research approaches, and future perspectives, are discussed in this editorial.

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## INTRODUCTION

Esophageal cancer is the eighth most common cause of cancer-related death worldwide<sup>[1]</sup>. Esophageal squamous cell carcinoma (ESCC) remains the predominant histology. Surgery is still the mainstay of treatment throughout the world, and an up to 50% five-year survival rate and < 5% surgical mortality rate can be achieved in select Asian centers<sup>[2]</sup>. Notwithstanding, multimodal treatment may achieve a better outcome, as overall survival improves modestly<sup>[3]</sup>. Most patients with localized disease will develop metastatic disease, with a minimal effects from combination chemotherapy<sup>[4]</sup>. After disease progression on first-line chemotherapy, there is no standard second-line treatment<sup>[5]</sup>. The unsatisfactory outcome in ESCC is mainly due to late diagnosis, the aggressiveness of this cancer, and lack of effective treatment strategies<sup>[6]</sup>.

Recently, tremendous progress has been made in cancer genomics and epigenomics with the advent of high-throughput techniques, such as next-generation sequencing. Three groups have reported the genetic landscape of human ESCC with whole genome sequencing and whole exome sequencing<sup>[7-9]</sup>. Genomic alterations include: (1) single nucleotide variants of many genes with a relatively significant frequency ( $\geq 5\%$ ), such as *p53*, *KMT2D*, *Notch1/2/3*, *FAT1/3*, *Syne1*, *EP300*, *Rb1*, *Nfe2l2*, *Cdkn2a*, *Ajuba*, *Crebbp*, *Kdm6A*,

*Fbxw7*, *MLL2/3*, *Pik3ca*, *Pten*, *Arid2*, *Pbrm1*, etc; (2) copy number alterations of many genes with a relatively significant frequency ( $\geq 5\%$ ), such as *CCND1*, *FGFs*, *CDKN2A*, *CDKN2B*, *Pik3ca*, *Dvl3*, *LRP5/6*, *KRas/MRas*, *EGFR*, *Akt1*, *Bcl2l1*, *Notch1/2/3*, *E2F1*, *SFRP4*, *SOS1/2*, *Birc5*, *Yap1*, *Sox2*, *Myc*, *IL7R*, etc; and (3) alterations in multiple signaling pathways, such as cell cycle regulation, apoptosis regulation, DNA damage control, histone modifications, as well as RTK-Ras-MAPK-PI3K-Akt, Hippo, Notch, Wnt, and Nfe2l2/Keap1 pathways. The overall mutation pattern appears similar to that of head and neck squamous cell carcinoma<sup>[10,11]</sup>, but different from that of esophageal adenocarcinoma<sup>[12,13]</sup> and lung squamous cell carcinoma<sup>[14]</sup>.

In addition to these descriptive data, smoking was not found to be related with signature mutations<sup>[7]</sup>, but the lack of alcohol consumption was associated with a cluster of gene mutations<sup>[9]</sup>. Viral integration was not found in the genomes of 88 subjects<sup>[9]</sup>. Trinucleotide signature analysis suggested DNA cytidine deaminase (*APOBEC3B*)-induced deamination was mainly responsible for mutations<sup>[8,15]</sup>. Moreover, mutations of single genes or gene clusters were associated with patient survival, for example, *EP300* mutation<sup>[7,9]</sup>. Certain genes, for example, *XPO1*, were explored as a therapeutic target<sup>[8]</sup>.

These landmark studies provided the research community with an enormous amount of information to better understand the molecular mechanisms of ESCC. This editorial is aimed to gain insights from such studies, and propose personalized and targeted therapy as a research direction in the future.

## INTERPRETATION OF GENOMICS DATA

### Driver genes and mutations

Currently available bioinformatics tools have been designed to prioritize gene mutations at the nucleotide, gene, pathway, and network levels. The number of nonsynonymous somatic mutations per ESCC averaged > 80. If a solid tumor ordinarily requires 5-8 hits (not necessarily 5-8 mutations) as suggested by classical epidemiologic studies, most of these mutations should be "passengers" instead of "drivers", which can offer selective growth advantage to the tumor cell<sup>[16]</sup>. Therefore, it is critical to identify which gene mutations are cancer drivers.

As driver mutations may occur at high or low frequencies<sup>[17]</sup>, it may not be safe to prioritize driver mutations according to their frequencies. However, as a clinically relevant parameter, a high frequency of a mutation does support its potential significance in carcinogenesis. In addition to mutated drivers, Epi-drivers are a class of driver genes that are not frequently mutated but aberrantly expressed in tumors through epigenetic alterations in DNA methylation or chromatin modification. Although epigenetics in ESCC has been studied for many years<sup>[18,19]</sup>, it is still

not clear how to differentiate epigenetic alterations that bring forth a selective growth advantage from those that do not<sup>[16]</sup>. According to Vogelstein *et al.*<sup>[16]</sup>'s 20/20 rule, only 125 mutated-driver genes of human cancers have been discovered to date, and the number is nearing saturation. Tamborero *et al.*<sup>[20]</sup> reported a list of 291 high-confidence cancer-driver genes and 144 candidate genes from 12 different types of cancer. Several databases have become available. For example, Network of Cancer Genes (NCG 4.0) contains 537 experimentally supported genes and 1463 candidate genes inferred using statistical methods<sup>[21]</sup>. The Candidate Cancer Gene Database contains cancer-driver genes from forward genetic screens in mice<sup>[22]</sup>. Considering tissue specificity of ESCC, there is a need to compile a cancer-driver gene list to support future research on ESCC therapy. However, it should be pointed out that cancer-driver genes may contain both driver mutations and passenger mutations in cancer. For example, APC mutations truncating the N-terminal amino acids are driver mutations, while those affecting other regions are passenger mutations. Even for the same driver gene (*e.g.*, *K-Ras*), different driver mutations (*e.g.*, mutations at codons 12, 13, and 61) have different impacts on carcinogenesis and clinical behaviors<sup>[23-25]</sup>. Because of these complexities, efforts need to be made in order to identify personalized driver genes in cancer<sup>[26]</sup>.

### Pathways and network

Increasing evidence suggests that dysregulation of cellular signaling pathways, rather than individual mutations, contributes to the pathogenesis of ESCC<sup>[27-29]</sup>. Driver genes usually do not work in isolation, but often function together to alter cellular processes<sup>[30]</sup>. There is a growing consensus that pathways rather than single genes are the primary target of mutations<sup>[31]</sup>. It is interesting that mutations in various components of a single pathway tend to be mutually exclusive<sup>[32]</sup>. Once driver genes or driver mutations are identified, the next step is to focus on driver pathways with genes grouped together according to the biochemical pathways that they play functional roles in. Pathway activity may be further validated by the downstream readouts, *e.g.*, mRNA and protein expression, morphology, and function. Incorporation of immunohistochemistry data, or even proteomics data, may help in evaluation of the pathway activity<sup>[33,34]</sup>.

One major challenge in analyzing genomics data of ESCC is the lack of information of esophagus-specific pathways. Pathway databases, *e.g.*, KEGG, are fairly incomplete and lack tissue and cell specificities. Applying such pathway information in analyzing ESCC data may generate misleading outcomes. For example, using ChIP-seq analyses, Sox2-regulated genes in ESCC cells are different from those in embryonic stem cells because in ESCC, Sox2 tends to interact with p63 as opposed to Oct4 in embryonic stem

cells<sup>[35]</sup>. Identifying bona fide target genes and using expression profiles of these genes to infer pathway activity in ESCC will be critical in the future<sup>[36]</sup>.

Few bioinformatics methods involve a procedure for taking account of pathway interactions, *i.e.*, pathways that are mutated in the same sample, and that are mutated together across a large subset of samples<sup>[8]</sup>. Similar to expression-based stratification, network-based stratification of tumor mutations can identify cancer subtypes to guide treatment and prognosis<sup>[37]</sup>. Categorizing ESCC into multiple subtypes according to its molecular alterations may be a practical step leading to final personalization of ESCC therapy. In fact, subtyping has been shown to be a successful approach in managing other cancers<sup>[38]</sup>.

### Drug selection

Selecting drugs according to genomics data has led to promising results in early studies on personalized and targeted therapy<sup>[39]</sup>. To date, most clinically approved targeted drugs are directed against kinases. Some of these have been utilized against ESCC (Table 1). Gefitinib, an epidermal growth factor receptor inhibitor, has been tested as a second-line treatment for esophageal cancer. In unselected patients it does not improve overall survival, but has palliative benefits in a subgroup of difficult-to-treat patients with a short-life expectancy<sup>[40]</sup>. Unfortunately, only a few cancer drivers have enzymatic activities that are targetable in this fashion, and whether a target is druggable becomes a research question<sup>[41]</sup>. Once a drug target is verified, drugs or experimental compounds may be developed. Several databases are available for search, including the Therapeutic Target Database<sup>[42]</sup> and DrugBank 4.0<sup>[43]</sup>.

If the target is not druggable, its regulatory proteins or functional pathway may be targeted. For example, cyclin D1 amplification is commonly seen in human ESCC. As cyclin D1 mainly functions through CDK activation, CDK4 and CDK6 can be targeted instead of cyclin D1<sup>[44]</sup>. *TP53*, which encodes p53, is the most commonly mutated gene in human ESCC. Instead of targeting *TP53*, many strategies have been tested to restore the functions of p53 by delivering wildtype *TP53*, targeting the MDM2-p53 interaction, restoring the functions of mutant p53, targeting p53 family proteins, or eliminating the mutation in p53<sup>[45,46]</sup>.

In addition to selecting drugs for targeted therapy, analysis of drug-metabolism genes in germ-line DNA can also optimize dosing and identify drug toxicity risk<sup>[47,48]</sup>. With the help of a database, such as Pharmacogenetics and Pharmacogenomics Knowledge Base, genetic variations can be associated with drug response<sup>[49]</sup>.

## ISSUES IN TARGETED THERAPY

### Cancer heterogeneity

Various combinations of drivers and pathways result in intratumoral, intermetastatic, intrametastatic, or

**Table 1 National clinical trials on targeted therapy of esophageal squamous cell carcinoma<sup>1</sup>**

Target	Agent	NCT number (phase)
EGFR	Erlotinib	NCT00045526 (II), NCT00030498 (I), NCT00397384 (I), NCT00524121 (II), NCT01013831 (I), NCT01561014 (I), NCT01752205 (III)
	Gefitinib	NCT00093652 (I / II), NCT00258297 (II), NCT00258323 (II), NCT00268346 (II), NCT00290719 (I) NCT01973725 (II)
	Icotinib	
	Lapatinib	NCT00239200 (II), NCT01666431 (II)
	Nimotuzumab	NCT02272699 (II / III), NCT01232374 (II), NCT01336049 (II), NCT01402180 (II / III), NCT01486992 (II), NCT01688700 (II), NCT01993784 (I / II), NCT02011594 (II), NCT02034968 (II), NCT02041819 (II)
	Panitumumab	NCT01077999 (II), NCT01262183 (II), NCT01627379 (III)
	PF00299804	NCT01608022 (II)
	Cetuximab	NCT02123381 (II), NCT00109850 (II), NCT00165490 (II), NCT00381706 (II), NCT00397384 (I), NCT00397904 (II), NCT00425425 (I / II), NCT00445861 (I / II), NCT00509561 (II / III), NCT00544362 (I / II), NCT00655876 (III), NCT00757549 (I), NCT00815308 (II), NCT01034189 (II), NCT01107639 (III)
	Cixutumumab	NCT01142388 (II)
	BKM120	NCT01626209 (I), NCT01806649 (II)
PI3K	BYL719	NCT01822613 (I / II)
	Rigosertib	NCT01807546 (II)
HDAC	Entinostat	NCT00020579 (I)
	Vorinostat	NCT00537121 (I), NCT01249443 (I)
HER3	LJM716	NCT01598077 (I), NCT01822613 (I / II)
VEGFR	Vandetanib	NCT00732745 (I)
	Sorafenib	NCT00917462 (II)
VEGFA	Bevacizumab	NCT01212822 (II)
PD-L1	MEDI4736	NCT01938612 (I)
Bcl-2 mRNA	Oblimersen	NCT00003103 (I / II)
CDK9	Alvocidib	NCT00006245 (II)
CRM1	Selinexor	NCT02213133 (II)
FGFR	AZD4547	NCT01795768 (II)
KIF11	Litroneisib	NCT01059643 (II)
TACSTD2	IMMU-132	NCT01631552 (I / II)

<sup>1</sup>"Esophageal squamous cell carcinoma" was searched at the website (www.clinicaltrials.gov). Targeted therapy has been or is being tried in 62/204 studies. Some of these agents target multiple molecules, for example, lapatinib (EGFR and ErbB2), rigosertib (PI3K and PLK), vandetanib (VEGFR, EGFR, and RET), and sorafenib (VEGFR, PDGFR and RAF).

interpatient heterogeneities. It may explain why the same treatment brings about either a favorable response or resistance in different patients, and why a patient that responds well initially can develop resistance over time. Intratumoral heterogeneity has been validated using single-cell RNA-seq of primary glioblastomas<sup>[50]</sup>. As the majority of cancer gene mutations appear in multiple regions of the same tumor, single-region sequencing may be adequate to identify the majority of cancer gene mutations<sup>[51]</sup>. It can be predicted that most cancer cells in the same tumor may share the major alterations. If this is proven true in ESCC, it will make treatment more predictable.

Intermetastatic and intrametastatic heterogeneity may not be a great concern. Despite many years of research, we have failed to identify a group of so-called metastasis genes. Metastasis is probably stochastic depending on the environment in the metastatic site<sup>[52]</sup>. Therefore, if we can understand genetic and epigenetic alterations in the primary tumor well, all cancer cells left at the primary site or metastatic sites would be expected to behave in the same way. Nevertheless, the prevalence of different patterns of tumor heterogeneity needs to be more robustly assessed in large patient cohorts, and new patterns will probably be identified as the wealth of genomic data of ESCC is analyzed<sup>[53]</sup>.

### Drug resistance

If carcinogenesis is regarded as an evolutionary process with successive new mutations driven by natural selection, chemotherapy, radiotherapy, and target therapy may all provide a potent source of artificial selection to alter clonal dynamics. Consequently, the antitumor therapy may lead to resistance<sup>[54]</sup>. Indeed, targeted therapy is associated with a high rate of resistance at the very beginning when vemurafenib, a *BRAF*<sup>V600E</sup> inhibitor, was clinically used for melanoma. Combination of a *BRAF*<sup>V600E</sup> inhibitor (dabrafenib) and a MEK inhibitor (trametinib) resulted in better response, yet did not prevent resistance from occurring. Distinct mechanisms include mutations in the target, reactivation of the targeted pathway, hyperactivation of alternative pathways, and cross-talk with the microenvironment<sup>[55]</sup>. Resistant cells may undergo a process called phenotype switching under the selection of targeted therapy<sup>[56]</sup>. Understanding these mechanisms has led to additional efforts in finding new therapies targeting the same target, the same pathway, or alternative pathways<sup>[57-59]</sup>.

Three strategies are feasible measures in the handling of drug resistance. Before treatment, both bioinformatics and experimental modeling can provide information concerning heterogeneity<sup>[60-62]</sup>. There is a need to develop clinically useful measures of heterogeneity<sup>[63]</sup>. Secondly, during treatment, limited success can be achieved with a single agent. The combination strategy may be the best way to refrain from the inevitable development of resistance to single drug-targeted therapies<sup>[31]</sup>. Thirdly, longitudinal tumor sampling will be essential to decipher the impact of tumor heterogeneity on cancer evolution, and developing minimally invasive methods to profile heterogeneous tumor genomes will play a major part in following clonal dynamics in real time<sup>[61]</sup>. For ESCC, repeated biopsy, circulating tumor DNA analysis<sup>[64,65]</sup>, and exfoliative cells<sup>[66,67]</sup> are all valid options for this purpose.

### Exceptional responders

As opposed to drug resistance, exceptional responders



are patients who have a unique response to treatments that are not effective for most other patients. The National Cancer Institute has embarked on the Exceptional Responders Initiative to understand the molecular underpinnings of exceptional responses to treatment in cancer patients. In the past, exceptional responders led to clinical breakthroughs in treatments of certain types of cancer, and understanding of novel molecular mechanisms of carcinogenesis<sup>[68]</sup>. It is foreseeable that careful characterization and follow-up of these exceptional responders will be of great value in the future practice of personalized and targeted therapy of ESCC.

### Side effects

As compared with traditional chemotherapy, targeted therapy is better tolerated. However, it does produce toxicities based on several major mechanisms, including on-target, off-target, hypersensitivity-related, and metabolite-induced toxicities. Vascular endothelial growth factor receptor inhibitors cause hypertension, and epidermal growth factor receptor inhibitors cause toxicities in tissues where they normally play an important functional role in tissue maintenance (*e.g.*, skin and gastrointestinal epithelia). Some of these on-target toxicities may serve as surrogate biomarkers for clinical response<sup>[69-73]</sup>. Considering these potential side effects, clinical oncologists should be prepared to educate the patients and undertake respective preventive and therapeutic measures.

## RESEARCH APPROACHES FOR TARGETED THERAPY

For genomics-guided research, cell line-based platforms have become an indispensable tool<sup>[74,75]</sup>. Clarification of genetic and epigenetic alterations of established ESCC cell lines would be great tools for preclinical drug development<sup>[76,77]</sup>, in particular, the KYSE series of ESCC cell lines that have been sequenced<sup>[7-9]</sup>. Patient-derived ESCC cells can be used for selection of potential individualized therapy<sup>[78,79]</sup>. These cells are particularly useful in identifying effective drug combinations for acquired resistance<sup>[57]</sup>.

Several models have been put into preclinical research and even clinical applications. A patient-derived xenograft model of ESCC is created when cancerous tissue from a patient's primary tumor is implanted directly into immunodeficient mice. This model provides solutions to the translational challenges that researchers and clinicians face in cancer drug research and selection<sup>[80,81]</sup>. Carcinogen-induced models, for example, the N-nitrosomethylbenzylamine-induced model, represent classical models for ESCC research. They mimic human ESCC in not only etiology and histopathology, but also in molecular alterations (*e.g.*, *TP53* mutations<sup>[82,83]</sup>). However, exactly how well this model can mimic human ESCC

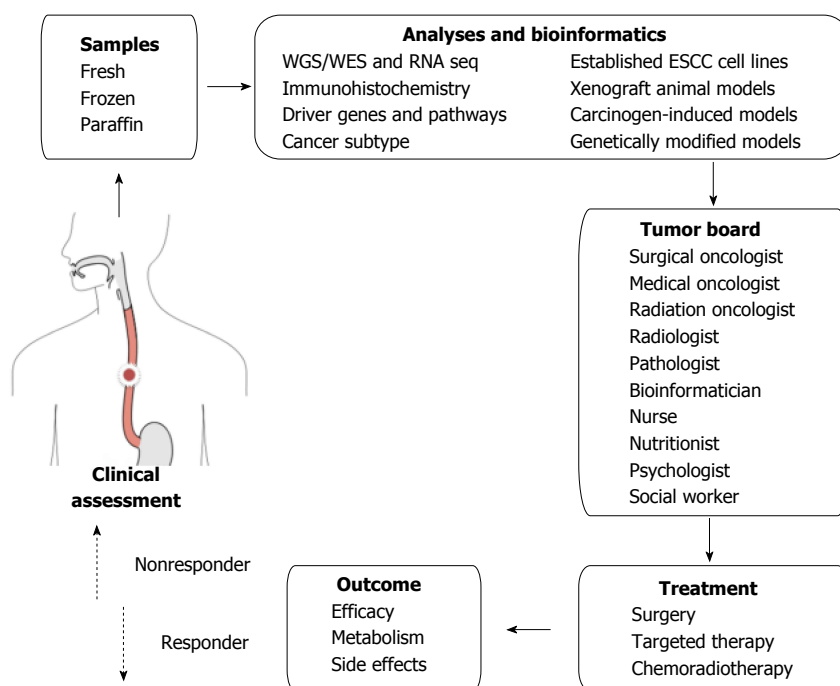
at the genomics level has not been well studied. Whole exome sequencing has already shown that carcinogen-induced and genetically engineered models lead to carcinogenesis through different routes. A carcinogen-induced model is particularly important in understanding the complex mutation spectra seen in human cancers<sup>[84]</sup>. It is encouraging that genomic alterations in 4-nitroquinoline 1-oxide-induced mouse tongue cancer are well preserved<sup>[83]</sup>.

Genetically engineered mouse models of human cancers have proven essential to dissect the molecular mechanisms behind carcinogenesis<sup>[85]</sup> and provide robust preclinical platforms for investigating drug efficacy<sup>[86]</sup> and resistance<sup>[87-89]</sup>. As an example, transgenic overexpression of *Sox2*, an amplified oncogene in ESCC<sup>[90]</sup>, drives the complete process of carcinogenesis in mice<sup>[91]</sup>. This model can readily be used for preclinical drug development for *SOX2*-overexpressing ESCC. Although it may be difficult to target *SOX2* itself, its downstream genes or pathways, such as the Akt/mTOR pathway, can be targeted<sup>[79]</sup>. Biochemical outcomes may be used for assessment of the efficacy of a *Sox2*-targeting therapy even when it does not reduce tumor incidence or size in mice. Genome engineering with CRISPR-Cas9 *in vivo* is an extremely promising technique in identifying cancer-driver genes and testing drug targets<sup>[92]</sup>. It may ultimately be used for human gene therapy in the future<sup>[93]</sup>.

As a hallmark of human cancer and a crucial determinant of variable response to treatment<sup>[75]</sup>, genomic heterogeneity calls for revision of clinical trial design currently in use in order to implement personalized therapy<sup>[94]</sup>. The majority of traditional prospective clinical trials are disease or histopathology based. Genomics-driven trials, for example, mutation-, pathway-, and subtype-based trials, will be more widely used in drug development<sup>[95]</sup>. Two genomics-based study designs are currently being utilized to develop targeted therapies, and for exploratory and multi-agent sequential design<sup>[96]</sup>. ESCC fits both study designs very well because the esophagus can be biopsied before and after treatment.

## FUTURE PERSPECTIVES

The biggest challenge in ESCC treatment is the translation of genomic discoveries into personalized therapies based on strategies sketched from patients' individual profiles<sup>[94]</sup>. The evasiveness of cancer cells has been a frustrating observation of clinical oncologists. Vogelstein *et al.*<sup>[16]</sup> proposed that "there is order in cancer," pointing to the need to tackle ESCC as a disease status with its own homeostatic mechanisms. From the perspective of ten hallmarks of human cancer<sup>[97]</sup>, Hanahan<sup>[98]</sup> proposed three strategically distinct "battlespace-guided plans" for cancer treatment: disruption of the enemy's many capabilities, defense against cancer's armed forces,



**Figure 1 Personalized and targeted therapy for esophageal squamous cell carcinoma.** The strategy is based on the concept that a patient's genetic makeup should guide his/her treatment. After a series of molecular analyses on tumor samples, bioinformatics is expected to identify driver genes, pathways, cancer subtype, and target drugs. A tumor board will synthesize all information and generate a personalized treatment plan. Nonresponders may be analyzed in a similar manner during subsequent surveillance and further treated. ESCC: Esophageal squamous cell carcinoma; WES: Whole exome sequencing; WGS: Whole genome sequencing.

and integration of the geographies of the battlefields. It is clear that combination therapy targeting multiple mechanisms would be the only option in the future. Using immunotherapy as an example, tremelimumab (anti-CTLA4) has been tested as a second-line therapy for esophageal cancer. Although the clinical response was not impressive, its biologic effect on T-cell activation seemed to be associated with clinical response<sup>[99]</sup>. Recent development of immunotherapy based on *ERBB2IP* mutation-specific CD4<sup>+</sup> T cells<sup>[100]</sup> and programmed-death ligand 1 (PD-L1) suppression is also quite promising. For patients in which pre-existing immunity is suppressed by PD-L1, blocking PD-L1 enhanced anti-cancer immunity (including one case of esophageal cancer)<sup>[101]</sup>. A realistic option in the near future can be a combination of target drugs and traditional chemoradiotherapy for ESCC. Target drugs are expected to kill cancer cells with specific genomic alterations, while traditional therapy acts in a much broader manner.

Technical issues continue to represent large hurdles for next-generation sequencing and bioinformatics, and they prevent us from gaining full insights into the mechanisms of carcinogenesis and metastasis of ESCC. Nonetheless, whole genome sequencing correlates with incomplete coverage of inherited disease genes, low reproducibility of genetic variation with the highest potential clinical effects, and uncertainty about clinically reportable sequencing findings<sup>[102]</sup>. Whole exome sequencing is particularly prone to errors, as only 61% of the mutated genes in ESCC are transcribed<sup>[8]</sup>. This

is similar to what has been observed in pancreatic cancer: only 63% of the expected 251 driver-gene mutations were identified, suggesting a 37% false-negative rate. Marked discrepancies in the detection of missense mutations in identical cell lines (57.38%) have been reported due to inadequate sequencing of GC-rich areas of the exome<sup>[103]</sup>. The protein-coding genes account for only about 1.5% of the total genome. Although the vast majority of the alterations in noncoding regions are presumably passengers, some of these may be drivers, for example, mutations in the Tert promoter<sup>[104,105]</sup>.

New computational and bioinformatics tools still need to be developed and improved due to low concordance of multiple variant-calling pipelines<sup>[106,107]</sup>. Directly comparing genome sequence reads may improve data quality as compared with initial alignment of reads to a reference genome<sup>[108]</sup>.

Apart from the logistic challenges, financial, social and ethical challenges are also posed by personalized and targeted therapy<sup>[39]</sup>. In addition to viewing a patient's cancer as a biologic phenomenon waiting for medical attention alone, personalized therapy emphasizes biopsychosocial care by including communication and information giving, psychologic and emotional well-being, enhancement of function, addressing financial and spiritual concerns, and providing symptom control and social support<sup>[109]</sup>. If we look at one specific patient's ESCC from all these perspectives, a tumor board should involve not only medical staff but also supporting staff (Figure 1).

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## Rectal cancer: An evidence-based update for primary care providers

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### Abstract

Rectal adenocarcinoma is an important cause of cancer-related deaths worldwide, and key anatomic

differences between the rectum and the colon have significant implications for management of rectal cancer. Many advances have been made in the diagnosis and management of rectal cancer. These include clinical staging with imaging studies such as endorectal ultrasound and pelvic magnetic resonance imaging, operative approaches such as transanal endoscopic microsurgery and laparoscopic and robotic assisted proctectomy, as well as refined neoadjuvant and adjuvant therapies. For stage II and III rectal cancers, combined chemoradiotherapy offers the lowest rates of local and distant relapse, and is delivered neoadjuvantly to improve tolerability and optimize surgical outcomes, particularly when sphincter-sparing surgery is an endpoint. The goal in rectal cancer treatment is to optimize disease-free and overall survival while minimizing the risk of local recurrence and toxicity from both radiation and systemic therapy. Optimal patient outcomes depend on multidisciplinary involvement for tailored therapy. The successful management of rectal cancer requires a multidisciplinary approach, with the involvement of enterostomal nurses, gastroenterologists, medical and radiation oncologists, radiologists, pathologists and surgeons. The identification of patients who are candidates for combined modality treatment is particularly useful to optimize outcomes. This article provides an overview of the diagnosis, staging and multimodal therapy of patients with rectal cancer for primary care providers.

**Key words:** Rectal cancer; Diagnosis; Treatment; Review; Primary care

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**Core tip:** Colorectal cancer is the third most common malignant neoplasm and second most common cause of cancer-death in the United States. It is essential for primary care providers to become familiar with the modifications and updates in the diagnosis and treatment of this common malignancy. This review focuses on the



advances made in the multidisciplinary approach to rectal cancer as well as minimally invasive surgical options as part of the management of rectal tumors.

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## INTRODUCTION

Colorectal cancer is the third most common non-cutaneous malignancy in the United States and the second most frequent cause of cancer-related deaths. In 2015, an estimated 132700 cases of colorectal cancer will be diagnosed in the United States and will account for 49700 deaths<sup>[1]</sup>. Of these cancers, 30 percent will arise in the rectum. The diagnosis, staging and treatment regimens for rectal cancer differ significantly from those for colon cancer and have undergone recent advances that are important for primary care providers, gastroenterologists and general surgeons to be aware of.

The work-up and management of rectal cancer requires detailed knowledge regarding its precise location. A National Cancer Institute consensus panel recommended that the rectum be defined as 12 cm or less from the anal verge using rigid proctoscopy (Figure 1)<sup>[2,3]</sup>. Anatomic considerations that distinguish rectal cancers from those that occur in the colon include the narrow and bony confines of the pelvis making surgical resection more difficult and the absence of serosa below the peritoneal reflection which facilitates deeper tumor growth in the perirectal fat, that may contribute to higher rates of locoregional failure<sup>[4]</sup>.

The mainstay of treatment for patients with rectal cancer has been curative surgical resection. Significant improvements in local control and survival have been seen with the implementation of total mesorectal excision (TME) and the addition of neoadjuvant chemoradiotherapy (CRT)<sup>[5-9]</sup>. Increased use of colonoscopic screening has thought to contribute to disease detection at an earlier stage, which may contribute to improved outcomes as well.

The aim of this review is to provide an evidence-based overview of the diagnosis, staging and multidisciplinary treatment of primary rectal cancer for primary care providers.

## DIAGNOSIS

### History and physical examination

Many symptoms associated with colorectal cancer have been described, with the main ones being rectal bleeding, diarrhea, and constipation (commonly named "change

in bowel habits"), as well as weight loss, abdominal pain, and anemia<sup>[10]</sup>. However, these symptoms are also common with benign conditions, therefore, clinicians must select patients at higher risk of colorectal cancer for further investigation. These risk factors include age  $\geq$  50 years, personal or family history of colorectal polyps and cancer, and history of inflammatory bowel disease. There is no reliable clinical information or test that has sufficient discrimination to provide the basis for referral decisions. Although primary care investigations such as fecal occult blood testing and estimation of hemoglobin are used to filter selected patients, symptomatic patients with risk factors for colorectal cancer should undergo a full colonoscopy.

Astin *et al.*<sup>[11]</sup> performed a systematic review to identify the risk of colorectal cancer in patients reporting symptoms to primary care. Positive predictive values for rectal bleeding from 13 papers ranged from 2.2% to 16%, with a pooled estimate of 8.1% in those aged  $\geq$  50 years. The authors recommended further investigation of rectal bleeding or anemia in primary care patient's  $\geq$  50 years.

Perhaps the most basic and informative test in patients with low rectal cancer is a digital examination (DRE). Important information can still be obtained from DRE, including the condition of the anal sphincters, distance from the anal verge with low-lying tumors, tumor fixation, and circumferential involvement. Nevertheless, DRE is not an adequate screening tool and even when rectal cancer is diagnosed, the associated findings do not correlate with the degree of tumor invasion.

Signs and symptoms associated with rectal cancer are non-specific but can guide primary care physicians in their referral decisions. Patient age, underlying inflammatory bowel disease and family history of colorectal cancer or polyps should influence this decision-making. Another important detail with patients that have significant family history is to consider referral to genetic counseling for appropriate risk assessment and timely notification of family members at risk.

### Endoscopic evaluation

When patients warrant endoscopic evaluation of the colon and rectum, a full colonoscopy is preferred to rule out the presence of synchronous polyps and cancers in the rest of the colon. Synchronous polyps or cancers may be present in 4% to 15% of patients<sup>[12]</sup>. Rigid proctoscopy will be performed in the surgeons' office to accurately measure the distance from the anal verge and to characterize the lesion. Unlike colon cancer where the tattooing is performed liberally and sometimes even circumferentially for easy intraoperative visualization, rectal cancer should be tattooed right at the lesion with one single injection for accurate endoscopic visualization and for future visualization when neoadjuvant therapy is to be given.

Approximately 10% of polypectomy specimens

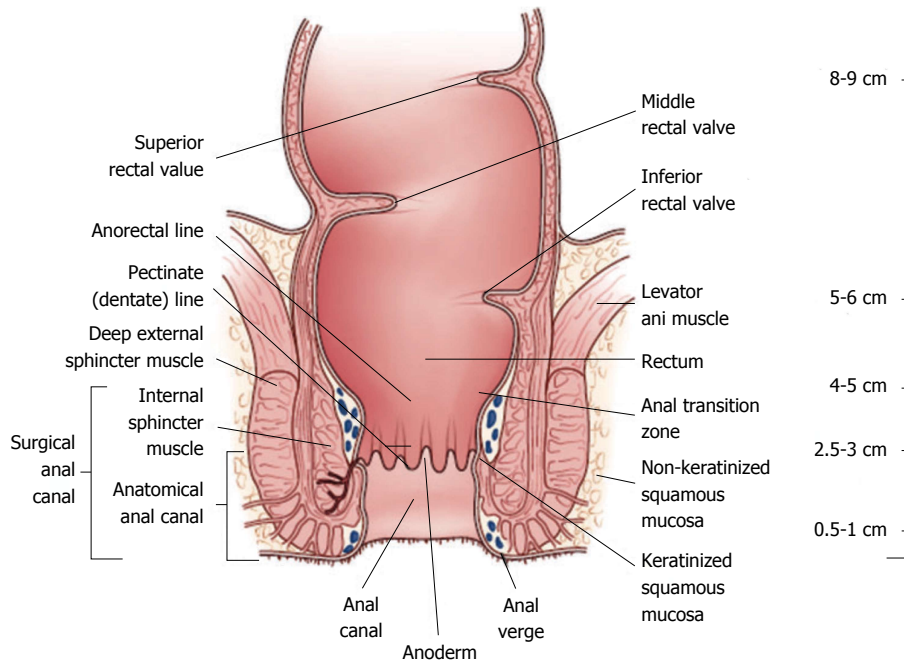


Figure 1 Rectal anatomy and landmarks of importance in the treatment of rectal cancer (Figure reproduced with permission from Apgar *et al*<sup>[3]</sup>).

**Table 1 Strengths of preoperative imaging studies for rectal cancer**

	CRM	T stage	N stage	EMVI	Peritoneum
ERUS	NA	+++	++	NA	NA
CT	+	++	-	+	+
MRI	+++	+++	++++	+++	++
PET/CT	NA	NA	+	NA	NA

ERUS: Endorectal ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography; EMVI: Extramural vascular invasion; NA: Not applicable.

harbor early colorectal carcinomas<sup>[13]</sup>. Endoscopic polypectomy alone is likely an adequate treatment for benign-appearing polyps  $\leq 2$  cm<sup>[14]</sup>. Patients with rectal polyps with malignant features (fixed, indurated, ulcerated), polyps  $> 2$  cm, flat or serrated adenomas, and polyps that are unable to be completely excised endoscopically should be referred to a surgeon for excision. Patients with submucosal lesions, polyps close to the anal canal, and polypectomy specimens harboring invasive carcinoma or dysplasia should also be sent for surgical consultation. A more emergent situation is when a patient is diagnosed with dysplasia or invasive malignancy in a polypectomy specimen. These patients should be assessed immediately by a surgeon to visualize and tattoo the polypectomy site before healing occurs.

### Histopathology

Rectal lesions that harbor invasive carcinoma, high-grade dysplasia or intramucosal carcinoma should be evaluated by a surgeon. Microscopic features associated with lymph node metastases, local recurrence

and poor prognosis include poorly differentiated, mucinous and signet ring histology; lymphovascular and perineural invasion, and tumor budding. Tumor budding is defined as the presence of individual cells and small clusters of tumor cells at the invasive front of carcinomas. Ulcerated, mucinous cancers and lesions with evidence of perineural and lymphovascular invasion are associated with local recurrence rates as high as 25%<sup>[15,16]</sup>. Chemoradiotherapy regimens are less effective in tumors with undifferentiated histology and lymphovascular invasion<sup>[17]</sup>.

### Radiologic evaluation

The preoperative staging assessment of rectal carcinoma has significant implications in terms of treatment. Patients with rectal carcinomas that have not breached the muscularis propria layer of the rectal wall may be adequately treated by resection alone. On the other hand, patients who present with transmural invasion or those who have lymph node metastases benefit from neoadjuvant CRT followed by resection. Imaging studies utilized to evaluate patients with rectal tumors include computed tomography (CT), magnetic resonance imaging (MRI) and endorectal ultrasound (ERUS) (Table 1). MRI and ERUS are especially useful to detect tumor invasion outside the rectal wall and predict the relationship of the tumor with the circumferential margins<sup>[18]</sup>. With the more recent addition of diffusion-weighted imaging, MRI has also emerged as a reliable indicator for assessing early response following neoadjuvant CRT.

ERUS is performed by a colorectal surgeon or gastroenterologist in an outpatient setting, requires minimal intestinal preparation, and results are highly operator dependant. MRI also requires minimal bowel

preparation, does not suffer from operator variability, and patients are not exposed to radiation. When ordering an MRI to work-up a patient with rectal cancer, one must assure that a "rectal cancer protocol" is being performed as this imaging technique differs from regular pelvic MRI's. MRI also has an increased cost when compared to ERUS.

Agreement between phase-array MRI and histopathology in predicting tumor stage has been established by a number of studies, including a prospective study by Brown *et al.*<sup>[19]</sup> that showed a 94% agreement between MRI and pathologic assessment of T stage. The multicenter MERCURY study directly compared the extramural depth of invasion measured by MRI and histopathology in 295 of 311 patients<sup>[20]</sup>. The mean difference between MRI and histopathology was 0.046 mm, thereby showing MRI to be equivalent to a histopathology assessment of depth to within 0.5 mm in terms of predicting depth of extramural tumor spread.

In a meta-analysis including data from 90 publications, Bipat *et al.*<sup>[21]</sup> found the sensitivity of ERUS and MRI for tumor invasion outside the rectal wall as high as 90% and 82%, respectively. However, the sensitivity for lymph node involvement was significantly lower at 67% and 66%, respectively. In a systematic review of 53 studies including 2915 patients<sup>[22]</sup>, the accuracy of ERUS was 87% for T-stage and 74% for lymph node involvement. For MRI, the corresponding numbers were 84% and 82%. Recent data has shown that 3-D reconstruction increases the accuracy of ERUS in assessing the depth of rectal wall and submucosal invasion and may help in selecting patients for radical resection<sup>[23]</sup>.

The addition of diffusion-weighted imaging [(DWI) a form of MRI based upon measuring the random Brownian motion of water molecules within a voxel of tissue] and dynamic contrast-enhanced imaging [(DCE) an MRI method that uses a contrast agent that enables the analysis of blood vessels], has recently shown to improve the accuracy in the local assessment of patients with rectal cancer, as well as the evaluation of treatment response after neoadjuvant therapy. Rao *et al.*<sup>[24]</sup> showed that addition of DWI to T2-weighted imaging improved accuracy of rectal cancer detection. Ichikawa and colleagues studied DWI in 33 colorectal cancer patients (14 with rectal cancer) and reported 91% sensitivity and 100% specificity<sup>[25]</sup>. DWI has also been utilized for detection of metastatic lymph nodes in rectal cancer. Ono *et al.*<sup>[26]</sup> reported 80% sensitivity, 77% specificity, and 78% accuracy in a series of 27 colorectal cancer patients (10 with rectal cancer). A recent study evaluating 129 patients showed 93% sensitivity, 81% specificity, and 87% accuracy in metastatic lymph node detection with combination of DWI and conventional MRI when compared with histopathologic examination after proctectomy with TME<sup>[27]</sup>. Diffusion-weighted imaging MRI has also been used to predict

pathologic response after chemoradiation. Engin *et al.*<sup>[28]</sup> showed that increase in apparent diffusion coefficient can predict therapy response. In many centers, DWI is now being used with T3 MRI protocols as an adjunctive to T2-weighted images. Dynamic contrast-enhanced MRI has been used in rectal cancer patients both for predicting response to therapy and for evaluation after neoadjuvant treatment. Kremser *et al.*<sup>[29]</sup> applied dynamic T1 mapping as a predictor of post-chemoradiotherapy tumor-response. Gollub *et al.*<sup>[30]</sup> showed that DCE MRI is reliable in predicting pathological complete response after chemotherapy. MRI with the use of a unique contrast agent, ultra-small superparamagnetic iron oxide (USPIO) which undergoes phagocytosis by macrophages in normal lymph nodes, is a promising technique to help detect lymph node metastasis. T2 images are obtained 24 h after USPIO injection and reduced signal is accepted as normal whereas loss of signal indicates involvement of the lymph node. Koh *et al.*<sup>[31]</sup> studied this technique in 25 patients with rectal cancer and reported improved accuracy with 65% sensitivity and 93% specificity. The use of USPIO has not been studied in a large comparative study nor has it been approved by the Food and Drug Administration for clinical use in the US.

CT scanning offers the opportunity in a single examination to stage rectal cancer both locally and distantly. It is readily available and relatively inexpensive and not prone to operator variability. When examining advanced rectal cancer, CT determines T stage with an accuracy of 79% to 94%; however, this falls to 52% to 74% when smaller tumors are evaluated<sup>[18]</sup>. The assessment of lymph node involvement with CT has a very poor sensitivity, which ranges from 22% to 73%<sup>[32]</sup>.

Overall, there is currently limited evidence in regards to the specificity and sensitivity of fluorodeoxyglucosepositron emission tomography (FDG-PET) in the initial staging of rectal cancer. PET/CT may be useful in detecting occult synchronous tumors or metastases at the time of initial presentation. However, this low-yield detection rate cannot justify the costs and radiation exposure for its routine use.

### Staging classification

Pathologic stage represents the most important prognostic factor for patients who have rectal cancer. The tumor-node-metastasis (TNM) system, as defined by the American Joint Committee on Cancer (AJCC), is the most commonly used staging system and is based on depth of local invasion, extent of regional lymph node involvement, and presence of distant sites of disease (Table 2)<sup>[33]</sup>. As the AJCC stage increases from stage I to stage IV, 5-year overall survival declines from greater than 90% to less than 10%<sup>[34,35]</sup>.

The American Society of Clinical Oncology guidelines suggest a preoperative baseline carcinoembryonic antigen (CEA) level<sup>[36]</sup>. If increased preoperatively, the CEA level should return to normal range postoperatively.

**Table 2 Tumor-node-metastasis staging system for rectal cancer (reproduced with permission from Greene *et al.*<sup>[33]</sup>)**

Primary tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the pericorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum
T4b	Tumor directly invades or is adherent to other organs or structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional node metastasis
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site (e.g., liver, lung, ovary, nonregional node)
M1b	Metastasis in more than one organ/site or the peritoneum

Serum levels of  $\geq 5.0$  ng/mL have an adverse impact on survival that is independent of tumor stage<sup>[36-39]</sup>. Elevated CEA levels that do not normalize following resection implies persistent disease and the need for further evaluation<sup>[40]</sup>. A CT scan of the chest, abdomen and pelvis to identify pulmonary and hepatic metastases should be performed. A chest CT is recommended because of the higher incidence of pulmonary metastases in rectal cancer patients compared to colon cancer patients<sup>[41]</sup>.

## TREATMENT

The goals for treating rectal cancer have broadened to include securing local and distant oncologic control; minimizing treatment-related morbidity and mortality; performing restorative anastomosis to achieve near normal continence and defecation; preserving genitourinary functions; and promoting rapid recovery after resection with prompt return to normal activities.

### Neoadjuvant therapy

Over the past two decades, neoadjuvant radiotherapy with or without sensitizing chemotherapy has been increasingly used together with surgical resection in the primary management of patients with rectal cancer. The rationale for radiotherapy is based on the finding that radiation inhibits cell proliferation, induces apoptotic cell death, and inhibits tumor growth<sup>[42]</sup>. The

rationale for giving chemotherapy with radiotherapy is that it potentiates local radiotherapy sensitization and has the potential to induce tumor downsizing, possibly improving rates of sphincter preservation and increase rates of pathological complete response (pCR)<sup>[43]</sup>.

In the US, neoadjuvant CRT is currently indicated for T3 and T4 rectal adenocarcinoma; and node positive tumors regardless of the T stage. There are several potential benefits to administering radiotherapy preoperatively, including decreased tumor seeding at operation, less acute toxicity, and increased likelihood of patient completion of the full treatment course<sup>[6,44,45]</sup>. One must have in mind that a major disadvantage is the potential for overtreatment of patients.

In the neoadjuvant setting, there are two possible approaches: short term radiation with 25Gy given in daily fractions of 5Gy and surgery the following week, or long term radiation treatment with chemotherapy in daily fractions of 1.8Gy five days per week, 50.4Gy in total, followed by surgery 6 to 8 wk later<sup>[46]</sup>. The latter treatment option, which has been defined as the standard of care for patients with locally advanced rectal cancer in the US, has the advantage of down staging of the tumor, particularly in advanced low rectal cancers<sup>[47]</sup>. The "long course" typically involves the administration of concurrent 5-FU-based chemotherapy<sup>[6,48,49]</sup>. The rationale for short-course radiotherapy is that the short time period for delivery of the dose may combat the effects of accelerated cellular repopulation, a phenomenon displayed by malignant cells exposed to radiotherapy. Short-course radiotherapy, which is widely used in Europe, does not result in apparent downsizing of tumors or downstaging in terms of nodal status, and has been associated with increased postoperative morbidity compared to "long course" radiotherapy<sup>[8]</sup>. Evidence from recent studies suggests that eliminating neoadjuvant radiotherapy may be feasible in selective patients with locally advanced rectal cancer. Specifically in patients with proximal rectal and chemosensitive tumors that may benefit from earlier and more intense systemic treatment. This regimen has the potential for reducing distant recurrence rates and avoiding the toxicity of pelvic radiotherapy, and is currently being studied in a randomized controlled trial<sup>[50]</sup>.

Several randomized control trials have investigated the value of both radiotherapy and chemotherapy in the management of rectal cancer. In this section we will summarize these results.

### Results of trials evaluating CRT and the impact of chemotherapy

Preoperative CRT is associated with a relative risk reduction in local recurrence of approximately 50% in patients with T3 and T4 rectal cancer compared with postoperative CRT. There is no significant difference in the overall rate of sphincter preservation or overall survival. In patients with T3 and T4 rectal cancer, the administration of chemotherapy in addition to



**Table 3 Vocabulary for the treatment of rectal cancer**

Anterior resection	Resection of rectum with an anastomosis above the pelvic peritoneal reflection
Low anterior resection	Resection of rectum with an anastomosis below the pelvic peritoneal reflection
TME	Total mesorectal resection. The adipose tissue at the posterior and lateral aspects of the rectum which contains the draining lymph nodes, is dissected down to the pelvic floor and resected
PME	Partial mesorectal excision. The mesorectum is divided 5 cm below the cancer as well as the distal rectum. PME is performed for cancers located in the upper rectum and rectosigmoid junction
TEM	Transanal endoscopic microsurgery. A specially designed proctoscope with an attached microscope permits local resection of premalignant lesions and selected cases of early rectal cancer up to 20 cm from the anal verge
TAE	Transanal excision. Lesions in the lower third of rectum can be resected transanally
APR	Abdominoperineal resection. Low rectal cancers that cannot be resected with a sphincter-saving procedure are resected with perianal tissue and the anal canal <i>en bloc</i> with the whole rectum and mesorectum
Adjuvant	Additional treatment (chemotherapy, radiation therapy or chemoradiation) given after surgical resection
Neoadjuvant CRT	Preoperative treatment Chemoradiotherapy. Chemotherapy drugs typically involve 5-fluorouracil, leucovorin and oxaliplatin. These are given in order to increase cancer cells sensitivity to the radiation. CRT is frequently offered to patients preoperatively (neoadjuvant) in order to reduce local recurrence but has not shown to improve overall survival
Intersphincteric resection	The internal anal sphincter muscle is resected in continuity with the lower rectum preserving the external anal sphincter in order to preserve anal function and avoid colostomy in cases of ultralow rectal cancer
CRM	Circumferential resection margin is the distance in mm from the mesorectal fascia (the resection plane) to the nearest tumor growth
DRM	Distal resection margin

TME: Total mesorectal excision; CRT: Chemoradiotherapy; TAE: Transanal excision; TEM: Transanal endoscopic microsurgery.

preoperative long-course radiotherapy, regardless of the timing of administration (preoperative or postoperative), is associated with a relative risk reduction in local recurrence of approximately 50% compared with patients who received long-course preoperative radiotherapy alone. This significant difference in local recurrence has not translated into a significant difference in overall survival<sup>[6,8,45,48,49]</sup>.

### Results of trials evaluating short-course radiotherapy

In patients with stage II and III rectal cancer, short-course radiotherapy with TME is associated with a relative risk reduction in local recurrence of 62% compared with patients who receive no neoadjuvant therapy. Patients who receive preoperative short-

course radiotherapy have increased postoperative morbidity, mainly wound complications and bowel dysfunction<sup>[7,8,51-55]</sup>.

### Results of trials evaluating CRT versus short-course radiotherapy

In patients with T3 and T4 rectal cancer, there is no significant difference in the rate of sphincter preservation or in local recurrence between patients assigned to preoperative CRT compared to preoperative short-course radiotherapy<sup>[56]</sup>.

The provision of chemotherapy and/or radiotherapy undoubtedly offers an oncological benefit in appropriately selected patients. The problem is that, in some instances, this benefit is at the cost of a 50% increase in some toxicity. Currently, there is no international consensus with regards to the indications for neoadjuvant CRT. International treatment guidelines are yet to be developed.

### Results of trials evaluating neoadjuvant chemotherapy alone

Several small, single-arm phase II trials have evaluated the outcomes of patients who receive neoadjuvant chemotherapy alone followed by TME and have reported down staging in 25%-58%, with 74%-84% disease-free survival and 85%-91% overall survival at between 4-5 years follow-up. Local recurrence rates at variable time intervals ranging from 48 to 75 mo have been reported in 0%-11.5% of patients. Postoperative complications have also been reported in up to 43% of patients in these small studies<sup>[57-61]</sup>.

### Operative treatment

Currently, radical resection with TME remains the standard curative operation for rectal cancer. Patients with tumors located at the upper or mid rectum will frequently undergo an anterior or low anterior resection (LAR), whereas many patients with a distal tumor will require abdominoperineal resection (APR) of the rectum with a permanent colostomy (Table 3). Whether to perform local excision (LE), a restorative procedure with anterior resection (AR), or APR with permanent colostomy remains a complex assessment that must take into account oncologic and technical considerations, patient preference, functional outcome, and surgeon experience. The level of the lesion and its relationship to the anal sphincters and pelvic floor is a primary consideration from a technical and oncologic standpoint. Additional factors include initial staging, response to neoadjuvant therapy, tumor histology, and margin status. Patient factors, particularly a narrow pelvis and obesity, can add significant technical difficulty. Other factors include gender, age, anal sphincter function, and patient's ability to manage a colostomy. Baseline bowel function, including incontinence, as well as sexual and urinary functions should be documented before any treatment modality.

**Table 4 Morphologic features of favorable and unfavorable T1 rectal cancers**

Favorable/low risk	Unfavorable/high risk
Well differentiated (G1-G2)	Poorly differentiated (G3)
SM 1	SM 2-3
Size < 3 cm	Size > 3 cm
< 40% wall circumferences	> 40% wall circumferences
No lymphovascular invasion	Lymphovascular invasion
No tumor budding	Tumor budding
No perineural invasion	Perineural invasion
No lymphocytic infiltration	Lymphocytic infiltration

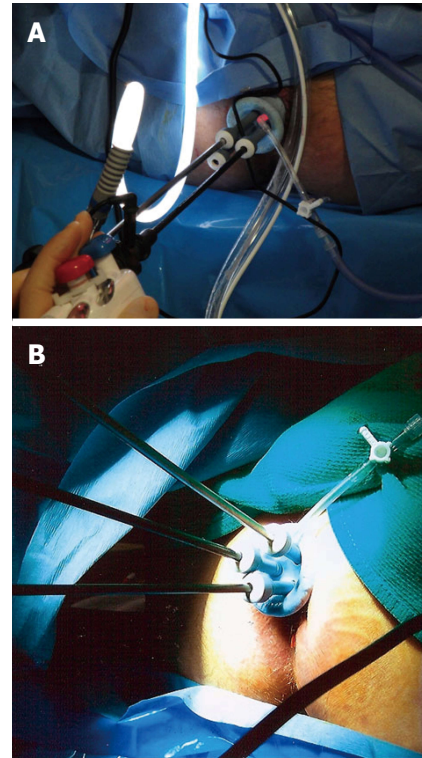
SM: Submucosal invasion.

### Local excision

Early rectal cancer is relatively uncommon in Western populations. The incidence of malignant colorectal polyps as a proportion of all adenomas removed varies between 2.6% and 9.7%<sup>[62]</sup>, with 3% to 8.6% of all resected colorectal adenocarcinomas staged as T1<sup>[63-66]</sup>. The role of LE for treatment of rectal cancer is highly controversial. While radical resection with TME continues to be the standard operation for most patients with rectal cancer, LE is an acceptable alternative with significantly less morbidity. Most surgeons restrict their curative intent use to selected patients with T1 disease (Table 4) or to those patients unfit for radical resection.

Surgeons continue to evolve techniques for LE. The most common technique for LE is transanal excision (TAE), which involves the excision of the rectal tumor with the assistance of an operating anoscope. This technique is exclusive for low-lying tumors and suffers from poor visualization. Transanal endoscopic microsurgery (TEM) is a modification of LE that combines the excellent visualization offered by a binocular stereoscope that is incorporated into an operating proctoscope, which permits an optimum view during the procedure thus enhancing the surgeon's ability to accurately perform full thickness excisions and to repair the rectal wall defect. TEM allows for improved endoanal access to the mid and upper rectum thus increasing the utility of LE. Disadvantages of TEM include costly equipment and slightly longer operating times. Atallah *et al*<sup>[67]</sup> recently reported their experience with using a single-incision laparoscopic surgery port for access to the rectum, replacing the conventional operative proctoscope, and using ordinary laparoscopic instruments (Figure 2). This approach is widely known as transanal minimally invasive surgery (TAMIS) and has been reported to be a safe and feasible alternative to TEM, providing its benefits at a fraction of the cost<sup>[68,69]</sup>.

One significant disadvantage of LE, including TEM, is the lack of information regarding the lymph node status of the mesorectum. Endoscopic posterior mesorectal resection (EPMR) is a recently described technique<sup>[70]</sup> that includes TAE or TEM of selected, favorable T1 rectal cancers followed by a minimally



**Figure 2** Operative setup for transanal minimally invasive surgery (Figure reproduced with permission from Atallah *et al*<sup>[67]</sup>).

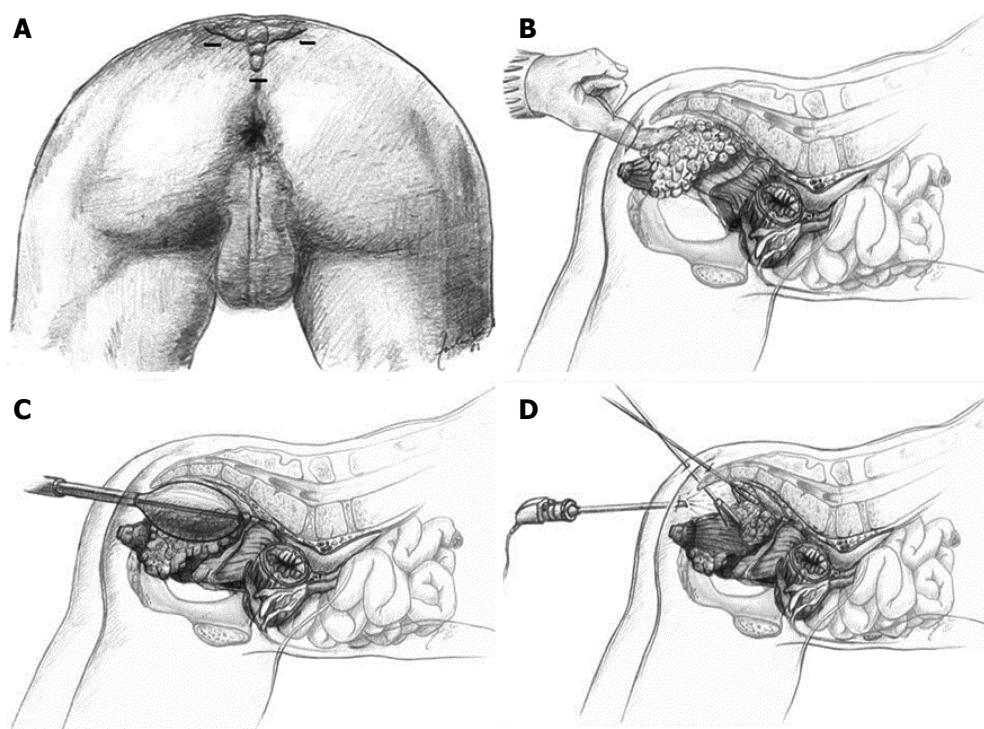
invasive transperineal resection of the posterior part of the mesorectum including all relevant lymphatic tissue (Figure 3). Its proponents claim this technique provides complete tumor staging with minimal morbidity after LE of T1 rectal cancers<sup>[71]</sup>.

Despite its decreased morbidity and mortality, LE has been associated with significantly higher local recurrence rates (12.5% vs 6.9% for T1 cancers and 22.1% vs 15.1% for T2 cancers)<sup>[72]</sup>. Compared to TAE, TEM and TAMIS offer a higher likelihood of achieving clear resection margins, lower recurrence rates and the ability to successfully excise more proximal tumors. Local recurrence after TEM and TAMIS has been reported mainly in single institution reviews which makes comparisons difficult. Recurrence rates range from 0% to 13% for patients with T1 tumors and from 0% to 80% for patients with T2 tumors<sup>[73-78]</sup>.

Significant disease progression can occur after any type of LE despite intense surveillance<sup>[79,80]</sup>, which may preclude curative salvage. The role of CRT and LE techniques in the treatment of rectal cancer is still under study.

### Radical resection

The determining factor in performing a sphincter-preserving operation is the ability to obtain adequate distal margin. For mid to low tumors or patients with difficult anatomy, the decision of whether to perform a sphincter preserving operation or not is generally only possible in the operating room when the rectum is completely mobilized. When performed



**Figure 3** Technique of endoscopic posterior mesorectal resection. A: Trocar positions; B: Access to the retrorectal space using the index finger; C: Establishment of a sufficient large operating space using a dissecting balloon trocar; D: Dissection of the mesorectum from the posterior wall of the rectum. Figure reproduced with permission from Zerz *et al*<sup>[70]</sup>.

for curative intent, both AR and APR involve TME to achieve adequate circumferential margin clearance. TME involves excision of the mesorectum following the anatomic planes of the pelvis. Dissection is performed sharply with the identification and preservation of the autonomic nervous system of the pelvis. TME has been repeatedly associated with a reduction in the local recurrence rate from 30%-40% to 5%-15% with the suggestion that surgical technique is a key factor<sup>[81,82]</sup>. TME has not shown significant differences in 30-d mortality, anastomotic leakage or overall operative morbidity when compared to pre TME-era controls with or without neoadjuvant therapy<sup>[83-85]</sup>.

### Minimally invasive techniques

Large comparative studies and multiple prospective randomized control trials have reported equivalence in short and long-term outcomes between open and laparoscopic resections for colon cancer<sup>[86-91]</sup> but laparoscopic AR with TME has not been well studied and whether it compromises long-term oncologic outcomes has not been refuted by the available literature. Laparoscopic rectal dissection is technically more demanding and may result in difficulties assessing and achieving negative surgical margins but does provide a clear and magnified view of the pelvis that helps with the sharp dissection for TME and assist in identification of vital pelvic structures including the ureters and autonomic nerves. Current data suggests that laparoscopic rectal cancer resection

may benefit patients with reduced blood loss, earlier return of bowel function, and shorter hospital length of stay<sup>[92,93]</sup>. There are two large, multicenter randomized controlled trials that are currently being conducted: the COLOR II trial in Europe and the ACOSOG-Z6051 trial in the US<sup>[94]</sup>. Both of these studies are comparing the laparoscopic and open approach for treatment of resectable rectal cancer.

In recent years, an increasing number of reports have been published on robotic colorectal surgery. Most of the interest has been in robotic TME for rectal cancer. Robotic-assisted laparoscopy can ease some of the limitations of conventional laparoscopy with improved visualization and maximal maneuverability provided by 360-degree reticulating arms. In colorectal surgery, robotic techniques are associated with longer operative times and higher costs compared with laparoscopic surgery<sup>[95]</sup>. Although operative morbidity and short-term oncologic outcomes are comparable to the laparoscopic approach, long-term outcomes remain unknown. Large prospective randomized clinical trials such as the international ROLARR trial are required to establish the benefits of robotic rectal surgery. The role of robotics in colorectal surgery is still largely undefined.

### Adjuvant therapy

The long term follow up of the European Organization for Research and Treatment of Cancer trial 22921 that compared adjuvant 5-FU-based chemotherapy



to no adjuvant treatment in patients with resectable T3-4 rectal cancer, reported no beneficial effects of adjuvant chemotherapy if the cancer did not respond to preoperative radiation or CRT<sup>[96]</sup>. The role of intraoperative and postoperative radiation has not been well studied and is limited to inadvertent intraoperative tumor perforations or involved resection margins if irradiation treatment was not given preoperatively<sup>[97-100]</sup>.

### Nonoperative treatment

Many studies have shown that neoadjuvant CRT is associated with significant pathological downstaging of rectal cancers, with up to 20% of patients having pCR<sup>[101-104]</sup>. Despite an apparent complete luminal and mural tumor response, up to 17% of tumors with histologically confirmed pCR harbor disease in the mesorectal lymph nodes<sup>[105]</sup>. Similarly, approximately 8% of patients with an apparent incomplete clinical response have pCR<sup>[106]</sup>. The challenge remains to identify those patients with a clinical complete response who have a true pCR.

Habr-Gama and colleagues from Sao Paulo, Brazil have pioneered the “watch and wait” approach where they enroll patients with pCR into a strict surveillance protocol without subsequent operative treatment. In their most updated experience including 67 patients with pCR, overall survival and disease-free survival rates at 5 years were 96% and 72%, respectively<sup>[107]</sup>. After a mean follow-up of 65 mo, recurrences were observed in 15 patients (21%). All endorectal recurrences were amenable to salvage therapy.

Although the results of Dr. Habr-Gama’s non-operative group appear promising, these data should be received with caution because others have shown that greater than 80% of complete clinical responders will recur locally within the first 10 mo of observation<sup>[108,109]</sup>. This implies that the nonoperative approach may only be appropriate for a subset of patients who have a durable pCR after neoadjuvant CRT. It is likely that combined radiological, biochemical and molecular biological markers will be required to accurately predict pCR as well as nodal status.

## CONCLUSION

Improved imaging techniques for staging, precise histopathologic assessments and feedback, and multidisciplinary treatment strategies have led to a greater understanding of the natural history of rectal cancer and improved outcomes. With accurate preoperative imaging techniques for staging, such as ERUS and MRI, patient selection for neoadjuvant CRT is constantly improving. Neoadjuvant CRT has been well studied and has been associated with significantly decreased local recurrence but no significant improvement in overall survival. A “watch and wait” approach in selected patients with pCR

after neoadjuvant CRT has been postulated but long-term results and expanded experiences are pending. Improved operative results with TME and the ongoing experience with laparoscopic and robotic-assisted techniques have also led to improved outcomes with faster recovery. LE with TAE, TEM or TAMIS should be performed selectively on T1 tumors with favorable clinical and histopathologic features. Management of rectal cancer can be complex and is optimized when approached in a coordinated manner by an experienced multidisciplinary cancer treatment team.

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## Gene polymorphisms associated with functional dyspepsia

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### Abstract

Functional dyspepsia (FD) is a constellation of functional upper abdominal complaints with poorly elucidated pathophysiology. However, there is increasing evidence that susceptibility to FD is influenced by hereditary factors. Genetic association studies in FD have examined genotypes related to gastrointestinal motility or sensation, as well as those related to inflammation or immune response. G-protein b3 subunit gene polymorphisms were first reported as being associated with FD. Thereafter, several gene polymorphisms including serotonin transporter promoter, interleukin-17F, migration inhibitory factor, cholecystocytine-1 intron 1, cyclooxygenase-1, catechol-o-methyltransferase, transient receptor potential vanilloid 1 receptor, regulated upon activation normal T cell expressed and secreted, p22PHOX, Toll like receptor 2, SCN10A, CD14 and adrenoceptors have been investigated in relation to FD; however, the results are contradictory. Several limitations underscore the value of current studies. Among others, inconsistencies in the definitions of FD and controls, subject composition differences regarding FD subtypes, inadequate samples, geographical and ethnical differences, as well as unadjusted environmental factors. Further well-designed studies are necessary to determine how targeted genes polymorphisms, influence the clinical manifestations and potentially the therapeutic response in FD.

**Key words:** Functional dyspepsia; Gene polymorphism; Genetic susceptibility; Pathophysiology

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**Core tip:** Functional dyspepsia is a common disorder with complex pathophysiology. Recent evidence has shown that certain gene polymorphisms might be implicated in its pathogenesis; however, results are inconsistent.

Further studies are required to develop new data that provide novel insights regarding the mechanisms of genetic susceptibility in functional dyspepsia.

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## INTRODUCTION

Functional dyspepsia (FD) is a common clinical condition, up to 25% of the population experience symptoms, characterized by the presence of one or more of the following: epigastric pain or burning, postprandial fullness, early satiation, with no anatomical abnormality (detected by gastroscopy) to explain the symptoms. These symptoms should be present for the last three months with symptom onset at least six months before diagnosis. Functional dyspepsia is classified into postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS); however, these two syndromes may overlap<sup>[1-3]</sup>.

While FD pathophysiology is not yet well elucidated<sup>[4]</sup>, several pathogenetic mechanisms have been proposed, including gastric motility and compliance dysfunctions (antral hypomotility, delayed or rapid gastric emptying, impaired gastric accommodation)<sup>[5-9]</sup>, visceral hypersensitivity<sup>[10-14]</sup>, and psychosocial disorders<sup>[15-18]</sup>. Moreover, evidence from randomized controlled trials suggests that eradication of *Helicobacter pylori* (*H. pylori*) leads to relief of dyspeptic symptoms in some patients. At the same time, studies have failed to confirm a temporal correlation between *H. pylori* infection and FD or the relation between *H. pylori* and FD subgroups<sup>[19-22]</sup>.

There is growing evidence that susceptibility to functional gastrointestinal disorders is influenced by hereditary factors. Genetic association studies in patients with FD have investigated candidate genes associated with G protein functions, inflammation and immune response, gastrointestinal sensation and control of adrenergic, serotonergic and cholecystokinergic functions; however, studies have shown inconsistent results in different populations. The results of currently available studies are summarized in Tables 1 and 2.

The aim of this review is to provide insights and critical appraisal on the existing evidence of certain gene polymorphisms implicated in FD pathogenesis.

## FAMILIAL AGGREGATION

Locke *et al.*<sup>[23]</sup> reported familial aggregation in adults with functional gastrointestinal disorders. Among 643

American subjects, the presence of a first-degree relative with abdominal pain or bowel problems was significantly associated with the diagnosis of either irritable bowel syndrome (OR = 2.3; 95%CI: 1.3-3.9) or dyspepsia (OR = 1.8; 95%CI: 1.05-3.0). Gathaiya *et al.*<sup>[24]</sup> showed that a positive family history of abdominal pain (OR = 4.7, 95%CI: 1.5-14.9; *P* = 0.008) and indigestion (OR = 3.4, 95%CI: 1.0-11.5; *P* = 0.04) were independently associated with FD raising the possibility of a genetic component in the disease, although shared environmental factors need to be considered. On the contrary, one study of 986 twin pairs in the United States showed a genetic contribution to gastro-esophageal reflux disease (GERD) and irritable bowel syndrome (IBS), but not to dyspepsia<sup>[25]</sup>. However, this study was limited by the inclusion of uninvestigated dyspeptics only.

## GENE POLYMORPHISMS AND FUNCTIONAL GASTROINTESTINAL DISORDERS

Evidence suggests that alterations in the brain-gut axis functionality is the main mechanism related to motor disorders, visceral hypersensitivity and autonomic dysfunction<sup>[26,27]</sup>. Several neurotransmitters such as serotonin 5-hydroxytryptamine (5-HT), substance P, VIP and CCK participate in the regulation of this axis. These mechanisms that affect both central and peripheral levels of brain-gut interaction may be influenced by genetic factors and some reports have suggested that genes and substances involved in the brain-gut axis might be the keys to unlock the mystery of IBS pathogenesis<sup>[27,28]</sup>.

Certain gene polymorphisms encoding adrenergic, serotonergic and opioidergic receptors, as well as genes involving proteins with immunomodulatory and/or neuromodulatory features have been investigated in functional disorders<sup>[29,30]</sup>. These studies have highlighted the importance of G-protein beta 3 subunit gene (C825T), polymorphisms in the promoter region of the serotonin reuptake transporter gene, cholecystokinin receptor (CCKAR), and high-producer tumor necrosis factor genotype in IBS<sup>[31]</sup>. Genetic variation in the *NPSR1* gene, which encodes neuropeptide S receptor, affects children's predisposition to recurrent abdominal pain (RAP), and the NPS-NPSR1 system which acts along the gut-brain axis to regulate inflammation, anxiety and nociception is suggested as a modulator of intestinal function that may influence individual risk for the development of functional gastrointestinal disorders<sup>[32]</sup>.

Candidate genotypes for FD have also been tested, leading to inconsistent results in different populations. GABARA6 genotypes have been associated with susceptibility to stress and gastric acid secretion. In a Chinese study, a positive correlation between

**Table 1** Genetic association studies in functional dyspepsia involving gene polymorphisms related to gastrointestinal motility or sensation

Ref.	Study origin	FD subjects/controls, <i>n</i>	Studied gene polymorphism	Disease association
Holtmann <i>et al</i> <sup>[39]</sup>	United States	STUDY A: 67/259 STUDY B: 56/112	GNB3 C825T (CC)	FD
Camilleri <i>et al</i> <sup>[40]</sup>	United States	41/47	GNB3 C825T (CC) or (TT)	Meal unrelated dyspepsia
van Lelyveld <i>et al</i> <sup>[42]</sup>	The Netherlands	112/336	GNB3 C825T 825T	FD
Tahara <i>et al</i> <sup>[41]</sup>	Japan	89/94	GNB3 C825T 825TT	FD in <i>H. pylori</i> (-)
Oshima <i>et al</i> <sup>[43]</sup>	Japan	68/761	GNB3 C825T 825TT	EPS
Shimpuku <i>et al</i> <sup>[44]</sup>	Japan	74/64	GNB3 C825T 825CC	PDS with impaired gastric emptying and feeling of hunger in FD
Park <i>et al</i> <sup>[45]</sup>	South Korea	102/148	GNB3 C825T 825CC	FD in children
Hwang <i>et al</i> <sup>[46]</sup>	South Korea	112/269	GNB3 C825T	No association
Camilleri <i>et al</i> <sup>[40]</sup>	United States	41/47	SERT-P(SLC6A4)	No association
van Lelyveld <i>et al</i> <sup>[42]</sup>	The Netherlands	112/336	SERT-P(SLC6A4)	No association
Toyoshima <i>et al</i> <sup>[62]</sup>	Japan	53/646	SLC6A45-HTTLPR allele	PDS
Arisawa <i>et al</i> <sup>[63]</sup>	Japan	223/172	SERT-P(SLC6A4)	No association
Park <i>et al</i> <sup>[45]</sup>	South Korea	102/148	SERT-P(SLC6A4)	No association
Hwang <i>et al</i> <sup>[46]</sup>	South Korea	112/269	SLC6A45-HTTLPR (S/S)	Inverse association with EPS in <i>H. pylori</i> (+)
Arisawa <i>et al</i> <sup>[63]</sup>	Japan	223/172	Pri-microRNA-325	FD
				Interacts with SLC6A4 in increasing susceptibility to FD, especially in <i>H. pylori</i> (-)
Camilleri <i>et al</i> <sup>[40]</sup>	United States	41/47	5-HT1A, 5-HT2A, 5HT2C	No association
van Lelyveld <i>et al</i> <sup>[42]</sup>	The Netherlands	112/336	HTR3A C178T	No association
Mujakovic <i>et al</i> <sup>[69]</sup>		NA	HTR3Ac-42C>T	Severe FD
			HTR3Ac-42T	
Tahara <i>et al</i> <sup>[68]</sup>	Japan	91/93	5HTR2A C 102T	No association
Camilleri <i>et al</i> <sup>[40]</sup>	United States	41/47	a2a, a2c adrenoreceptor	No association
Hwang <i>et al</i> <sup>[46]</sup>	South Korea	112/269	a2a	No association
Camilleri <i>et al</i> <sup>[40]</sup>	United States	41/47	CCK1, CCK promoter	No association
Hwang <i>et al</i> <sup>[46]</sup>	South Korea	112/269	CCK1R intron 779 T>C	No association
Tahara <i>et al</i> <sup>[73]</sup>	Japan	124/119	CCK-1 intron 1 779T	PDS in males
Tahara <i>et al</i> <sup>[107]</sup>	Japan	109/98	TRPV1 G315C 315CC	Inverse association with FD, EPS, PDS, <i>H. pylori</i> (+)
Hwang <i>et al</i> <sup>[46]</sup>	South Korea	112/269	TRPV1 G945C CC	Inverse association with FD, PDS, EPS especially in <i>H. pylori</i> (+)
Arisawa <i>et al</i> <sup>[108]</sup>	Japan	297/345	SCN10A 3218CC	Inverse association with FD
Arisawa <i>et al</i> <sup>[108]</sup>	Japan	297/345	SCN10A 2884A>G	Inverse association with FD, EPS, PDS especially in <i>H. pylori</i> (-)
Arisawa <i>et al</i> <sup>[108]</sup>	Japan	297/345	SCN10A 3275T>5	Inverse association with FD, EPS, PDS especially in <i>H. pylori</i> (-)
Tahara <i>et al</i> <sup>[100]</sup>	Japan	91/94	COMT	FD

FD: Functional dyspepsia; EPS: Epigastric pain syndrome; PDS: Postprandial distress syndrome.

GABARA6 genotypes and functional heartburn provides an insight into the contribution of genetic factors to disease development<sup>[33]</sup>.

#### G-protein b3 subunit gene polymorphism (C825T)

G-proteins are important in stimulus-response coupling of almost 80% of membrane receptors that are linked to intracellular effector systems<sup>[34]</sup>. G-protein B3 (GNB3) subunit gene polymorphism (C825T) is related to alternative G-protein activity and signal transduction. The 825T allele is associated with enhanced G-protein activation, while the C allele

is predictive of diminished G-protein activation<sup>[35,36]</sup>. Studies have shown an association between GNB3 status and depression<sup>[37]</sup>, altered activation of  $\alpha$ 2-adrenoreceptors<sup>[36]</sup> and increased immune cell activation<sup>[38]</sup>. The altered signal transduction related to the CC or TT allele may contribute to the abnormalities in gastroduodenal sensory and motor function observed in FD.

Holtmann *et al*<sup>[39]</sup> suggested, for the first time, a role for the homozygous 825CC GNB3 genotype in dyspepsia in Caucasians. Blood donors with gastrointestinal symptoms and two different clinic-

**Table 2** Genetic association studies in functional dyspepsia involving gene polymorphisms related to inflammation or immune response

Ref.	Study origin	FD subjects/ controls, <i>n</i>	Studied gene polymorphism	Disease association
Tahara <i>et al</i> <sup>[78]</sup>	Japan	108/99	CD 14	No association
Arisawa <i>et al</i> <sup>[82]</sup>	Japan	90/188	MIF G173C	EPS, especially in <i>H. pylori</i> (+)
Arisawa <i>et al</i> <sup>[82]</sup>	Japan	90/188	173C	
Arisawa <i>et al</i> <sup>[82]</sup>	Japan	90/188	IL-17A	No association
Arisawa <i>et al</i> <sup>[82]</sup>	Japan	90/188	IL17F	EPS in <i>H. pylori</i> (+)
			7488T	
Tahara <i>et al</i> <sup>[86]</sup>	Japan	134/112	RANTES promoter C-28G	Reduced risk for PDS, especially in <i>H. pylori</i> (+)
			G carrier	
Tahara <i>et al</i> <sup>[94]</sup>	Japan	111/106	TLR2	Inverse association with FD and PDS in <i>H. pylori</i> (+)
			-196 to -174	
Tahara <i>et al</i> <sup>[94]</sup>	Japan	111/106	MBL2	No association
Tahara <i>et al</i> <sup>[95]</sup>	Japan	89/95	C242T p22PHOX	Inversely related to FD in <i>H. pylori</i> (+)
Arisawa <i>et al</i> <sup>[96]</sup>	Japan	87/185	COX-1	EPS in females
Park <i>et al</i> <sup>[99]</sup>	South Korea	89/180	NOS	FD
			T allele	

FD: Functional dyspepsia; EPS: Epigastric pain syndrome; PDS: Postprandial distress syndrome.

based groups of patients with dyspepsia showed an association between homozygous 825C carrier status and unexplained upper abdominal complaints (OR = 2.2, 95%CI: 1.4-3.3). Furthermore, Camilleri *et al*<sup>[40]</sup> showed that meal-unrelated dyspepsia was associated with the homozygous 825 T or C alleles of GNB3 protein in a United States community study. In this study, DNA was extracted from 41 dyspeptic patients and 47 healthy controls. Homozygous C genotype was identified in 67% of meal-unrelated dyspeptics vs 43% of controls, and homozygous T genotype in 20% of meal-unrelated dyspeptics and 3% of controls. On the other hand, a Japanese study<sup>[41]</sup> with 89 dyspeptic and 94 asymptomatic individuals concluded that homozygous GNB3 825T status was associated with dyspepsia in the absence of *H. pylori* infection (CC vs TT, OR = 5.73, 95%CI: 1.27-25.82; CC vs others, OR = 3.08, 95%CI: 1.02-9.25 after adjustment for sex and age). van Lelyveld *et al*<sup>[42]</sup> reported that T allele carriers of GNB3 C825T polymorphism were associated with dyspepsia (OR = 1.60, 95%CI: 1.03-2.49) in a population study in the Netherlands that enrolled 112 FD patients and 336 sex- and age-matched controls. Furthermore, Oshima *et al*<sup>[43]</sup> showed that the homozygous 825T allele status influences the susceptibility to epigastric pain syndrome-like dyspepsia (OR = 2.00, 95%CI: 1.07-3.76, adjusted for gender and age) in a population of 68 dyspeptics and 761 controls. However, no significant relationship was found between GNB3 polymorphism and PDS-like symptoms. On the contrary, Shimpuku *et al*<sup>[44]</sup> detected a significant relationship ( $P = 0.045$ ) between GNB3 825CC genotype and PDS in 74 FD patients with impaired gastric emptying compared to 64 controls. GNB3 825CC genotype was also significantly associated ( $P = 0.0485$ ) with a feeling of hunger compared with the other GNB3 825 genotypes. Furthermore, Park *et al*<sup>[45]</sup> reported that the CC genotype of GNB3 C825T

may be associated with FD and diarrhea-predominant IBS in Korean children. However, Hwang *et al*<sup>[46]</sup>, found no association between this genotype and FD in another Korean study.

These contrasting observations might be explained by the different genotypic composition of populations in different countries and different racial groups. The frequency of 825TT allele seems to be higher in Japanese subjects than in Caucasians. In addition, the definition of FD or sample selection may also affect the outcome and may at least partially explain the differences detected in the aforementioned two Korean studies<sup>[45,46]</sup>. Moreover, the effect of type II error cannot be excluded in relatively small sample sizes. Conclusively, even though the association between GNB3 C825T polymorphism and FD has been investigated by various studies, its role in FD pathogenesis is not yet clear.

### Genes of the serotonergic system

#### Serotonin transporter protein (SERT or SLC6A4):

Serotonin (5-HT) is a brain neurotransmitter linked to the development of migraine, depression and other neuropsychiatric disorders. 95% of the body's serotonin is found in the gut, synthesized in enterochromaffin cells and this hormone affects motor and sensory functions in the GI tract<sup>[47]</sup> due to seven subclasses of 5-HT receptors, differentiated on the basis of molecular mechanisms, structure and function<sup>[48]</sup>. The action of 5-HT in the gut is terminated by reuptake via the 5-HT transporter (SERT) which is encoded by a single gene on chromosome 17q11 composed of 14 exons<sup>[49]</sup>. There is a 44-bp insertion/deletion in the 5'-flanking promoter region which generates a short and a long allele. The short (S) allele of SERT gene has been implicated with lower transcriptional efficiency and lower reuptake of serotonin than the long allele (L)<sup>[50]</sup>.



Several studies have investigated the relationship between SERT polymorphisms and both behavioral and psychological disorders<sup>[51-55]</sup>, and IBS<sup>[56-61]</sup>. Few studies have investigated the association between the SERT gene and FD. Among these studies, no significant association was detected in studies from the United States<sup>[40]</sup> and the Netherlands<sup>[42]</sup>. On the contrary, in a Japanese study<sup>[62]</sup> that included 53 dyspeptics and 646 controls, the SERT L carriers were at increased risk of PDS (OR = 2.32, 95%CI: 1.23-4.37) compared to the SS genotype, when adjusted for sex and age. However, in another larger Japanese study<sup>[63]</sup>, neither SLC6A4 -185 A>C nor 463 G>T was associated with susceptibility to FD. However, the authors found that the rs5981521 T allele in pri-miR-325, targeting the SLC6A4 3'-untranslated region (UTR) was a risk factor for the development of FD, especially in *H. pylori* negative patients. This allele also interacts with SLC6A4 polymorphisms in increasing susceptibility to dyspepsia in Japan. At the same period, a study from Korea<sup>[45]</sup> showed that polymorphisms of the 5'-flanking controlled SERT gene linked polymorphic region (5HTTLPR) gene were not associated with FD in Korean children. In another Korean study<sup>[46]</sup> that recruited 112 FD patients and 269 controls, the frequency of S/S genotype of SLC6A4 5-HTTLPR polymorphism, was significantly lower than that of L/L + L/S genotype in FD compared to controls ( $P < 0.05$ ). After stratification according to *H. pylori* infection status, the S/S genotype was protective for EPS subtype in *H. pylori*-positive patients compared to controls (adjusted OR 0.46; 95% CI 0.22-0.99;  $P = 0.048$ ).

**5-HT receptor genes:** Of special interest among the seven subclasses of 5-HT receptors<sup>[48]</sup> is 5-HT<sub>3</sub> receptor, as 5-HT<sub>3</sub> receptor antagonism leads to a reduction in dyspeptic symptoms and anxiety relief. It is present in central nervous system and enteric neurons, as well as in the mucosal terminals of extrinsic primary afferents. Five different subunit genes have been identified for this receptor, termed A-E; the 5-HT<sub>3A</sub> subunit being the most important in the receptor formation<sup>[64-67]</sup>.

Very few studies have investigated the association between genes controlling serotonergic functions and FD. Camilleri *et al.*<sup>[40]</sup> found no association for 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, while van Lelyveld *et al.*<sup>[42]</sup> did not reveal any association between HTR3A C178T polymorphism and FD. In addition, Tahara *et al.*<sup>[68]</sup> suggested that 5-HT<sub>2A</sub> receptor T102C polymorphism is unlikely to be associated with susceptibility to dyspeptic symptoms. On the contrary, a study in a Caucasian population of 592 dyspeptics<sup>[69]</sup> showed that HTR3A c.-42T allele carriers of HTR3A c.-42C>T Single Nucleotide Polymorphism were more prevalent in patients with severe dyspepsia (OR = 1.50, 95%CI: 1.06-2.20). This association appeared to be stronger in females (OR = 2.05, 95%CI: 1.25-3.39) and patients

homozygous for the long (L) variant of the 5-HTTLPR genotype (OR = 2.00, 95%CI: 1.01-3.94); females with 5-HTTLPR LL genotype showed the strongest association (OR = 3.50, 95%CI: 1.37-8.90).

### Genotypes altering adrenergic and cholecystokinergic functions

**Adrenergic agents**, mainly  $\alpha_2$  agents, affect motor and sensory function of the human gastrointestinal tract. Three  $\alpha_2$  adrenoceptor (AR) subtypes have been identified: 2A, 2B and 2C. Prejunctional  $\alpha_2A$ - and  $\alpha_2C$ -adrenoreceptors contribute to a negative feedback regulation of norepinephrine release from sympathetic nerves. Genetic disorders of  $\alpha_2$  mechanisms can influence functions such as intestinal motility and pain sensation, as well as anxiety and somatization<sup>[70,71]</sup>.

Cholecystokinin (CCK) is a hormone secreted after meal ingestion and signals satiation through peripheral or central actions. CCK receptors found in the gastrointestinal tract and the central nervous system also influence gastric emptying and accommodation, while they regulate satiety *via* the same connections<sup>[72]</sup>.

Camilleri *et al.*<sup>[40]</sup> detected no association between polymorphisms of candidate genes for  $\alpha_2A$ ,  $\alpha_2C$  adrenoreceptors or CCK-1 receptors and CCK promoter with FD. In addition, Hwang *et al.*<sup>[46]</sup>, also found no significant association between ADRA2A- 1291C>G or CCK-1R intron 779T>C with dyspeptic symptoms. In contrast to the aforementioned observations, Tahara *et al.*<sup>[73]</sup> suggested that Japanese male 779 T carriers of CCK-1 intron 1 are exposed to a higher risk of PDS.

### Genes associated with inflammation or immune response

**CD14:** CD14 mediates the inflammatory response in the first line of host defense by recognition of Lipopolysaccharide, a main component of the outer cell wall of *H. pylori*<sup>[74]</sup>. Soluble CD14 levels tend to be higher in *H. pylori* positive than in *H. pylori* negative patients<sup>[75]</sup>. In addition, FD patients with extensive gastric mucosal inflammation accompanied by a high density of *H. pylori* show increased CD14 expression<sup>[76]</sup>. The TT genotype of the CD14 C-159T polymorphism has been related to high density of the CD14 receptor and high soluble CD14 levels<sup>[77]</sup>. Due to the important role of CD14 in inflammation, polymorphisms in the CD14 gene promoter may influence gastric inflammation and susceptibility to FD. Tahara *et al.*<sup>[78]</sup> investigated the association between CD14 promoter C-159T polymorphism and FD among 108 dyspeptic and 99 non-dyspeptic Japanese individuals. They suggested that this CD14 polymorphism is not associated with dyspepsia, while there was a weak correlation between TT genotype and PDS in male patients. There has been no study so far in Caucasians investigating the association between CD14 gene polymorphisms and FD.

**Macrophage migration inhibitory factor:** Macrophage migration inhibitory factor (MIF), isolated from T lymphocytes, inhibits the random migration of macrophages<sup>[79]</sup>. MIF is an important regulator of innate immunity both directly and *via* stimulation of the expression of proinflammatory cytokines by immune cells<sup>[80]</sup>. Polymorphisms (G-173C and -794 CATT) identified in the *MIF* gene promoter, were correlated with alteration of MIF genes transcription levels *in vitro*. It seems that MIF also plays a significant role in inflammation related to *H. pylori* infection<sup>[81]</sup>. A study from Japan<sup>[82]</sup> examined the association between MIF gene polymorphisms and FD. Investigators showed that MIF-173C allele carriers were at significantly increased risk of developing epigastric pain syndrome; this association being more prominent in *H. pylori* infected subjects.

**Interleukin-17:** Interleukin-17 (IL-17) family members coordinate local tissue inflammation by inducing the release of proinflammatory and neutrophil-mobilizing cytokines. Furthermore, IL-17A and -17F function in a similar way: they are involved in the recruitment and activation of neutrophils<sup>[83]</sup>. On the basis of this pathophysiology, Arisawa *et al.*<sup>[82]</sup> revealed that polymorphisms of the IL-17A and IL-17F genes were not related to FD susceptibility, overall. However, the IL-17F 7488T allele was positively associated with developing EPS in *H. pylori* infected subjects.

**RANTES promoter:** RANTES is a powerful chemotactic agent for T lymphocytes and monocytes<sup>[84]</sup> contributing to the inflammatory response in *H. pylori* gastritis<sup>[85]</sup>. The effect of RANTES promoter C-28G polymorphism on the risk of functional dyspepsia has been investigated in 134 dyspeptic and 112 non-symptomatic Japanese subjects<sup>[86]</sup>. Although the frequency of RANTES promoter polymorphisms did not differ among dyspeptics and controls, overall, a significant association was revealed between G carriers and a reduced risk of PDS; this association being more prominent in *H. pylori* positive subjects.

**Toll like receptor 2 and mannan-binding lectin genes:** Toll like receptor 2 (TLR2) and mannan-binding lectin (MBL) protein play significant roles in innate immune system activation<sup>[87]</sup>. It was reported that TLR2 is expressed in gastric epithelial cells infected by *H. pylori*<sup>[88]</sup>. MBL activates complement and also functions as an opsonin<sup>[89,90]</sup>.

The expression of MBL in the mucosa is up-regulated in *H. pylori*-related gastric mucosa inflammation<sup>[91]</sup> and two studies reported a possible association between MBL2 haplotype and susceptibility to *H. pylori* infection, as well as an increased risk of gastric malignancy<sup>[92,93]</sup>. With regard to FD, absence of the correlation between TLR2 -196 to -174 del and MBL2 codon 54 G/A polymorphisms and the syndrome has been shown in a Japanese population<sup>[94]</sup>. However, it was suggested

that TLR2 -196 to -174 del carrier status, but not MBL2 codon 54 G/A, was inversely related to the risk of PDS in *H. pylori*-infected subjects.

**C242T p22PHOX:** Superoxide has been associated with the pathogenesis of *H. pylori*-related diseases through induction of inflammation. Nicotinamide adenine dinucleotide phosphate oxidase, a major source of superoxide plays a crucial role in *H. pylori* gastritis. Tahara *et al.*<sup>[95]</sup> did not identify any significant association between C242T polymorphism of p22PHOX, an essential component of nicotinamide adenine dinucleotide phosphate oxidase and FD. However, C242T carriers were at lower risk of developing FD in the sub-group of *H. pylori*-infected patients.

**Cyclooxygenase-1:** Cyclooxygenase (COX) is the key enzyme in the conversion of arachidonic acid to prostaglandins (PGs), which are implicated in many physiological gastric processes and contribute to gastrointestinal integrity. There is only one study investigating COX-1 genotypes and dyspepsia. Arisawa *et al.*<sup>[96]</sup> revealed that T-1676C polymorphism in the COX-1 gene promoter was significantly associated with the development of EPS in female subjects.

**Nitric oxide synthase:** Nitric oxide (NO) is a major neurotransmitter that mediates gastric accommodation or relaxation and meal-induced satiety<sup>[97]</sup>. Therefore, it has been postulated that impairment of this system can lead to FD. There are three different NOS isoforms: neuronal NOS (nNOS), inducible (iNOS), and endothelial NOS (eNOS)<sup>[98]</sup>. In the only available study of FD, Park *et al.*<sup>[99]</sup> suggested that the genotype frequencies of eNOS and iNOS were not significantly different between FD patients and controls. However, the nNOS gene polymorphism was associated with susceptibility to FD and influences satiation in dyspeptics.

#### Catechol-o-methyltransferase gene

Catechol-o-methyltransferase (COMT) is an important enzyme in the brain-gut axis regulating pain sensitivity, and the presence of COMT gene val158met has been associated with dyspepsia<sup>[100]</sup>.

#### Capsaicin/vanilloid receptor (transient receptor potential vanilloid 1 receptor)

TRPV1 receptor is expressed in the gastrointestinal tract, and is a member of the sensory ion channel superfamily. Studies using capsaicin provide evidence that TRPV1 regulates gastrointestinal sensation. Capsaicin administration into the alimentary tract causes pain in humans<sup>[101]</sup> and mice<sup>[102]</sup>. Hammer *et al.*<sup>[103]</sup> suggested that capsaicin application produces symptoms originating from the upper abdomen; being more severe in FD. Continuous capsaicin desensitization has also been reported to be beneficial in patients with FD<sup>[104]</sup>. Capsaicin suppresses gastrointestinal hyperalgesia by desensitization/inactivation and

downregulation of TRPV1<sup>[105]</sup>, indicating that the up-regulation of TRPV1 may be implicated in the pathogenesis of FD.

G315C gene polymorphism alters TRPV1 protein levels and the variant 315C of TRPV1 G315C increases TRPV1 mRNA and protein expression and results in maximal response to capsaicin<sup>[106]</sup>. However, a significant inverse association was detected between TRPV1 315CC genotype and FD, EPS, PDS and *H. pylori* positive FD in Japan<sup>[107]</sup>. Furthermore, a significant inverse correlation between C carrier of TRPV1 945G>C polymorphism status and PDS has been detected and C/C genotype *H. pylori*-positive subjects were at reduced risk of EPS<sup>[46]</sup>.

### SCN10A

Visceral hypersensitivity has been implicated in the pathogenesis of functional gastrointestinal disorders. C-fibers contribute to visceral sensory impulse transmission from the gastrointestinal tract to the central nervous system. SCN10A gene encodes the tetrodotoxin-resistant (TTX-r) sodium channel, Na(V) 1.8/SNS (sensory-neuron specific), which has been identified on C-fibers. Arisawa *et al.*<sup>[108]</sup> investigated the association between FD and SCN10A non-synonymous polymorphisms (2884 A>G, 3218 C>T and 3275 T>C) among 297 dyspeptics and 345 symptom-free controls. They concluded that subjects with the 2884 G allele, 3275 T allele and no 3218 T allele of SCN10A were at reduced risk of FD, for both the EPS and PDS subtypes, especially in *H. pylori*-negative patients.

## LIMITATIONS OF THE AVAILABLE STUDIES

Genetic association studies in FD have yielded diverse and inconsistent results for the investigated candidate genes. These inconsistencies might be explained by subject composition differences and can be influenced by unadjusted environmental factors. In addition, the definition of FD has changed over time and FD patients were inconsistently defined. The selection procedure for controls is also questionable in functional disease studies, as symptom-free subjects are not necessarily equal to healthy controls<sup>[109]</sup>. Moreover, in most of these studies the association between the candidate genotype and FD has been investigated in small samples without appropriate sample size estimation; therefore type II error cannot be excluded. Finally, the majority of the studies have been conducted in Asia, and Western populations are underrepresented.

## CONCLUSION

Functional dyspepsia is a disorder with complex pathophysiology including gastrointestinal motor abnormalities, altered visceral sensation, psychosocial and genetic factors. Genetic factors may influence

susceptibility to FD in the presence of exogenous factors, such as *H. pylori* infection. Studies investigating possible associations between FD and genotypes related to gastrointestinal motility and sensation, as well as to inflammation or immune response are limited by several caveats and they have reached inconclusive results. Properly designed, multicenter, adequately powered, worldwide conducted studies enrolling well defined disease subjects and controls are warranted to investigate potential associations between pre-specified gene polymorphisms and FD. The results of such studies may shed light on the pathogenesis of the syndrome and may potentially lead to genetic diagnostic tools and ultimately to novel therapeutic options for FD.

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## Primary biliary cirrhosis: Clinical and laboratory criteria for its diagnosis

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### Abstract

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic granulomatous, and destructive inflammatory lesion of small intralobular and septal bile ducts, which is likely to be caused by an autoimmune mechanism with a the presence of serum antimitochondrial antibodies and a potential tendency to progress to cirrhosis. Despite the fact that the etiology of this disease has been

unknown so far, there has been a considerable body of scientific evidence that can reveal the clinical and laboratory signs of PBC and the individual components of its pathogenesis and elaborate diagnostic criteria for the disease and its symptomatic therapy. Deficiencies in autoimmune tolerance are critical factors for the initiation and perpetuation of the disease. The purpose of this review is to summarize the data available in the literature and the author's findings on clinical and laboratory criteria for the diagnosis of PBC. This review describes the major clinical manifestations of the disease and the mechanisms of its development. It presents the immunological, biochemical, and morphological signs of PBC and their significance for its diagnosis. A great deal of novel scientific evidence for the problem of PBC has been accumulated. However, the inadequate efficiency of therapy for the disease lends impetus to the quest for its etiological factors and to further investigations of its pathogenetic mechanisms and, on this basis, to searches for new methods for its early diagnosis.

**Key words:** Primary biliary cirrhosis; Clinical criteria; Laboratory criteria; Immunological signs; Biochemical signs; Morphological signs

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**Core tip:** Primary biliary cirrhosis is a chronic autoimmune cholestatic liver disease. This review summarizes current literature data and our own experiences on clinical and laboratory criteria for the diagnosis of primary biliary cirrhosis. Thanks to advances in biochemistry, molecular biology and genetics, it became possible to present these data with regard to the pathophysiological mechanisms of their development.

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## INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic autoimmune cholestatic liver disease characterized by a striking female predominance, high-titer serum antimitochondrial autoantibodies (AMAs), disease-specific antinuclear autoantibodies (ANAs), and an autoimmune-mediated progressive granulomatous destruction of small and medium-sized intralobular and septal intrahepatic bile ducts, leading to cirrhosis and ultimately liver transplantation or death<sup>[1-5]</sup>. Deficiencies in autoimmune tolerance are critical factors for the initiation and perpetuation of the disease<sup>[6]</sup>. Immunologically, PBC is distinguished by immune-mediated destruction of the intrahepatic bile ducts and the presence of high-titer antimitochondrial autoantibodies<sup>[3]</sup> directed against a highly specific epitope within the lipoic acid binding domain of the pyruvate dehydrogenase E2 subunit (PDC-E2)<sup>[7]</sup>. The natural history of the disease is 10 to 20 years<sup>[6]</sup>.

According to the presence or absence of cirrhosis, the mean survival was 9.17 years (95%CI: 6.79-11.56) and 10.7 years (95%CI: 9.27-12.14), respectively ( $P = 0.03$ )<sup>[8]</sup>. Mortality from PBC is 2.2% of all deaths due to liver cirrhosis<sup>[9]</sup>.

### History of PBC

In 1851, Addison *et al*<sup>[10]</sup> were the first to observe an association of skin changes with liver disease in women. Elevated serum cholesterol levels in these patients and the presence of cutaneous xanthelasmas served as a basis for employing the term "xanthomatous biliary cirrhosis" to denote this disease<sup>[11,12]</sup>. Almost 100 years ago, its clinical picture was described in detail and the term "primary biliary cirrhosis" was offered<sup>[11,12]</sup>. In 1965, a group of morphologists under the supervision of H. Popper proposed the term "chronic non-purulent destructive cholangitis"<sup>[13]</sup>.

### Epidemiology of PBC

PBC is encountered in all parts of the world among people of all races and nationalities<sup>[14,15]</sup>. No differences were observed in the geographical distributions of the disease<sup>[15]</sup>. According to different authors, the prevalence of PBC is 4-14 cases per 100000 population<sup>[15-17]</sup>. PBC is mostly found in patients in Northern Europe, the United Kingdom, and the northern United States. The prevalence of familial PBC was later reported to be 6.4% in United Kingdom<sup>[18]</sup>, and between 3.8% and 9.0% in a number of studies from North America, Europe, and Japan<sup>[5]</sup>. In Asia, Japan is the only country with a known prevalence of PBC, at 27-54 per million<sup>[19,20]</sup>. The annual incidence rates range between 0.7 and 49 cases per million persons, while

the global prevalence rates range between 6.7 and 402 cases per million persons<sup>[3,5,6,20-24]</sup>. A study conducted by Gershwin *et al*<sup>[25]</sup> indicated that having a first-degree relative with PBC was significantly associated with increased risk of PBC, with an odds ratio of 10.7. This is supported by the high concordance rate of PBC among first-degree relatives and homozygous twins (approximately 60%)<sup>[26,27]</sup>. A pairwise concordance rate of 0.63 for PBC in monozygotic twin pairs has been published, which is one of the highest reported in autoimmunity<sup>[28]</sup>. Specifically, the high concordance for PBC in monozygotic twins, family clustering, and female predominance suggest that genetic factors may play an important role in the development of PBC<sup>[29-31]</sup>.

PBC is a typical female disease that occurs from 40-60 years of age<sup>[32,33]</sup>. Much less is logged PBC in persons under the age of 25 years<sup>[34,35]</sup>. There is a high female:male incidence ratio (8:1), with suggestions of a significant role for X chromosome defects in PBC, based on the observation that women with PBC have a significantly enhanced monosomy X frequency in peripheral white blood cells compared to age-matched healthy women<sup>[36]</sup> and that the X chromosome loss is preferential<sup>[37]</sup>. Smoking behavior, age at menarche, age at first pregnancy, gravidity, and number of children were not significantly different between PBC cases and controls<sup>[15]</sup>.

### Etiology and pathogenesis of PBC

The etiology of PBC is still unknown<sup>[1,38]</sup>. Currently, it is believed that PBC is likely to be triggered by a combination of environmental factors, including infection in a genetically susceptible individual<sup>[2,7]</sup>. Many authors regard the disease as impaired immunoregulation with a loss of tolerance of histocompatibility antigen-enriched tissues. PBC is characterized by T-cell-mediated destruction of small bile duct epithelial cells<sup>[39]</sup>. This leads to ductulopenia and persistent cholestasis, by developing end-stage hepatic-cell failure. Recent data point towards apoptosis as a leading mechanism for ductopenia<sup>[40]</sup>. The loss of bile ducts leads to decreased bile secretion and the retention of toxic substances within the liver. This results in further hepatic damage, fibrosis, cirrhosis, and eventually liver failure<sup>[35]</sup>. A vital question in the pathogenesis of PBC is why biliary epithelial cells (BECs) in particular are the primary target of disease despite the ubiquitous presence of the pyruvate dehydrogenase complex (PDC) autoantigen in all tissue cells<sup>[39]</sup>. How and why the bile ducts are involved in this process remains unknown. Viruses<sup>[41,42]</sup>, bacteria, xenobiotics<sup>[43]</sup>, and human immunoregulatory defect may be possible PBC triggers that initiate the immunopathological cascade. Infection, either viral or bacterial, can either directly induce BEC apoptosis or more probably trigger an immune attack on epithelial cells as a result of molecular mimicry<sup>[28]</sup>. Initiating mimotopes of the vulnerable epitope of the E2 subunit of the pyruvate

**Table 1** Classification of the clinical course of primary biliary cirrhosis

Clinical classification		Clinical stages		
Ref.				
Sasaki <i>et al</i> <sup>[44]</sup> , 1985	Asymptomatic stage	Itching stage	Stage of jaundice	End-stage
Poupon <sup>[45]</sup> , 1991	Preclinical, asymptomatic stage	Clinical stage	End-stage	-

dehydrogenase comple (PDC-E2) autoantigen can be derived from microbes that utilize the PDC enzyme or, alternatively, environmental xenobiotics/chemical compounds that modify the structure of native proteins to make them immunogenic. A further alternative as a source of antigen is PDC-E2 derived from apoptotic cells. In the effector phase the biliary ductular cell, by reason of its proclivity to express the antigen PDC-E2 in the course of apoptosis, undergoes a multilineage immune attack comprised of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and antibody<sup>[39]</sup>. The liver can encounter a number of pathogenic microorganisms and their by-products from the gut by acting as a traffic hub. Kouroumalis *et al*<sup>[28]</sup> propose a pathogenetic model for PBC, which plays an important role in primary dysfunction of endothelial cells overproducing endothelin-2.

The pathogenesis of PBC involves environmental factors, genetic predisposition and loss of immune tolerance<sup>[6]</sup>. In recent years, it has become univocally accepted that an inappropriately activated immune response is one of the most important factors in PBC. A number of models for the disease have been proposed to systematize the ideas on its development mechanisms<sup>[1,28,39]</sup>. However, the existing concepts of PBC cannot fully explain the specificity of biliary epithelial injury, the expression of mitochondrial PDC-E2 of bile duct epithelial cells as autoantigens, and the higher prevalence of this disease in women than men (9:1).

### Clinical course of PBC

A few classifications of PBC have been proposed according to its clinical course (Table 1).

The development of PBC is preceded by a long asymptomatic period. The wide use of computer-aided screening biochemical and immunological studies has significantly increased the detection of asymptomatic patients. In this period, there are generally no physical signs of PBC, at the same time anti-mitochondrial autoantibodies are detectable in the serum of virtually all patients (95%)<sup>[1]</sup>. The fact that AMAs are detectable many years before PBC manifests itself is indicative of their primary immunopathological role rather than a secondary phenomenon that occurs in the presence of cholestasis. The production of AMAs is not an epiphenomenon, and an understanding of the mechanism of AMAs induction will shed light on the etiology of PBC. The clinical manifestations of PBC may mask those of other diseases. The blood of PBC patients shows a moderate increase in the

activity of gamma-glutamyltransferase ( $\gamma$ -GT), alkaline phosphatase (ALP), 5'-nucleotidase (5'-NT), and leucine aminopeptidase (LAP). The level of serum cholesterol and aminotransferases in this period are within normal limits. Morphological examination of liver biopsy specimens allows a diagnosis of one of the stages of PBC. The signs of hepatocellular insufficiency or those of portal hypertension and their complications (esophageal varices, ascites, hepatic encephalopathy and others) occur only in the advanced stages of the disease<sup>[46]</sup>.

Il'ichenko *et al*<sup>[47]</sup> identify the following course options PBC: (1) Asymptomatic PBC (10.9%); (2) AMA-positive (classical) PBC (85.4%). It is characterized by the typical clinical manifestations of the disease, such as skin itching and jaundice, by the biochemical signs of cholestasis and serum AMAs at diagnostic titers of higher than 1:40; (3) AMA-negative PBC (14.6%). It is characterized by decreased biochemical and immunological activities and the lower frequency of extrahepatic manifestations, which has no impact on the prognosis of liver cirrhosis and the time of its progression; and (4) PBC-autoimmune hepatitis (AIH) overlap-syndrome (PBC/AIH) (9.4%); Patients are observed to have simultaneously signs of PBC and AIH (those of cholestasis and cytolysis and the presence of autoantibodies (antimitochondrial, antinuclear, anti-smooth muscle autoantibodies); their liver biopsy specimens exhibit the morphological signs of non-purulent destructive cholangitis, as well as bridging necroses and plasma cells.

### The main clinical signs of PBC and its complications

Weakness, fatigue, daytime somnolence; Pruritus; Weight loss; Xanthelasma; Skin hyperpigmentation; Jaundice; Hepatomegaly and less - splenomegaly; Malabsorption syndrome; Osteodystrophy, osteoporosis; Cholelithiasis; Extrahepatic manifestations of autoimmune nature.

### Weakness, fatigue, daytime somnolence, pruritus

Primary biliary cirrhosis is latent and oligosymptomatic disease for many years<sup>[48,49]</sup>. The disease begins quietly and is long manifested only by weakness, malaise, fatigue, daytime somnolence and/or low working efficiency.

Fatigue and pruritus are the most common symptoms of PBC, but the majority of patients are asymptomatic at first presentation<sup>[50]</sup>. Fatigue is considered to be a specific manifestation of PBC<sup>[51,52]</sup>. The pathogenesis of fatigue in PBC is unclear, but preliminary studies suggest

it has central mechanisms and may have peripheral manifestations<sup>[53]</sup>. Comorbidities and depression might have played a role in its pathogenesis<sup>[52]</sup>. The asthenic sign of PBC is more pronounced than that of other chronic liver diseases.

The leading and early pathognomonic symptom of PBC is the appearance of skin itching that may be the only manifestation of the disease within a few months and even few years. The skin shows multiple scratched traces that further display hyperpigmentation portions. The itching is characterized by extension (local or systemic), degrees (moderate or severe), and duration (persistent or transient). Itching may be excruciating, may seriously impair quality of life and even induce suicidal ideation in the most severe cases<sup>[54]</sup>. Itching is more marked at night and frequently enhanced in contact with fabrics and also in warmth.

Itching is not relieved with symptomatic (anti-histamine, sedative) agents; it often causes excruciating insomnia, emotional changes, and depression. Poor sleep at night leads to daytime somnolence, chronic fatigue and reduced ability to work. Durazzo *et al*<sup>[33]</sup> indicate that female sex hormones may cause constitutional symptoms (malaise, anorexia, weight loss, and fatigue). The patients frequently seek the advice of a dermatologist, an allergist, or a neurologist.

The molecular mechanism of itch signal transduction in cholestasis is largely unclear. It may be caused or potentiated by compounds that accumulate in the circulating blood during cholestasis<sup>[54]</sup>. Increased concentrations of bile salts<sup>[1]</sup>, histamine, female steroid hormones and their metabolites<sup>[33]</sup>, endogenous opioids, and lysophosphatidic acid (LPA)<sup>[55-57]</sup> have been controversially discussed as potential pruritogens in cholestasis<sup>[58-60]</sup>. Fatigue, pruritus, and Sjögren's syndrome are more common in women than men, but other signs and symptoms do not differ in the two sexes<sup>[61]</sup>. Females experience pruritus as a single symptom more often than males. It has been suggested that female sex hormones may be linked with pruritus<sup>[33]</sup>.

It has been suggested that opiates and their receptors are involved in the development of itchy skin in cholestatic liver diseases. Excessive amount of endogenous lipophilic bile acids are likely to promote the release of so far unknown substances that stimulate opioid receptors. There are data on the use of opiate agonists to alleviate itching<sup>[62]</sup>.

It has been assumed that retention of bile salts, with their deposition in the skin, is related in some way to the development of the pruritus. This assumption is confirmed by two facts: (1) Purified bile acids have produced itch when injected into the skin<sup>[63]</sup>; and (2) The bile salt binding agent cholestyramine effectively relieves the itch of cholestasis, and concomitantly lowers serum bile salt concentrations. The poor correlation between serum bile salt concentration and pruritus may thus be due to variation in bile salt composition<sup>[63]</sup>. The various bile salts might differ in their ability to provoke pruritus. Unconjugated

bile acids were found to be more pruritogenic than conjugates, and the dihydroxy bile salts (particularly unconjugated chenodeoxycholate) are more effective pruritogens than the trihydroxy (cholic acid) salts<sup>[63]</sup>. 50%-85% of unconjugated bile acids and less than 20% of sulfate esters are detected in the skin of PBC patients<sup>[64]</sup>. Large differences in the retention of either sulfated or nonsulfated fractions could correlate with pruritus. The intensity of itching may depend on the ratio of sulfated (conjugated, glucuronized) and nonsulfated (unconjugated, nonglucuronized) bile cells accumulating in the skin of PBC patients.

Most recently, novel itch-specific neuronal pathways, itch mediators and their relevant receptors have been identified<sup>[55]</sup>. Screening plasma samples of a large group of patients with various cholestatic conditions revealed LPA as the active itchy compound<sup>[57]</sup>. LPA is a very potent signaling lipid that can activate cells through various LPA receptors. Subsequently, authors could demonstrate that cholestatic patients with pruritus have highly elevated levels of serum autotaxin (ATX), the enzyme that converts lysophosphatidylcholine into LPA<sup>[54,57]</sup>.

Kremer *et al*<sup>[59]</sup> highlight that increased serum ATX levels are specific for pruritus of cholestasis, but not pruritus of uremia, Hodgkin's disease, or atopic dermatitis. Oude Elferink *et al*<sup>[57]</sup> hypothesize that during cholestasis, expression of ATX is induced and gives rise to increased local formation of LPA near demyelinated nerve endings of itch fibers. LPA then activates these neurons through one of the LPA receptors, which in turn potentiates action potentials along itch fibers<sup>[54,57]</sup>.

Serum autotaxin activity correlates with pruritus intensity, but its causal relationship, expression pattern and exact mode of action during cholestasis remain to be established.

Analysis of available scientific data may suggest the following hypothetical scheme of the mechanism responsible for developing skin itching in patients with PBC (Figure 1).

In a small number (8.6%) of patients, itching may be absent in the whole period of the disease practically until terminal complications develop<sup>[47]</sup>.

Varying skin changes are observed. Skin elasticity loss and dryness, scratching traces, hyperpigmentation, xanthomas, and xanthelasmas engage attention when examining patients with PBC.

### **Skin hyperpigmentation in PBC**

According to Sherlock *et al*<sup>[65]</sup>, skin hyperpigmentation is due to excessive melanin biosynthesis in the melanocytes. Its initial reaction is catalyzed by the copper-containing enzyme tyrosinase. According to one of the hypotheses, elevated serum copper levels in patients with PBC may lead to the enhanced activity of a tyrosinase reaction and the increased biosynthesis of melanins whose skin deposition does induce hyperpigmentation<sup>[66]</sup>. Copper accumulation imparts a bronzed color to the skin.

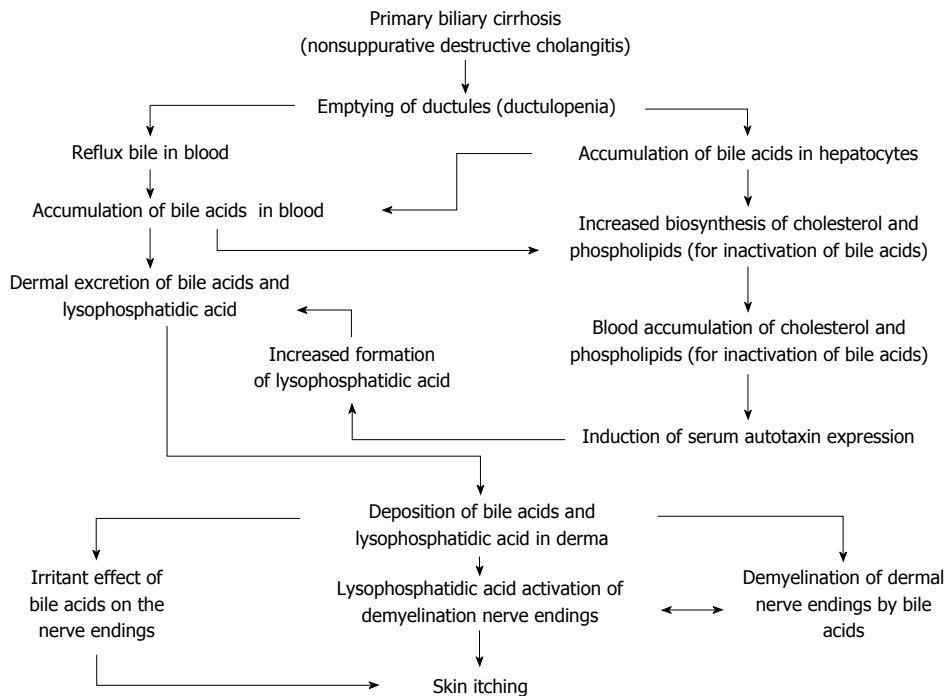


Figure 1 Scheme of mechanism of development of pruritus in primary biliary cirrhosis.



Figure 2 Xanthelasma on both eye lids in a patient with primary biliary cirrhosis.

### Xanthelasma

Xanthelasma vary in shape, may be solitary or multiple, flat, pale yellow color slightly raised above the skin. In PBC, there are xanthelasma on the upper or lower eyelids (Figure 2), as well as in the palmar creases, under the breasts, in the areas of the joints, tendons, buttocks, etc<sup>[67-69]</sup>.

According to the data obtained by Ahrens *et al*<sup>[70]</sup>, xanthelasma is formed in elevated blood concentration of cholesterol (more than 400 mg/dL) persisting for at least three months. Xanthelasma may disappear after normalization of cholesterol levels and in end-stage disease due to the progression of hepatocellular damage<sup>[65]</sup>.

### Jaundice

Jaundice is an important clinical symptom of PBC, but may be absent long (for 2 years or more). Jaundice develops in the end-stage disease<sup>[61]</sup>. In its early

stages, jaundice is generally undulating. Later on, occur its steady progression with the simultaneously increased blood levels of conjugated bilirubin. Patients with PBC show individual variations in bilirubin levels. Jaundice and skin itching concurrently occur in about one fourth of patients<sup>[32]</sup>. When jaundice is an initial manifestation of PBC, the patients exhibit a more rapid development of its end-stage, lower survival rates, and an earlier outcome than do those with its anicteric type<sup>[47,71]</sup>.

### Hepatomegaly

In the early stages of PBC, a moderate enlargement of the liver is detectable in 70%-80% of cases. The liver slowly increases in size during the whole period of the disease. The enlarged liver is associated with its compensatory regeneration in response to the decreased hepatocytes' functional ability caused by the excess accumulation and toxic action of bile acids. Hepatomegaly suggests a lesion of the liver on the one hand and the preserved regenerative ability of the organ on the other. In most cases, the liver is of moderate consistency, its surface is smooth and, in end-stage PBC, finely tuberos, painless on palpation.

The enlarged spleen is untypical of PBC, observed in its end stages in 20% of cases, and indicative of portal hypertension.

### Malabsorption and slowly progressive weight loss

In PBC, reduced secretion of bile acids gives rise to steatorrhea (fecal fat excretion of more than 7 g/d). Intestinal bile salts deficiency impairs fat absorption in PBC patients<sup>[72]</sup> and result in bacterial overgrowth. DiBaise *et al*<sup>[73]</sup> suggest that bacterial overgrowth plays



a significant role in the development of steatorrhea in patients with PBC and that an assessment for bacterial overgrowth should be performed on persons with steatorrhea in PBC. The severity of steatorrhea is associated with reduced bile acid outputs and concentrations ( $r = 0.82$ ;  $P < 0.0001$ ), degree of cholestasis (serum bilirubin;  $r = 0.88$ ;  $P < 0.001$ ) and advanced histologic stages ( $P < 0.005$ )<sup>[74]</sup>. All patients with a total serum bilirubin level of more than 4.5 mg/dL had severe steatorrhea (fecal fat excretion was above 25 g/d).

The results obtained Ros *et al*<sup>[72]</sup> indicate that overt pancreatic failure is uncommon in PBC and that fat maldigestion and steatorrhea, regardless of what degree, are due mainly to low intestinal bile salt levels secondary to bile secretory failure. The stool in the patients with PBC usually shows admixtures of incompletely digested fats. Despite steatorrhea, the patients with PBC have constipation. The latter seems to be related to the inadequate effect of a small amount of intestinal bile acids on the small and large intestinal motility.

The activity of pancreatic ALP in the serum of patients with PBC does not correlate with the severity of steatorrhea and that of amylase is within the normal range.

Skin itching accompanied by sleep disorder, as well as malabsorption of fats and fat-soluble vitamins lead to a slowly progressive weight loss in patients with PBC.

The clinical picture may be determined by complications and comorbidities.

### **Osteodystrophy, osteoporosis**

Metabolic bone disease has been recognized as an important complication of chronic liver disease particularly in PBC and after liver transplantation<sup>[75]</sup>. It includes osteoporosis and more rarely osteomalacia, which is more frequent in severe malabsorption and advanced liver disease.

The molecular mechanisms of osteoporosis in patients with PBC are associated with the impaired enterohepatic circulation of bile acids.

Decreased small intestinal bile acid concentrations in PBC can lead to impaired absorption of fats and fat-soluble vitamins, resulting in deficiencies in vitamins A, D, E, and K<sup>[76]</sup>.

Malabsorption of calcium ions and fat-soluble vitamin D in the small intestine gives rise to osteodystrophy<sup>[77,78]</sup>. The latter may manifest as bone pain in early stages of PBC; in its severe form, osteoporosis develops. Osteoporosis is a systemic skeletal disease characterized by low bone mass and bone tissue microarchitectural deterioration, resulting in increased bone fragility and fracture risk<sup>[79-81]</sup>.

Osteopenia is a recognized complication of cholestatic liver diseases, usually ascribed to metabolic bone diseases such as osteomalacia or osteoporosis, with a prevalence of 10% to 56%, depending on the

nature of liver disease<sup>[82]</sup>. PBC is the condition causing osteopenia more frequently than other cholestatic liver disease<sup>[78]</sup>. Regardless of the etiology of osteoporosis in PBC patients, they have an increased risk of spontaneous or low-trauma fracturing leading to significant patient morbidity, deterioration of quality of life, and even patient mortality<sup>[75]</sup>. Osteopenia predisposes to atraumatic fractures, particularly in PBC patients undergoing orthotopic liver transplantation and treated with high corticosteroid doses. There are pathological fractures of vertebrae and ribs commonly and those of the pelvis and long bones more rarely.

Kehayoglou *et al*<sup>[83]</sup> have reported that the mean absorption of calcium ions is significantly less in patients with PBC than in controls. Impaired calcium absorption correlated well with increased fecal fat excretion and less well with the intensity of jaundice. The degree of osteoporosis depends on physical activity, nutritional, hormonal, and genetic factors.

The pathogenesis of bone disease in both adults and children with chronic cholestasis is not completely understood. Various potential inciting factors that either directly or indirectly alter bone mass are insulin-like growth factor 1 (IGF-I) deficiency, hyperbilirubinemia, hypogonadism, alcohol, subnormal 25-hydroxyvitamin D<sub>3</sub> levels, vitamin D receptor genotypes, vitamin K, osteoprotegerin and receptor activator of nuclear factor Kb ligand interactions and concurrent use of drugs like cholestyramine, furosemide, glucocorticoids and immunosuppressive agents<sup>[78]</sup>.

Hypogonadism is an established risk factor for osteoporosis in chronic liver disease.

The pathogenesis of osteoporosis in patients with PBC is complex and is likely to be multifactorial<sup>[75,84]</sup> and involves impairments of vitamin D<sub>3</sub> absorption and metabolism<sup>[85]</sup>, decreased intestinal calcium ion absorption<sup>[86,87]</sup>, genetic predisposition<sup>[88]</sup>, and impact of corticosteroid therapy<sup>[89]</sup> (Figure 3).

Deficient entry of bile acids into the intestinal lumen substantially diminishes the absorption of fats and fat-soluble vitamins (A, D, and K)<sup>[90]</sup>. Vitamin D<sub>3</sub> deficiency and metabolic bone disease are common complications of PBC. In patients with severe cholestasis, malabsorption of dietary vitamin D is an important contributing factor to vitamin D<sub>3</sub> deficiency<sup>[91]</sup>. Along with insufficient vitamin D absorption, there is a lower formation of 1,25-dihydroxyvitamin D<sub>3</sub> on cytochrome P<sub>450</sub> (competitive inhibition of monooxygenases due to the enhanced biosynthesis of bile acids)<sup>[1]</sup>. This all brings about inadequate calcium ion absorption in the small bowel and impaired phosphorus-calcium metabolism.

Lower serum IGF-I levels were seen in patients with chronic liver diseases<sup>[92]</sup>. IGF-I is synthesized by the liver. It is a bone collagen and osteoblast stimulator. IGF-I together with other genetic and environmental factors may be involved in the complex regulation of bone mineral density (BMD) in PBC.

Genetic factors have been implicated in the path-

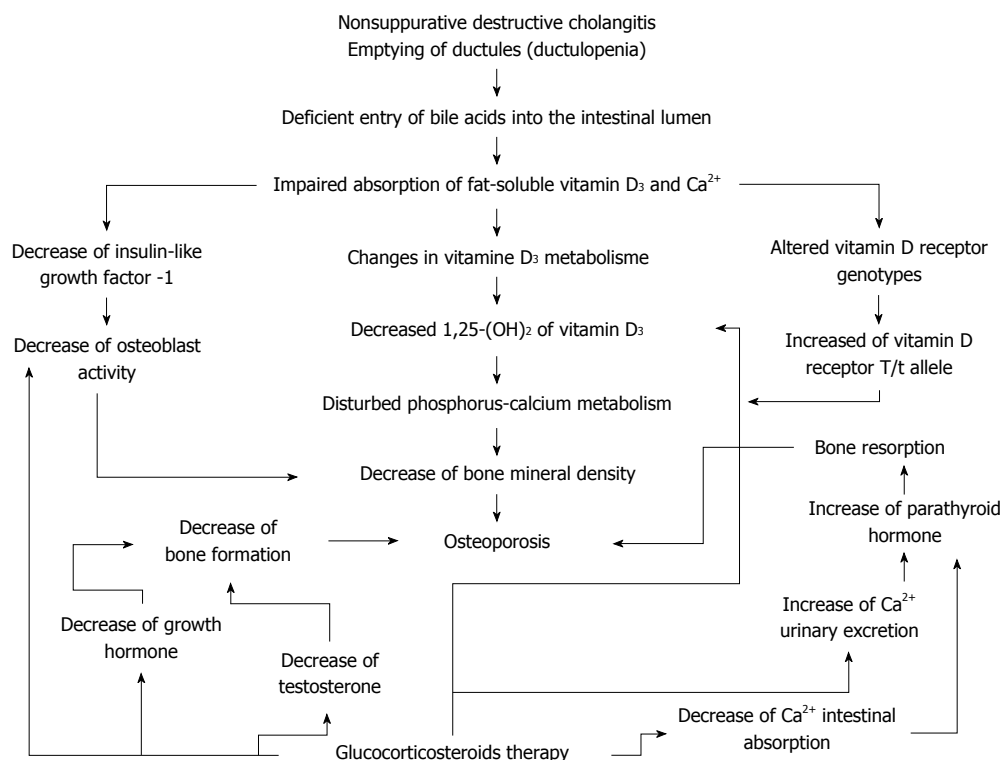


Figure 3 The scheme of the development of osteoporosis in primary biliary cirrhosis.

ogenesis of osteoporosis, which is a common disorder in PBC. IGF-I gene microsatellite repeat polymorphism was found to be associated with osteoporosis and lower BMD in PBC<sup>[88]</sup>.

Reduced tissue sensitivity to circulating vitamin D due to altered vitamin D receptor genotypes may play a role in the development of hepatic osteodystrophy. Vitamin D receptor allelic polymorphisms, designated B/b, A/a, and T/t alleles, correlate with BMD. The risk of developing a vertebral fracture increased 2- to 3-fold with the presence of a T/t allele<sup>[93]</sup>.

Prolonged steroid therapy of PBC patients may results in clinically significant bone loss with an increase in fracture risk by greater than 2-fold<sup>[94]</sup>. Glucocorticosteroids (GCSs) diminish intestinal calcium absorption, by reducing the production of 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub>, increasing urinary calcium excretion, and depressing canalicular reabsorption. As a result, there is a compensatory increase in parathyroid hormone production and bone resorption. In addition, GCSs directly increase parathyroid hormone release and enhance its sensitivity. Furthermore, GCSs inhibit bone formation indirectly, by suppressing the synthesis of testosterone in gonads and by decreasing the production of growth hormone, IGF, and accordingly type 1 collagen, as well as directly by suppressing the function of osteoblasts<sup>[95,96]</sup>. Steroids exert a direct effect on bone cells by increasing osteoclastic activity by increasing IL1 and IL6 and decreasing differentiation, recruitment and life span of osteoblasts<sup>[78]</sup>.

García-Suárez *et al*<sup>[97]</sup> suggest that serum leptin

is associated with BMD. Szalay *et al*<sup>[98]</sup> found a lower serum leptin level and a higher soluble leptin receptor in patients with PBC, which could not be explained by the difference in body mass index. As leptin is associated with BMD, it may be hypothesized that leptin is involved in the complex regulation of bone metabolism in PBC. There is a clear increase in serum leptin levels according to its histological stage<sup>[97]</sup>.

The best course of management for PBC patients is to review the individual risk factors for osteoporosis, to obtain a bone mass measurement, and to prescribe age- and disease-specific therapies<sup>[75,78]</sup>. The development of bone densitometry has allowed assessment of bone mass and then contributed in estimating the fracture risk<sup>[78]</sup>. The risk of fracture shows a correlation with BMD but no fracture threshold is determined<sup>[75]</sup>.

BMD measurement is the best way to assess the presence and severity of osteopenia in PBC patients, while laboratory tests give important information about the metabolic status of the bone. Newer diagnostic modalities have improved the detection of hepatic osteodystrophy and vitamin D repletion, calcium supplementation and bisphosphonates seem promising<sup>[78]</sup>.

### Extrahepatic manifestations of PBC

There are different immune extrahepatic diseases (Table 2) in PBC, which make its diagnosis difficult. PBC is associated with a large variety of other diseases, like arthropathy, CREST syndrome, autoimmune thyroiditis, and so on, which in addition will or will not

**Table 2 The most common extrahepatic diseases associated with primary biliary cirrhosis**

Autoimmune hepatitis
Sjogren's syndrome (Sicca syndrome) <sup>[99]</sup>
CREST syndrome
Raynaud's phenomenon
Rheumatoid arthritis
Autoimmune thyroiditis
Scleroderma
Pulmonary fibrosis
Polymyositis
Sarcoidosis <sup>[100]</sup>
Hemolytic anemia
Celiac disease
Inflammatory bowel disease

produce symptoms<sup>[61]</sup>.

According to Gu *et al*<sup>[101]</sup>, the most common comorbidities were Sjögren's syndrome (9.14%), rheumatoid arthritis (3.95%) and type 2 diabetes (2.54%). Concomitant autoimmune diseases, such as Sicca syndrome, scleroderma and Raynaud's phenomenon, have been shown to be less prevalent in men. These findings suggest that females are more likely to suffer concomitant autoimmune disease than males<sup>[33]</sup>.

The coexistence of PBC and AIH in PBC patients has been described as overlap syndrome<sup>[102-109]</sup>. The prevalence of typical PBC possessing features of AIH has been reported to range from 5% to 19%<sup>[109-111]</sup>. PBC/AIH overlap-syndrome has the histological features of AIH and PBC, with AMAs, ANAs, or smooth muscle antibodies (SMAs)<sup>[61,112]</sup>. Recent studies have shown that PBC patients with features of PBC-AIH overlap may have a rapid progression toward cirrhosis and liver failure and greater risk of varices, ascites, portal hypertension, transplantation<sup>[113,114]</sup>. Steroids or immunosuppressive therapies may be effective in these patients<sup>[17,104,107-109,115-117]</sup>. The combination therapy with UDCA and corticosteroids was more effective for PBC-AIH<sup>[118]</sup>.

Hepatocellular carcinoma (HCC) in PBC is rare<sup>[119,120]</sup>. However, PBC in the advanced stage, corresponding to PBC stage IV, was shown in the past to be associated with an increased incidence of HCC<sup>[121]</sup>. Hepatobiliary malignancies had a relative risk of 46 ( $P < 0.0001$ ) for women and 55 ( $P < 0.0001$ ) for men<sup>[120]</sup>. Whereas it is a relatively rare complication of cirrhotic PBC in women, HCC is a relatively common cause of death in male PBC patients with cirrhosis<sup>[33,61,119,122]</sup>. As far as the development of HCC is concerned, instead, PBC patients should undergo the usual surveillance reserved to other categories of cirrhotic patients, according to published guidelines for the management of HCC<sup>[121]</sup>. Such surveillance should start only when PBC patients have reached stage IV disease.

The risk of extrahepatic malignancies is higher than that of hepatocellular carcinoma, but it is not influenced by the histologic stage of the liver

disease<sup>[123-125]</sup>. For stomach and pancreas cancers, the results of one study that only examined male patients with PBC indicated that PBC patients had increased risk of stomach cancer and pancreatic cancer, whereas the results of other studies of mixed-sex patients showed no significant association<sup>[124-126]</sup>. Therefore, despite inconsistent results, the meta-analysis could not be conducted for assessing the association. PBC was not significantly associated with increased risk of other cancers<sup>[126]</sup>.

PBC patients might benefit from more aggressive surveillance for hepatobiliary malignancies during their lifetime<sup>[120]</sup>. The risk of HCC development may be an additional reason to consider earlier transplantation in these patients.

## LABORATORY SIGNS OF PBC

### Immunological signs of PBC

In recent years, it has become univocally accepted that an inappropriately activated immune response is one of the most important factors in PBC<sup>[6]</sup>. Substantial amounts of data to date have illustrated that autoimmunity plays a critical role in the pathogenesis of PBC<sup>[6]</sup>. There is concrete evidence that apoptosis is possibly the most important mechanism of biliary epithelial cells loss<sup>[28]</sup>. Enhanced BEC apoptosis is a critical step in ductular destruction in PBC<sup>[127,128]</sup>. BECs apoptosis in PBC is assumed to cause tissue-specific autoimmune reactions, as evidenced by the detection of AMAs<sup>[2,3]</sup>. High titers of antibodies against mitochondrial elements are characteristic of the disease<sup>[28]</sup>.

Walker *et al*<sup>[129]</sup> were the first to find AMAs in the sera of patients with PBC. AMAs are detectable in different diseases; but anti-M2 AMAs are predominantly PBC-specific<sup>[130]</sup>. The highly PBC-specific autoantibody, AMA-M2, recognizes components of the oxo-acid dehydrogenase complex, which are ubiquitously expressed on the inner mitochondrial membrane, including the E2 subset of the pyruvate dehydrogenase complex (PDC-E2)<sup>[6]</sup>. The serological hallmark of PBC is the presence of high-titer (1:40 and more) AMAs directed against the E2 subunit of 2-oxo-acid dehydrogenase enzymes, chiefly PDC-E2 enzyme complexes located on the inner mitochondrial membrane<sup>[39,131,132]</sup>. These reflect the presence of autoreactive T and B cells to the culprit antigens.

The presence of serum AMAs and autoreactive B cells strongly endorses the concept of an autoimmune pathogenesis of PBC<sup>[133-135]</sup>. A. Lleo and colleagues<sup>[40,136]</sup> demonstrate that PDC-E2 with antigenic reactivity is only detectable in apoptotic blebs of human intrahepatic BECs. Interestingly, *in vitro* caspase cleavage of PDC-E2 has been shown to generate immunologically active protein fragments<sup>[137]</sup>. The antigens are released from apoptotic blebs of the BECs, or come from molecular mimicry of infectious agents, or from alteration by xenobiotics<sup>[138]</sup>.

AMAs can be detected even before clinical and morphological symptoms or biochemical abnormalities. Although most patients with PBC have AMAs against PDC-E2, there is no direct correlation between the titer of AMAs and disease severity, as well as histological progression of BECs damage<sup>[39,61]</sup>. AMAs titer measurement is, first of all, of diagnostic value.

The activity of antibodies to antigens of varying specificities (exogenous and autologous antigens) is associated with different classes of immunoglobulins - A, D, E, G, and M. In addition to high titers of circulating AMAs, PBC patients have high levels of serum IgM that are not related to titers of AMAs. IgM levels in PBC patients average  $6.27 \pm 0.66$  g/L (normal human plasma IgM concentrations are 1-2 g/L)<sup>[139]</sup>. The mechanism of IgM elevation is still unclear in PBC, but abnormal Ig class switching may be involved<sup>[6]</sup>. The appearance of IgM antibodies is the earliest immune response to antigen.

Specific IgA-type AMAs that have specificity for PDC-E2 can be detected in almost all body fluids of patients with PBC, including saliva, urine and bile<sup>[140,141]</sup>. The mechanism responsible is likely that a greater concentration of IgA in the bile ducts can make cells more susceptible to apoptosis through constant transcytosis, resulting in subsequent bile duct damage<sup>[142]</sup>. The presence of IgA-anti-PDC-E2 in sera or saliva might be associated with the progression of PBC<sup>[143]</sup>.

In addition to AMAs, PBC sera can exhibit other disease-specific autoantibodies, particularly ANAs and SMAs<sup>[144]</sup>.

Disease-specific ANAs are present in about one third of patients with PBC<sup>[144]</sup>. ANAs belong mainly to the IgG class. PBC-specific ANAs reactants include nuclear pore glycoproteins of the inner nuclear membrane, Gp210<sup>[145,146]</sup> and p62<sup>[146,147]</sup>. This subtype of PBC-specific ANAs has been shown to correlate with disease severity and progression<sup>[144,148,149]</sup>. High blood ANA concentrations in patients with PBC have the least favorable prognosis. Rigopoulou *et al*<sup>[150]</sup> have found that AMA-IgG3 is associated with a more severe disease.

The study conducted by Granito *et al*<sup>[151]</sup> has identified Sp140 protein as a new, highly specific autoantigen in early-stage PBC. Anti-Sp140 antibodies were present in 15% of PBC patients with a higher frequency in AMA-negative cases (53% vs 9%,  $P < 0.0001$ ). Anti-Sp140 positivity was not associated with a specific clinical feature of PBC. Anti-Sp140 antibodies were found together with anti-Sp100 antibodies in 90% of cases and with anti-promyelocytic leukemia protein antibodies in 60% of cases<sup>[151]</sup>.

The detection of antinuclear antibodies is of diagnostic and prognostic value. That of antibodies against nuclear envelop antigens in the presence of clinical signs of the disease, but in the absence of AMAs can verify the diagnosis of PBC and suggest its

unfavorable course.

SMAs against contractile proteins are found less frequently than ANAs. The SMAs are detected simultaneously with the latter ones in about one third of patients. Smooth muscle antibodies are not organ- or species-specific. F-actin serves as an autoantigen for these antibodies. SMAs in PBC belong mainly to the IgM class.

AMA-negative PBC patients account for about 10% to 15%<sup>[47]</sup>. When AMAs are not detected, then ANAs (autoantibodies against Gp210 and others) can be detected in 50% of AMAs-negative patients. Routine biochemical tests are not different from AMA-positive patients, but usually higher ANAs, SMAs, and IgG concentrations are detected. Serum IgM levels were lower in AMA-negative patients when compared with AMA-positive PBC patients<sup>[152,153]</sup>. Histologically, it is PBC<sup>[61]</sup>.

The proportion of AMA-negative patients has been minimized due to the development of sensitive detection technology of autoantibodies<sup>[154,155]</sup>. AMAs are highly specific for PBC and can be detected in nearly 100% of patients when sensitive diagnostic methodologies based on recombinant antigens are used<sup>[154]</sup>.

The production of AMA-IgM from peripheral blood mononuclear cells from PBC patients is reduced after exposure to UDCA<sup>[156]</sup>.

### Genetic and immunological characteristics of PBC

The etiology of PBC remains enigmatic, recent evidence has strengthened the importance of genetic factors in determining the susceptibility to the disease<sup>[5]</sup>. Hirschfield *et al*<sup>[157-159]</sup> have recently reported that PBC is associated with the mutation of genes, such as *HLA*, *IL12A*, *IL12RB2*, and *IRF5-TNPO3*. Mells *et al*<sup>[160]</sup> have a correlation of PBC with 12 new candidate genes, including *STAT4*, *DENND1B*, *CD80*, *IL7R*, *CXCR5*, *TNFRSF1A*, *CLEC16A*, and *NFKB1*. Active genetic research is expected to provide significant achievements for prevention and early diagnosis of PBC.

Class II human leukocyte antigen (HLA) genes fully return to attract interest thanks to recent genome-wide association studies (GWAS), which clearly demonstrates that the major components of the genetic architecture of PBC are within the HLA region. PBC is exceptional among autoimmune diseases in having controversially variable associations with alleles of the major histocompatibility complex (MHC, HLA); only a weak and regional association with HLA DRB1\*08 has been widely confirmed<sup>[161]</sup>, although there is growing evidence on a protective association with HLA DRB1\*11 and \*13<sup>[162,163]</sup>.

Investigations by Qin *et al*<sup>[164]</sup> suggest that distinct HLA class II genetic variants conferred both a predisposition and a resistance to PBC. HLA-DQB1 (\*02, \*04, \*0401, \*0402 and \*0601) and HLA-DRB1 (\*01, \*03, \*0405, \*07, \*08, \*0801, and \*0803) were



**Table 3** Levels of bile acids, cholesterol, lecithin, and orthophosphate in the hepatic bile portion and serum of patients with primary biliary cirrhosis

Parameters		Parameter values in the groups		mean <sub>1</sub> /mean <sub>2</sub>
		Control (mean <sub>1</sub> ± SE)	Patients with PBC (mean <sub>2</sub> ± SE)	
Bile acids (g/L)	Bile	3.9 ± 0.8	0.65 ± 0.02	6.0
	Blood	0.012 ± 0.008	0.054 ± 0.008	-0.2
Cholesterol (μmol/L)	Bile	0.91 ± 0.06	0.38 ± 0.08	2.4
	Blood	4.2 ± 0.6	11.8 ± 1.6	-0.4
Lecithin <sup>1</sup>	Bile	2.1 ± 0.3	0.5 ± 0.1	4.6
Orthophosphate <sup>1</sup>	Bile	1.2 ± 0.3	0.30 ± 0.07	4.0

<sup>1</sup>The data given in the table show the average statistical integral intensity of the corresponding <sup>31</sup>P-NMR spectral signals in conventional units. Patients with primary biliary cirrhosis (*n* = 16) and control (*n* = 14).

identified as risk factors for PBC, whereas HLA-DQB1 (\*0301, \*06, \*0602 and \*0604), and HLA-DRB1 (\*11, \*1101, \*13 and \*1501) were potent protective factors. Also, DR8 was identified to be a predisposing factor. A total of 13 studies contained data on serological HLA-DR from 5400 subjects (788 cases of PBC and 4612 controls). A high degree of heterogeneity was found to exist between DR8 and PBC risk ( $I^2 = 54.8\%$ ,  $P = 0.011$ )<sup>[164]</sup>. These results expand the repertoire of HLA-Class II genes with potential roles in PBC pathogenesis, however follow-up biological studies are needed to confirm these associations<sup>[164]</sup>.

PDC-E2 specific T-cells are present in the liver of PBC patients<sup>[165,166]</sup>, mostly during the earliest disease stages<sup>[167,168]</sup> that are essential in the pathogenesis and diagnosis of this disease<sup>[168-170]</sup>. Autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells are demonstrably involved in the pathogenesis of PBC and, histologically, infiltration of presumably autoreactive T cells in the liver and periductular spaces is one of the major features of PBC<sup>[171,172]</sup>. CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes reactive with subsets recognize epitopes of PDC-E2 have been identified in the peripheral blood and liver biopsy samples of PBC patients<sup>[28,165,167,168]</sup>. Several experiments using murine models have indicated a central role of CD4<sup>+</sup> T cells in the pathogenesis of PBC<sup>[173]</sup>. CD4<sup>+</sup>CD25<sup>high</sup> regulatory T (Treg) cells play a critical role in self-tolerance and the prevention of autoimmune disease. Patients with PBC display a relative reduction of circulating CD4<sup>+</sup>CD25<sup>high</sup> Tregs compared to controls<sup>[174,175]</sup>. This may be associated with decreased estrogen levels in patients with PBC. Previous studies have examined gender differences in the immune system, and suggest that estrogen and androgen may modulate the immune system. Women have significantly higher CD4<sup>+</sup> T lymphocyte counts and a higher CD4<sup>+</sup>/CD8<sup>+</sup> ratio than men<sup>[33,176]</sup>.

The frequency of circulating Tregs can increase after 1 year of treatment with UDCA<sup>[177]</sup>.

Compared to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells play a more

significant role in mediating the destruction of the bile duct<sup>[6]</sup>. BECs apoptosis is considered to result from the attack of effector cells like CD8 T cells<sup>[169]</sup>. CD8<sup>+</sup> T cells may mediate bile ductular injury in the presence of Treg function loss<sup>[178]</sup>. There is concrete evidence that apoptosis is possibly the most important mechanism of BECs loss. Apoptosis is considered to result from the attack of effector cells like CD8<sup>+</sup> T cells<sup>[169]</sup>. Markers of ongoing apoptosis have been reported within affected portal tracts<sup>[179,180]</sup>. Interestingly, *in vitro* caspase cleavage of PDC-E2 has been shown to generate immunologically active protein fragments<sup>[137]</sup>.

It is thought that activated CD4<sup>+</sup> T cells can recognize peptide PDC-E2163-176 while activated CD8<sup>+</sup> T cells can recognize peptide PDC-E2159-167 and PDC-E2165-174 in PBC<sup>[6,181,182]</sup>. HLA-A2-restricted CD8<sup>+</sup> T cell lines reactive with PDC-E2 residues 159-167 have been characterized<sup>[181,182]</sup>. Interestingly, CD8<sup>+</sup> T cells from livers of PBC patients demonstrate cytotoxicity against PDC-E2 159-167 pulsed autologous cells<sup>[169]</sup>.

### Biochemical signs of PBC

PBC is characterized by changes in many blood biochemical parameters. Patients' sera show the enhanced activity of alkaline phosphatase (ALP),  $\gamma$ -glutamyltransferase (gamma glutamyltranspeptidase,  $\gamma$ -GT), 5'-nucleotidase (5'-NT), and leucineamino-peptidase (LAP), the higher levels of bile acids, cholesterol, phospholipids, copper,  $\gamma$ -globulins, and bilirubin, and the lower level of total protein mainly at the expense of albumin fractions.

### Bile acids, cholesterol, lecithin in PBC

In PBC, there is a decline in the levels of bile acids, cholesterol, and lecithin in the hepatic bile portion and their simultaneous rises in hepatocytes and blood (Table 3). This suggests that enterohepatic bile acid circulation is impaired in PBC.

### Bile acids in PBC patients

Intrahepatic cholestasis in PBC is a multifactorial process that leads to biochemical disorders and damages to subcellular structures, with changes in the metabolism of bile acids and their transmembrane transport that is done by carrier proteins in the sinusoidal and canalicular membranes<sup>[183,184]</sup>.

Serum bile acid concentrations increase in patients with PBC (Table 3) just in its asymptomatic stage, which is associated with the occurrence of skin itching, the first clinical sign of the disease<sup>[66]</sup>. All fractions of conjugated bile acids appear in appreciable amounts in the blood of patients with PBC. Unconjugated bile acids are rarely detectable in the serum of PBC patients.

Autoimmune pathological processes in PBC gradually lead to ductulopenia and impair the mechanism responsible for the metabolism of bile acids. The increased number of desolated bile ductules result in

impaired bile excretion and insufficient entry of bile acids into the duodenum. As a response, the hepatocyte increases the synthesis of bile acids. A reduction in intestinal bile acid levels *via* a feedback mechanism induces a compensatory hepatocyte increase in the biosynthesis of bile acids and cholesterol. Cholesterol is the major substrate for bile acid biosynthesis. But ductulopenia does not diminish. The hepatocytic concentration of bile acids gradually elevates and their entry into the intestinal lumen remains insufficient, giving rise to a closed vicious circle that results in the accumulation of bile acids in the liver cells. Due to the increased hepatocytic level of bile acids, their reabsorption from the portal venous bed decreases, leading to the entry and progressive accumulation of bile acids in systemic circulation.

Bile acids, unconjugated ones in particular, are potent detergents that are able to impair cell membranes and have irritant effects on nerve endings. *In vitro* experiments have shown that several bile acids cause hepatocyte injury with a concomitant generation of hydroperoxide by mitochondria<sup>[185,186]</sup> and also induce hepatocyte apoptosis in a time- and concentration-dependent manner *via* reactive oxygen species (ROS) generation by mitochondria<sup>[187]</sup>.

The blood of patients with PBC shows a higher ratio of trihydroxy-/dihydroxychoholic acids and a lower glycine/taurine coefficient<sup>[188]</sup>. According to Greim *et al*<sup>[189,190]</sup>, cholic (trihydroxychoholic) bile acid has lower detergent properties than dihydroxy- (deoxychoholic and chenodeoxychoholic) bile acids.

The conjugation, sulfation, and glucuronidation of bile acids are directed towards decreasing their detergent and irritant effects on somatic cells and nerve endings. These also lead to their enhanced release from the systemic circulation through the skin, kidneys, and bowel. García-Marín *et al*<sup>[191]</sup> have indicated that taurine-conjugated bile acids stimulate the production of micelles with cholesterol and phosphatidylcholine (lecithin).

Thus, the higher ratio of trihydroxy-/dihydroxychoholic bile acids and the appearance of glycine- or taurine-conjugated, sulfated, and glucuronidated bile acids in the systemic circulation in PBC may be considered as the body's compensatory and detoxifying response to cholestasis and considerably increased choholic acids in circulating blood.

Atypical, non-physiological bile acids appear in the blood and urine of patients with PBC<sup>[192]</sup>. The latter have more potent detergent effects on cell membranes and a stronger irritant effect on nerve receptors than primary and secondary bile acids. The atypical, non-physiological bile acids can be released from the systemic circulation through the skin, taking part in the mechanisms of skin itching<sup>[66]</sup>. The intensity of the latter may depend on the amount of atypical bile acids in the skin of patients with PBC.

### **Lipid metabolic disturbance**

Biochemical studies have revealed that hepatic bile portions from PBC patients contain lower levels of not only bile acids, but also phosphatidylcholine (lecithin) and cholesterol (Table 3). The hepatic bile portions from the patients with PBC and those in the comparison group show a ratio of lipid components (bile acids, lecithin, and cholesterol) of 6:4.6:2.4 (Table 3), which is indicative of high bile lithogenicity in patients with PBC. The appearance of gallbladder concrements complicates PBC in 35%-40% of cases. As a rule, is formed pigment gallstones.

The altered concentration of phosphatidylcholine in the hepatic bile portions can be induced by a change in the relative amounts of bile acids<sup>[193]</sup>. Hepatocytic lecithin secretion is acid-dependent (dependent on the secretion of bile acids)<sup>[194]</sup>. The regulatory effects of bile acids on the liver and biliary tract are largely dependent on the hydrophobic-hydrophilic balance of the recirculating bile acid pool<sup>[195]</sup>.

The hepatic biopsy specimens from patients with PBC show a 1.5-fold ( $P = 0.044$ ) increase in the total amount of phospholipids<sup>[196]</sup>. Moreover, the same membranes contain the lower levels of lysophosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine<sup>[1,196]</sup>. There is a 2-fold decrease in the ratio of cholesterol to phospholipids in the hepatocyte membranes of PBC patients.

The blood of patients with early-stage PBC contains higher levels of phospholipids, cholesterol<sup>[197,198]</sup>, and bile acids<sup>[199-201]</sup> (Table 3). The change in the content of cholesterol and phospholipids in the blood of patients with PBC is associated with their increased formation in the liver and regurgitation into blood flow. Cholesterol and phospholipids are able to bind bile acids and to inactivate their solubilizing effect. Hyperlipidemia in PBC is a compensatory reaction of the organism in response to cholestasis. The high levels of cholesterol and phosphatidylcholine in the blood of PBC patients are apparently associated with the neutralization of the detergent effect of excess bile acids.

In PBC, serum cholesterol levels markedly increase with worsening of cholestasis, and decrease in the late disease stages, despite a severe reduction in biliary secretion<sup>[197]</sup>. At the same time, there were no significant differences in the levels of major lipoprotein classes in healthy individuals and patients with PBC<sup>[66]</sup> (Table 4).

Marked hypercholesterolemia that is typical for longstanding cholestasis is unassociated with an excess risk of cardiovascular disease<sup>[197]</sup> and the risk of developing atherosclerosis in PBC<sup>[202]</sup>.

PBC can serve as a model disease, showing that only hypercholesterolemia is insufficient to develop atherosclerosis and cardiovascular diseases.

Blood triglyceride levels in PBC are virtually unchanged.

**Table 4 Lipoproteins of blood in primary biliary cirrhosis patients and control group**

Lipoproteins		Parameter values in the groups	
		Control ( <i>n</i> = 21)	PBC ( <i>n</i> = 18)
β-lipoproteins	mean ± SD	50.1 ± 6.0	56.4 ± 16.6
	Range	39.2-59.1	34.8-86.4
α-lipoproteins	mean ± SD	28.4 ± 6.5	23.9 ± 12.2
	Range	15.5-42.7	6.4-46.8
Pre-β-lipoproteins	mean ± SD	20.5 ± 7.5	18.2 ± 8.5
	Range	10.5-34.7	6.3-43.5

### Hyperbilirubinemia

Elevated blood levels of many biliary constituents are observed in patients with PBC. However, the higher bilirubin concentrations are untypical of early-stage PBC<sup>[203]</sup>. The blood total and conjugated bilirubin levels are increased in patients with PBC in the stage of its obvious clinical symptoms and rarely reach high values. Hyperbilirubinemia results from intrahepatic cholestasis when, in the absence of extrahepatic obstruction, conjugated bilirubin comprises a high fraction of the total bilirubin<sup>[204]</sup>. Significant individual variations in bilirubin levels are noted in patients with PBC. However, the blood bilirubin concentrations generally correspond to the stage of the disease and the activity of the pathological process. In PBC, the levels of bilirubin are elevated mainly by its conjugated fraction. This suggests that the normal hepatocytic activity of glycosylating enzymes is retained to produce conjugated bilirubin.

The development of hyperbilirubinemia in PBC is associated with impaired bilirubin excretion. The elevation of conjugated bilirubin may result from obstructed bile flow, altered bile ductular integrity, or reduced production of bile due to defective activity of bile efflux transporters<sup>[204]</sup>. The most probable pathway for the leak of bile back into the systemic circulation is *via* the intercellular tight junctions<sup>[204]</sup>. Damage to small and medium-sized bile ducts in PBC and the resultant increased pressure in the biliary system produce reflux of conjugated bilirubin into the plasma. Bile reflux can occur from the bile capillary lumen or hepatocyte into the Disse space and then into blood. Bilirubin accumulations are noted in liver biopsies<sup>[205]</sup>.

In the late stages of the disease, hyperbilirubinemia may be caused by not only bile reflux, but also by increased hemolysis. Enhanced hemolysis is favored by excess serum bile acids in patients with PBC. As potent detergents, bile acids can solubilize the cytoplasm membranes of not only erythrocytes, but also other blood cells, causing them to hemolyze. This favors the development of a number of hematological complications in the end stage of the disease. The magnitude of hyperbilirubinemia at this time is characterized by not only the conjugated fraction of bilirubin, but also by its unconjugated one. Unconjugated and conjugated hyperbilirubinemia is

usually the result of the so-called hepatocellular injury, such as cirrhosis.

Conjugated bilirubin may be also detected in the urine of PBC patients. Moreover, urobilinogen is excreted in the urine in proportion with the amount of bile entering the duodenum<sup>[32]</sup>.

Progressively increased serum bilirubin concentrations in PBC are the best indicator for prognostic purposes<sup>[61,206]</sup>. A study conducted in 1979 claimed that when total bilirubin, a prognostic factor of PBC, is 10 mg/dL or greater, the average survival period is 1.4 years<sup>[207]</sup>. Pretreatment levels of serum bilirubin, bilirubin levels during follow-up, and the occurrence of normal levels of serum bilirubin were significantly associated with prognosis<sup>[208]</sup>.

A number of studies have reported that the Mayo risk score is an effective means of predicting prognoses in PBC patients<sup>[209,210]</sup>. The Mayo Clinic published a study that excluded the invasive technique of biopsy, and predicted prognosis based only on clinical and biochemical factors, including age, serum bilirubin level, serum albumin level, prothrombin time, and existence of peripheral edema<sup>[211]</sup>.

Total bilirubin, the Mayo risk score, and the revised International AIH Group score are significantly important as prognostic factors of PBC<sup>[206]</sup>.

### Activity of ALP, γ-GT, and 5'-NT

Serum ALP and γ-GT levels were markedly elevated in PBC patients<sup>[101]</sup>. The activity of these enzymes increases in patients with PBC already in the asymptomatic stage of the disease<sup>[212,213]</sup>. ALP and γ-GT increase to 10 or more times the upper limit of normal in PBC patients<sup>[61]</sup>. In PBC, there is an increased activity mainly of the hepatic fraction of ALP. The amount of ALP does not correlate with disease progression or stage of the disease<sup>[61]</sup>. Laboratory studies show a rise in the serum ALP level following the onset of pruritus<sup>[203]</sup>. The biochemical levels of ALP and γ-GT were reported to be slightly higher in symptomatic males compared to asymptomatic males, but both were higher than in females<sup>[214]</sup>.

The enhanced activity of ALP and 5'-NT is most likely associated with the increased biosynthesis of phospholipids in PBC patients. The biosynthesis of phospholipids occurs with using of orthophosphate which formed by hydrolysis of organic phosphorus compounds under the action of phosphatases: ALP and 5'-NT. ALP activates the hydrolysis of glycerophosphate, glucose-1-phosphate and glucose-6-phosphate. 5'-NT does that of adenosine monophosphate, guanosine monophosphate, cimetidine monophosphate and uridine monophosphate. It is theorized that ALP and 5'-NT give rise to orthophosphate, where required<sup>[215]</sup>. Based on this theory, the enhancement in the activity of ALP and 5'-nucleotidase in PBC indicates the higher needs of hepatocytes in the orthophosphate

The decreased quantity of orthophosphate in

the hepatic bile portion (Table 3) from PBC patients indicates a reduction in its secretion in bile and simultaneously its reduction in liver cells since the secretion of orthophosphate from hepatocytes to bile capillary are passively effected by the concentration gradient. The lower orthophosphate concentrations (despite the enhanced activity of ALP and 5'-NT) indicate the intensive utilization of a phosphorus group in the metabolic processes which take place in hepatocytes in PBC. One of the possible ways the increased using of orthophosphate in patients with PBC is increase the biosynthesis of phospholipids.

The enhanced activity of ALP and 5'-NT is presumably due to their increased biosynthesis in the hepatocytes<sup>[216-218]</sup>. The mechanism by which cholestasis results in an elevated serum ALP level is thought to involve the induction of ALP synthesis as a result of the enhanced translation of ALP mRNA<sup>[219]</sup>. To enhance the synthesis of these enzymes, it is necessary to increase the delivery of amino acids to the cells. This can be achieved by increasing the activity of  $\gamma$ -GT in PBC patients. Loginov<sup>[212]</sup> has noted that the change in blood  $\gamma$ -GT activity in patients with PBC outstrips the increase in the activities of ALP and 5'-NT. Some PBC patients are observed to have an early increase in the activity of  $\gamma$ -GT (> 3 upper limits of normal) before enhancing the activity of ALP. When the effect of alcohol or medications is ruled out, this test is highly sensitive in identifying cholestasis in PBC patients.

Elevated serum ALP and  $\gamma$ -GT levels, together with a positive AMA/AMA-M2, can help diagnose PBC<sup>[219]</sup>.

### **Aspartate aminotransferase and alanine aminotransferase in PBC**

During the formation of the PBC, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities remain normal or only modestly is increased<sup>[46,203,219]</sup>. The normal (or slightly enhanced) activity of aminotransferases for several years suggests the preserved integrity and normal permeability of the cytoplasmic membrane of hepatocytes in most patients with PBC. The biochemical level of ALT is reported to be slightly higher in symptomatic males than asymptomatic males, but both were higher than in females<sup>[33,214]</sup>. From biological and physiological points of view, the differences between men and women may be explained by differences in the presence of risk factors, protective/aggravating effects of sexual hormones, variances linked to genetics and various corporal structures<sup>[33,220]</sup>.

During the last decade many studies have been designed to identify non-invasive markers capable of providing accurate information about liver fibrogenesis activity and liver fibrosis stage in patients with chronic, potentially progressive hepatic diseases<sup>[35,221]</sup>.

Sebastiani *et al*<sup>[222]</sup> analyze the value of AST/ALT ratio as an indicator of cirrhosis in patients with

PBC. This study includes 160 patients with PBC and laboratory and liver histology data are available for 121 patients. The authors analyze the clinical and laboratory data and follow-up outcomes: liver-related death, liver transplantation and survival. The AST/ALT ratio was also used for assessment in alcohol-induced liver cirrhosis prediction of oesophageal varices and ascites presence. It is suggested that the AST/ALT ratio increases in patients who develop liver cirrhosis, regardless of its cause. The reason for the increased AST/ALT ratio is unknown. It is suggested that the sinusoidal clearance of AST decreases in cirrhotic patients<sup>[223-225]</sup>. The higher AST/ALT ratio in PBC patients may be due to decreased hepatocyte adhesion<sup>[1]</sup>. A quantitative method used to evaluate adhesive interactions of hepatocytes has revealed decreased intercellular interactions, particularly at the site of tight junction, probably due to cholestasis and its related elevated pressure in the bile capillaries<sup>[226]</sup>. The divergence of hepatocytes can be accompanied by the increased permeability of the liver cell cytoplasmic membrane for AST.

Nyblom *et al*<sup>[223]</sup> reported the use of this ratio for discrimination between cirrhotic and non-cirrhotic patients with a sensitivity of 82% and a specificity of 79%, for a cut-off value 1.1. The explanation for such high sensitivity is that a large number of cirrhotic patients were included, while sampling variability in the liver biopsies contributed to low specificity. The study concluded that the AST/ALT ratio is of clinical value as a predictor of cirrhosis in patients with PBC, but not as a prognostic factor.

Alempijevic *et al*<sup>[35]</sup> observed lower sensitivity (47.5%) and specificity (75%) for the AST/ALT ratio for staging the disease that could be explained by different study design. The authors found a statistically significant correlation between PBC stage and AST, ALT to platelet ratio, ALT/platelet count, AST/ALT, ALT/AST and ALT/cholesterol ratios, with the values of Spearman's rho of 0.338, 0.476, 0.404, 0.356, 0.351 and 0.325, respectively. The best sensitivity and specificity were shown for AST/ALT, with an area under ROC of 0.660. The authors suggest that the potential predictive value of aminotransferase and platelet count ratios in predicting the stage of PBC may be used to evaluate PBC evolution, despite their limited sensitivity and specificity, especially when considering their availability and cost effectiveness<sup>[35]</sup>.

LAP is highly active in the liver where it is present mainly in the biliary epithelium. It is an enzyme involved in protein metabolism. The enhanced serum activity of LAP, like 5'-NT and, to a lesser extent,  $\gamma$ -GT, is specific to all forms of intra- and extrahepatic cholestasis. In PBC, there is increased LAP activity, which may indicate bile duct epithelial damage<sup>[71]</sup>.

### **Copper metabolic changes**

The liver is known to play an important role in the



**Table 5** Classification of morphological stages of primary biliary cirrhosis

Ref.	Morphological stages of PBC			
	I	II	III	IV
Popper <i>et al</i> <sup>[233]</sup> , 1970	Cholangitis	Ductular proliferation	Precirrhosis	Cirrhosis
Scheuer <sup>[234]</sup> , 1974	Ductular damage	Ductular proliferation	Scarring	Cirrhosis
Ludwig <i>et al</i> <sup>[235]</sup> , 1978	Portal stage	Periportal stage	Septal stage	Cirrhosis

PBC: Primary biliary cirrhosis.

metabolism of copper, giving rise to hepatocytic protein-copper complexes and its biliary excretion<sup>[227]</sup>. In health, about 80% of copper is excreted from the body into bile and feces.

Liver copper levels are significantly increased in PBC compared with other liver chronic diseases<sup>[228,229]</sup>. In PBC, liver cell copper retention occurs as a complication of impaired biliary copper excretion<sup>[230]</sup>. Elevated blood copper concentrations are due to hepatocyte adhesion impairment and reflux of biliary components into blood. The copper levels may exceed 25 mg/100 g of dry liver tissue (normal range up to 6 mg/100 g) in PBC<sup>[229]</sup>. Despite the retention of copper in hepatocytes, liver cell function is well preserved<sup>[230]</sup> and there are no clinical signs of toxic effects of copper on liver cells.

In PBC patients with increased liver copper concentrations, the latter do not correlate with the biochemical parameters of liver damage<sup>[230]</sup>, but generally do with the stage of the disease<sup>[231]</sup>. The characteristic organelle (nuclear vacuolation and steatosis) changes associated with copper toxicity in Wilson's disease are not observed in PBC<sup>[230]</sup>. A Kayser-Fleischer ring is not detected in patients with PBC. Copper-associated proteins are found in the disease<sup>[227]</sup>. Detection of protein-binding copper in PBC is suggestive of the normal hepatocytic metabolism of copper and its reflux with biliary components into blood. This can account for that there is no Kayser-Fleischer ring or toxic effect of copper on the body.

### Decreased protein synthetic function

The liver is the only site for the synthesis of albumin, fibrinogen, prothrombin, and some other blood coagulation factors. Furthermore, the liver plays a leading role in producing  $\alpha$ -globulins, a major portion of  $\beta$ -globulins, heparin, and enzymes. In patients with early-stage PBC, the blood levels of albumins and globulins are within the normal range. Liver cell synthetic function is well preserved in PBC patients. All the patients have normal prothrombin times and subnormal serum albumin concentrations<sup>[230]</sup>. With progression of PBC observed increased levels of  $\gamma$ -globulins, IgM in particular. At the same time, there is simultaneously a relative decline in blood albumin. A

test for an alkaline albumin fraction is a highly sensitive method in hepatocellular failure. In health, the alkaline fraction accounts for about 3% of a total of albumin. The half-life of the alkaline fraction is longer than that of other albumin fractions; therefore, when its hepatic synthesis is impaired, the percentage of the alkaline fraction is increased (as high as 50% in end-stage cirrhosis). Unfortunately, this test is not practically used in the diagnosis of PBC.

In end-stage disease, impairments in fat-soluble vitamin absorption and hepatic protein synthetic function can result in vitamin K deficiency, coagulopathies, and decreased vitamin A levels, which can promote visual disorder.

The most marked biochemical changes suggesting hepatocytic protein synthetic dysfunction are usually found in more advanced stage (3-4) PBC.

Thus, cholestasis in PBC is accompanied by the enhanced activity of ALP, LAP,  $\gamma$ -GT, 5'-NT; hypercholesterolemia, elevated levels of bile acids, phospholipids, and  $\beta$ -lipoproteins, and hyperbilirubinemia.

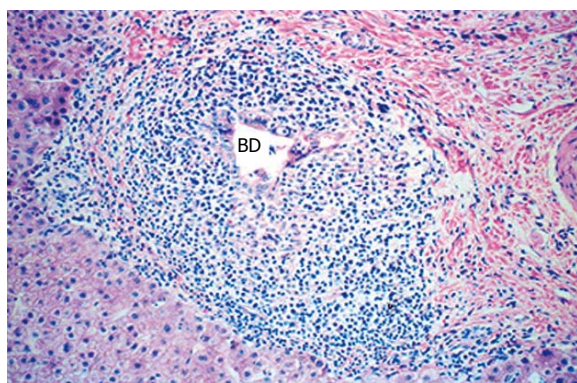
## MORPHOLOGICAL SIGNS OF PBC

Imaging procedures are not helpful for the diagnosis of PBC, except for liver histology. Histologically, PBC is characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts. These changes occur at different rates and with varying degrees of severity in different patients. The loss of bile ducts leads to decreased bile secretion and the retention of toxic substances within the liver, resulting in further hepatic damage, fibrosis, cirrhosis, and eventually liver failure<sup>[3,232]</sup>.

Histological findings after liver biopsy included focal and piecemeal necrosis, portal, periportal, and lobular inflammation, fibrosis and overall inflammation and liver cell damage ("histological activity")<sup>[230]</sup>. The histological manifestations are damaged BECs and infiltration of T cells, B cells, macrophages, eosinophils and natural killer cells in the portal area<sup>[3,6]</sup>. In the field nucleopore frequently detected complexes of autoantibodies with Gp210, p62 and sp100, which to form a ring around the nucleus<sup>[146,227]</sup>. Liver histology in PBC patients is of interest for the assessment of the diagnosis and for staging of the disease<sup>[61]</sup>.

According to its morphological characteristics, PBC is classified in four stages ranging from florid bile duct lesions, ductular proliferation, and fibrosis to liver cirrhosis (Table 5). Stage I-IV disease classification was used by an experienced pathologist who assessed liver biopsies<sup>[236]</sup>.

Stage I is defined by the localization of inflammation to the portal triads. In stage II, the number of normal bile ducts is reduced, and inflammation extends beyond the portal triads into the surrounding parenchyma. Fibrous septa link adjacent portal triads in stage III, while stage IV represents end-stage liver disease characterized by obvious cirrhosis



**Figure 4 Stage I primary biliary cirrhosis.** Inflammatory infiltrate consisting of mononuclear cells around the bile duct (BD). Hematoxylin eosin staining, magnification  $\times 125$ .

with regenerative nodules. The morphogenesis of PBC displays a gradual transition from early to later stages. Liver biopsy specimens from PBC patients can frequently exhibit the histological features of different stages of the disease<sup>[227,237]</sup>. According to Klöppel *et al.*<sup>[238]</sup>, despite this fact, in most cases liver puncture biopsy enables one to reveal the signs characterizing this or that stage of PBC. The histological findings after liver biopsy in PBC patients point to the predominant histological pattern of one of the disease stages.

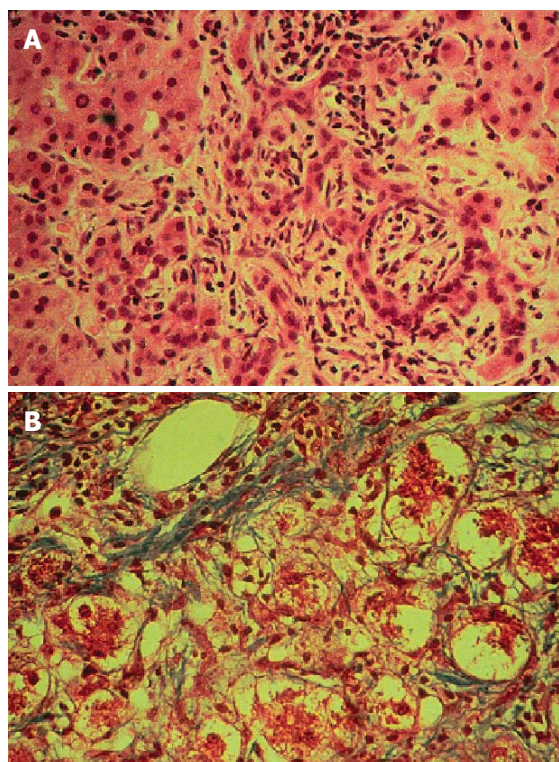
In stages I - II PBC, biopsy specimens show different phases of bile duct injury (Figure 4). According to G. Roschlau, these changes may precede the clinical manifestations of PBC<sup>[239]</sup>. Early injuries develop in the interlobular ducts 45-75  $\mu\text{m}$  in diameter. Dystrophy of ductal epithelial cells should be considered the earliest sign. Their cytoplasm becomes granular or homogenic eosinophilic, turgid, vacuolated; the nuclei get pycnotic. There is further necrosis of a small canalicular segment, but its outlines are still retained and finally the wall is destroyed, giving rise to a pattern of destructive cholangitis<sup>[227]</sup>.

Lymphoid/plasma cell infiltration is observed in the periportal fields around the epithelium lining the bile ducts. Moreover, the epithelial cells appear compressed, in the basal part in particular. Examination of inflammatory infiltrates reveals the elevated CD4 lymphocyte levels exceeding the CD8 lymphocyte counts (4:1).

There are also large lymph follicles, overexuberant portal tract infiltration, sometimes with an impurity of xanthoma cells, as well as some histiocytic and epithelioid cell granulomas.

Damage to the septal or interlobular bile ducts is a pathognomonic sign of PBC, but such changes are rarely found in the puncture biopsy specimens<sup>[240]</sup>. The hepatocytes in this stage have a usual structure; the stellate reticuloendothelial cells are hyperplastic.

Proliferation of ductuli that penetrate the terminal plate, periductal fibrosis, and sclerotic processes to form blind septa are dominant in stages II - III PBC (Figure 5A and B).



**Figure 5 Stages II-III primary biliary cirrhosis.** A: Wide septa formed by proliferating ductuli. Lymphocytic infiltration. Hematoxylin eosin staining, magnification  $\times 200$ . B: Hyaline inclusions. Swelling and hepatocellular injury due to the damaging effect of bile acids. van Gieson's, magnification  $\times 200$ .

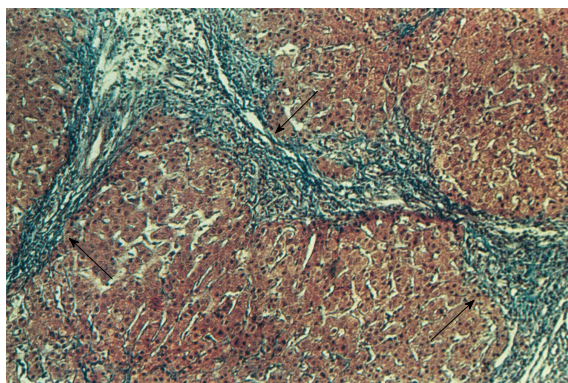
Fibrosis of the portal fields may lead to portal hypertension just as long before liver cirrhosis develops. The epithelium of some proliferating canaliculi is dystrophic. This may be regarded as the presence of signs of an exacerbation or progredient course of disease. Bile ducts not in all portal tracts are detected. Their sites show scars or small groups of epithelial cells. Bile thrombi are seen rarely. The parenchymal structure is usually preserved. Lobular necroses are detectable at the site of destroyed hepatocytes in the liver lobule.

Stage IV PBC is characterized by a pattern of significant micronodular cirrhosis. At the same time, there may also be signs typical of earlier-stage PBC, as well as granulomas. Inflammatory infiltration is found predominantly around the latter of the remaining bile ducts and resolves after their destruction. The developed cirrhosis in PBC patients is often difficult and sometimes impossible to differentiate from liver cirrhosis of another etiology.

Thus, only the last stage of PBC complies with the conventional criteria for cirrhosis. In the others, there is no diffuse fibrosis or nodular transformation, the necessary signs of cirrhosis, which are apparent from its definition. Much significance is attached to piecemeal and bridging parenchymal necrosis in the development of the final stage of liver cirrhosis (Figure 6)<sup>[241]</sup>.

In cholestasis, extralysosomal copper is often present in the hepatocellular cytoplasm<sup>[231]</sup>. Orsein-





**Figure 6 Stages IV primary biliary cirrhosis.** Cirrhotic transformation. Silver impregnation method for Gomori, magnification  $\times 200$ .

positive granules are found in biopsy specimens in cholestatic liver diseases<sup>[242]</sup>. In PBC, these granules are found in the cytoplasm of periportal hepatocytes. As shown by the test with rubean-hydrogen acid, copper is found in combination with these granules. The detection of orsein-positive granules in combination with copper in the cytoplasm of periportal hepatocytes is an additional informative sign in favor of PBC<sup>[242]</sup>.

For diagnosis, staining for copper and for copper-associated protein may assist in the differentiation of PBC from chronic active hepatitis<sup>[231]</sup>. Symptomatic male patients with PBC had more stainable copper deposits in the histological samples than asymptomatic males.

Hepatic damage may produce different consequences in men and women in ongoing primitive diseases and during acquired conditions<sup>[33,220]</sup>. The only histological difference identified is that symptomatic female patients had more piecemeal necrosis of the liver and that symptomatic males had more stainable copper storage than asymptomatic males<sup>[33]</sup>. Additionally, symptomatic females were reported to have more pseudoxanthomatous transformation than asymptomatic females<sup>[33,243]</sup>.

In PBC patients, liver biopsy is used as the gold standard for assessing liver fibrosis. There are four stages of fibrosis in PBC<sup>[244]</sup>: (1) no fibrosis (stage I); (2) periportal fibrosis (stage II); (3) bridging fibrosis (stage III); and (4) cirrhosis (stage IV).

Many fibrosis experts would therefore consider serum fibrosis tests with an ROC area of 0.85-0.90 to be as good as liver biopsy for staging fibrosis<sup>[35,245]</sup>.

Biochemical markers and their ratios do correlate with different sensitivity to and specificity of PBC disease stage. The use of biochemical markers and their ratios in clinical evaluation of PBC patients may reduce, but not eliminate, the need for liver biopsy<sup>[35]</sup>.

New technology has been developed based on the fact that liver stiffness increases as liver fibrosis progresses<sup>[246]</sup>. Transient elastography is a new modality developed for non-invasive evaluation of

liver stiffness. Liver stiffness correlates well with the histological stage of fibrosis. Changes in liver fibrosis stage may thus be estimated non-invasively using transient elastography<sup>[247]</sup>. Nevertheless, further studies are needed to confirm the value of this method in different chronic liver diseases.

## DIAGNOSIS OF PBC

Diagnosis of PBC is based on clinical, laboratory and morphological criteria (Figure 7). In the early stages of PBC, its diagnosis presents no great problems, particularly if at examination a middle-aged woman is detected to have skin itching, more than 6-mo increases in the activity of ALP and/or  $\gamma$ -GT, an AMAs titer of over 1:40, and morphological changes corresponding to nonsuppurative destructive cholangitis<sup>[66,248]</sup>. Currently, the diagnosis of PBC is often made when the patient is still asymptomatic, with abnormal liver biochemistry and/or AMAs<sup>[248,249]</sup>. The symptomatic patients may have fatigue, generalized pruritus, osteoporosis, fat-soluble vitamin deficiencies and portal hypertension<sup>[245,250]</sup>.

Owing to easy-to-use biochemical tests, such as quantification of ALP,  $\gamma$ -GT, bile acids, AST, ALT, and total bilirubin, and other tests for AMAs, ANAs, SMAs, IgM, and IgG, PBC is often diagnosed in its early stages<sup>[206]</sup>. The early criteria for PBC are (1) skin itching; (2) a positive test for AMAs; (3) ALP levels at least two times higher than the upper limit of normal (ULN) and/or  $\gamma$ -GT levels at least five times higher than the ULN; and (4) a liver biopsy specimen showing florid bile duct lesions<sup>[61,118]</sup>.

AMAs detection with evidence of normal ALP activity suggests a lifetime risk for PBC. There is a concurrent increase in ALP and  $\gamma$ -GT activities. The enhanced activity of  $\gamma$ -GT only is not highly specific for PBC and may be caused by alcohol or drugs. That of ALP only may also be due to bone changes, pregnancy, or familial cholestasis.

Chronic cholestasis is a disruption in bile synthesis and outflow for more than 6 mo. Biochemical tests cannot differentiate intra- and extrahepatic cholestasis. Abdominal ultrasonography (AUS), magnetic resonance cholangiopancreatography (MRCP), endoscopic ultrasound (EUS), endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy used to improve bile outflow are performed to evaluate intra- and extrahepatic bile ducts. ERCP is particularly important in the differential diagnosis of primary sclerosing cholangitis (PSC). Laparoscopy allows the presence of stage IV PBC to be specified.

MRCP and EUS are a great advantage in interpreting the biliary system. ERCP may cause pancreatitis (3%-5%), bleeding (2%), and cholangitis (1%), at sphincterotomy in particular. ERCP-related mortality is 0.4%<sup>[251]</sup>.

Liver biopsy is indicated when serum AMAs are

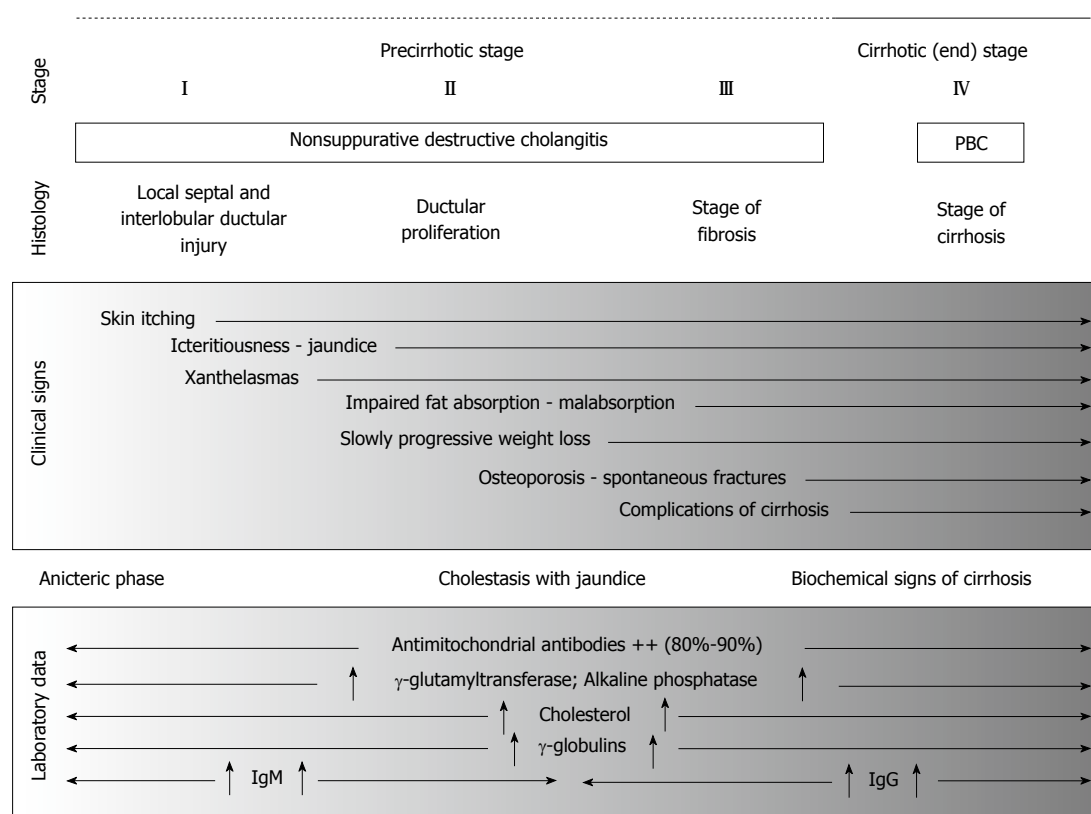


Figure 7 Schematically summarizes the clinical, biochemical, immunological, and morphological signs of primary biliary cirrhosis.

absent in the typical pattern of the disease, when its stage and activity should be determined, or when the overlap syndrome is suggested<sup>[252]</sup>. The PBC-associated morphological changes are characterized by a peculiar mosaicism of lesions and a stereotypy of liver tissue reactions. This is due to the fact that sequential progression from one to another stage may be manifested in other segments of the organ.

## DIFFERENTIAL DIAGNOSIS

PBC should be differentially diagnosed with extrahepatic cholestasis, sarcoidosis, drug-induced hepatitis, PSC, and AIH. Moreover, the diagnosis of PBC in the absence of AMAs may pose particular problems<sup>[47,152]</sup>.

In asymptomatic PBC, it may be difficult to make a differential diagnosis of Paget's disease characterized by enhanced serum ALP activity. Determination of the activities of  $\gamma$ -GT and ALP isoforms permits a reliable diagnosis. Enhanced  $\gamma$ -GT activity is typical of PBC rather than Paget's disease. The determination of hepatic and osseous ALP isoforms reveals an increase in the former in PBC and in the latter in Paget's disease.

AUS, EUS, MRCP, and ERCP data are of decisive importance in differentially diagnosing PBC and extrahepatic cholestasis-accompanied diseases.

The detection of tissue granulomas may suggest cholestatic sarcoidosis. In sarcoidosis, serum AMAs are absent and the Kveim-Siltzbach test is positive

(75%). Great importance is attached to biochemical functional tests and morphological examination of liver biopsy specimens. Thus, well-formed granulomas with minimal bile duct injury are found in sarcoidosis. On the contrary, there may be biliary epithelial changes, mild hepatocyte necrosis, and lymphoid cell infiltration just in the early stages of PBC.

Drug-induced hepatitis may also have a clinical picture similar to that of PBC. In these cases, hepatic damage is associated with the use of drugs and characterized by an acuter onset and rapid development of jaundice (about 4-6 wk).

The etiology of liver diseases concurrent with cholestasis caused by exogenous factors is established by carefully collecting the history data of a patient and those around him; that in viral hepatitis is determined using the serological markers of hepatitis B, C, D and other viruses.

Patient age, the presence or absence of AMAs and other autoantibodies (most commonly, perinuclear antineutrophilic cytoplasmic antibodies), and ERCP or MRCP evidence are of importance for the differential diagnosis of PBC and PSC. The latter affects mainly young or middle-aged men. PSC is characterized by either none or low (< 1:40) AMA titers.

After ERCP and MRCP, PSC patients are noted to have an impaired typical structure of bile ducts as unevenness in the lumen of the common bile duct, deformations of the extra- and intrahepatic bile ducts, and the appearance of well-defined irregular



ity segments, by alternating stenosis and saccular enlargements.

The differential diagnosis of AIH frequently presents problems in advanced-stage PBC. There are simplified criteria for the diagnosis of AIH, such as serum ANAs, SMAs (1:80 or more), antibodies to soluble liver antigen, or autoantibodies to liver/kidney microsomal antigen in a titer of  $\geq 1:40$ ; elevated IgG levels above 1.1 of the upper limit of normal; liver tissue morphological changes corresponding to chronic hepatitis, as well as no markers for hepatitis viruses<sup>[252]</sup>.

Furthermore, PBC is characterized by higher IgM levels than IgG levels, which also enables PBC and AIH to be differentiated<sup>[253,254]</sup>.

When ANAs and SMAs at the diagnostic titer are detected in a PBC patient, the overlap syndrome should be diagnosed<sup>[255]</sup>. Histological examination shows the signs of the two diseases, such as intrahepatic bile duct injury, lymphocytic plasma cell infiltrates, bridging hepatocyte necrosis, etc.

## CONCLUSION

Incidence and prevalence rates of PBC are increasing over time<sup>[15]</sup>. During the last several years, advanced biochemical assays, further delineation of specific liver histological findings, more effective serum autoantibody detection methods and improved diagnostic abilities have led to higher prevalence estimates worldwide<sup>[2,6,22,29,256]</sup>. PBC is diagnosed more frequently than it was a decade ago because of its greater recognition by physicians, the widespread use of automated blood testing and the AMA test, which is relatively specific for the diseases<sup>[35,245]</sup>.

Due to advances in testing methodology, many cases of PBC are diagnosed early in the asymptomatic phase, and early diagnosis of PBC provides a much better prognosis. Early diagnosis and treatment of PBC of UDCA is effective in delaying the development of hepatic fibrosis and esophageal varix<sup>[3,206,257-259]</sup>.

Further improvements in immunological assays for detecting autoantibodies (to bile duct epithelial proteins) will be able to optimize a diagnostic search in cholestatic liver damages in the absence of AMAs, in very advanced-stage PBC, and bile duct injuries of unknown origin.

Clinicians should be practically alert to the need for early diagnosis, during the long latent period of the disease, in the susceptible middle-aged female population to ensure that such subjects do gain benefit from disease-retarding therapy, at whatever stage their disease may be<sup>[260]</sup>. This is already happening to some degree, if we compare what PBC was like 20 years ago<sup>[261]</sup> to what it is today<sup>[262]</sup>.

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## Geographic differences in low-dose aspirin-associated gastroduodenal mucosal injury

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### Abstract

Aspirin, even at low doses, has been known to cause upper gastro-intestinal complications, such as gastroduodenal ulcers, despite the definite benefits from its antithrombotic effects. *Helicobacter pylori* (*H. pylori*) is major pathogen responsible for gastroduodenal ulcer

formation. There have been conflicting results about the potential interaction between these two ulcerogenic factors and the geographic areas involved. In Western countries, the prevalence of gastroduodenal ulcers is consistently higher in *H. pylori*-positive low-dose aspirin (LDA) users than in *H. pylori*-negative ones, suggesting that *H. pylori* infection exacerbates LDA-induced gastroduodenal mucosal injury in these geographic areas. Meanwhile, previous studies from Japan have generally reported a similar prevalence of LDA-induced gastroduodenal mucosal injury regardless of the presence of *H. pylori* infection, indicating that the infection is not an overall exacerbating factor for drug-induced injury. *H. pylori* infection could have a synergistic or antagonistic interaction with LDA use in adverse gastroduodenal events depending on gastric acid secretion. It is well-recognized that the net effect of *H. pylori* infection on gastric acid secretion shows considerable geographic variation at the population level. While gastric acid secretion levels were not decreased and were well-preserved in most patients with *H. pylori* infection from Western countries, the majority of Japanese patients with *H. pylori* infection exhibited decreased gastric acid secretion. Such large geographic differences in the net effect of *H. pylori* infection on gastric acid secretion could be at least partly responsible for the geographically distinct interaction between LDA use and *H. pylori* infection on adverse gastroduodenal lesions.

**Key words:** *Helicobacter pylori*; Low-dose aspirin; Gastric acid secretion; Gastroduodenal ulcers; Geographic variation

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**Core tip:** There have been conflicting results about the potential interaction between these low-dose aspirin (LDA) use and *Helicobacter pylori* (*H. pylori*) infection on the gastroduodenal ulcers and the geographic areas involved. *H. pylori* infection could have a synergistic

or antagonistic interaction with LDA use in adverse gastroduodenal events depending on gastric acid secretion. Large geographic differences in the net effect of *H. pylori* infection on gastric acid secretion could be at least partly responsible for the geographically distinct interaction between LDA use and *H. pylori* infection on adverse gastroduodenal lesions.

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## INTRODUCTION

Use of aspirin at relatively low doses (usually ranging from 75 to 325 mg/d) is now widespread as an anti-thrombotic drug for the prevention of cerebrovascular and cardiovascular diseases<sup>[1-3]</sup>. Although the use of low-dose aspirin for primary and secondary prophylaxis is still less prevalent in Asia than in Western countries<sup>[4]</sup>, aspirin use has also become a common clinical practice among Asian patients with atherothrombotic diseases or multiple cardiovascular risk factors<sup>[5]</sup>. However, despite the well-defined benefits from the antithrombotic effects, aspirin, even at a low dose, has been recognized to yield upper gastro-intestinal (GI) complications such as gastroduodenal ulcers<sup>[6]</sup>. The identification of high-risk groups for low-dose aspirin (LDA)-induced GI mucosal injury and targeted administration of gastro-protective drugs to the high-risk groups is essential for stabilizing prolonged LDA administration, considering that numerous people are now taking aspirin prophylactically.

*Helicobacter pylori* (*H. pylori*) is major pathogen responsible for the formation of peptic ulcers in the upper GI tract. Currently, *H. pylori* infection and the use of non-steroidal anti-inflammatory drugs (NSAIDs), including LDA, have been identified as the two major causes of peptic ulcers<sup>[7]</sup>. It is well-recognized that the *H. pylori* infection rate is still higher in Asian than in Western populations, although the infection rate is decreasing in Asian countries<sup>[8]</sup>. Considering that there could be a potential interaction between these two ulcerogenic factors<sup>[9]</sup>, the prevalence of adverse gastroduodenal mucosal injury in LDA users could be modulated by *H. pylori* infection rates that are different in Asia and Western countries.

As most LDA-induced ulcers are asymptomatic and small, ulcer complications (bleeding and perforation) are the real clinical problems in LDA-induced adverse upper GI events. However, as occurrences of ulcer complications are relatively rare in chronic LDA users<sup>[6]</sup>, endoscopic ulcers are frequently employed as an endpoint for LDA-induced upper GI injury in clinical studies. A recent review of the available literature

suggested that endoscopic ulcers could be a possible surrogate endpoint for upper GI injury<sup>[10]</sup>.

In this study, we surveyed the prevalence of gastroduodenal mucosal injury (mainly endoscopic ulcers and erosions) in chronic LDA users from various parts of the world; we compared the incidence between *H. pylori*-positive and -negative subjects; and we then attempted to estimate future trends of LDA-induced upper GI injury in Asia in the post-*H. pylori* era, where infection rates have decreased globally.

## DIFFERENCES IN THE PREVALENCE OF LOW-DOSE ASPIRIN-INDUCED GASTRODUODENAL INJURY BETWEEN WESTERN COUNTRIES AND JAPAN

There have been 4 prospective studies reporting the prevalence of endoscopic ulcers/erosions in chronic LDA users in European and North American countries<sup>[11-14]</sup>: 2 studies reported the prevalence of endoscopic ulcers<sup>[12,13]</sup>, 1 study reported the combined prevalence of endoscopic ulcers and/or erosions<sup>[11]</sup>, and the remaining study reported the prevalence of ulcers as well as ulcers and/or erosions<sup>[14]</sup>. Overall, these studies reported a relatively high prevalence of endoscopic ulcers from 10.7% to 39.1% and a high combined prevalence of endoscopic ulcers/erosions (47.8% and 68%). However, because of the widespread availability of endoscopic examinations, there have been a relatively large number of studies, 7 in total, reporting the prevalence of endoscopic ulcers and/or erosions among chronic LDA users in Japan<sup>[15-21]</sup>. All studies presented the prevalence of endoscopic ulcers<sup>[15-21]</sup>, and 4 studies also presented the combined prevalence of endoscopic ulcers and/or erosions<sup>[16,18,19,21]</sup>. These studies showed a low prevalence of endoscopic ulcers, from 4% to 20%, although the combined prevalence of endoscopic ulcers and/or erosions was relatively high in the 4 studies, ranging from 29.2% to 57.3%. Taking all the results reported from each geographic area, 101 of 478 of chronic LDA users (21.1%) from Western countries and 266 of 3685 chronic LDA users (7.2%) from Japan suffered from peptic ulcers (Table 1). Thus, although it may be difficult to make a direct comparison due to variations in the study subjects (inclusion and exclusion criteria, proportion of concomitant administration of proton pump inhibitors: PPI) and the study design (e.g., the definition of ulcer, retrospective or prospective sampling) among these studies, there appear to be differences in the prevalence of peptic ulcers among LDA users between Western countries and Japan; that is, the prevalence is likely to be lower in Japan than in Western countries. Meanwhile, the combined prevalence of endoscopic ulcers and/or erosions in LDA users is more comparable between Western countries and Japan (47.8% to 68% vs 29.2% to 57.3%) (Table 1).

**Table 1** Differences in the prevalence of endoscopic ulcers and erosions in low-dose aspirin users between Western countries and Japan

Ref.	Countries	Study design	Sample size	Definition of ulcers	Prevalence of ulcers	Prevalence of ulcers and/or erosions
Kordecki <i>et al</i> <sup>[11]</sup> , 1997	Poland	Prospective	96	Greater than 5 mm	-	68.0%
Pilotto <i>et al</i> <sup>[12]</sup> , 2004	Italy	Prospective	245		25.7%	-
Yeomans <i>et al</i> <sup>[13]</sup> , 2005	Australia, United Kingdom Canada, Spain, Israel	Prospective	187	Greater than 3 mm	10.7%	-
Niv <i>et al</i> <sup>[14]</sup> , 2005		Prospective	46	Greater than 3 mm	39.1%	47.8%
Subtotal			478 ulcers, 142 ulcers and/or erosions		21.1%	61.2%
Shiotani <i>et al</i> <sup>[15]</sup> , 2009	Japan	Prospective	305	Greater than 5 mm	12.4%	-
Nema <i>et al</i> <sup>[16]</sup> , 2009	Japan	Prospective	236	Greater than 5 mm	11.0%	48.4%
Kawai <i>et al</i> <sup>[17]</sup> , 2010	Japan	Prospective	101	Greater than 3 mm	15.8%	-
Tamura <i>et al</i> <sup>[18]</sup> , 2011	Japan	Prospective	150	Greater than 3 mm	4.0%	37.3%
Fujisawa <i>et al</i> <sup>[19]</sup> , 2011	Japan	Retrospective	1213	Greater than 5 mm	5.9%	57.3%
Kawamura <i>et al</i> <sup>[20]</sup> , 2013	Japan	Retrospective	226	Greater than 5 mm	6.2%	-
Uemura <i>et al</i> <sup>[21]</sup> , 2014	Japan	Prospective	1454	Greater than 5 mm	6.5%	29.2%
Subtotal			3685 for ulcers, 3053 for ulcers and/or erosions		7.2%	36.1%

## DIVERSE EFFECTS OF *H. PYLORI* INFECTION ON LOW-DOSE ASPIRIN-INDUCED GASTRODUODENAL INJURY IN WESTERN COUNTRIES AND JAPAN

There have been some studies comparing *H. pylori*-negative and -positive subjects in terms of the prevalence of gastroduodenal mucosal injury among chronic LDA users. These comparisons of the presence or absence of *H. pylori* infection within the same study design should be more reliable than those of the prevalence of events among different studies (Table 2).

Four studies from Western countries have reported the prevalence of different outcomes of LDA-related adverse gastroduodenal events (endoscopic ulcers, endoscopic ulcers and/or erosions, and upper GI bleeding) separately in *H. pylori*-negative and -positive subjects<sup>[11,12,22,23]</sup>. Feldman *et al*<sup>[22]</sup> conducted a prospective comparative study in the United States in which either low-dose aspirin (81 or 325 mg/d) or placebo was administered to 61 healthy volunteers for 45 d. They found that erosive disease from LDA (erosions and/or ulcers) occurred in 50% of *H. pylori*-positive subjects, which was significantly higher than that observed in *H. pylori*-negative subjects (16%) ( $P = 0.02$ ). Lanis *et al*<sup>[23]</sup> conducted a case-controlled study in Spanish patients, in which 98 chronic users of LDA with peptic ulcer bleeding were enrolled as cases and 147 chronic users without the upper GI lesions were also enrolled as controls. They found a *H. pylori* infection rate of 90% in the cases, a rate that was significantly higher than in the controls (69%) ( $P < 0.01$ ) and which corresponded to the prevalence of upper GI bleeding in 46% of *H. pylori*-positive subjects

and in 18% of *H. pylori*-negative ones. Similarly, Pilotto *et al*<sup>[12]</sup> conducted an observational study in Italy, in which 245 chronic LDA users were enrolled and the prevalence of endoscopic ulcers was evaluated. A significantly higher prevalence of peptic ulcers was observed in *H. pylori*-positive than *H. pylori*-negative subjects (37% vs 16%,  $P < 0.01$ ). In addition, Kordecki *et al*<sup>[11]</sup> conducted a prospective observational study in Poland, in which 96 chronic LDA users were enrolled and the prevalence of endoscopic ulcers and/or erosions was evaluated. They found a very high prevalence of the lesions in *H. pylori*-positive patients compared with that in *H. pylori*-negative patients (75% vs 35%,  $P < 0.01$ ). These studies consistently found large differences in the adverse gastroduodenal events between *H. pylori*-negative and -positive LDA users; there is, a significantly higher prevalence in *H. pylori*-positive LDA users than *H. pylori*-negative ones. Thus, *H. pylori* infection clearly exacerbates LDA-induced gastroduodenal mucosal injury in Western countries.

In contrast, the interaction between *H. pylori* infection and LDA use in gastroduodenal mucosal injury is considerably different in Japan than in Western countries. Five studies conducted in Japan investigated the prevalence of endoscopic ulcers in LDA users in the presence and absence of *H. pylori* infection<sup>[15,18,21,24,25]</sup>. In a prospective observational study among 305 chronic LDA users, Shiotani *et al*<sup>[15]</sup> reported a similar prevalence of endoscopic ulcers between *H. pylori*-positive and -negative subjects (13% vs 12%). Likewise, Iijima *et al*<sup>[25]</sup> and Watanabe *et al*<sup>[24]</sup> independently reported a similar prevalence of endoscopic ulcers in a relatively small number of LDA users between *H. pylori*-positive and -negative subjects (17% vs 13% and 20% vs 19%, respectively). In



**Table 2** Diverse effects of *Helicobacter pylori* infection on low-dose aspirin-induced gastroduodenal injury between Western countries and Japan *n* (%)

Ref.	Countries	Subjects	Study design	Outcomes	Prevalence of outcomes ( <i>H. pylori</i> -positive vs -negative)
Kordecki <i>et al</i> <sup>[11]</sup> , 1997	Poland	96 chronic LDA users	Observational	Endoscopic ulcers and erosions	49 (75) vs 11 (35)
Feldman <i>et al</i> <sup>[22]</sup> , 2001	United States	61 healthy volunteers	Interventional study (LDA vs placebo)	Endoscopic ulcers and erosions	11 (50) vs 4 (16)
Lanas <i>et al</i> <sup>[23]</sup> , 2002	Spain	245 chronic LDA users	Case-control	Upper gastrointestinal bleeding	88 (46) vs 10 (18)
Pilotto <i>et al</i> <sup>[12]</sup> , 2004	Italy	245 chronic LDA users	Observational	Endoscopic ulcers	41 (37) vs 21 (16)
Hart <i>et al</i> <sup>[31]</sup> , 2010	Australia, United Kingdom, Canada, Spain,	206 chronic LDA users	Observational	Endoscopic erosions	27 (40) vs 78 (64)
Shiotani <i>et al</i> <sup>[15]</sup> , 2009	Japan	305 chronic LDA users	Observational	Endoscopic ulcers	22 (13) vs 16 (12)
Tamura <i>et al</i> <sup>[18]</sup> , 2011	Japan	150 asymptomatic chronic LDA users	Observational	Endoscopic ulcers	3 (4.4) vs 3 (3.7)
Watanabe <i>et al</i> <sup>[24]</sup> , 2011	Japan	75 chronic LDA users	Observational	Endoscopic ulcers	7 (20) vs 8 (19)
Uemura <i>et al</i> <sup>[21]</sup> , 2014	Japan	1454 chronic LDA users	Observational	Endoscopic ulcers	59 (8.4) vs 35 (4.6)
				Endoscopic erosions	132 (19) vs 293 (39)
Iijima <i>et al</i> <sup>[25]</sup> , 2015	Japan	100 chronic LDA users	Observational	Endoscopic ulcers	10 (17) vs 5 (13)

*H. pylori*: *Helicobacter pylori*; LDA: Low-dose aspirin.

addition, Tamura *et al*<sup>[18]</sup> reported a relatively low overall prevalence of endoscopic ulcers in 150 asymptomatic chronic LDA users, in which the prevalence was similar between *H. pylori*-positive and -negative subjects (4.5% vs 3.7%). Finally, in a multicenter, large-scale study comprising 1,454 chronic LDA users, Uemura *et al*<sup>[21]</sup> demonstrated a higher but non-significant prevalence of endoscopic ulcers in *H. pylori*-positive subjects compared with *H. pylori*-negative ones (8.4% vs 4.6%), in which a multivariate regression analysis indicated a weak but significant positive association between *H. pylori* infection and LDA use for the risk of gastroduodenal ulcers. Nonetheless, this study also demonstrated a much lower prevalence of endoscopic erosions in *H. pylori*-positive subjects compared with *H. pylori* -negative ones (19% vs 39%,  $P < 0.0001$ ). Thus, these studies from Japan generally reported a similar prevalence of LDA-induced gastroduodenal mucosal injury regardless of *H. pylori* infection status<sup>[21]</sup>.

Consequently, while the difference in the prevalence of LDA-induced gastroduodenal injury between Western countries and Japan was relatively small in *H. pylori*-negative subjects (e.g., 16% to 35% vs 4% to 19%), the difference became larger in *H. pylori*-positive subjects (e.g., 37% to 75% vs 5% to 20%). Thus, it seems that *H. pylori*-positive Japanese subjects are more resistant to LDA-induced gastro-duodenal mucosal injury than *H. pylori*-positive Westerners. Although there has recently been a marked decline in *H. pylori* infection rates among the general Japanese population, especially among the young and middle-aged populations, the infection rate is still high (60%) in the elderly<sup>[26]</sup>. Hence, the fact that there are no additional exacerbating effects of *H. pylori* infection on gastroduodenal mucosal injury in Japanese LDA users will likely result in a notably lower prevalence of

gastroduodenal ulcers in Japanese LDA users overall.

The potential diverse effect of PPI administration on the LDA-induced gastroduodenal mucosal injury between *H. pylori*-negative and -positive subjects need to be addressed when comparing the prevalence between the Japanese and Westerners because many of these studies comprised a portion of LDA users with co-treatment of PPI. However, thus far, there has been no consistent conclusion on this issue; that is, although a study reported that PPI treatment is more efficient to suppress LDA-induced adverse gastroduodenal lesions in *H. pylori*-negative subjects than in *H. pylori*-positive ones<sup>[27]</sup>, another study reported the opposite result<sup>[28]</sup>, and the remaining studies have indicated that the treatment is efficient to the same degree regardless of the infection status<sup>[29,30]</sup>. In addition, the different association of *H. pylori* infection with LDA-induced adverse gastroduodenal lesions between Western and Japanese subjects seems to persist in the three studies in which patients with co-treatment of PPI were excluded<sup>[11,22,25]</sup>. Thus, the inclusion of PPI users could have a minimal impact on the geographic difference in LDA-induced adverse gastroduodenal lesions according to *H. pylori* infection status.

It should also be noted that the prevalence of exclusive endoscopic erosions is significantly lower in *H. pylori*-positive subjects than in *H. pylori* -negative ones not only in Japan but also in Western countries. Uemura *et al*<sup>[21]</sup> reported a significantly lower prevalence of endoscopic erosions in Japanese *H. pylori*-positive LDA users compared with *H. pylori*-negative ones as described above (19% vs 39%,  $P < 0.0001$ ). Similarly, in a multinational study comprising Canada, Australia, England, and Spain, Hart *et al*<sup>[31]</sup> reported a significantly lower prevalence of endoscopic erosions in *H. pylori*-positive LDA users compared with

that in *H. pylori*-negative ones (40% vs 64%,  $P = 0.03$ ). Hence, *H. pylori* infection may play a protective role in the formation of gastroduodenal erosions in LDA users regardless of the geographic area; however, the infection affects the subsequent process of ulcer formation differently in Western countries and Japan. The infection could exacerbate the small, eroded mucosal injury (erosions) initially created by LDA more aggressively in Western countries than in Japan.

## ESSENTIAL ROLE OF GASTRIC ACID IN PROVOKING ASPIRIN-INDUCED GASTRODUODENAL MUCOSAL INJURY

Gastric acid plays an essential role as an aggressive factor in upper GI mucosal injury through hydrochloric (HCl) acid back-diffusion into the epithelium, which is also true for aspirin-induced gastric mucosal damage<sup>[7,32]</sup>. In a previous animal model study, parenteral aspirin produced extensive gastric mucosal injury in the presence of luminal acid (pH 1.3), but did not induce gastric mucosal injury with the intragastric instillation of saline (pH 3.7), suggesting that aspirin-induced gastric mucosal injury only occurs in the presence of acid<sup>[33]</sup>. In addition, we recently clarified that individual gastric acid secretion levels in human chronic LDA users is pivotal in determining the extent of aspirin-induced gastric mucosal injury. Whereas the administration of LDA showed only a modest increase in the risk of gastric mucosal injury in the absence of a sufficient level of gastric acid secretion, the dosing greatly elevated the risk in those with sufficient gastric acid secretion<sup>[34]</sup>. Similarly, Nishino *et al.*<sup>[35]</sup>, using 24-h pH monitoring in healthy volunteers, demonstrated that the extent of LDA-related gastroduodenal mucosal injury is positively associated with gastric acidity. Furthermore, the preventive effect of a potent inhibitor of gastric acid secretion, a PPI, on aspirin-induced gastroduodenal mucosal injury also supports this point of view<sup>[27-30]</sup>. Taken together, these studies in an animal model and in humans indicated an essential role of gastric acid in provoking LDA-induced gastroduodenal mucosal injury.

## BIPHASIC EFFECT OF *H. PYLORI* INFECTION ON LOW-DOSE ASPIRIN-INDUCED GASTRODUODENAL INJURY

*H. pylori* infection is known to diversely affect gastric acid secretion; that is, infection could yield an elevation, decline, or no change in gastric acid secretion according to the distribution of inflammation or atrophy within the stomach<sup>[36]</sup>. Given the essential role of gastric acid in the formation of LDA-induced gastroduodenal injury, such diverse effects of *H. pylori* infection on the gastric acid secretion could modulate

the interaction between *H. pylori* infection and LDA use on adverse gastroduodenal lesions.

*H. pylori* infection may be synergistic with LDA use in adverse gastroduodenal events through several plausible mechanisms. NSAIDs/aspirin reduce mucosal capillary blood flow and cause ischemic changes by inducing the adhesion of neutrophils to endothelial cells, leading to neutrophil-mediated tissue injury<sup>[7,37,38]</sup>. Hence, as the neutrophil chemotaxis induced by *H. pylori* infection in the gastric mucosa may exacerbate gastric mucosal injury in aspirin users as shown in a previous animal model study that indicated that although *H. pylori* infection potentiated aspirin-induced gastric mucosal injury, pre-treatment with anti-neutrophil serum attenuated the synergistic action<sup>[39]</sup>. Otherwise, *H. pylori* infection could potentiate LDA-induced gastric mucosal injury by hampering the gastric adaptation to the agent. The normal gastric mucosa could become more tolerant or adapted in response to the repeated administration of noxious agents such as aspirin<sup>[40,41]</sup>. A previous study demonstrated that the presence of *H. pylori* infection significantly impaired such an adaptive response to aspirin, and its eradication restored the response<sup>[42]</sup>. Thus, *H. pylori* infection and aspirin could synergistically potentiate the gastric mucosal injury.

However, such a synergistic interaction could only occur in the presence of sufficient amounts of gastric acid. In the absence of sufficient amounts of gastric acid, this potential synergistic effect could be completely offset, and the *H. pylori* infection could even repress aspirin-induced gastric mucosal injury. Our recent study noted this biphasic effect of *H. pylori* infection on LDA-induced gastric injury<sup>[43]</sup>. In that study, we determined *H. pylori* status and individual gastric acid secretion levels in 93 chronic LDA users, and we classified the drug users into 3 groups: *H. pylori*-negative subjects, *H. pylori*-positive non-hyposecretors, and *H. pylori*-positive hyposecretors. Consequently, setting *H. pylori*-negative patients as the reference, *H. pylori* infection was positively associated with intensive gastric mucosal injury among non-hyposecretors with an odds ratio (OR) of 4.2 and 95%CI of 1.1-17.1, whereas the infection was negatively associated with the injury among hyposecretors with an OR of 0.3 and a 95%CI of 0.1-0.9<sup>[43]</sup>. These findings were supported by those of another study conducted by Shiotani *et al.*<sup>[15]</sup> in Japanese patients, which demonstrated that corpus atrophy serologically defined by the serum pepsinogen value, a well-known surrogate for hypochlorhydria, decreased the risk of aspirin-induced ulcers.

It is well-recognized that the net effect of *H. pylori* infection on gastric acid secretion shows considerable geographic variation at the population level. Gastric acid secretion levels were not decreased and were well-preserved in most patients with *H. pylori* infection from Western countries<sup>[44-46]</sup>. In contrast, the majority

of Japanese patients with *H. pylori* infection exhibited decreased gastric acid secretion<sup>[47,48]</sup>, and the overall gastric acid secretion in *H. pylori*-positive Japanese subjects was half of that in *H. pylori*-negative ones<sup>[49]</sup>. In addition, gastric acid secretion further declines with age in the *H. pylori*-positive Japanese subjects with the advance of gastric atrophic changes<sup>[47]</sup>. Accordingly, gastric acid secretion is profoundly decreased in *H. pylori*-positive Japanese subjects, especially in the elderly, who are frequently administered anti-thrombotic therapy with LDA for the prevention of cerebrovascular and cardiovascular diseases. Thus, such a large geographic difference in the influences of *H. pylori* infection on gastric acid secretion could at least partly account for the different prevalence of adverse gastroduodenal lesions observed in *H. pylori*-positive LDA users in Western countries and Japan.

### HOMOLOGY OF *H. PYLORI*-INDUCED GASTRITIS IN JAPAN AND OTHER EAST ASIAN COUNTRIES

Thus far, there have been no reports from other Asian countries regarding either the prevalence of gastroduodenal ulcers in LDA users and the presence or absence of *H. pylori* infection or the effects of *H. pylori* infection on gastric acid secretion. The distribution of inflammation and mucosal atrophy determine the outcomes of *H. pylori* infection on gastric acid secretion<sup>[36,50]</sup>. There are some studies evaluating international comparisons of histological findings in *H. pylori*-positive subjects. In comparisons among 4 Asian countries (Japan, China, Thailand, and Vietnam), histological findings were similar between *H. pylori*-positive Chinese and Japanese subjects, which were different from those of Thai and Vietnamese subjects<sup>[51,52]</sup>. Another study indicated that the pattern of *H. pylori* infection-induced gastritis may differ between Korean and Japanese patients compared with Americans<sup>[53]</sup>. These results indicated histological homology in *H. pylori*-infected subjects among East Asian countries. This notion is also supported by the fact that a high prevalence of non-cardiac gastric carcinoma is common in *H. pylori*-positive subjects from East Asian countries<sup>[54]</sup>. The strain diversity of *H. pylori* could be responsible for the histological homology in East Asia. For example, *H. pylori*-related virulence factor cytotoxin-associated antigen A (CagA), particularly the more virulent East Asian subtype, is highly prevalent in East Asian countries<sup>[55]</sup>. Hence, gastric acid secretion should supposedly decrease in the presence of the infection in other East Asian populations, such as in Japan, implying that LDA-induced gastroduodenal mucosal injury could be suppressed in *H. pylori*-positive aspirin users in these geographic areas.

### IDENTIFICATION OF HIGH-RISK GROUPS OF ADVERSE GASTRODUODENAL EVENTS AMONG *H. PYLORI*-POSITIVE LDA USERS

The vulnerability of chronic LDA-users to the drug-induced gastric mucosal injury seemed to be largely defined by the individual gastric acid secretion level of *H. pylori*-positive patients. Accordingly, a *H. pylori*-positive gastric hypersecretor could be classified in a high-risk group for adverse gastroduodenal events; hence, these patients should be assigned to concomitant treatment with PPIs even without conventional risk factors (e.g., a history of peptic ulcers) when physicians commence prolonged LDA therapy. Otherwise, eradication therapy might lead to significant long-term preventive effects on LDA-induced gastric mucosal injury among *H. pylori*-positive chronic LDA users with gastric hypersecretion, as in patients with ulcer history<sup>[56,57]</sup>. The extraction of high-risk groups from the general *H. pylori*-positive LDA users by estimating the individual's gastric acid secretion level would be particularly effective in a country such as Japan where the *H. pylori* infection rate is high in the elderly<sup>[26]</sup> and the majority of *H. pylori*-positive subjects exhibit decreased acid secretion levels<sup>[48-50]</sup>.

Gastric acid secretion levels can be roughly estimated by simple serum measurements of pepsinogen concentrations<sup>[58,59]</sup>, and we found that *H. pylori*-positive LDA users with a pepsinogen (PG) I/II ratio of 3.3 or higher, a surrogate marker of gastric hypersecretion, had an extremely high risk for drug-induced gastropathy<sup>[25]</sup>. Moreover, endoscopic findings could also provide some useful information for estimating gastric acid secretion levels. We reported that among non-LDA users, erosion and hematin formation in the antrum could be useful markers for gastric hypersecretors; in particular, the co-existence of these findings identified *H. pylori*-positive gastric hypersecretors with a specificity of 95%<sup>[60]</sup>. Accordingly, the implementation of these tests for *H. pylori*-positive patients before the commencement of prolonged LDA treatment would be helpful to identify a high-risk group of adverse gastroduodenal events.

### FUTURE TREND OF LOW-DOSE ASPIRIN-INDUCED GASTRODUODENAL INJURY IN ASIA

Aspirin is one of the most effective antiplatelet agents for long-term vascular disease prevention in those with a high risk of cardio/cerebrovascular diseases<sup>[61]</sup>, and the administration of aspirin for prevention is consistently recommended by international guidelines<sup>[1-3]</sup>. The mortality and morbidity of acute

coronary diseases are increasing in Asia, and the dominant pattern of cerebrovascular disease in Asia has shifted from hemorrhagic stroke to ischemic stroke<sup>[4]</sup>. Hence, although prophylactic aspirin is still underutilized in Asian countries and is at present prescribed at a 20% to 30% lower rate than in Western countries<sup>[4,5]</sup>, it will be expected to further increase in Asia in the near future. In addition, the accumulating evidence supporting the benefits of aspirin in the prevention of colorectal and other cancers<sup>[62,63]</sup> will likely enhance the demand for aspirin use worldwide further.

In recent decades, the *H. pylori* infection rate has declined in Asian countries, especially among the younger populations; furthermore, the infection rate is likely to also substantially decrease in the elderly<sup>[8]</sup>. Accompanying this change, the prevalence of ordinary *H. pylori*-positive peptic ulcer is declining in Japan<sup>[64]</sup>. However, during this period, the proportion of drug (mainly NSAIDs/aspirin)-induced ulcers has increased<sup>[65,66]</sup>. Considering that *H. pylori* infection could play a protective role in LDA-induced gastroduodenal mucosal injury in the majority of infected subjects in East Asia, we believe that the prevalence of LDA-induced ulcer will not decrease despite the declining rate of *H. pylori* infection. Rather, the increasing use of the drug is likely to increase the prevalence of LDA-induced ulcers in Asian countries. Currently, because *H. pylori* infection could have diverse effects on the LDA-induced adverse gastroduodenal lesions, especially in Eastern Asia, appropriate measures to extract high-risk groups among these patients and administer the concomitant gastro-protective drugs to the targeted subjects need to be established.

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## Basic Study

# Hepatoprotective effect of *Geranium schiedeanum* against ethanol toxicity during liver regeneration

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## Abstract

**AIM:** To evaluate the effect of an extract of *Geranium schiedeanum* (Gs) as a hepatoprotective agent against ethanol (EtOH)-induced toxicity in rats.

**METHODS:** Male Wistar rats weighing 200-230 g were subjected to a 70% partial hepatectomy (PH); they were then divided into three groups (groups 1-3). During the experiment, animals in group 1 drank only water. The other two groups (2-3) drank an aqueous solution of EtOH (40%, v/v). Additionally, rats in group 3 received a Gs extract daily at a dose of 300 mg/kg body weight intragastrically. Subsequently, to identify markers of liver damage in serum, alanine aminotransferase, aspartate aminotransferase, albumin and bilirubin were measured by colorimetric methods. Glucose, triglyceride and cholesterol concentrations were also determined. In addition, oxidative damage was estimated by measuring lipid peroxidation [using thiobarbituric-acid reactive substances (TBARS)] in both plasma and the liver and by measuring the total concentration of antioxidants in serum and the total antioxidant capacity in the liver. In addition, a liver mass gain assessment, total DNA analysis and a morpho-histological analysis of the liver from animals in all three groups were performed and compared. Finally, the number of deaths observed in the three groups was analyzed.

**RESULTS:** Administration of the *Geranium schiedeanum* extract significantly reduced the unfavorable effect of ethanol on liver regeneration (restitution liver mass: PH-EtOH group 60.68% *vs* PH-Gs-EtOH group 69.22%). This finding was congruent with the reduced levels of hepatic enzymes and the sustained or increased levels of albumin and decreased bilirubin in serum. The extract also modified the metabolic processes that regulate glucose and lipid levels, as observed from the serum measurements. Lower antioxidant levels and the liver damage induced by EtOH administration appeared to be mitigated by the extract, as observed from the TBARS (PH-EtOH group 200.14 mmol/mg *vs* PH-Gs-EtOH group 54.20 mmol/mg;  $P < 0.05$ ), total status of antioxidants (PH-EtOH group 1.43 mmol/L *vs* PH-Gs-EtOH group 1.99 mmol/L;  $P < 0.05$ ), total antioxidant capacity values, liver mass gain and total DNA determination (PH-EtOH group 4.80 mg/g *vs* PH-Gs-EtOH 9.10 mg/g;  $P < 0.05$ ). Overall, these processes could be related to decreased mortality in these treated animals.

**CONCLUSION:** The administered extract showed a hepatoprotective effect, limiting the EtOH-induced hepatotoxic effects. This effect can be related to

modulating oxido-reduction processes.

**Key words:** *Geranium schiedeanum*; Liver regeneration; Ethanol; Free radicals; Nuclear factor erythroid-2-related factor 2

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**Core tip:** The geranium is an alternative preventive agent to protect the liver from diverse substances that cause cellular damage, such as ethanol (EtOH). In this paper, according to the phytochemical studies, administering geranium and its compounds, primarily tannins, provided evidence of potentially being protective against liver damage caused by EtOH.

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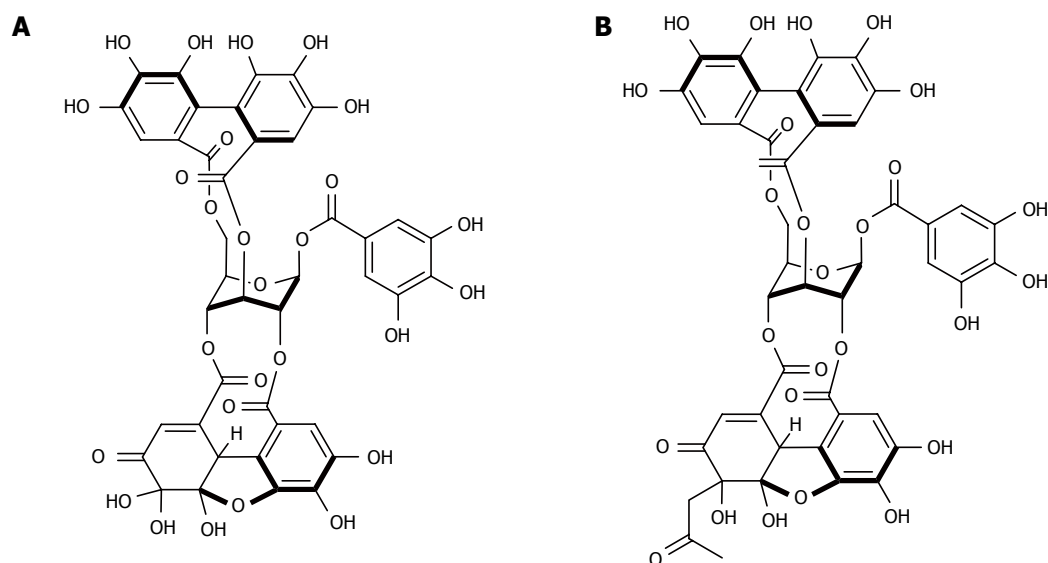
## INTRODUCTION

When there is a lesion in the liver, hepatic regeneration normally presents *via* a complex process that can be stimulated by diverse processes. Experimentally, the most utilized hepatic lesion model is one that is surgically induced through a partial hepatectomy (PH), which restores the liver function and hepatic mass<sup>[1-5]</sup>. Through this procedure, the anatomical (80%) and functional restitution of the regenerating liver occurs approximately 8 d after the PH. Thus, it is a good model for studying the liver regenerative process under physiological and pathological conditions<sup>[1,6,7]</sup>.

Because the liver is the main organ that metabolizes ethanol (EtOH), it suffers the most important harmful effects due to both the molecule and the products of its metabolism, including acetaldehyde and free radicals, which significantly contribute to alcohol-related liver disease<sup>[8-10]</sup>. Various studies have been conducted to identify the adverse effects of EtOH administration on the physiology of this organ and during the liver regeneration process. Alterations in oxidative stress (OS), hepatic metabolism and histological changes, among others<sup>[10-14]</sup> have been identified, with OS being the main component in the physiopathology of alcohol-related liver disease.

Conversely, the use of herbal treatments is increasingly being used to treat diverse pathologies, including hepatopathies. There are reports of the hepatoprotective effects of diverse plants and natural





**Figure 1** Chemical structure of geraniin (A) and of acetonil-geraniin (B).

extracts against agents that induce the production of free radicals, such as EtOH. The bioactivity of these extracts has been shown to be directly related to the sequestering capacity of free radicals<sup>[15-22]</sup>, a property that situates them as an excellent antioxidant.

One order of plants that contain substances with potential antioxidant properties and can therefore function as a hepatoprotector is the geranium (Geraniales)<sup>[23,24]</sup>. Studies are lacking that demonstrate the capacity of this order. Among the compounds that have been attracting attention to these plants are the hydrolyzable tannins, which contain the dehydrohexahydroxydephenyl group, such as geraniin (Figure 1A). These tannins can be isolated as condensed derivatives with acetone and, from some geraniums (Family: Geraniaceae) such as acetonil-geraniin (Figure 1B), compounds that exhibit different characteristics from their precursors under biological conditions<sup>[25]</sup>.

Within the genus *Geranium*, 423 accepted species are distributed into the following three subgenera: *Erodioidea*, *Geranium*, and *Robertium*. To date, eight different species have been classified in the state of Hidalgo, Mexico<sup>[26]</sup>, and only three have been studied so far. Phytochemical studies on these species indicate the invariable presence of geraniin<sup>[23,24]</sup>. From this compound, adducts have been described by condensation with ascorbic acid or acetone<sup>[27]</sup>; however, these acetone condensates are known to be much more stable under pH conditions with solute concentrations similar to those of plasma; thus, they can be adequate alternatives for pharmacological studies<sup>[25,28]</sup>. Currently, tannins are well known for their antioxidant properties<sup>[27,29]</sup>. Tannin-protein complexes in the gastrointestinal tract provide persistent antioxidant activity, yielding the hypothesis that studying an additional genus could reveal a novel, useful hepatoprotective agent to prevent alcohol-related liver damage.

In this study, we evaluated the hepatoprotective

effect of the acetone-water extract of *Geranium schiedeanum* (Gs) on the toxicity induced by EtOH on partial post-hepatectomy liver regeneration in rats.

## MATERIALS AND METHODS

### Animals

We utilized male Wistar rats with an initial body weight (BW) of 200-230 g that were obtained from the Escuela Superior de Medicina (ESM) Bioterium of the Instituto Politécnico Nacional. The rats were housed in cages in the Bioterium (ESM). They were maintained at a temperature of 22 °C with 12-h/12-h light-dark cycles and received standard rat-pellet food (Purina de México, S.A.) and water *ad libitum* prior to the treatments. After 14 d of adaptation, the procedure was initiated. The protocol and the experimental procedures were conducted according to the Mexican Official Norm for the use and care of laboratory animals (NOM-062-ZOO-1999, México)<sup>[30]</sup>.

### Obtaining the extract

We obtained the extract as previously described<sup>[21]</sup>. In brief, 1 kg of the dried and ground aerial parts of *Geranium schiedeanum* were extracted by maceration over 7 d with 20 L acetone-water (at a ratio of 7:3) and were concentrated by reduced pressure until obtaining a volume of 3 L, which was extracted with CHCl<sub>3</sub>, yielding obtaining 12.75 g of F-CHCl<sub>3</sub> and 105 of F-Ac. Twenty grams of F-Ac was submitted to chromatography in a column with Sephadex LH-20, utilizing mixtures of H<sub>2</sub>O-MeOH (1:0; 9:1; 4:1; 7:3; 3:2; 1:1; 2:3; 3:7; 1:4; 1:9, and 0:1) with 300 mL in each one (Gayosso-de-Lucio *et al.*<sup>[21]</sup> 2014). The fractions were grouped based on their chromatographic profiles using thin-layer chromatography (TLC), and subsequent chromatographies (silica gel and C-18) achieved identification of the following four

majority components: ellagic acid, gallic acid, 3-O-a-L arabinofuranoside-7-O-a-L-ramnopyranoside of Kaempferol, and geranium acetonitrile. Notably, the latter represents approximately 40% of the F-Ac; thus, we suggest that it is the active compound<sup>[28]</sup>.

### Surgical procedures

Surgical removal of two-thirds of the liver using a technique known as a PH was performed according to the procedure described by Higgins and Anderson<sup>[1]</sup>. The surgical procedures were performed between 08:00 and 10:00 am under light anesthesia with ethyl ether. As a surgical control, we utilized sham rats on which we only carried out laparotomy, without removing the hepatic mass.

### Experimental design

After the surgical procedure, the rats were housed individually. They were grouped ( $n = 5-6$ , for each experimental group) in the following manner: (1) Control group (sham); (2) group with PH; (3) group with PH plus intragastric (ig) administration of EtOH (PH-EtOH); (4) group receiving a hepatectomy, the Gs extract and EtOH (PH-Gs-EtOH); and (5) group receiving a PH and the Gs extract (PH-Gs). The rats in all groups received food and water throughout the treatment period.

EtOH-treated animals received an ig dose of 1.5 g/kg BW (an EtOH solution at 40% in isotonic saline solution), equivalent to blood alcohol values between 75 and 150 mg/dL, which have been reported to be capable of inhibiting the liver regenerative process, as reported previously<sup>[7,31,32]</sup>. The geranium extract dose was 300 mg/kg BW ig, as reported previously<sup>[21]</sup>. All treatments (EtOH solution and geranium extract) were administered daily for 7 d.

### Serum and liver samples

On day 8, the animals were sacrificed by decapitation after being previously anesthetized with pentobarbital sodium (40 mg/kg BW). Blood samples were obtained and centrifuged in a clinical centrifuge to obtain the sera, which were frozen at  $-70^{\circ}\text{C}$  for later use. The liver was isolated, weighed, rapidly placed in cold phosphate-buffered saline solution (PBS) solution with a phosphate tampon, pH 7.5), and washed to completely eliminate the blood. The liver was placed in 9 volumes of cold buffer (sucrose 0.25 mol/L, TRIS 10 mmol/L, EGTA 0.3 mmol/L, and bovine serum albumin 0.2%, pH 7.4). The liver was homogenized using a homogenizer with a piston-type driver with a Teflon tip. The homogenate was divided into aliquots and frozen at  $-70^{\circ}\text{C}$  until later use. The total concentration of protein of the homogenate was determined by the method of Lowry<sup>[33]</sup>, utilizing bovine serum albumin (BSA) solution as a standard.

### Parameters of liver regeneration

Liver regeneration was determined by calculating the

liver restitution weight and determining the total DNA concentration. After the rats were killed, the liver of each animal was resected and washed as previously described. To estimate the percentage of restitution of the hepatic mass, we employed a previously reported procedure<sup>[34]</sup>. For this, we proceeded as follows: the resected liver was weighed, and that weight was divided by 0.7 to obtain the estimate of the initial weight (pre-PH) of each liver. The percentage of restituted hepatic mass was calculated using the following formula: remnant liver weight/initial liver  $\times 100$ <sup>[34]</sup>. The results of each group were utilized to calculate the average restitution of hepatic mass in each corresponding group. The DNA concentration was determined in liver samples according to the technique of Labarca and Paigen<sup>[35]</sup> as modified by Ramírez-Farías *et al.*<sup>[6]</sup>.

### Liver histology

Hepatic samples from each group were used for the light microscopy. Samples were fixed with formaldehyde (10% in isotonic solution), embedded in wax, and stained with hematoxylin-eosin. Biopsy specimens were coded and read blindly without knowledge of the other data by independent observers at two different laboratories (J.A.M.G and J.B.R.). The criteria used to analyze the morphological abnormalities were the same as those reported by Morales-González *et al.*<sup>[10]</sup> as follows: fatty infiltration (+, mild; ++, moderate; +++, severe; and +++++, very severe); inflammation (+, zonal localization, focal inflammatory cells; ++, moderate, not restricted to one zone of the acinus; +++, diffuse); and hepatocellular disorganization (+, isolated foci in zone 3 of the liver acinus; ++, more widespread; and +++, definitively diffused in the hepatic acini).

### Determination of enzymes and metabolites in serum

The activities of serum alanine aminotransferase [ALT; expansion coefficient (EC) 2.6.1.2] and aspartate aminotransferase (AST, EC 2.6.1.1) were measured colorimetrically using diagnostic kits (Spinreact de México, SA de CV), following the manufacturer's instructions; the results are reported in units/L.

Serum concentrations of glucose, triacylglycerides, cholesterol, bilirubin, and albumin were determined by spectrophotometric techniques using diagnostic kits (Spinreact de México, SA de CV), following the instructions provided by the manufacture; the results are reported in mg/dL, except for albumin, which is reported in g/dL.

### Total antioxidant status in serum

The total antioxidant status (TAS) was determined utilizing the Randox Kit (Randox Laboratories Ltd., United Kingdom) and is reported in mmol/L.

### Total antioxidant capacity in the liver

The total antioxidant capacity (TAC) was determined

**Table 1** Liver regeneration parameters in each experimental group after 7 d of treatment with partial hepatectomy, ethanol, and the *Geranium schiedeanum* extract

Group	Mortality (%)	Resected liver mass (g)	Final liver weight (g)	Restitution of liver mass (%)	mgDNA/g liver
Sham	-	-	9.410 ± 0.47	100	4.40 ± 0.35
PH	0	6.185 ± 0.30	6.705 ± 0.29	75.89 ± 3.5	9.20 ± 0.20 <sup>a</sup>
PH-EtOH	33 <sup>c,e</sup>	6.374 ± 0.29	5.525 ± 0.27 <sup>a,c,e</sup>	60.68 ± 2.8 <sup>c,e</sup>	4.80 ± 0.15 <sup>c,e</sup>
PH-Gs-EtOH	0	6.797 ± 0.33	6.721 ± 0.31	69.22 ± 1.4	9.10 ± 0.27 <sup>a</sup>

Values are expressed as the mean ± SE in each experimental group ( $n = 5-6$ ). <sup>a</sup> $P < 0.05$  vs sham group; <sup>c</sup> $P < 0.05$  vs PH group; <sup>e</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

**Table 2** Histopathological changes induced by partial hepatectomy, ethanol and *Geranium schiedeanum* extract treatment in liver cells

Group	Fatty change	Inflammation	Hepatocellular disorganization
Sham	0	0	0
PH	+	0	0
PH-EtOH	++/+++	++/+++	0
PH-Gs-EtOH	+	+	+ / ++

Histopathological parameters were evaluated as described in the Material and Methods. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

using a BioAssay Systems DTAC-100 (CA, United States), and the result is reported in  $\mu\text{mol/mg}$  (Trolox).

#### Determination of thiobarbituric acid reactive substances

We determined thiobarbituric acid reactive substances (TBARS) using the DTBA-100 Assay Kit (BioAssay Systems, CA, United States), following the manufacturer's instructions and reporting results in  $\mu\text{mol/mg}$  of protein.

#### Assay of hepatic enzymes

Enzymes in the samples of liver homogenate were determined according to standard techniques described previously<sup>[6,10,36-38]</sup>. We determined the specific activity of the following enzymes: ALT (EC 2.6.1.2) and AST (EC 2.6.1.1). The result is expressed as  $\mu\text{mol/min}$  per milligram of protein.

#### Statistical analysis

The results were analyzed using Sigma Plot ver. 12.3 statistical program software. The results are expressed as the mean ± SE, as required. We carried out a statistical analysis using Student's *t*-test and/or analysis of variance (ANOVA). We considered differences among the groups to be statistically significant when  $P < 0.05$ .

## RESULTS

#### Effect of Gs extract on liver regeneration

The PH-Gs group did not show differences compared

with the PH group in terms of any study indicator, which demonstrated that the Gs extract does not exert a toxic effect, in agreement with the previously reported results<sup>[21]</sup>.

#### Effect of ethanol and Gs extract on the survival and parameters of liver regeneration

Survival as well as changes in liver regeneration indicators and DNA concentrations in hepatic tissue for each study group are depicted in Table 1. The PH-EtOH group presented a significant diminution in hepatic indicators (Table 1) compared with the PH group. Similarly, we observed that the PH-EtOH group resulted in a 30% mortality rate compared with the PH group ( $P < 0.05$ ). In contrast, administration of the Gs extract (PH-Gs-EtOH) significantly diminished mortality ( $P < 0.05$ ) compared with the PH-EtOH group.

With respect to the weight gain of the liver, the experimental group treated with EtOH in combination with the Gs extract (PH-Gs-EtOH) demonstrated a restored weight gain in the liver, obtaining values comparable to those of the PH group; the difference between the groups was 6.67%. Conversely, the PH-EtOH group had an increase of only 60.68% in restoring hepatic mass with the latter significantly lower than the pH group (75.89%;  $P < 0.05$ ). Additionally, we observed that treatment with EtOH significantly diminished the concentration of DNA compared with the PH group (4.80 mg DNA/g vs 9.20 mg DNA/g,  $P < 0.05$ ). In contrast, the PH-Gs-EtOH group showed a value of 9.10 mgDNA/g.

#### Effect of Gs extract on histological indicators (fatty change, inflammation, and hepatocellular disorganization)

In Table 2, we can observe the effect exerted by Gs on histological changes during liver regeneration and ethanol administration. A significant increase in the parameters of fatty change and inflammation in the PH-EtOH group was observed compared with the PH group. In the PH-Gs-EtOH group, however, administering geranium significantly diminished the increases in fatty change and inflammatory histological parameters caused by ethanol. In addition, the geranium extract moderately increased hepatocellular

**Table 3** Concentrations of serum metabolites in diverse study groups

Group	Glucose (mg/dL)	Cholesterol (mg/dL)	Triacylglycerols (mg/dL)
Sham	120 ± 10.50	57 ± 7.80	76 ± 3.0
PH	112 ± 5.30	41 ± 2.50	44 ± 4.05 <sup>a</sup>
PH-EtOH	86 ± 4.20 <sup>a,c,e</sup>	34 ± 4.3 <sup>a,e</sup>	43 ± 6.0 <sup>a</sup>
PH-Gs-EtOH	124 ± 8.45	55 ± 2.81 <sup>c</sup>	39 ± 0.88 <sup>a</sup>

Metabolites are expressed as mean ± SE ( $n = 5-6$ ). <sup>a</sup> $P < 0.05$  vs sham group; <sup>c</sup> $P < 0.05$  vs PH group; <sup>e</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

**Table 4** Serum concentrations of albumin and bilirubin in different study groups

Group	Albumin (g/dL)	Bilirubin (mg/dL)
Sham	3.03 ± 0.12	0.07 ± 0.06
PH	2.85 ± 0.15	0.13 ± 0.01 <sup>a</sup>
PH-EtOH	2.72 ± 0.05 <sup>a,c</sup>	0.15 ± 0.06 <sup>a</sup>
PH-Gs-EtOH	3.00 ± 0.10	0.11 ± 0.08 <sup>a</sup>

Metabolites are expressed as mean ± SE ( $n = 5-6$ ). <sup>a</sup> $P < 0.05$  vs sham group; <sup>c</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

disorganization.

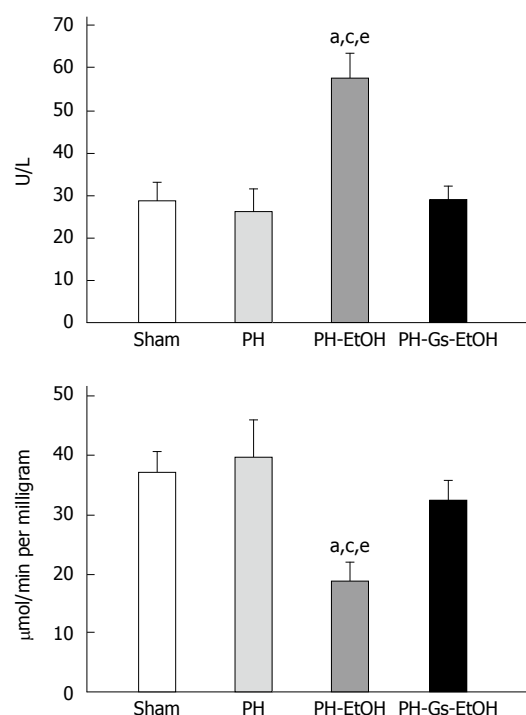
### Effects of treatment with Gs extract on serum concentrations of glucose, triacylglycerides, and cholesterol

Table 3 illustrates the concentrations of serum metabolites whose metabolism primarily occurred in the liver on 8 d in all of the experimental groups. In the PH group, the glucose levels exhibited similar levels to those of the sham group 8 d post-surgery. In contrast, the PH-EtOH group presented a decrease in serum glucose levels compared with those of the sham (120 mg/dL;  $P < 0.05$ ) and the PH (112 mg/dL;  $P < 0.05$ ) groups. In contrast, the PH-Gs-EtOH group had normalized glucose levels, which differed from the PH-EtOH group (124 mg/dL vs 86 mg/dL,  $P < 0.05$ ).

The concentration of serum cholesterol in the PH-EtOH group (34 mg/dL) was statistically significant compared with those obtained in the sham (57 mg/dL;  $P < 0.05$ ) and the PH (41 mg/dL;  $P < 0.05$ ) groups. Conversely, the concentration of serum cholesterol in the PH-Gs-EtOH group (55 mg/dL) was found to be similar to the sham group (57 mg/dL). Independently to the experimental group, the concentration of triacylglycerides was found to be lower than that of the sham group (Table 3).

### Effects of treatment with Gs extract on serum concentrations of albumin and bilirubin

The results of the metabolic integrity of the liver are presented in Table 4. Similar concentrations of bilirubin and albumin in serum were present in the sham group and the PH group. In contrast, in the PH-EtOH group,



**Figure 2** Aspartate aminotransferase activity in serum (upper panel) and in the liver (lower panel) in distinct study groups. Values are expressed as mean ± SE in each experimental group;  $n = 5-6$ . <sup>a</sup> $P < 0.05$  vs sham group; <sup>c</sup> $P < 0.05$  vs PH group; <sup>e</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

a significant decrease in the serum concentration of albumin (2.72 g/dL vs 3.03 g/dL,  $P < 0.05$ ) and an increase in bilirubin (0.15 mg/dL vs 0.07 mg/dL,  $P < 0.05$ ) occurred compared with the PH group. However, in the PH-Gs-EtOH group, we found that the albumin concentration increased to 3.0 g/dL but that the bilirubin value decreased to 0.11 mg/dL.

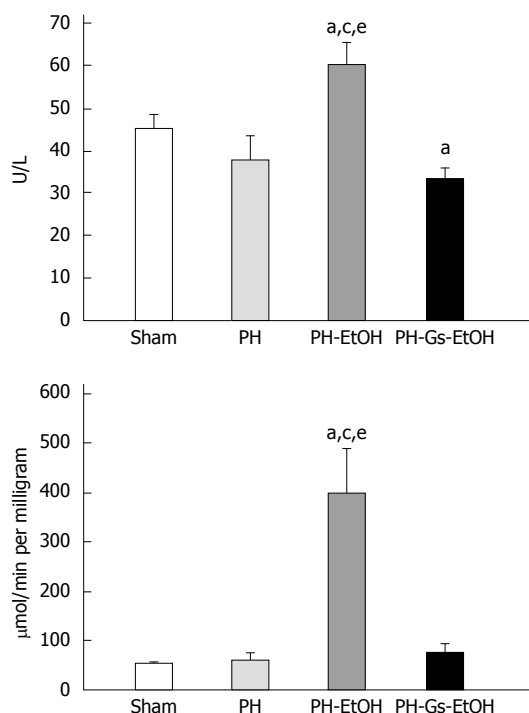
### Activity of ALT and AST in serum and the liver after treatment with Gs

The effect of the Gs extract was evaluated through determining the activity of ALT and AST because these enzymes classically reflect liver function that is dependent on morphofunctional integrity.

Figure 2 depicts the AST activity in serum (upper panel) and the liver (lower panel) in the diverse experimental groups. Whereas the AST activity was not different in the PH group compared with the sham group, alcohol administration in rats with PH induced a significant increase in AST activity in serum on day 7 post-surgery (Figure 2, upper panel;  $P < 0.05$ ). In addition, AST activity in the liver presented the following behavior (Figure 2, lower panel): the PH-EtOH group showed a decrease in AST activity compared with the sham (50%;  $P < 0.05$ ) and the PH (53%;  $P < 0.05$ ) groups. In contrast, the PH-Gs-EtOH group presented levels similar to those in the sham group.

Figure 3 illustrates the ALT activity in serum (upper panel) and in the liver (lower panel) in the





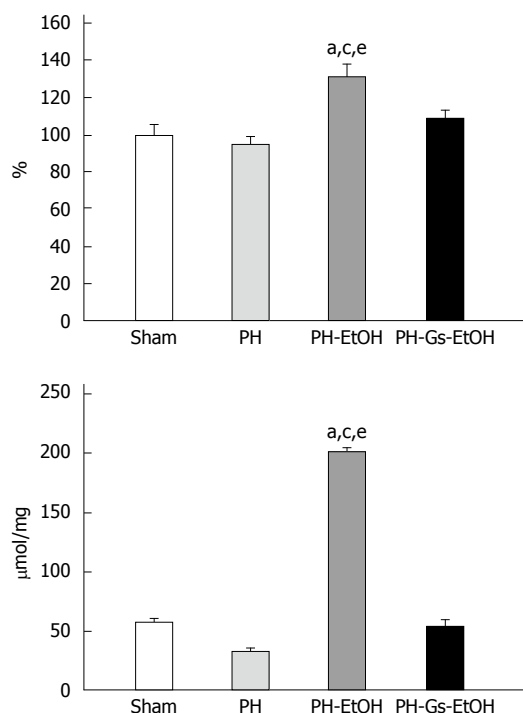
**Figure 3** Alanine aminotransferase enzymatic activity in serum (upper panel) and in the liver (lower panel) in distinct study groups. Values are expressed as the mean  $\pm$  SE ( $n = 5-6$ ). <sup>a</sup> $P < 0.05$  vs sham group; <sup>c</sup> $P < 0.05$  vs PH group; <sup>e</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

experimental groups. In both cases, there was a significant increase in ALT activity in the PH-EtOH group compared with the sham and PH groups. In serum (upper panel), the activity of the enzyme in the sham group was 45 U/L, whereas a decrease was observed in the PH group (37.5 U/L) and an increase was observed in the PH-EtOH group (60 U/L;  $P < 0.05$ ). ALT activity in the liver (lower panel) in the sham group was 53.23  $\mu\text{mol/min per milligram}$  without a significant difference in the PH group (60.08  $\mu\text{mol/min per milligram}$ ); conversely, the PH-EtOH group reported a significant increase compared with the two prior groups (399.75  $\mu\text{mol/min per milligram}$ ;  $P < 0.05$ ).

In contrast, rats in the PH-Gs-EtOH group exhibited a significant decrease in serum ALT levels, reaching values comparable to those reported for the sham group. When comparing the ALT levels of this group with those of the group administered EtOH, we observed the following findings: serum (upper panel), PH-EtOH group 60 U/L vs PH-Gs-EtOH group 33 U/L;  $P < 0.05$ ; liver (lower panel), PH-EtOH group 399.75  $\mu\text{mol/min per milligram}$  vs PH-Gs-EtOH group 75.20  $\mu\text{mol/min per milligram}$ ;  $P < 0.05$ .

#### Effect of Gs extract on the TBARS concentration in rats with PH and treatment with EtOH

To evaluate the damage produced by reactive oxygen species (ROS), we determined the TBARS concentration in the liver and serum of animals treated with EtOH and the Gs extract. Regarding the TBARS



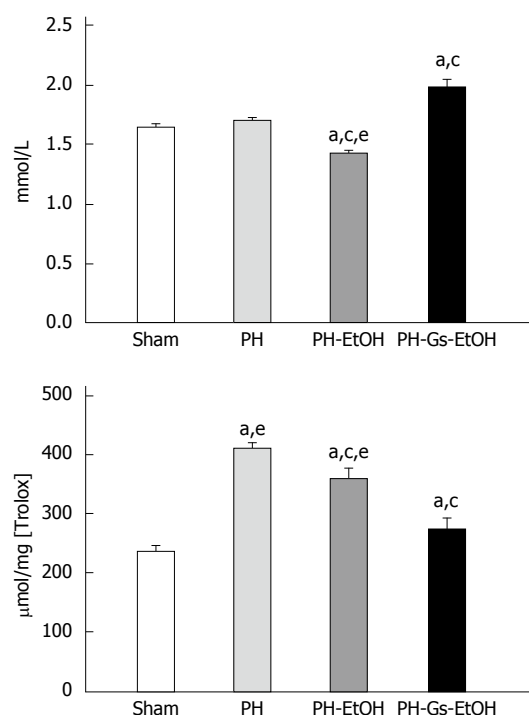
**Figure 4** Thiobarbituric acid reactive substances concentration in serum (upper panel) and in the liver (lower panel) during liver regeneration and intoxication with ethanol. Values are expressed as the mean  $\pm$  SE ( $n = 5-6$ ). <sup>a</sup> $P < 0.05$  vs sham group; <sup>c</sup> $P < 0.05$  vs PH group; <sup>e</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

concentrations in serum (Figure 4, upper panel), the PH-EtOH group presented a significant increase of 32% relative to the sham group. In contrast, when the Gs extract was administered to EtOH-intoxicated rats, there were no differences between the serum TBARS concentrations compared with those observed in the PH and sham groups. The hepatic concentrations of TBARS in the different study groups are presented in Figure 4 (lower panel). As observed in the figure, there was an increase in TBARS in the PH-EtOH group of 200.14 mmol/mg, which was 3.5 and 6.1 times greater in comparison with the sham group (56.07 mmol/mg) and the PH group (32.38 mmol/mg), respectively. In contrast, the PH-Gs-EtOH group demonstrated a decrease in the hepatic concentration of TBARS (54.20 mmol/mg); this result was significant compared with the PH-EtOH group (54.20 mmol/mg vs 200.14 mmol/mg,  $P < 0.05$ ).

#### Effect of treatment with Gs extract on the TAS and TAC concentrations

In Figure 5, the levels of TAS are shown as quantified in serum (upper panel) and TAC as determined in the liver (lower panel).

The level of TAS diminished significantly due to the administration of EtOH (1.43 mmol/L) compared with the sham group (1.65 mmol/L;  $P < 0.05$ ) and the PH group (1.7 mmol/L;  $P < 0.05$ ). Administration of the Gs extract resulted in a concentration of 1.99 mmol/L in the PH-Gs-EtOH group; this finding was significant



**Figure 5** Effect of the *Geranium schiedeanum* extract on the concentration of the total antioxidant status in serum (upper panel) as well as the total antioxidant capacity in liver (lower panel) on liver regeneration and intoxication with ethanol. Values are expressed as the mean  $\pm$  SE ( $n = 5-6$ ). <sup>a</sup> $P < 0.05$  vs sham group; <sup>b</sup> $P < 0.05$  vs PH group; <sup>c</sup> $P < 0.05$  vs PH-Gs-EtOH group. PH: Partial hepatectomy; EtOH: Ethanol; Gs: *Geranium schiedeanum*.

compared with the sham group, as can be observed in Figure 5 (upper panel).

In Figure 5 (lower panel), the levels of TAC determined in the liver are illustrated. The regenerative process increased the TAC levels, as observed in the PH group compared with the sham group as follows: 409  $\mu\text{mol/mg[Trolox]}$  vs 237  $\mu\text{mol/mg[Trolox]}$ , respectively. Conversely, EtOH administration diminished the TAC levels in the PH-EtOH group (360  $\mu\text{mol/mg[Trolox]}$ ) compared with the PH group. Finally, in the PH-Gs-EtOH group, we found a values of 275  $\mu\text{mol/mg[Trolox]}$ .

## DISCUSSION

Chronic degenerative diseases are increasingly exhibiting an increase in morbidity and mortality. Among these pathologies, cirrhosis of the liver and liver cancer have been found to represent a public health problem in Mexico and worldwide<sup>[39]</sup>. One of the diverse causal agents of these diseases is chronic consumption of alcohol; according to the World Health Organization, alcohol consumption has increased in recent years<sup>[40,41]</sup>. A good model for studying the mechanisms of damage due to EtOH and the hepatoprotective effect of diverse agents is liver regeneration induced by PH in rats<sup>[1-3]</sup>. Liver regeneration is a highly regulated process in which diverse molecular changes and metabolic adjustments intervene, which have been characterized by an increase in DNA synthesis and cellular

proliferation<sup>[42]</sup>. Acute EtOH administration negatively alters PH-induced liver regeneration, resulting in a decrease in cellular regeneration parameters in the liver remnant<sup>[10,43]</sup>. However, a 1-1.5-g/kg dose of EtOH has been shown to strongly inhibit thymidine kinase and thymidylate synthase enzymatic activity in the remnant liver of rats subjected to PH<sup>[10,44]</sup>. These enzymes are closely related to the synthesis of DNA. Due to the high morbidity and mortality represented by hepatic cirrhosis, studying therapeutic agents that can prevent or limit the damage caused by EtOH to the liver is necessary. A relatively new field consists of using phytochemicals that protect the liver from damage caused by EtOH. In a recent study, we reported the chemical composition and protective effects that are possessed by the Gs extract on damage caused by thioacetamide to the liver<sup>[21]</sup>. Therefore, in this work, we evaluated the use of the Gs extract as an alternative in liver lesions utilizing the liver regeneration model in rats, seeking to form part of the pioneering reports aiming to show the potential relevance of using phytochemical extracts to treat hepatopathies.

Weight gain correlates with DNA concentration, both of which are cellular proliferation parameters (Table 1). The increase in DNA concentration is due to the activity of thymidine kinase and thymidylate synthase enzyme, which increase in the early phase of PH-induced liver regeneration<sup>[10,45]</sup>. One of the mechanisms of damage by EtOH to the hepatocyte, which inhibits the proliferative process of this organ, is the production of free radicals<sup>[6,46-48]</sup>; this inhibition is reverted by the use of diverse antioxidant agents such as vitamins<sup>[6,47,48]</sup>. The results demonstrate that in animals with only PH, the DNA concentration is twice as high as that of the sham group (Table 1). The concentration of DNA diminished in EtOH-treated PH rats, which correlates with a gain in liver weight; however, in the PH-Gs-EtOH group, weight gain and DNA concentrations were restored to levels similar to the PH group. This result and the previous studies support the possible protective effect of antioxidants on liver regeneration because of their capacity to eliminate the free radicals formed by EtOH metabolism; however, there are differences in the protective capacity of each antioxidant. Thus, it is noteworthy that there are differences in their physicochemical properties, dose, administration route, and mechanism of action; thus, it is necessary to conduct more studies that are targeted at finding the best protective agents. Our data demonstrate the protective effect of Gs extract on inhibiting the effects of EtOH on liver regeneration, which has not been previously reported, to the best of our knowledge.

Morphologically, on 8 d after PH, cellular changes were no longer observed. The administration of ethanol during liver regeneration resulted in the presence of fat drops and moderate-to-severe grade inflammation (Table 2). However, when the Gs

extract was administered, the accumulations of fat drops and inflammation were similar to those in the group in which only PH was performed. Additionally, structural changes in hepatocellular disorganization occurred because of the Gs extract administration. These changes were associated with weight gain of the remnant liver and the concentration of DNA in the hepatocytes (Table 1) and were in direct correlation with the proliferative process, which agrees with the previously reported results<sup>[10,14]</sup>.

A good indicator of the harmful effect of EtOH on hepatocyte function during liver regeneration are the serum metabolites, such as glucose, triacylglycerides, and cholesterol, which directly regulate this organ.

We previously demonstrated that the regenerative process of the liver in itself results in a decrease in serum concentrations of glucose and cholesterol and an elevation in triacylglyceride levels that return to their basal levels after completing the regenerative process<sup>[10,46]</sup>. However, a high dose of EtOH (5 g/kg) administered immediately after PH has been shown to increase serum levels of glucose and triacylglycerides<sup>[38]</sup>. In contrast, administering a low dose of EtOH (1.5 g/kg) at different post-PH liver regeneration time points causes a decrease in serum concentration of these metabolites<sup>[10,46]</sup>. Some reports<sup>[10,34]</sup> have demonstrated that EtOH consumption increases lipid deposits in the liver and diminishes protein synthesis; thus, this finding can be associated with a decrease in serum cholesterol in the EtOH-treated rats compared with the sham group. Orrego *et al.*<sup>[34]</sup> also suggested a reduction in cholesterol transport through the organ due to the decrease in the proteins required for transport. Our results demonstrate that treatment with EtOH (1.5 g/kg) for 7 d caused a decrease in serum concentrations of the three metabolites (glucose, cholesterol, and triacylglycerides) to levels lower than those of the sham group. This decrease in triacylglyceride concentration was correlated with a decrease in liver regeneration (liver weight and DNA concentration gain) because the increase in serum triacylglyceride levels was found to be a consistent characteristic of hepatic proliferation advancement, which has been attributed to liver permeability for the lipids<sup>[49]</sup>. In our study, administration of the Gs extract normalized the serum concentrations of these metabolites (Table 3), which is in agreement with findings by Nakanishi *et al.*<sup>[50]</sup>, who compared the protective effect of three compounds (geraniin, ellagic acid, and gallic acid) on liver damage caused by carbon tetrachloride, D-galactosamine, and thioacetamide. Three hepatotoxic compounds were found to increase serum levels of triacylglycerides and cholesterol, but pre-treatment with geraniin and ellagic acid avoided this increase, instead exhibiting a protective effect against the cellular damage in the liver caused by carbon tetrachloride, D-galactosamine, and thioacetamide. Similarly, it was reported that the compounds contained in the Gs extract were

gallic acid, ellagic acid, and acetonyl-geraniin, and a flavonoid, 3-O $\alpha$ -L-arabinofuranoside-7-O- $\beta$ -D-ramnoside of kaempferol, which possesses a protective effect according to previous results<sup>[21]</sup>.

EtOH has been thought to be an important cause of damage to hepatic cells. The hepatic metabolites (albumin and bilirubin) are indicators of good liver functioning and can be utilized to identify the protective effect of the Gs extract. Serum concentrations of albumin and bilirubin and AST and ALT enzymatic activities are the most sensitive evidence of diagnosing hepatic diseases<sup>[51]</sup>. In this study, we observed that rats treated with EtOH presented with a decrease in serum albumin concentration and an increase in bilirubin concentration (Table 4), which is in agreement with the results reported previously by our group<sup>[10,46]</sup>. These studies demonstrate a hepatotoxic effect of EtOH; the decrease in serum albumin is possibly directly related to a decrease in ATP generated in the liver; this effect is due to EtOH consumption and not to the PH, in which the level of this protein is similar to the control group<sup>[10]</sup>. In our study, we observed that in the PH-Gs-EtOH group, serum concentrations of albumin and bilirubin were normalized (Table 4). The latter is most likely due to the protective effect of the Gs extract on liver damage caused by EtOH during liver regeneration, which, in turn, is most likely due to the antioxidant effect of the extract's components. Various reports have demonstrated the protective effect of antioxidants (*e.g.*, Vitamin C, Vitamin E, glycine, geraniin, and ellagic acid) on the diverse toxic, free radical-generating agents that cause damage to the liver, including the following agents: EtOH, carbon tetrachloride, D-galactosamine, and thioacetamide<sup>[10,46,50,52]</sup>. Among the biological results, we found a normalization of albumin and bilirubin levels in serum. In our work, we found a protective effect in the liver of the Gs extract, which is similar to the findings reported for other antioxidants, because of the components of the extract, most likely the acetonyl-geraniin component.

The release of AST, lactic dehydrogenase, ALT, glutamate dehydrogenase (GDH), and ornithine transcarbamylase enzymes by the hepatocyte and the increase in their serum enzymatic activity post-PH of 70% has been reported previously<sup>[6,10,37,46,53]</sup>. The increase of enzymatic activity in serum during liver regeneration has been interpreted in the following two ways: first, as a consequence of a necrotic event in the liver and second, as an increase in the permeability of the cellular and mitochondrial membrane.

In previous studies, alcohol administration in early stages of liver regeneration in rats with PH has been shown to diminish serum activity of these enzymes, and this activity was not related to liver necrosis but with the selective release of these enzymes during liver regeneration<sup>[10]</sup>. This activity could be a mechanism of interorgan signaling, which depends on the dose and the EtOH administration route and on the liver regeneration stage being studied. In fact, the increase

in serum activities of some enzymes post-PH could be due to a release from the damaged cells or from cells with alterations in permeability, resulting in greater synthesis and enzyme release<sup>[10]</sup>.

The enzymatic rise in serum in late stages of liver regeneration when EtOH was administered daily for 1 wk can most likely be attributed to damage to the structural integrity of the liver cells. As a result, these enzymes are found in the cytoplasm and are released into the circulation after cellular damage; EtOH could possibly damage other organelles such as the mitochondria, causing their enzymes [GDH, AST, and malate dehydrogenase (MDH)] to be released into the blood. This finding indicates that this xenobiotic causes damage to the plasma membrane as well as to the mitochondrial membrane<sup>[6,37,46,54]</sup>. In the PH-Gs-EtOH group, we observed a decrease in the activity of AST and ALT enzymes in serum, suggesting a possible capacity of the extract to preserve the normal structure of the liver (Figures 2 and 3).

Our results correlate with previous reports by various authors. Gayosso-De-Lucio *et al.*<sup>[21]</sup> reported that Gs pre-treatment for 3 d resulted in a decrease in serum AST and ALT enzymatic activity after elevation by thioacetamide administration, concluding that this extract protects against liver damage caused by thioacetamide. Nakanishi *et al.*<sup>[50]</sup> compared the protective effect of the three compounds (geraniin, ellagic acid, and gallic acid) on the damage caused to the liver by carbon tetrachloride, D-galactosamine, and thioacetamide; they found that geraniin and ellagic acid significantly decreased serum levels of both enzymes, which had been increased by exposure to these hepatotoxic agents. Both studies were conducted when the liver was not regenerating; however, our study was performed when this process was present, induced by PH, and when EtOH and the extract were administered at the same time.

Some mechanisms by which EtOH inhibits liver regeneration are known to be due to the increase of free radicals produced by EtOH metabolism, causing cellular damage as well as altering liver functions<sup>[55]</sup>. Our results suggest that the Gs extract eliminates free radicals and consequently eliminates the inhibiting effect of EtOH on liver regeneration, thereby diminishing damage in the cellular membrane and consequently lowering the concentration of lipid peroxidation in serum as well as in the liver, as can be observed in Figure 4, which correlates with findings reported by various authors<sup>[21,50,52]</sup>.

Alterations in TAS, together with an important increase in the concentration of TBARS, are characteristic of OS<sup>[56]</sup>. As noted, our results indicate that OS was caused by the administration of EtOH, similar to the findings reported by our group<sup>[6]</sup>. Some investigators have used these to understand other types of xenobiotics, such as paracetamol and thioacetamide<sup>[21,57]</sup>.

The administration of xenobiotics (EtOH, paracetamol, thioacetamide) during PH-induced liver

regeneration promotes an increase in antioxidant mechanisms as a system of defense in an attack of the ROS that are produced during the OS generated by these hepatotoxins<sup>[20]</sup>. The decrease in TAS (serum) and increase in TAC (liver) found in our study (Figure 5) agree with previous studies, indicating that EtOH favors OS in this model, as demonstrated by the high TBARS levels found (Figure 4). The increase in the TBARS concentration indicates severe damage in cellular membranes throughout the organism, in experimental models with laboratory animals, and with alcohol-related liver disease. Ingesting antioxidant supplements has been shown to significantly diminish the TBARS levels in serum and restore antioxidant mechanisms to their normal levels in the liver<sup>[6,46]</sup>. Our results suggest that the Gs extract increases the antioxidant defenses (TAS and TAC), importantly diminishing OS (TBARS), thus improving liver function (*e.g.*, albumin, bilirubin, and triacylglycerides) and the liver's proliferative capacity (weight and DNA gain).

The biological importance of this work consists of contributing some advances in the mechanisms of action implied in the hepatoprotective effect of Gs against the toxic action of alcohol. We found that Gs possesses an antioxidant effect *in vitro* that is most likely also expressed *in vivo*, as proposed for other species<sup>[58]</sup>, which indicates the need to conduct more studies to confirm this effect. In addition, to our knowledge, there are no biological or pharmacological reports about its use. Our results also constitute, to our knowledge, the first report of the protective effect of Gs (most likely through the acetonyl-geraniin component) on damage caused by EtOH on liver regeneration. This study contributes new knowledge on a potential therapeutic alternative to treat alcohol-related liver damage and is relevant to public health because this damage constitutes one of the main causes of morbidity and mortality worldwide.

In summary, we have addressed the protective effect shown by the geranium extract on the damage caused by ethanol on liver regeneration. However, knowing the cellular or molecular mechanisms of action of the phytochemicals is important and will be an interesting area for future studies. Various reports have demonstrated that the polyphenol compounds act to activate the nuclear factor erythroid-2-related factor 2 (Nrf-2) transcription factor and/or directly as free radical scavengers. Thus, molecular and cellular studies will be needed to study the specific effect of the components of Gs, such as acetonyl-geraniin, on the regulation of the Keap1-Nrf2-ARE pathway and the principal antioxidant enzymes and their gene expression, amount of protein and specific activity.

## COMMENTS

### Background

Hepatopathies associated with consuming alcohol comprise a group of diseases of interest due to their great social impact. Plant extracts have been



poorly applied as a protective agent against the damage caused by alcohol consumption. This article describes the hepatoprotective effect of an extract of *Geranium schiedeanum* (Gs) on a model of regenerative liver.

### Research frontiers

Studying the effect of natural products on limiting the damaging effect of alcohol consumption and the mechanisms for this protective effect is currently a hot topic.

### Innovations and breakthroughs

This article presents an innovative approach for evaluating the hepatoprotective effects of plant extracts. This approach includes biochemical markers of metabolism, antioxidative performance and damage in serum and the liver and observations on the mortality of the studied animals.

### Applications

The results presented in this article strongly suggest the use of geranium extract as a hepatoprotective agent. Moreover, this application can be extended to other processes in which limiting oxidative phenomena can be beneficial.

### Terminology

Partial hepatectomy is an operation consisting of removing the median and left lateral lobes of the liver. Liver regeneration after partial hepatectomy in the rat has been widely employed as an experimental model to study mammalian cell proliferation.

### Peer-review

The authors investigated the protective effect of a Gs extract (exGs) in regenerative livers of rats receiving ethanol as hepatotoxin. For this purpose, they measured alanine aminotransferase, aspartate aminotransferase, albumin, bilirubin and some markers of carbohydrates and lipid metabolism in plasma as well as a biochemical, particularly antioxidant profile and histological consequences in the liver. Rats treated chronically with ethanol and exGs showed a significant decrease in mortality, oxidative stress and biochemical parameters of liver damage compared with rats that were not treated with exGs. In addition, the treatment with exGs was associated with an increase in liver regeneration.

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## Basic Study

# TLR2 and TLR4 polymorphisms influence mRNA and protein expression in colorectal cancer

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**Author contributions:** Proença MA planned and conducted the study, collected and interpreted the data, drafted and wrote the manuscript; de Oliveira JG collected data on genotyping of *TLR2* and *TLR4* polymorphisms in the control group; Cadamuro ACT collected data on immunohistochemistry of both *TLR2* and *TLR4* proteins; Succi M collected data on CRC samples; Netinho JG collected data on CRC samples; Goloni-Bertolo EM and Pavarino EC planned the study; Silva AE conceived and planned the study and critically revised the manuscript.

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## Abstract

**AIM:** To evaluate the effect of promoter region polymorphisms of toll-like receptor (*TLR*)2-196 to -174del and *TLR4*-1607T/C (rs10759932) on mRNA and protein expression in tumor tissue and of *TLR4*+896A/G (rs4986790) on colorectal cancer (CRC) risk.

**METHODS:** The *TLR2*-196 to -174del polymorphism was investigated using allele-specific polymerase chain reaction (PCR) and the *TLR4*-1607T/C and *TLR4*+896A/G by PCR-restriction fragment length polymorphism (RFLP). We genotyped 434 DNA samples from 194 CRC patients and 240 healthy individuals. The mRNA relative quantification (RQ) was performed in 40 tumor tissue samples by quantitative PCR TaqMan assay, using specific probes for *TLR2* and *TLR4* genes, and *ACTB* and *GAPDH* reference genes



were used as endogenous controls. Protein expression was analyzed by immunohistochemistry with specific primary antibodies.

**RESULTS:** No association was found for *TLR4-1607T/C* and *TLR4+896A/G* by three statistical models (log-additive, dominant and recessive). However, based on dominant and log-additive models, the polymorphic variant *TLR2-196* to *-174del* was associated with increased CRC risk [dominant: odds ratio (OR) = 1.72, 95%CI: 1.03-2.89;  $P = 0.038$  and log-additive: OR = 1.59, 95%CI: 1.02-2.48;  $P = 0.039$ ]. *TLR2* mRNA expression was increased in tumor tissue (RQ = 2.36) when compared to adjacent normal tissue (RQ = 1;  $P < 0.0001$ ), whereas the *TLR4* mRNA showed a basal expression (RQ = 0.74 *vs* RQ = 1,  $P = 0.452$ ). Immunohistochemistry analysis of TLR2 and TLR4 protein expression was concordant with the findings of mRNA expression. In addition, the *TLR2-196* to *-174del* variant carriers showed mRNA relative expression 2.19 times higher than wild-genotype carriers. The TLR2 protein expression was also higher for the *TLR2-196* to *-174del* variant carriers [ $117 \pm 10$  arbitrary unit (a.u.) *vs*  $95 \pm 4$  a.u.,  $P = 0.03$ ]. However, for the *TLR4-1607T/C* polymorphism no significant difference was found for both mRNA ( $P = 0.56$ ) and protein expression ( $P = 0.26$ ).

**CONCLUSION:** Our findings suggest that *TLR2-196* to *-174del* polymorphism increases *TLR2* mRNA expression and is associated with higher CRC risk, indicating an important role in CRC genetic susceptibility.

**Key words:** Toll-like receptor 2; Toll-like receptor 4; Colorectal cancer; Protein expression; Gene expression; Genetic polymorphisms

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**Core tip:** This study investigated the influence of the toll-like receptor (*TLR*)2 and *TLR4* functional polymorphisms on mRNA and protein expression levels in colorectal cancer samples and the association of these polymorphisms with the risk of developing this neoplasm. Increased expression of TLR2 (mRNA and protein) in tumor tissue was observed compared with adjacent normal tissue. Moreover, for the first time, the polymorphism *TLR2-196* to *-174del* was associated with a higher risk of developing this type of cancer, and *TLR2-196* to *-174del* allele carriers showed mRNA relative expression approximately two times higher than wild-genotype carriers. Thus, functional polymorphism in *TLR2* may change gene expression levels, accentuating inflammation and aggravating the development of colorectal cancer.

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## INTRODUCTION

Chronic inflammation has emerged as one of the main risk factors and features of cancer. It can affect any stage of tumorigenesis, generating a microenvironment conducive to tumor development and progression, and promoting the survival, proliferation and migration of cancer cells<sup>[1-3]</sup>. Thus, many cancers can arise from local irritation, inflammation and chronic infection<sup>[4,5]</sup>. The inflammatory process occurs through a network of chemical signals that initiate and maintain a host immune response in order to heal the affected tissue<sup>[4]</sup>. The activation of innate and adaptive immune responses is the main mechanism involved in the homeostasis alteration caused by tumors in adjacent tissues<sup>[6,7]</sup>.

Colorectal cancer (CRC) is one of the main examples of the inflammation-cancer association<sup>[4]</sup>. Moreover, experimental models have provided evidence that innate immune system chemical mediators and bacterial toxins play key roles in CRC development<sup>[8,9]</sup>. CRC reports show a yearly worldwide incidence of approximately 1 million cases and a mortality rate of over 500000, representing the second or third leading cause of cancer-related death in many countries<sup>[10,11]</sup>. In 2012, the estimated incidence of CRC in Brazil was 16368 cases in men and 17581 cases in women, and 8549 men and 9058 women died of the disease<sup>[12]</sup>. Compared to the hereditary form, sporadic CRC (SCRC) is the most common type, accounting for more than 90% of cases.

As the intestine is under a constant inflammatory process due to the presence of microorganisms and their pathogen-associated molecular patterns (PAMPs)<sup>[13]</sup>, changes in proteins or receptors involved in the inflammatory and immune responses may contribute to an increased risk of developing cancer<sup>[14]</sup>. In this respect, the toll-like receptor (*TLR*) family that encodes type I transmembrane proteins plays an essential role in pathogen recognition by the extracellular matrix<sup>[15]</sup>, leading to activation of innate and adaptive immune responses and to a process of controlled inflammation<sup>[10,16]</sup>. The first step in the interaction of commensal bacteria with the intestinal epithelium is their recognition by TLR2 and TLR4 receptors, which recognize lipoproteins and PAMPs, and lipopolysaccharides (LPS) of Gram-negative bacteria, respectively<sup>[17,18]</sup>. TLRs activate the nuclear factor kappa B (NF- $\kappa$ B) pathway, the main regulatory inflammation signaling pathway, and this activation is involved in the pathogenesis of CRC<sup>[19,20]</sup>. Certain TLRs have been reported to play a role in bowel diseases, and it is believed that one of these roles is to induce



**Table 1** Characteristics of patients with colorectal cancer and controls *n* (%)

Variables	CRC	Controls	OR (95%CI)	P value
Individuals, <i>n</i>	194	240		
Age (yr)			0.4513 (0.3063-0.6649)	
< 60	72 (37.1)	136 (56.7)		< 0.0001
≥ 60	122 (62.9)	104 (43.3)		
mean ± SD	62 ± 12	56 ± 18		
Variation	24 to 88	20 to 93		
Gender			0.8619 (0.5898-1.259)	0.4988
Female	89 (45.9)	119 (49.6)		
Male	105 (54.1)	121 (50.4)		
Smoking habit			1.910 (1.299-2.808)	0.0013
Non-smokers	101 (52.1)	87 (36.3)		
Smokers	93 (47.9)	153 (63.7)		
Alcoholic habit			0.4963 (0.3305-0.7454)	0.0010
Non-alcoholic	114 (58.8)	178 (74.1)		
Alcoholics	80 (41.2)	62 (25.9)		

CRC: Colorectal cancer; OR: Odds ratio.

cell death by apoptosis of neoplastic cells<sup>[10]</sup>.

Genes *TLR2* (4q32) and *TLR4* (9q33.1) are highly polymorphic, which may cause changes in protein expression or function<sup>[21]</sup>, resulting in a differentiated inflammatory response that in turn can influence the progression of several cancer types, such as CRC<sup>[22-26]</sup>.

Among the *TLR2* polymorphisms, a 22 bp deletion at position -196 to -174 of the promoter region appears to produce a reduction in gene transcription activity<sup>[21]</sup>. Variations in the *TLR2* gene have been associated with susceptibility to various infectious and inflammatory diseases<sup>[27]</sup> and some types of cancer associated with inflammation<sup>[26,28]</sup>. Similarly, the *TLR4* gene also shows polymorphisms located in the promoter region, such as -1607T/C (rs10759932), still poorly studied in association with cancer<sup>[29-31]</sup> and no reported studies on CRC. A *TLR4* polymorphism that has been largely studied and has received special attention consists of the substitution of an aspartic acid residue for glycine in amino acid 299 (Asp<sup>299</sup>Gly) corresponding to *TLR4* +896A/G polymorphism (rs4986790), but the results regarding its association with infectious diseases and cancer are still controversial<sup>[26,32-35]</sup>. However, alterations in *TLR4* expression levels have been reported to influence the innate immune response and to be potentially related to variation in the promoter sequence, with susceptibility to human diseases such as cancer<sup>[35]</sup>.

Therefore, we aimed to evaluate whether common gene variants involved in the inflammatory response, such as *TLR2*-196 to -174del and *TLR4* -1607T/C occurring in the promoter region influence gene expression in tumor tissue and whether these functional polymorphisms together with the *TLR4* +896A/G were associated with colorectal cancer risk.

## MATERIALS AND METHODS

### Approval and consent

This study was approved by the Ethics in Research Committee IBILCE/UNESP, nº027/11 (protocol: 0009.0.229.000-11). All participants gave written informed consent, and the epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions on current and past occupation, smoking habits, alcohol intake and family history of cancer or adenomatous polyps and lesions.

### Study populations

This case-control study comprised 434 individuals (Table 1). The case group (CRC) consisted of 194 samples from patients with a confirmed diagnosis of sporadic CRC by clinical histopathological parameters, 160 of which were studied based on samples of peripheral blood and 40 on samples of biopsies or surgical fragments (6 patients had both biopsy and blood samples) and normal adjacent mucosa (105 men and 89 women; mean age: 62 ± 12 years). All CRC samples were collected between December 2010 and August 2012 at the Cancer Institute (ICA) and the Proctology Service of the Base Hospital in São José do Rio Preto, SP, Brazil and all required information on clinical histopathological parameters was obtained from the patients' medical records. The inclusion criterion was as follows: patients with sporadic cancer and the exclusion criterion was patients with hereditary cancer. The control group (C) consisted of 240 healthy blood donors (121 men and 119 women; mean age: 56 ± 18 years), according to the criteria described in a previous study<sup>[26]</sup>, whose DNA samples from leukocytes were stored in our laboratory.

With regard to risk factors, statistically significant differences were found between the group aged younger than 60 years and the group greater than or equal to 60 years, and between non-smokers and smokers and non-alcoholics and alcoholics. Gender was not significantly different between the groups, according to Fisher's exact test (Table 1).

### Nucleic acid extraction

DNA was extracted from peripheral blood leukocytes of the CRC group according to the technique of Miller *et al.*<sup>[36]</sup>, with modifications (using Ficoll-Paque™ PLUS to separate the blood phases), and stored at -20 °C for subsequent genotyping. Simultaneous extraction of total RNA and DNA from tissue samples was performed, using the Trizol® reagent protocol.

### Polymorphism genotyping

To investigate the *TLR2*-196 to -174del polymorphism,

**Table 2** Polymerase chain reaction-restriction fragment length polymorphism conditions, primers sequences, restriction enzyme and fragment sizes

Polymorphisms	Primers (5'-3')	Enzymes T°/time	Fragment (bp)	Ref.
<i>TLR2</i> -196 to -174 <del>del</del>	F: CACGGAGGCAGCGAGAAA R: CTGGGCCGTGCAAAGAAG	-	<i>ins</i> : 286 <i>del</i> : 264	[59]
<i>TLR4</i> +896A/G (rs4986790)	F: AGCATACTTAGACTACCACCTCGATG R: GTTGCCATCCGAAATATAAGAAAAG	<i>Bst</i> XI 37 °C / 3 h	A: 131 G: 108; 23	[60]
<i>TLR4</i> -1607T/C (rs10759932)	F: TTTGTATAATTGACTACCATTCGCT R: CATTTTTCACATCTTCACCAGC	<i>Hha</i> I 37 °C / 3 h	T: 139 C: 117; 22	[30]

*ins*: Wild-type allele (insertion); *del*: Polymorphic allele (deletion); bp: Base pairs; T°: Temperature; F: Forward; R: Reverse.

allele-specific PCR was performed, and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) was used to assess the *TLR4* +896A/G and *TLR4* -1607T/C polymorphisms (Table 2). For both PCR techniques, the reaction solution contained the following: 1X buffer, 0.10 µmol/L of dNTPs, 0.5 µmol/L of MgCl<sub>2</sub>, 0.5 µmol/L of each primer, 1 U of Taq DNA polymerase, 13.3 µL of ultrapure Milli-Q H<sub>2</sub>O, and 200 ng of genomic DNA. The material was processed in an automatic thermocycler and for *TLR4* +896A/G and *TLR4* -1607T/C the material was also subjected to enzymatic digestion. The amplification products of polymorphism *TLR2*-196 to -174~~del~~ and the digestion products of *TLR4* polymorphisms +896A/G and -1607T/C were visualized on 3% agarose 1000 gel (Invitrogen®) stained with ethidium bromide in the presence of a 100 bp molecular marker. To ensure greater genotyping reliability, a positive control was included in all reactions, consisting of a sample that was heterozygous for the polymorphism under evaluation.

#### mRNA relative quantification by quantitative PCR

A reverse transcriptase reaction was performed using a High Capacity cDNA kit (Applied Biosystems). The cDNA was validated by PCR amplification of a 613 bp fragment of the *ACTB* gene (β-actin). A quantitative PCR (qPCR) reaction was performed by means of a TaqMan® gene expression assay (Applied Biosystems), using specific probes for genes *TLR2* (Hs\_00610101m1 inventoried) and *TLR4* (Hs\_01060206m1 inventoried). Both reference genes *ACTB* (Catalog#: 4352935E) and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) (Catalog#: 4352934E) (Applied Biosystems) were used as endogenous controls in all analyses.

The reactions were performed in triplicate, using 25 ng of cDNA in StepOnePlus™ Real-Time PCR equipment (Applied Biosystems), and in all experiments a negative control was used to determine contamination. Relative quantification (RQ) was calculated using the 2<sup>-ΔΔCt</sup> method<sup>[37]</sup> compared to both reference genes, using four pools of normal adjacent tissue samples as a calibrator, grouped relating to polymorphism *TLR2*-196 to -174~~del~~ and *TLR4* -1607T/C genotypes, located in the promoter region (pool1 and pool4: *ins*/

*ins* + T/T; pool2: T/C + C/C; pool3: *ins*/*del*).

RQ was also calculated for the samples stratified by polymorphism genotypes in the promoter region (*TLR2*-196 to -174~~del~~ and *TLR4* -1607T/C). They were grouped according to genotypes for each polymorphism separately (at least one polymorphic allele vs wild homozygote). The tumor tissue samples with wild genotype were used as a calibrator in comparison with those with at least one polymorphic allele.

#### Protein expression by immunohistochemistry

Immunohistochemical analysis was performed using a total of 20 tumor and normal adjacent samples. Consecutive 4 µm-thick sections were cut from each trimmed paraffin block. Deparaffinized tissue slides were submitted to antigen retrieval, using a high-temperature antigen-unmasking technique. The sections were incubated with specific primary antibodies: rabbit polyclonal antibody anti-*TLR2* (06-1119, 1:50 dilution; Millipore) and mouse monoclonal anti-*TLR4* (76B357.1, 1:200 dilution; Abcam). Next, the slides were incubated with biotinylated secondary antibody (Picture Max Polymer Detection Kit, Invitrogen) for 30 min, following the manufacturer's protocol. Immunostaining was carried out with 3,3'-diaminobenzidine tetrahydrochloride containing 0.005% H<sub>2</sub>O<sub>2</sub> and hematoxylin counterstain. Placental mucosa and appendix tissue were used as positive controls for proteins *TLR2* and *TLR4*, respectively. Immunostaining was evaluated in the epithelial cytoplasm by densitometric analysis according to an arbitrary scale from 0 to 255 arbitrary unit (a.u.), performed with AxioVision software under a Zeiss-Axioskop II light microscope. A total of 60 points equally distributed in each of the regions were scored, and values were expressed as mean ± SE.

#### Statistical analysis

SNPStats software was used to perform multiple logistic regression to evaluate the association of polymorphisms with CRC risk, including a log-additive model (major allele homozygotes vs heterozygotes vs minor allele homozygotes), a dominant model (major allele homozygotes vs heterozygotes + minor allele homozygotes), and a recessive model (major

**Table 3** Allele and genotype frequencies of *TLR2* and *TLR4* polymorphisms and multiple logistic regression analysis between case and control groups *n* (%)

Polymorphisms	Statistical Models	Genotypes /alleles	C	CRC	P value
<i>TLR2</i> -196 to -174del			<i>n</i> = 240	<i>n</i> = 188	
		<i>ins/ins</i>	200 (83.0)	144 (77.0)	
		<i>ins/del</i>	36 (15.0)	39 (21.0)	
		<i>del/del</i>	4 (2.0)	5 (3.0)	
		<i>ins</i>	436 (0.9)	327 (0.9)	
		<i>del</i>	44 (0.1)	49 (0.1)	
	Dominant	<i>ins/ins</i>	200 (83.3)	144 (76.9)	0.038
		<i>ins/del + del/del</i>	40 (16.7)	44 (23.1)	
	OR (95%CI)		1.72(1.03-2.89)		
	Recessive	<i>ins/ins + ins/del</i>	236 (98.3)	183 (97.3)	0.360
		<i>del/del</i>	4 (1.7)	5 (2.7)	
	OR (95%CI)		1.90 (0.48-7.58)		
<i>TLR4</i> -1607T/C	Log-additive	<i>ins/ins</i>	200 (83.0)	144 (77.0)	0.039
		<i>ins/del</i>	36 (15.0)	39 (21.0)	
		<i>del/del</i>	4 (2.0)	5 (3.0)	
	OR (95%CI)		1.59 (1.02-2.48)		
			<i>n</i> = 208	<i>n</i> = 190	
		T/T	166 (79.0)	154 (81.0)	
		T/C	39 (19.0)	33 (17.0)	
		C/C	3 (2.0)	3 (2.0)	
		T	371 (0.9)	341 (0.9)	
		C	45 (0.1)	39 (0.1)	
	Dominant	T/T	166 (79.0)	154 (81.0)	0.860
		T/C + C/C	42 (21.0)	36 (19.0)	
<i>TLR4</i> +896A/G	OR (95%CI)		0.95 (0.56-1.63)		
	Recessive	T/T + T/C	205 (98.0)	187 (98.0)	0.940
		C/C	3 (2.0)	3 (2.0)	
	OR (95%CI)		0.93 (0.14-5.95)		
	Log-additive	T/T	166 (79.0)	154 (81.0)	0.860
		T/C	39 (19.0)	33 (17.0)	
		C/C	3 (2.0)	3 (2.0)	
	OR (95%CI)		0.96 (0.59-1.55)		
			<i>n</i> = 240	<i>n</i> = 190	
	Dominant	A/A	224 (93.3)	172 (90.5)	0.520
		A/G	16 (6.7)	18 (9.5)	
	OR (95%CI)		1.28 (0.60-2.73)		
		A	464 (0.97)	349 (0.95)	
		G	16 (0.03)	17 (0.05)	

The data are adjusted for age, gender, smoking and drinking status. CRC: Colorectal cancer; C: Controls; OR: Odds ratio.

allele homozygotes + heterozygotes vs minor allele homozygotes)<sup>[38]</sup>. Age, gender, smoking and drinking as covariates were adjusted to obtain statistical significance between groups for all polymorphisms evaluated. The GraphPad InStat (version 3.00) software was used to perform Fisher's exact test for assessing the effect of combined genotypes, and the HaploView software (version 4.0) was used to analyze the distribution of haplotype frequencies.

*TLR2* and *TLR4* mRNA and protein expression analysis was performed using the GraphPad Prism software (version 6.01), and the gene expression analysis results were validated and confirmed by ExpressionSuite software (Life Technologies, version 1.0). RQ values were used for statistical analysis. For protein expression, the means of densitometry analysis of tumor and normal adjacent samples were compared. Continuous data distribution was evaluated using D'Agostino and Pearson's omnibus normality test. Student's *t*-test or correspondent nonparametric

tests, such as the Mann-Whitney and Wilcoxon's signed rank test, were used for comparisons between groups. The Benjamini-Hochberg correction<sup>[39]</sup> was applied to the analysis. The probability level of  $P \leq 0.05$  was considered statistically significant in all analyses.

## RESULTS

### *TLR2* and *TLR4* polymorphisms

The allele and genotype frequency distributions of polymorphisms *TLR2* and *TLR4* (Table 3) were consistent with the Hardy-Weinberg equilibrium in the control (C) group (data not shown).

*TLR2*-196 to -174del was associated with increased CRC risk by both the dominant [odds ratio (OR) = 1.72, 95%CI: 1.03-2.89;  $P = 0.038$ ] and the log-additive models (OR = 1.59, 95%CI: 1.02-2.48;  $P = 0.039$ ), while *TLR4* -1607T/C and *TLR4* +896A/G were not (Table 3). With regard to polymorphism *TLR4* +896A/G, it was not detected in individuals with a homozygous

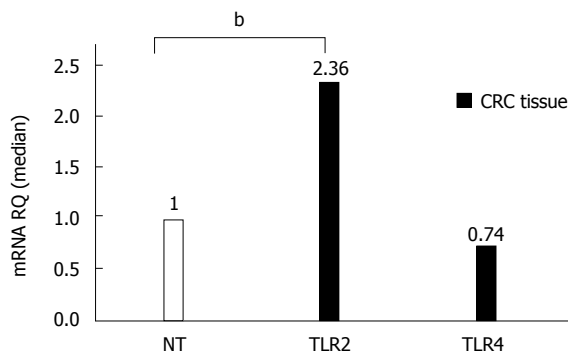
**Table 4** Combined effect of polymorphisms *TLR2* -196 to -174 del, *TLR4* +896 A/G and *TLR4* -1607 T/C on the risk of colorectal carcinoma

Risk genotypes	C	CRC	OR (95%CI)	P value
	n = 240	n = 188		
None	104	136	1.00 (reference)	
<i>TLR2</i> ins/del or del/del	4	3	0.98 (0.21-4.48)	1.000
<i>TLR4</i> +896 A/G				
<i>TLR2</i> ins/del or del/del	11	11	1.31 (0.54-3.13)	0.650
<i>TLR4</i> -1607 T/C or C/C				
<i>TLR4</i> +896 A/G/	2	2	1.31 (0.18-9.44)	1.000
<i>TLR4</i> -1607 T/C or C/C				

CRC: Colorectal cancer; C: Controls; OR: Odds ratio.

**Table 5** Haplotype frequency distribution of variants -1607 T/C and +896 A/G of *TLR4* gene in the case and control groups

Haplotypes <sup>1</sup>	C	CRC	$\chi^2$	P value
<b><i>TLR4</i> -1607/+896</b>				
T-A	0.861	0.849	0.207	0.649
C-A	0.108	0.103	0.052	0.819
T-G	0.031	0.048	1.414	0.234

<sup>1</sup>C-G haplotype was not found. CRC: Colorectal cancer; C: Controls.**Figure 1** Distribution of the median mRNA relative quantification values of genes *TLR2* and *TLR4*. Using the Wilcoxon's signed rank test, a statistically significant difference was found for *TLR2* ( $P < 0.0001$ ), but not for *TLR4* ( $P = 0.452$ ), when comparing the relative quantification (RQ) in colorectal cancer tissue (CRC) with adjacent normal tissue pools (NT). The reference genes *ACTB* and *GAPDH* were used as endogenous controls.

polymorphic genotype G/G in either group, precluding analysis by the three statistical models.

We intended to evaluate the combined effect of the three polymorphisms (*TLR2*-196 to -174del, *TLR4* +896A/G and *TLR4* -1607T/C) on the risk of CRC. However, no individuals with the combination of these three variant alleles were observed in our study subjects, and the combinations of two variant alleles showed no statistically significant difference between the control and the CRC group (Table 4). To further investigate the polymorphisms in gene *TLR4*, we also performed a haplotype analysis of polymorphisms -1607T/C and +896A/G (Table 5). Haplotype CG was not found, and the other haplotypes showed

**Table 6** *TLR2* and *TLR4* mRNA relative quantification values in colorectal carcinoma, stratified according to wild and polymorphic genotype

	<i>TLR2</i> -196 to -174 del		<i>TLR4</i> -1607 T/C	
	ins/ins	ins/del <sup>2</sup>	T/T	T/C + C/C
n (%) <sup>1</sup>	27 (73.0)	10 (27.0)	31 (83.8)	6 (16.2)
Median	3.57	6.95	0.75	0.86
Range	0.49-13.59	0.62-14.69	0.20-23.89	0.47-2.27
P value <sup>3</sup>	0.035		1.000	

<sup>1</sup>3 tumor tissue samples which were not genotyped were excluded from the analysis; <sup>2</sup>No individuals with a del/del genotype in tumor tissue samples were found; <sup>3</sup>Results of the nonparametric Mann-Whitney *U* test. ins: Wild-type allele (insertion); del: Polymorphic allele (deletion).

no statistical difference in the frequencies of allele combinations between the CRC and C groups.

### mRNA and protein expression

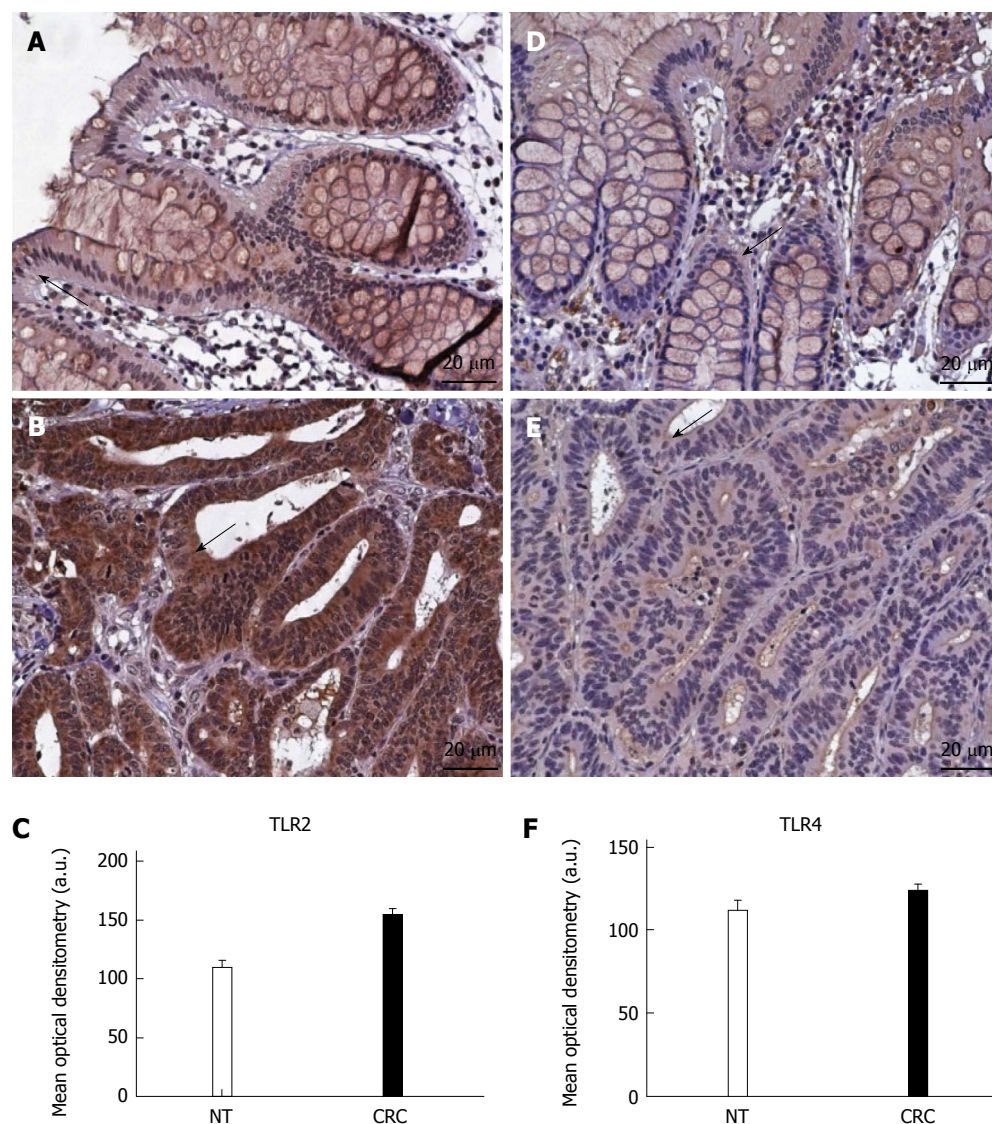
We observed significantly increased *TLR2* relative gene expression in tumor tissue (RQ = 2.36) compared to adjacent normal tissue (RQ = 1;  $P < 0.0001$ ) (Figure 1). With regard to the *TLR4* gene, however, we did not find statistically significant differences between the relative expression in normal and tumor tissue that showed basal relative gene expression (RQ = 0.74,  $P = 0.452$ ) (Figure 1).

In the immunohistochemical analyses of *TLR2* and *TLR4* protein expression, we considered only the epithelial cytoplasm. In normal adjacent tissues, this analysis showed weak or moderate cytoplasm staining in the epithelium for both proteins (Figure 2A and D). In tumor tissue, although a strong cytoplasm immunostaining pattern in the epithelium was found for the *TLR2* protein (Figure 2B), the *TLR4* protein showed weak or moderate expression (Figure 2E). The mean optical densitometry values for *TLR2* in tumor tissue ( $154 \pm 5$  a.u.) were statistically higher than those in normal adjacent tissues ( $109 \pm 6$  a.u.,  $P < 0.0001$ ; Figure 2C), but no difference was found for *TLR4* (CRC =  $123 \pm 4$  a.u., NT =  $111 \pm 6$  a.u.,  $P = 0.117$ ; Figure 2F). Thus, these results are concordant with the findings of mRNA expression.

### Stratification of functional polymorphisms and influence on gene and protein expression

In order to evaluate the influence of the functional polymorphisms *TLR2*-196 to -174del and *TLR4* -1607T/C on mRNA and protein expression, the samples were grouped according to genotypes, considering the presence of at least one polymorphic allele compared to wild genotype samples (Table 6). For the mRNA expression analysis, the wild genotype group was used as a calibrator (RQ = 1) to calculate the relative gene expression. Individuals with at least one polymorphic allele *TLR2*-196 to -174del had more than two times higher *TLR2* expression in tumor tissue (RQ = 2.19;  $P = 0.03$ ) compared to those with the wild





**Figure 2** Toll-like receptor 2 and toll-like receptor 4 protein expression in intestinal mucosa (cytoplasm staining). Moderate expression of TLR2 in normal adjacent mucosa (NT), predominantly in epithelial cells (arrowhead) (A); compared with intense immunostaining in colorectal cancer (B); Low expression of TLR4 in normal adjacent mucosa, predominantly in the epithelium (arrowhead) (D); Moderate TLR4 immunostaining in the epithelial cells (arrowhead) of colorectal cancer (E); Harris' Hematoxylin counterstain. Bar: 20  $\mu$ m. Densitometry analyses (mean  $\pm$  SE) (C and F);  $P < 0.001$ . All images are from the same patient. a.u.: Arbitrary unit; CRC: Colorectal cancer.

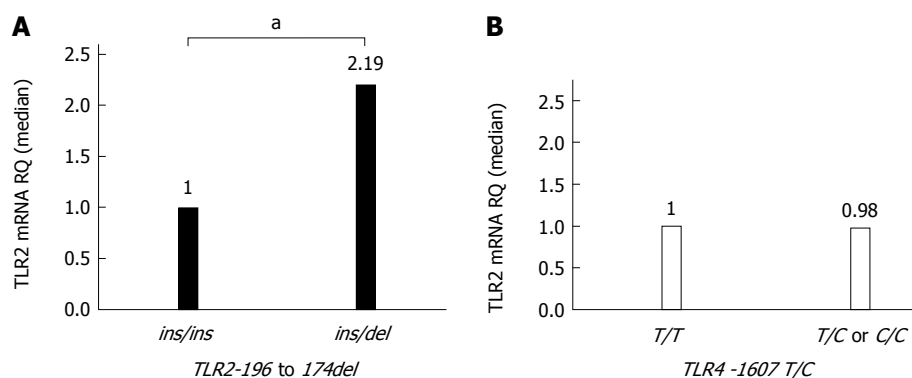
genotype (Figure 3A). However, for the *TLR4* -1607T/C polymorphism, this analysis showed no statistically significant difference in gene expression between individuals with the polymorphic allele ( $RQ = 0.98$ ;  $P = 0.56$ ) and those with the wild allele in homozygosis (Figure 3B).

A similar result was observed when protein expression was compared according to genotypes. Individuals carrying the polymorphic genotype had higher protein expression ( $117 \pm 10$  a.u. vs  $95 \pm 4$  a.u.,  $P = 0.03$ ) than those carrying the wild homozygous genotype for the *TLR2*-196 to -174del polymorphism (Figure 4). However, for the *TLR4* -1607T/C polymorphism no significant difference was found ( $139 \pm 10$  a.u. vs  $128 \pm 4$  a.u.,  $P = 0.26$ ) (Figure 4).

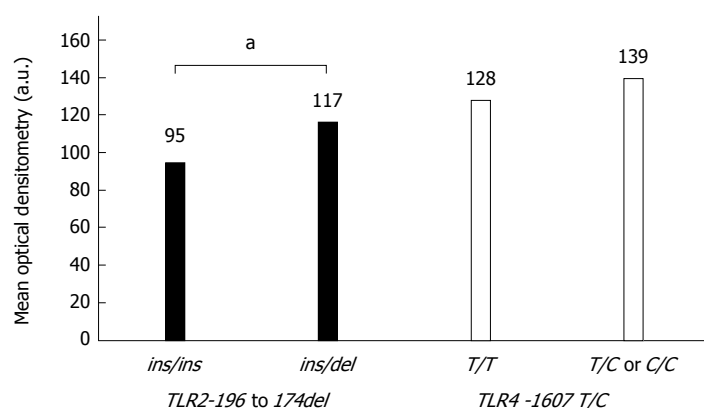
## DISCUSSION

To the best of our knowledge, this is the first report of an evaluation of Toll-like receptor polymorphisms *TLR2*-196 to -174del, *TLR4* -1607T/C and *TLR4* +896A/G in a group of patients with sporadic CRC, which showed that the presence of polymorphic allele *TLR2*-196 to -174del is associated with increased risk of developing this type of cancer, and influences mRNA and protein expression in tumor tissue of patients with CRC compared to those with wild genotype. However, no change in *TLR4* mRNA and protein expression or an association with polymorphisms *TLR4* -1607T/C and +896A/G were found.

The association between CRC risk and the polymor-



**Figure 3** Toll-like receptor 2 (A) and toll-like receptor 4 (B) mRNA relative quantification, using tumor tissue samples from a wild-genotype colorectal cancer group as a calibrator, compared to those with at least one polymorphic allele. Using Wilcoxon's signed rank test, a statistically significant difference was found for the gene expression of *TLR2* ( $P = 0.037$ ), but not for *TLR4* ( $P = 1.000$ ). The reference genes *ACTB* and *GAPDH* were used as endogenous controls. CRC: Colorectal cancer.



**Figure 4** Densitometry values of immunohistochemistry analysis for toll-like receptor 2 and toll-like receptor 4 proteins, stratified according to polymorphic and wild genotypes. Carriers of the polymorphic genotype del/del for *TLR2*-196 to -174del polymorphism showed higher protein expression than those with homozygous wild genotype ins/ins ( $P = 0.03$ ). No statistical difference was found for the values of *TLR4* protein. *ins*: Wild-type allele (insertion); *del*: Polymorphic allele (deletion).

phic variant *TLR2*-196 to -174del was demonstrated by both the dominant and the log-additive statistical models. Other studies showed an association between the *TLR2*-196 to -174del variant and breast cancer in the Greek population<sup>[40]</sup>, with gastric cancer in the Brazilian<sup>[26]</sup> and the Chinese<sup>[41]</sup> populations, and with bladder<sup>[42]</sup>, prostate<sup>[43]</sup> and cervical cancer<sup>[44]</sup> in a northern Indian population. However, no association between gastric cancer was found in the Japanese population<sup>[45]</sup>, nor did we find any studies reporting the presence of this polymorphism in CRC.

In the present study, polymorphisms *TLR4* +896A/G and *TLR4* -1607T/C were not associated with susceptibility to CRC. Studies on the functional polymorphism *TLR4*-1607T/C and cancer are scarce. This polymorphism has been described as relatively common, with a frequency of over 5% for the polymorphic allele C<sup>[30]</sup>, as found in the present study (10% in both groups). This polymorphism was associated with a protective effect for gastric<sup>[30]</sup> and prostate<sup>[31]</sup> cancer. In contrast, a positive association was found for prostate cancer risk in a North American population<sup>[29]</sup>.

The *TLR4* +896A/G variant is located in a coding

region and causes a substitution of the amino acid 299 glycine for asparagine (Asp<sup>299</sup>Gly). Some studies have indicated that this variant is associated with a change in the extracellular domain structure of the *TLR4* receptor and suggest that the polymorphic allele G is associated with an attenuated immune response to LPS and lower secretion levels of pro-inflammatory cytokines<sup>[46-48]</sup>, justifying, to a certain extent, the lack of association between this polymorphism and the risk of CRC.

Our study found a low frequency of the *TLR4* +896 G allele and no homozygous G/G subjects were detected in either the CRC or the control group. The rarity of this genotype has also been observed in other populations, such as those of Croatia<sup>[22]</sup>, northern India<sup>[44]</sup> and Greece<sup>[40]</sup>. In line with our results, other studies have also reported absence of the polymorphic homozygous G/G genotype, such as one conducted in Spain<sup>[45]</sup> and a previous study from our laboratory in the Brazilian population<sup>[26]</sup>.

A lack of association with the *TLR4* +896A/G polymorphism has also been reported in CRC by some studies conducted in Spanish<sup>[49]</sup> and Chinese<sup>[50]</sup> populations, in addition to other types of cancer,

such as cervical in northern India<sup>[44]</sup> and prostate in Sweden<sup>[51]</sup>. This lack of association may be due to this polymorphism having no effect, or only a reduced effect, on the biological development of CRC, therefore unnoticeable in the analyses of these samples<sup>[49]</sup>. Thus, due to the low frequency of the G variant and the rarity of G/G homozygotes, much larger samples may be needed to allow a more robust conclusion on the association of this polymorphism with the development of cancer, although other authors did find an association between this variant and CRC in populations of Croatia<sup>[22]</sup> and Europe<sup>[52]</sup>. In another, recently completed study by our research group, although no homozygous G/G subjects were detected, an association between *TLR4* +896A/G and the risk of gastric cancer and chronic gastritis was observed<sup>[26]</sup>.

An evaluation of the combined effect of the three polymorphisms (*TLR2*-196 to -174del, *TLR4* +896A/G and *TLR4* -1607T/C) on CRC risk showed that none of the combinations of two variant alleles produced any significant differences between the CRC and control groups, which suggests that these combinations do not affect the risk of developing CRC. However, the above-mentioned recent study conducted by our research group showed that the combination of genotypes *TLR2*-196 to -174 ins/del and del/del with *TLR4* +896A/G leads to a higher risk of developing gastric cancer<sup>[26]</sup>.

In addition, haplotype analysis of *TLR4* polymorphisms -1607T/C and +896A/G showed no statistically significant differences in the distribution of allele combination frequencies between the CRC and control groups, suggesting that the possible formation of haplotypes with these gene polymorphisms does not affect CRC risk. In line with our findings, other studies also failed to find an association between *TLR4* gene haplotypes and either CRC risk<sup>[53]</sup>, or chronic gastritis and intestinal metaplasia<sup>[54]</sup>. However, an association between other *TLR4* gene haplotypes with some types of cancer, such as *TLR4* G-C (<sup>299</sup>Gly-<sup>399</sup>Thr) with increased gastric cancer risk<sup>[26]</sup>, and Asp<sup>299</sup>-Ileu<sup>399</sup> with increased gastritis and precancerous lesions risk<sup>[34]</sup> were reported.

The mRNA relative expression analysis of genes *TLR2* and *TLR4* in tumor and adjacent normal tissues from patients with CRC showed a 2.36-fold increased gene expression of *TLR2* in tumor tissue, unlike *TLR4* which showed basal relative gene expression. Furthermore, in line with this result, the immunohistochemistry assay showed that only the TLR2 protein was overexpressed in tumor tissue, demonstrating a change in the expression of this receptor in CRC.

The TLR2 receptor operates in the recognition of microorganisms in the intestinal mucosa, leading to their activation and triggering an inflammatory process in the organ microenvironment. Therefore, TLR2 overexpression may cause a more accentuated inflammatory response, recruiting MyD88 for the TLR/TIR domain and thereby inducing pro-inflammatory cytokine production by a classical signaling pathway.

The IKK protein is activated in a process that involves IRAK-1 and TRAF6. The IKK complex catalyzes IκB phosphorylation and degradation by the proteasome, allowing NF-κB displacement to the nucleus. In the nucleus, NF-κB regulates pro-inflammatory cytokine expression and molecule adhesion<sup>[55]</sup>, thus facilitating tumor progression.

High *TLR2* expression in tumor tissue of patients with CRC has been reported, while no difference was found regarding *TLR4* expression<sup>[10,16]</sup>. In gastric cancer, increased expression of *TLR2*, but not of *TLR4* mRNA<sup>[56]</sup> was also reported, and was also associated with *Helicobacter pylori* infection<sup>[57]</sup>.

When the mRNA and protein expression of TLR2 and TLR4 from CRC patients were stratified according to genotypes (wild and carrying at least one variant allele), it became clear that in the subgroup of patients with at least one polymorphic allele of *TLR2*-196 to -174del, the expression of TLR2 mRNA and protein was significantly higher than that of wild genotype carriers.

In contrast, another study conducted in a Japanese population, found that the polymorphic genotype del/del decreases the transactivation of responsive promoters, causing a decrease in gene transcription and thus a decrease in gene expression<sup>[21]</sup>. However, considering the important role of this gene in the induction of inflammatory processes and the association between polymorphism *TLR2*-196 to -174del and several types of cancer, taken together these data suggest that this deletion must increase the level of gene transcription in tumor tissue, enhancing the inflammatory process and favoring cancer progression. Therefore, these are novel findings, as they indicate for the first time the influence of the *TLR2*-196 to -174del polymorphism on increased expression of *TLR2* gene in CRC. Another study has reported the importance of polymorphisms on gene expression levels in ulcerative colitis<sup>[58]</sup>.

Unlike *TLR4* -1607T/C polymorphism, we did not find any influence of the polymorphic allele C on relative gene expression in CRC tumor tissue. This polymorphism was shown by a functional luciferase expression assay not to influence gene transcription<sup>[35]</sup>. This result confirms the lack of association between this functional polymorphism and CRC risk, indicating that its presence does not cause significant changes in gene transcription.

Considering that in most cases CRC has a good prognosis and is treatable when diagnosed at an early stage, it is of the utmost importance to establish molecular markers capable of identifying risk groups and providing early diagnosis in individuals with increased risk of developing this neoplasm. Overall, our results indicate that the *TLR2* gene plays an important role in colorectal carcinogenesis, highlighting the importance of the *TLR2*-196 to -174del polymorphism in increasing gene expression and possibly triggering a stronger inflammatory response, which in turn enhances the risk of tumor progression.



In conclusion, *TLR2* mRNA and protein expression are increased in CRC tissue. In addition, the functional polymorphism *TLR2*-196 to -174del influences mRNA and protein expression in tumor tissue of patients with CRC promoting its increase in relation to wild-type genotype carriers, thus emphasizing their important role in colorectal carcinogenesis. Furthermore, the polymorphic variant *TLR2*-196 to -174del is associated with increased CRC risk and may contribute to the identification of CRC risk groups. However, there is no evidence of an association between CRC and *TLR4* -1607T/C and *TLR4* +896A/G polymorphisms or of these polymorphisms on *TLR4* gene expression. In future studies, these genes should be evaluated in other intestinal diseases, such as in precancerous lesions, as well in other populations to better understand their importance and function in colorectal carcinogenesis.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Colorectal cancer is one of the main inflammation-cancer association models, therefore it is interesting to evaluate polymorphisms in genes related to the inflammatory process which are associated with the development of some cancers, such as toll-like receptors (TLRs). *TLR2* and *TLR4* genes have promoter region polymorphisms which may change the levels of gene transcription. However, only a few studies have investigated the influence of functional polymorphisms on changes in mRNA or protein expression levels in colorectal cancer and their role in carcinogenesis.

### Research frontiers

Epidemiological studies on the association between polymorphisms and susceptibility to disease such as cancer frequently present conflicting results. However, together with other analyses such as gene and protein expression this may help clarify their effects on gene regulation at the transcription level.

### Innovations and breakthroughs

This study showed, for the first time, that the presence of polymorphic allele *TLR2*-196 to -174del is associated with an increased risk of developing colorectal cancer, and this polymorphism enhances mRNA and protein expression in tumor tissue from patients with colorectal cancer compared to those with the wild genotype.

### Applications

The data show that carriers of the *TLR2*-196 to -174del polymorphism constitute a risk group for the development of colorectal cancer. Thus, considering the high incidence of this cancer in the Brazilian population, an understanding of the mechanisms involved in activation of the immune and inflammatory response mediated by these receptors is important in the development of preventive and therapeutic strategies for this neoplasm.

### Terminology

*TLR2*: toll-like receptor 2, lipoprotein bacterial receptor, gene located on chromosome 4; *TLR4*: toll-like receptor 4, lipopolysaccharide receptor, gene mapped on chromosome 9.

### Peer-review

The study explores the role of *TLR2* and *TLR4* polymorphism and colorectal cancer risk. The article is meaningful and well written.

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## Basic Study

# Effect of endogenous cholecystokinin on the course of acute pancreatitis in rats

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## Abstract

**AIM:** To examine the effects of pancreatic rest, stimulation and rest/stimulation on the natural course of recovery after acute pancreatitis.

**METHODS:** Acute hemorrhagic pancreatitis (AP) was induced in male rats by intraductal infusion of 40  $\mu$ L/100 g body weight of 3% sodium taurocholate. All rats took food *ad libitum*. At 24 h after induction of AP, rats were divided into four groups: control (AP-C), pancreas rest (AP-R), stimulation (AP-S), and rest/stimulation (AP-R/S). Rats in the AP-C, AP-R and AP-S groups received oral administration of 2 mL/kg body weight saline, cholecystokinin (CCK)-1 receptor antagonist, and endogenous CCK release stimulant, respectively, twice daily for 10 d, while those in the AP-R/S group received twice daily CCK-1 receptor antagonist for the first 5 d followed by twice daily CCK release stimulant for 5 d. Rats without any treatment were used as control group (Control). Biochemical and

histological changes in the pancreas, and secretory function were evaluated on day 12 at 24 h after the last treatment.

**RESULTS:** Feeding *ad libitum* (AP-C) delayed biochemical, histological and functional recovery from AP. In AP-C rats, bombesin-stimulated pancreatic secretory function and HOMA- $\beta$ -cell score were significantly lower than those in other groups of rats. In AP-R rats, protein per DNA ratio and pancreatic exocrine secretory function were significantly low compared with those in Control rats. In AP-S and AP-R/S rats, the above parameters recovered to the Control levels. Bombesin-stimulated pancreatic exocrine response in AP-R/S rats was higher than in AP-S rats and almost returned to control levels. In the pancreas of AP-C rats, destruction of pancreatic acini, marked infiltration of inflammatory cells, and strong expression of  $\alpha$ -smooth muscle actin, tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  were seen. Pancreatic rest reversed these histological alterations, but not atrophy of pancreatic acini and mild infiltration of inflammatory cells. In AP-S and AP-R/S rats, the pancreas showed almost normal architecture.

**CONCLUSION:** The favorable treatment strategy for AP is to keep the pancreas at rest during an early stage followed by pancreatic stimulation by promoting endogenous CCK release.

**Key words:** Acute pancreatitis; Pancreatic stimulation; Cholecystokinin; Pancreatic rest; Pancreatic function

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**Core tip:** In experimental acute hemorrhagic pancreatitis, feeding *ad libitum* without any treatment delayed biochemical, histological and functional recovery. Both pancreatic rest made by blocking cholecystokinin (CCK)-1 receptor and pancreatic stimulation caused by eliciting endogenous CCK release improved biochemical and histological alterations, except pancreatic secretory function. The favorable treatment strategy for acute pancreatitis (AP) is to keep the pancreas at rest during an early stage followed by pancreatic stimulation. Thus, high-protein meals should be avoided during the early phase after AP but protein meals may be important at later times to stimulate recovery of pancreatic function.

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## INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease occurring in the pancreas. It is assumed that in-

appropriately activated trypsin triggers a chain of intracellular zymogen activation in the pancreas, resulting in AP<sup>[1,2]</sup>. Regardless of the underlying causes, vigorous intravenous hydration is the first important treatment principle of AP to stabilize blood pressure and intravascular volume, and prevent hypovolemic shock<sup>[3,4]</sup>. In addition to fluid resuscitation, traditional treatment consists of initial fasting to suppress synthesis and secretion of pancreatic enzymes, and avoid activation of proteolytic enzymes<sup>[5]</sup>. Food intake would elicit endogenous release of cholecystokinin (CCK) that stimulates pancreatic enzyme synthesis and secretion<sup>[6,7]</sup>, which may aggravate damage of the pancreas<sup>[8,9]</sup>. Similarly, previous studies have demonstrated that exogenous injection of cerulein or CCK-8, even at physiological doses, worsens the mortality and morbidity in AP in rats and mice<sup>[10,11]</sup>. Indeed, fasting decreased endogenous CCK concentrations and ameliorated the severity of AP<sup>[12]</sup>. In addition, we have demonstrated that CCK-1-receptor-deficient Otsuka Long-Evans Tokushima Fatty (OLETF) rats do not develop severe AP, although plasma CCK levels rise up to 4-14-fold over the preloading values after the onset of AP<sup>[13]</sup>. In concert with these observations, numerous studies have shown that potent and specific CCK-1 receptor antagonists reduce the severity of pancreatitis in animal experiments<sup>[8,9,14]</sup> and clinical trials<sup>[15,16]</sup>. These results suggest that pancreatic rest may promote healing, decrease pain, and reduce secretion and complications.

However, patients with AP maintain an accelerated basal metabolic rate, protein catabolism increases by 80% and energy expenditure by 20%, and therefore have increased caloric needs, therefore, nutritional support is especially important<sup>[17,18]</sup>. Although parenteral nutrition (PN) was traditionally used to maintain pancreatic rest by avoiding gastrointestinal (GI) hormone release and supporting nutritional needs, avoidance of using the GI tract in patients with AP exacerbated the severity of AP, leading to greater incidence of complications and prolonged hospitalization<sup>[19,20]</sup>. Enteral nutrition (EN), in comparison to PN, significantly reduces systemic infections, pancreatic infections, surgical interventions, length of hospital stay, and mortality. It is generally accepted that EN is significantly superior to total PN regarding mortality, infectious complications, and organ failure<sup>[19,20]</sup>. It is conceivable that EN may improve outcome in patients with AP if given early<sup>[21,22]</sup>. Indeed, a randomized clinical study has revealed that immediate oral feeding in patients with mild AP may accelerate recovery<sup>[21,22]</sup>. However, there is no report regarding recovery of pancreatic function by oral nutrition from an early stage after AP. Moreover, it is unknown whether early feeding in AP improves histological alterations, or pancreatic exocrine and endocrine function. It is reported that recovery to normal does not necessarily occur after AP and that progression to chronic pancreatitis is possible in a considerable number of cases<sup>[23]</sup>.



In the present study, we examined pancreatic histology and function in post-pancreatitis rats after feeding with a normal rat diet, keeping the pancreas at rest by blocking CCK-1 receptor, or stimulating the pancreas by eliciting endogenous CCK release.

## MATERIALS AND METHODS

### Animals

Male Wistar rats weighing 230-250 g were used in all experiments. The animals were kept in a temperature- ( $23 \pm 2^\circ\text{C}$ ) and humidity- ( $55\% \pm 5\%$ ) controlled room with a 12-h light/dark cycle (lights on at 07:00 am). The animals were provided *ad libitum* standard rat chow consisting of (as a percentage of calories) 61% carbohydrate, 26% protein, and 13% fat (3.596 kcal/g diet: Oriental Yeast, Tokyo, Japan) and tap water.

### Animal care guidelines

Our institutional Animal Welfare Committee approved the experimental protocol, and rats received humane care according to the guidelines of our institution. All experiments were performed according to the guidelines of the Ethics Committee of Animal Care and Experimentation at University of Occupational and Environmental Health, Japan. Animals were kept under specific pathogen-free conditions.

### Induction of AP

Pancreatitis was induced in overnight-fasted rats according to the method of Aho *et al.*<sup>[24]</sup> by retrograde intraductal infusion of 40  $\mu\text{L}$ /100 g body weight of 3% taurocholic acid sodium salt (NaTc) (Sigma, St. Louis, MO, United States) dissolved in saline. Intraductal infusion was performed under steady manual pressure over a period of 30 s<sup>[13]</sup>. Rats without intraductal infusion were used as untreated normal controls (Control group).

### Pancreatic rest and stimulation

At 24 h after induction of acute hemorrhagic pancreatitis, rats were divided into four different treatment groups: standard rat chow (AP-C); standard rat chow with pancreatic rest (AP-R); standard rat chow with pancreatic stimulation (AP-S); and standard rat chow with pancreatic rest, followed by pancreatic stimulation (AP-R/S). Rats in the AP-C group received 2 mL/kg body weight saline orally (po) *via* an orogastric tube twice daily (09:00 and 21:00 h) for 10 d; the AP-R group received 50 mg/kg body weight of CCK-1 receptor antagonist loxiglumide<sup>[25]</sup> (kindly supplied by Kaken Pharmaceutical Co., Tokyo, Japan) dissolved in 2 mL distilled water po twice daily for 10 d; the AP-S group received 25 mg/kg body weight protease inhibitor camostat (a generous gift from Ono Pharmaceutical Co., Osaka, Japan), which is known to stimulate endogenous CCK release<sup>[26-28]</sup>, dissolved in 2 mL distilled water po

twice daily for 10 d; and the AP-R/S group received 50 mg/kg body weight loxiglumide twice daily for the first 5 d followed by 25 mg/kg body weight camostat twice daily for the next 5 d. Rats were fed *ad libitum*. On day 12 at 24 h after the last treatment and overnight fasting, pancreatic exocrine function and histological examination of the pancreas were performed.

Based on our previous studies, we used CCK-1 receptor antagonist loxiglumide to make the pancreas rest<sup>[25]</sup> and synthetic protease inhibitor camostat to stimulate the pancreas *via* endogenous CCK release (pancreas stimulation)<sup>[26-28]</sup>.

### Exocrine secretory function

Rats were weighed before the experiment, and anesthesia was induced by subcutaneous (sc) injection of sodium pentobarbital (50 mg/kg body weight) after an overnight fast. After collecting blood for measurement of serum concentrations of glucose and insulin, the left jugular vein, and bile and pancreatic ducts were cannulated, and the pylorus was ligated. The bile was returned into the duodenum during the experiment. Pancreatic fluid secretion was obtained by replacing a calibrated tube attached to the free end of the pancreatic cannula every 10 min, and the volume and protein concentrations were determined<sup>[26,27,29,30]</sup>. The abdominal wound was covered with a saline-moistened gauze, and body temperature was maintained between 37 and 38  $^\circ\text{C}$  with a heating pad throughout the experiment. After collection of basal fluid flow, bombesin (Protein Research Institutes, Osaka, Japan) was infused into the jugular vein at a dose of 5 nmol/kg body weight/h using a syringe pump (Razal Scientific Instruments, Stanford, CT, United States) for 1 h at a rate of 1 mL/h. Pancreatic fluid was collected every 10 min.

Since there is a possibility that CCK-1 receptor antagonist loxiglumide accumulates and modifies CCK-mediated pancreatic fluid and protein secretion<sup>[29,30]</sup>, and since camostat stimulates endogenous CCK release and downregulates CCK receptor<sup>[28]</sup>, we used bombesin stimulation to determine pancreatic exocrine function. CCK-1 receptor antagonist is known not to inhibit the action of bombesin on rat pancreatic secretion<sup>[31]</sup>.

### Endocrine function and insulin resistance

Insulin secretion was calculated by homeostasis model assessment insulin secretion (HOMA- $\beta$ -cell) with the following formula: fasting insulin ( $\mu\text{U/mL}$ )  $\times$  20/[fasting glucose (mmol/L) - 3.5], as described by Matthews *et al.*<sup>[32]</sup>. HOMA- $\beta$ -cell function positively correlates with the ratio of change in insulin and glucose. Insulin resistance was calculated by homeostasis model assessment insulin resistance (HOMA-IR) with the following formula: fasting insulin ( $\mu\text{U/mL}$ )  $\times$  fasting glucose (mmol/L)/22.5, as described by Matthews *et al.*<sup>[32]</sup>. With such a method, high HOMA-IR score denotes low insulin sensitivity (insulin resistance).

### Measurement of pancreatic contents

After the last collection of pancreatic fluid, rats were killed, and the pancreas was excised and weighed after being trimmed free of fat, mesentery, and lymph nodes. Portions of each pancreatic tissue with similar anatomic orientation were used for histologic examination. Pieces of pancreatic tissue (100–200 mg wet weight) were homogenized in saline using a motor-driven Teflon-coated glass homogenizer at 3000 rpm (eight passes). The homogenates were filtered through three layers of gauze and then sonicated for 1 min for measurement of pancreatic contents of protein, DNA, amylase, lipase and insulin.

### Histological examination

A portion of the pancreatic tissue was fixed overnight in 10% formaldehyde solution for hematoxylin and eosin (HE) staining, immunostaining, and light microscopic examination. The pathologist, without awareness of the treatment, examined all histologic samples in a single-blind fashion.

### Immunohistochemistry

Paraffin-embedded pancreatic tissue sections were prepared on glass slides. Sections for interleukin (IL)-1 $\beta$  were pretreated in microwaves in citrate buffer (pH 6.0) for 12 min, while sections for tumor necrosis factor (TNF)- $\alpha$  immunohistochemistry were incubated in protease K for 10 min (for antigen retrieval). The sections for  $\alpha$ -smooth muscle actin (SMA) immunohistochemistry were used without pretreatment. These sections were treated with graded alcohol solutions and incubated for 15 min in 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activities. Nonspecific staining was blocked by incubating with bovine serum for 10 min at room temperature. Each section was incubated with goat anti-mouse TNF- $\alpha$  antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) and rabbit anti-human IL-1 $\beta$  antibody (Santa Cruz Biotechnology) at a dilution of 1:10 at room temperature for 1 h. The sections for  $\alpha$ -SMA immunohistochemistry were incubated with mouse anti-human  $\alpha$ -SMA antibody (Dako Corporation, Carpinteria, CA, United States) at a dilution of 1:50 at room temperature for 30 min. In TNF- $\alpha$  immunohistochemistry, bound antibody was detected with rabbit anti-goat antibody (Dako Corporation) in dilution 1:400. In IL-1 $\beta$  or  $\alpha$ -SMA immunohistochemistry, bound antibodies were detected with the peroxidase-labeled streptavidin-biotin method (LSAB Kit; Dako Corporation). Then, these sections were stained with diaminobenzidine (DAB). Counterstaining was performed with Mayer's hematoxylin, and the sections were mounted.

### Assays

Serum glucose concentrations were determined by the glucose-oxidase method using a glucose kit (Glucose-E reagent; International Reagents, Kobe, Japan)<sup>[33]</sup>.

Insulin concentrations in the serum and pancreatic homogenates were measured by radioimmunoassay using the double-antibody method<sup>[34]</sup> with a commercially available radioimmunoassay kit (ShionRIA; Shionogi Pharmaceutical, Osaka, Japan) using crystalline rat insulin as a reference standard. The protein concentration in pancreatic homogenates and pancreatic fluid was determined by the method of Lowry *et al.*<sup>[35]</sup>. DNA content was measured using the fluorescent dye H-33258 (Hoechst AG, Germany) according to Labarca *et al.*<sup>[36]</sup>. Amylase activity in pancreatic homogenates was determined by a chromogenic method with blue-dyed starch polymer<sup>[37]</sup> and expressed in Somogyi units (SU). Lipase activity was determined according to the method of Whitaker<sup>[38]</sup> and expressed in international units (IU).

### Statistical analysis

Results are expressed as the mean  $\pm$  SE of at least six rats per group. Data were analyzed with the use of analysis of variance followed by Tukey's test using commercial software StatView (Abacus Concepts/Brain Power, Berkeley, CA, United States).  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Pancreatic wet weight and protein and DNA content

In AP-C rats on day 12 after induction of AP, pancreatic wet weight and pancreatic content of protein and DNA were significantly lower than those in the Control rats (Table 1). However, protein per DNA ratio (an indicator of cell size) in AP-C rats was similar to that in the Control rats. Pancreatic rest for 10 d (AP-R) significantly increased pancreatic wet weight, and contents of protein and DNA compared with those in AP-C rats, but were significantly lower than those in Control rats. In addition, protein per DNA ratio in AP-R rats was significantly low compared with that in AP-C, AP-S and AP-R/S rats. In AP-S and AP-R/S rats, pancreatic wet weight and pancreatic contents of protein and DNA were recovered to the levels in Control rats (Table 1). There were no differences in pancreatic wet weight, and contents of protein and DNA between AP-S and AP-R/S rats. Protein per DNA ratio in AP-R/S rats was similar to that in Control rats, whereas that in AP-S rats tended to be higher than Control or AP-R/S rats, although the difference was not statistically significant (Table 1).

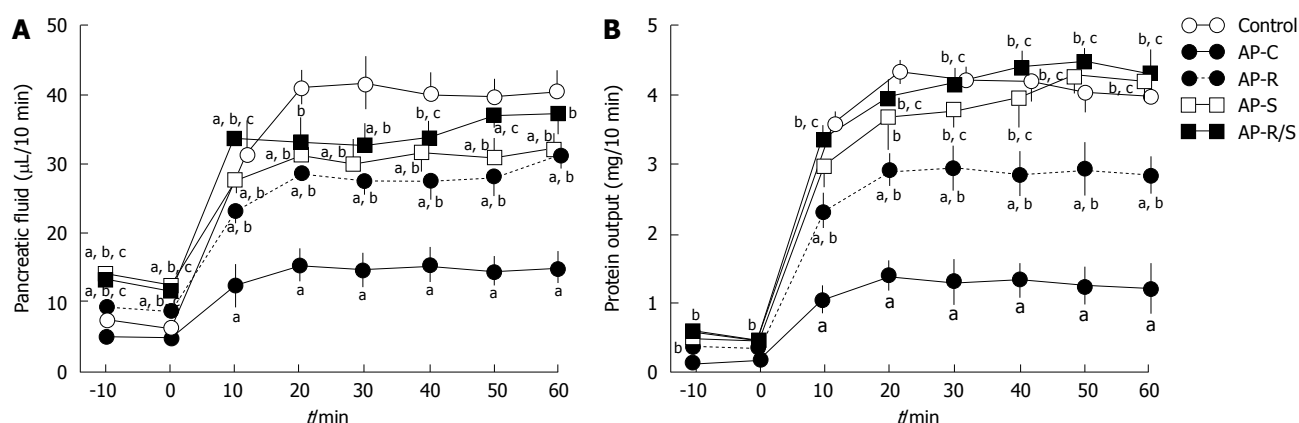
### Exocrine function in response to bombesin stimulation

Basal pancreatic fluid secretion in AP-C rats was not significantly different from that in the Control rats, whereas it was significantly increased in AP-R, AP-S and AP-R/S rats compared with that in AP-C or Control rats (Figure 1A). Basal protein output in AP-C rats was significantly lower than that in Control, AP-S and AP-R/S rats (Figure 1B). Pancreatic fluid secretion and

**Table 1** Effect of pancreatic rest or stimulation on the recovery of the pancreas after acute pancreatitis

Parameters	Control	AP-C	AP-R	AP-S	AP-R/S
Pancreatic wet weight (mg/rat)	1120 ± 30	698 ± 58 <sup>1</sup>	881 ± 37 <sup>1,2</sup>	1074 ± 69 <sup>2,3</sup>	1079 ± 66 <sup>2,3</sup>
Pancreatic contents					
Protein (mg/pancreas)	132 ± 8	50 ± 9 <sup>1</sup>	89 ± 4 <sup>1</sup>	131 ± 18 <sup>2,3</sup>	126 ± 13 <sup>2,3</sup>
DNA (mg/pancreas)	4.2 ± 0.2	1.7 ± 0.3 <sup>1</sup>	3.3 ± 0.1 <sup>1,2</sup>	3.5 ± 0.4 <sup>2</sup>	4.1 ± 0.4 <sup>2</sup>
Protein/DNA (mg/mg)	31.1 ± 0.8	31.3 ± 2.2	27.2 ± 1.3 <sup>1,2</sup>	37.7 ± 1.4 <sup>3</sup>	31.3 ± 1.3 <sup>3</sup>
Amylase					
(103 SU/pancreas)	91.2 ± 17.7	12.3 ± 5.2 <sup>1</sup>	46.7 ± 7.8 <sup>1,2</sup>	67.7 ± 13.4 <sup>2,3</sup>	73.4 ± 12.7 <sup>2,3</sup>
(SU/mg protein)	681 ± 105	240 ± 49 <sup>1</sup>	520 ± 96 <sup>2</sup>	502 ± 72 <sup>2</sup>	509 ± 78 <sup>2</sup>
(103 SU/mg DNA)	20.4 ± 3	7.0 ± 1.2 <sup>1</sup>	14.2 ± 3.2 <sup>2</sup>	20.0 ± 3.7 <sup>2</sup>	17.6 ± 2.5 <sup>2</sup>
Lipase					
(103 U/pancreas)	11.6 ± 1.2	4.5 ± 1.2 <sup>1</sup>	8.0 ± 0.6 <sup>1,2</sup>	11.4 ± 1.9 <sup>2</sup>	12.4 ± 1.6 <sup>2</sup>
(U/mg protein)	123.3 ± 22.7	77.4 ± 8.0 <sup>1</sup>	86.3 ± 5.9 <sup>1</sup>	91.7 ± 6.3 <sup>2</sup>	97.9 ± 7.1 <sup>2</sup>
(103 U/mg DNA)	3.7 ± 0.6	2.6 ± 0.4 <sup>1</sup>	2.7 ± 0.1 <sup>1</sup>	3.4 ± 0.3 <sup>2</sup>	3.1 ± 0.3 <sup>2</sup>
Insulin content					
(nmol/pancreas)	20.2 ± 1.2	12.8 ± 0.8 <sup>1</sup>	18.9 ± 1.5 <sup>2</sup>	16.8 ± 0.6 <sup>2</sup>	17.2 ± 0.5 <sup>2</sup>
(nmol/mg DNA)	4.95 ± 0.22	8.56 ± 1.10 <sup>1</sup>	5.56 ± 0.57 <sup>2</sup>	5.25 ± 1.00 <sup>2</sup>	4.80 ± 0.55 <sup>2</sup>

<sup>1</sup>Significant difference *vs* control; <sup>2</sup>Significant difference *vs* AP-C; <sup>3</sup>Significant difference *vs* AP-R. Values are the mean ± SE of 6-8 rats. At 24 h after induction of acute hemorrhagic pancreatitis by retrograde intraductal infusion of 40  $\mu$ L/100 g body weight of 3% NaTc, rats were divided into four groups. Rats in the AP-C group received 2 mL/kg body weight saline twice daily for 10 d; the AP-R group received 50 mg/kg body weight CCK receptor antagonist loxiglumide twice daily for 10 d; the AP-S group received 25 mg/kg body weight protease inhibitor camostat twice daily for 10 d; and the AP-R/S group received 50 mg/kg body weight loxiglumide twice daily for the first 5 d followed by 25 mg/kg body weight camostat twice daily for the next 5 d. Rats were fed *ad libitum*. On day 12 at 24 h after the last treatment and an overnight fasting, pancreatic exocrine function and histological examination of the pancreas were performed.



**Figure 1** Pancreatic fluid secretion (A) and protein output (B) in response to bombesin stimulation in the four different treatment groups on day 12 after induction of acute pancreatitis. Results are the mean ± SE of 6-8 experiments. <sup>a</sup>*P* < 0.05 *vs* control; <sup>b</sup>*P* < 0.01 *vs* AP-C; <sup>c</sup>*P* < 0.05 *vs* AP-R. AP: Acute pancreatitis.

protein output in AP-C rats in response to bombesin infusion were significantly lower than those in other groups of rats (Control, AP-R, AP-S and AP-R/S) (Figure 1A and B). In AP-R rats, bombesin-stimulated pancreatic fluid secretion and protein output were significantly higher than those in AP-C rats but lower than other treatment groups (AP-S and AP-R/S) and Control. Pancreatic fluid secretion during bombesin infusion in AP-S and AP-R/S was almost similar to those in Control rats (Figure 1A and B).

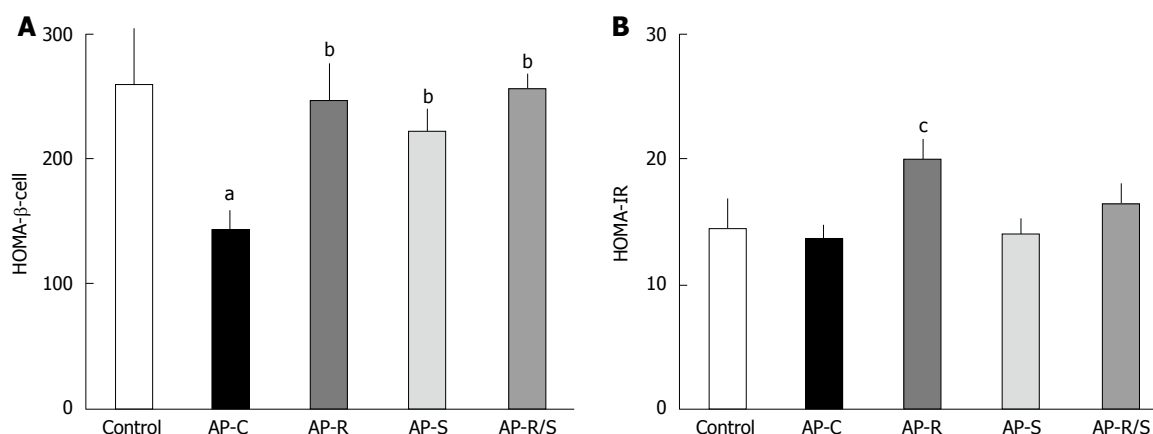
### Endocrine function and insulin resistance

HOMA- $\beta$ -cell score (an indicator of  $\beta$ -cell function) in AP-C rats on day 12 after induction of pancreatitis was significantly low compared with that in the Control and AP-R, AP-R and AP-R/S groups (Figure 2A). HOMA- $\beta$ -

cell score in AP-R, AP-S and AP-R/S rats was similar to that in Control rats (Figure 2A). In contrast, HOMA-IR score (an indicator of insulin resistance) in AP-R rats was significantly high compared with other groups of rats, whereas that in AP-C, AP-S and AP-R/S rats was similar to that in the Control rats (Figure 2B). In AP-R/S rats, HOMA-IR score tended to be higher than Control rats, although the difference was not statistically significant (Figure 2B).

### Pancreatic contents of amylase, lipase and insulin

In AP-C rats, total pancreatic contents of amylase and lipase, and concentrations of these enzymes relative to protein or DNA were lower than those in Control, AP-R, AP-S and AP-R/S rats. In AP-R rats, total amylase content was significantly lower than that



**Figure 2** HOMA-β-cell (A) and HOMA-IR (B) in the four different treatment groups on day 12 after induction of acute pancreatitis. Results are the mean  $\pm$  SE of 6-8 experiments. <sup>a</sup> $P < 0.05$  vs control, AP-R, AP-S and AP-R/S; <sup>b</sup> $P < 0.01$  vs AP-C; <sup>c</sup> $P < 0.05$  vs control, AP-C, AP-S and AP-R/S. AP: Acute pancreatitis.

in the Control, AP-S and AP-R/S rats (Table 1). Total pancreatic amylase content and concentrations relative to protein or DNA in AP-S and AP-R/S rats were similar to those in the Control rats. Pancreatic insulin content in AP-C rats on day 12 after induction of AP was significantly lower than that in the Control, AP-R, AP-S and AP-R/S rats. However, insulin concentration relative to DNA in AP-C rats was significantly higher than that in other groups due to a decrease in DNA content. Pancreatic rest (AP-R) or stimulation (AP-S and AP-R/S) significantly increased pancreatic insulin content to that in the Control rats (Table 1).

### Histological changes

Representative photomicrographs of randomly selected sections of the pancreas taken on day 12 after induction of AP in four different treatment groups are shown in Figure 3 using the same magnification. In the pancreas of AP-C rats, destruction of pancreatic acini, tubular complexes and marked infiltration of inflammatory cells, mainly lymphocytes, were seen (Figure 3A). Pancreas rest (AP-R) for 10 d greatly reversed these histological alterations (Figure 3B), but atrophy of pancreatic acini and mild infiltration of inflammatory cells were still observed in the pancreas. These histological findings in AP-R rats were consistent with the low value of protein per DNA ratio (an indicator of cell size) compared with other groups of rats (Table 1). In AP-S (Figure 3C) and AP-R/S (Figure 3D) rats, the pancreas showed almost normal architecture.

### Expression of $\alpha$ -SMA and cytokines

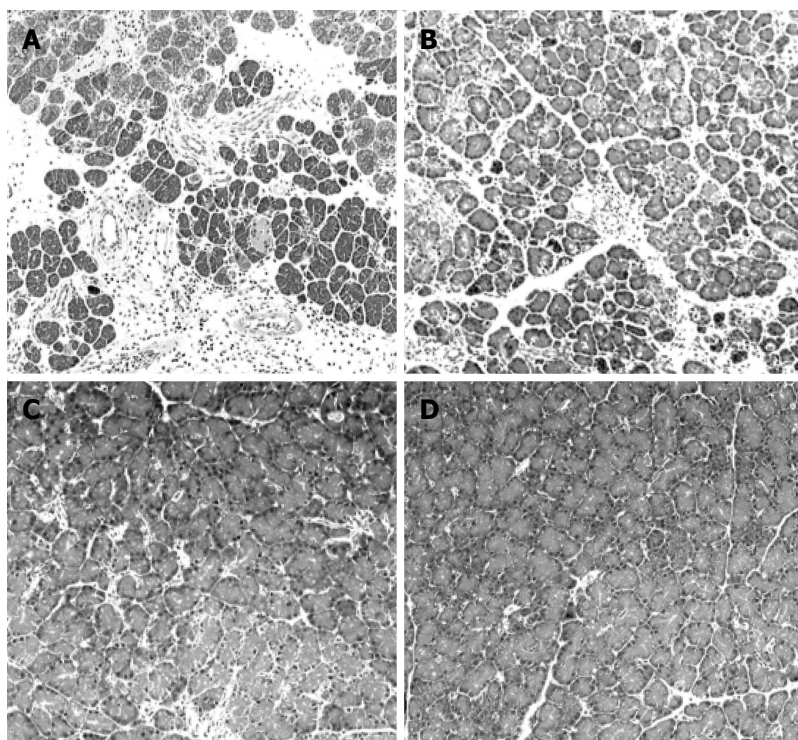
Immunohistochemical studies of pancreatic tissues of AP-C rats showed strong expression of  $\alpha$ -SMA in the degenerative regions (Figure 4A). In AP-R rats (Figure 4D),  $\alpha$ -SMA expression was markedly suppressed compared with AP-C rats. In AP-S (Figure 4G) and AP-R/S (Figure 4J) rats,  $\alpha$ -SMA was only detected in pancreatic ducts. TNF- $\alpha$  and IL-1 $\beta$  were strongly expressed in inflammatory cells in the pancreas of AP-C

rats (Figure 4B and C), while they were not detected in the pancreas of AP-R (Figure 4E and F), AP-S (Figure 4H and I) and AP-R/S rats (Figure 4K and L).

## DISCUSSION

Food intake elicits GI hormone release such as secretin and CCK<sup>[6,7]</sup>, which may stimulate the damaged pancreas in post-pancreatic states and further aggravate pancreatic inflammation. In particular, it is reported that the increased secretion of CCK is involved in aggravation of AP following administration of trypsin inhibitor<sup>[39]</sup>. Thus the principle of the traditional treatment for AP is to rest the pancreas by giving the patient nothing po but parenterally<sup>[5]</sup>. However, no advantages of PN were reported on the total hospital stay or incidences of complications of pancreatitis<sup>[19]</sup>. Absence of food in the intestine may cause intestinal atrophy resulting in bacterial translocation and multiple organ failure (MOF)<sup>[20]</sup>. Nowadays, it is generally accepted that total oral abstinence from food with PN is not beneficial to patients with severe AP, but may in fact be harmful. On the other hand, EN maintains the gut barrier, with consequent decreased bacterial translocation, which is in turn associated with fewer septic complications, and reduced surgical procedures and length of hospital stay<sup>[20]</sup>. EN within 48 h of admission was feasible and improved the clinical outcomes in mild as well as in predicted severe or severe AP by reducing complications<sup>[18]</sup>. In mild AP, immediate oral feeding is feasible and safe, and may accelerate recovery without adverse GI events<sup>[21,22]</sup>. Meta-analysis of observational data from 165 individuals from 8 randomized trials revealed that EN started within 24 h of admission reduced complications compared to EN started after 24 h of admission<sup>[21]</sup>. However, there is no report regarding recovery of pancreatic function by oral nutrition from an early time point after AP. Also, it is unknown whether after an initial attack of AP, the inflamed gland heals completely, or whether the disease progresses to chronic pancreatitis<sup>[23,40]</sup>. It is reported





**Figure 3** Representative photomicrographs of the pancreas in the four different treatment groups on day 12 after induction of acute pancreatitis. A: The pancreas of AP-C rat (*ad libitum* feeding with saline administration) showed destruction of pancreatic acini, tubular complexes and marked infiltration of inflammatory cells, mainly lymphocytes; B: The pancreas of AP-R rat (pancreatic rest) showed minimal histologic alterations with atrophic pancreatic acini and mild inflammatory cell infiltration; The pancreas in AP-S (C) (pancreatic stimulation) and AP-R/S rats (D) (pancreatic rest for the first 5 d followed by pancreatic stimulation for 5 d) showed almost normal architecture. Original magnification  $\times 25$ . AP: Acute pancreatitis.

that recovery to normal does not necessarily occur after AP and that progression to chronic pancreatitis is possible at a considerable percentage<sup>[40]</sup>.

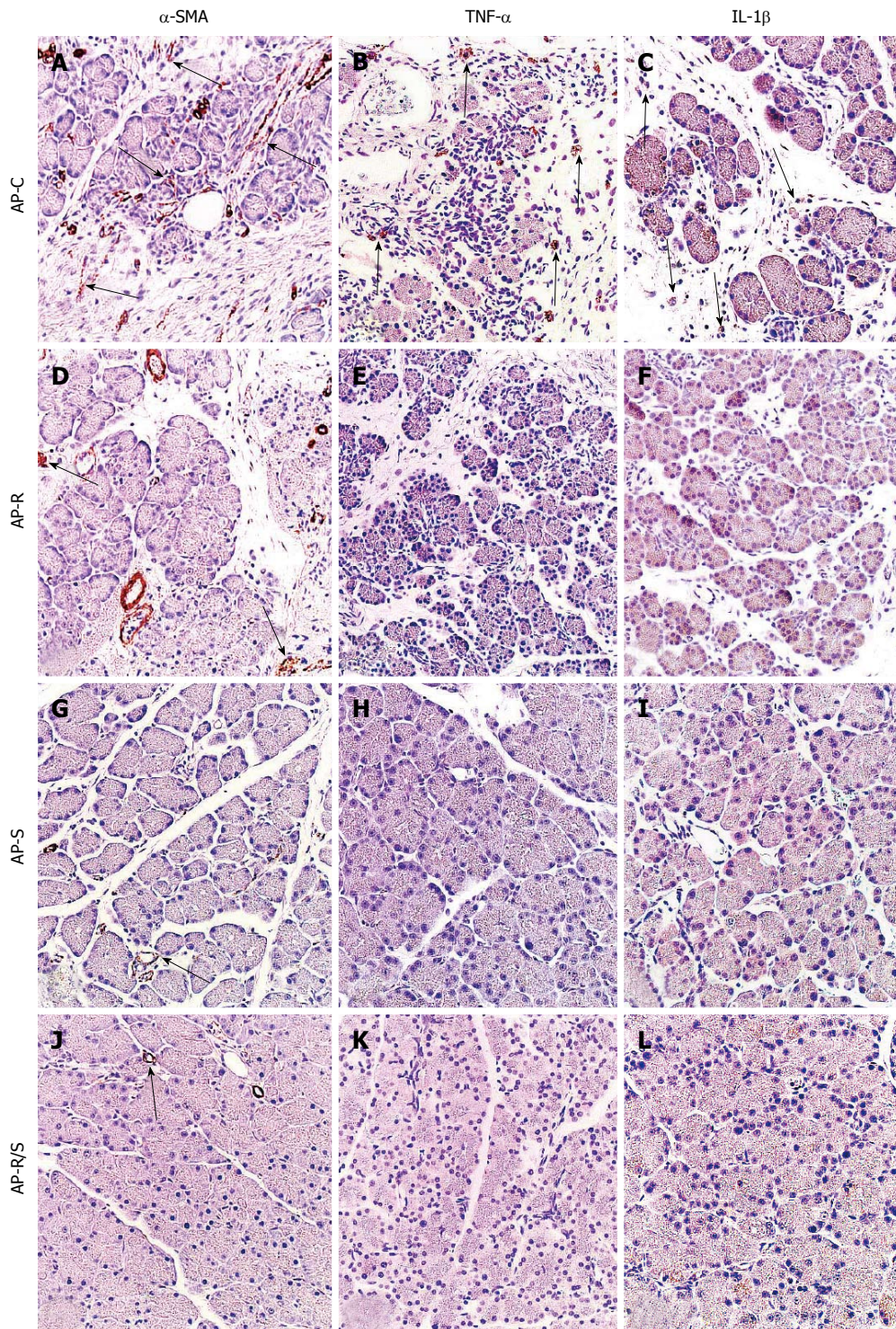
In the present study, we investigated the effects of pancreatic rest by oral administration of CCK-1 receptor antagonist loxiglumide<sup>[25]</sup> and pancreas stimulation *via* endogenous CCK release induced by po protease inhibitor camostat<sup>[26-28]</sup> on the recovery of pancreatic secretory function, and biochemical and histological changes of the pancreas after acute hemorrhagic pancreatitis. Oral administration of CCK-1 receptor antagonist loxiglumide with a dose of 50 mg/kg body weight inhibited pancreatic exocrine secretion for more than 12 h<sup>[29]</sup>. Thus, every 12-h administration of loxiglumide might have completely blocked the effect of endogenously released CCK on the pancreas (pancreatic rest). On the other hand, basal plasma CCK concentrations in randomly fed rats were  $2.59 \pm 0.13$  pmol/L, and increased to the peak of  $27.6 \pm 4.1$  pmol/L 1 h after an oral administration of 20 mg/kg body weight camostat<sup>[27]</sup>, and plasma CCK concentrations at 24 h after oral administration of 100 mg/kg body weight camostat were  $6.57 \pm 0.67$  pmol/L, further increased to  $14.24 \pm 1.63$  pmol/L after consecutive camostat administration for 10 d<sup>[28]</sup>. Our previous studies clearly indicate that po camostat is a strong stimulant for endogenous CCK release.

In AP-C rats that were provided *ad libitum* standard rat chow consisting of (as a percentage of calories)

61% carbohydrate, 26% protein and 13% fat with no other treatment, biochemical, histological and functional recovery from AP was delayed and incomplete, even 12 d after the attack of AP compared with that in AP-R, AP-S and AP-R/S rats. Although a previous study has revealed that EN downregulates splanchnic cytokine production and modulates the acute phase response<sup>[18]</sup>, pancreatic histology and immunohistochemistry in AP-C rats suggested the presence of continued inflammatory changes in the pancreas even on day 12 after induction of AP. We found that plasma CCK levels in NaTc-induced pancreatitis rats increased from  $1.6 \pm 0.2$  pmol/L to  $22.9 \pm 2.4$  pmol/L at 12 h after intraductal infusion of NaTc, and remained elevated levels of  $11.0 \pm 1.0$  pmol/L even 24 h after<sup>[13]</sup>. However, the sensitivity and responsiveness of the pancreas to CCK stimulation are decreased at an early stage of AP<sup>[41,42]</sup>, therefore, the injured pancreas after AP does not respond to CCK stimulation. Thus, it is difficult to believe that the increase in plasma CCK concentrations after AP leads to exacerbation of AP. Moreover, physiological increases in plasma CCK concentrations after *ad libitum* feeding in post-pancreatitis rats seem to be too low to stimulate proliferation and growth of the damaged pancreas, and thus the recovery in AP-C rats might be delayed.

High plasma CCK concentrations are reported in patients with AP<sup>[43]</sup> as well as in various animal models of AP<sup>[8,13]</sup>. However, it is not clear whether the increase in plasma CCK concentrations is the result





**Figure 4** Representative immunohistochemical studies of the pancreas in the four different treatment groups on day 12 after induction of AP. A: The pancreas of AP-C rats showed strong  $\alpha$ -SMA expression in the degenerative regions; In AP-S (G) and AP-R/S rats (J),  $\alpha$ -SMA was only detected in pancreatic ducts. In AP-C rats, TNF- $\alpha$  (B) and IL-1 $\beta$  (C) were strongly expressed in inflammatory cells in the pancreas. In AP-R, AP-S and AP-R/S rats, TNF- $\alpha$  (E, H and K) and IL-1 $\beta$  (F, I and L) were not detected in the pancreas; D: Pancreatic rest (AP-R) markedly reduced  $\alpha$ -SMA expression. Arrows in A, D, G and J indicate  $\alpha$ -SMA expression in the pancreas. Arrows in B indicate TNF- $\alpha$  expression in the pancreas. Arrows in C indicate IL-1 $\beta$  expression in the pancreas. Original magnification  $\times 50$ .

or cause of AP. Administration of excessive doses of CCK or its analog cerulein causes AP<sup>[44,45]</sup>, and even physiological doses worsen AP<sup>[10]</sup>. On the other hand, CCK-1 receptor antagonists have not only preventive but also protective effects on experimental models of

AP<sup>[8-11,14]</sup>. Moreover, in the OLETF rats that are missing the CCK-1 receptor, in spite of significant increase in plasma CCK concentrations after AP, biochemical, histological and functional changes are mild compared with those in the control Long-Evans Tokushima



Otsuka (LETO) rats<sup>[13]</sup>. Consistent with these reports that suggest involvement of CCK in the progress of AP, the present study demonstrated that blockade of the CCK stimulation (AP-R) accelerated the recovery compared to that in saline-treated AP-C rats. However, biochemical, histological and functional recovery in this group was still incomplete compared to that in AP-S and AP-R/S groups. Moreover, consecutive blockade of the CCK-1 receptors for 10 d appeared to delay biochemical, histological and functional recovery, since these parameters were low compared with the untreated Control rats. In addition, pancreatic rest for 10 d caused atrophy of pancreatic acini evaluated by a decrease in protein per DNA ratio, although it suppressed  $\alpha$ -SMA and cytokines expression. Since the recovery of the injured pancreas in AP-R rats was faster than that in AP-C rats, but delayed compared with AP-S and AP-R/S rats, the regeneration process of the damaged pancreas might be under the influence of endogenous CCK at some point after AP, as previously reported<sup>[46,47]</sup>. It is conceivable therefore that the pancreatic rest should be limited only at early time points to accelerate the regeneration process of the damaged pancreas after AP. However, our previous study in cerulein-induced mild AP revealed that loxiglumide, even when given only for 3 d after the onset of AP, suppressed the spontaneous recovery of pancreatic weight and protein content evaluated on day 8<sup>[46]</sup>. The difference between the present and the previous observation might be due to the difference in severity of AP (hemorrhagic vs edematous pancreatitis) or to the magnitude of the elevation of endogenous CCK after AP. These differences suggest that pancreatic rest is not necessary after mild AP.

Endogenously released as well as exogenously administered CCK causes hyperplasia and hypertrophy of the pancreas, and increases pancreatic enzyme content in normal rats<sup>[26,46]</sup>. Similarly, in cerulein-induced post-pancreatitis rats, repeated sc injections of cerulein increased all parameters within 5 d and induced pancreatic growth thereafter when given after 3 d of rest<sup>[47]</sup>. Moreover, Evander *et al.*<sup>[10,39]</sup> and Jurkowska *et al.*<sup>[47]</sup> also demonstrated in NaTc-induced post-pancreatitis rats that soybean trypsin inhibitor (SBTI) restores the pancreas to normal after 10 d with cellular hypertrophy when started after 3 d of rest. In contrast, repeated sc injections of CCK-8 for 6 d started from 24 h after induction of cerulein-pancreatitis suppressed the spontaneous recovery of pancreatic wet weight<sup>[46]</sup>. These different results can be explained by the differences in the start of cerulein/CCK-8 or trypsin inhibitor administration after induction of AP. Both cerulein- and NaTc-induced AP rats are resistant to cerulein/CCK stimulation during an early stage of pancreatitis<sup>[41,42]</sup>. It is possible, therefore, that the repeated sc injections of CCK or endogenous CCK release during early post-acute pancreatitis ( $\leq 3$  d after onset of AP) might have

no influence on the pancreas due to cerulein/CCK resistance, and thus delayed the expected hypertrophic and hyperplastic response of the pancreas. After 3 d of rest, the post-AP pancreas might respond to CCK stimulation with hypertrophy and hyperplasia. In the present study, we used the synthetic trypsin inhibitor camostat as a stimulant for endogenous CCK release. Orally administered camostat not only elicits endogenous CCK release by inhibiting trypsin activity in the intestine, but also inhibits circulating proteases such as trypsin, kallikrein, thrombin, plasmin, and Ci esterase after being absorbed from the intestine<sup>[48]</sup>. Stimulation of the pancreas by endogenous CCK from an early time after induction of AP (AP-S) markedly decreased the expression of  $\alpha$ -SMA and cytokines, as well as infiltration of inflammatory cells in the pancreas, and almost completely recovered pancreatic wet weight, pancreatic contents of protein, DNA and enzymes, and histology. Since the protein per DNA ratio tended to increase compared with that in Control rats, endogenously released CCK might have induced hypertrophy of the pancreas. However, pancreatic fluid secretion to bombesin stimulation was significantly low compared with that in Control rats. These results suggest that stimulation of the pancreas from an early stage after induction of AP also slightly delays the recovery.

In normal rats and mice, po administration of camostat induces pancreatic hypertrophy and hyperplasia<sup>[28,49]</sup>. However, oral administration of camostat for 10 d from an early stage after induction of AP had no significant trophic effects on the pancreas compared with untreated Control rats, although it increased pancreatic weight, and protein and enzyme contents compared with those in the AP-C rats. A similar result was observed in postpancreatitis rats injected with CCK-8 from the early stage after induction of cerulein-pancreatitis<sup>[46]</sup>. Since po administration of protease inhibitor stimulates endogenous CCK release<sup>[26-28]</sup>, and endogenous CCK is shown to be an exacerbatory factor in AP<sup>[8,9,10,39]</sup>, it is possible that camostat exerted an anti-protease effect but had neither hypertrophic nor hyperplastic effects on the damaged pancreas during the early stage (CCK resistant stage<sup>[41,42]</sup> after induction of AP. In contrast, however, Song *et al.*<sup>[50]</sup> demonstrated a trophic effect of endogenous CCK by feeding with 0.1% camostat-containing diet for 7 d on pancreatic regeneration in severe model of acute hemorrhagic pancreatitis that was induced in rats by two intraperitoneal injections of cerulein under water-immersion stress for 5 h, once a day for three successive days.

Endogenously released as well as exogenously administered CCK plays an important role in the growth of the normal pancreas<sup>[51]</sup> and pancreatic regeneration in postpancreatitis rats<sup>[46,50]</sup>. We investigated the effect of pancreatic rest by CCK-1 antagonist for the first 5 d followed by stimulation by endogenous CCK for the next

5 d after induction of acute hemorrhagic pancreatitis (AP-R/S). By this treatment, biochemical parameters, and pancreatic endocrine and exocrine functions were completely recovered to normal. Moreover, the pancreas showed almost normal architecture and  $\alpha$ -SMA and cytokines expression was completely inhibited. Thus, it is clear that the combination of CCK-1 receptor antagonist with an endogenous CCK release stimulant further accelerates recovery from acute hemorrhagic pancreatitis.

Pancreatitis is a complex syndrome consisting of exocrine and endocrine derangement. In the present study, we found that not only exocrine pancreas but also endocrine pancreas evaluated by HOMA- $\beta$ -cell score and pancreatic insulin content were deranged in NaTc-induced AP rats. Pancreatic rest by CCK-1 receptor antagonist, and pancreatic stimulation by endogenous CCK release stimulant significantly recovered pancreatic insulin content. Thus pancreatic rest or stimulation after AP appears to improve pancreatic endocrine function, although there is a possibility that long-term CCK-1 receptor antagonist treatment causes insulin resistance as our previous reports<sup>[52,53]</sup>.

In summary, long-term pancreas rest, or pancreatic stimulation from an early stage after induction of AP seems to delay the recovery. The most favorable strategy for the treatment of acute hemorrhagic pancreatitis is to maintain the pancreas at rest during an early stage for only a short period, followed by pancreatic stimulation. Although it is difficult to translate the present observation made in a particular animal model to humans, these results suggest that a high-protein meal should be avoided during an early time after AP but protein meals may be important during later times to stimulate recovery of pancreatic function.

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## COMMENTS

### Background

Acute pancreatitis (AP) is an inflammatory disease occurring in the pancreas. Regardless of the underlying causes, intravenous hydration, pancreatic rest, and nutritional support are important. Enteral nutrition, compared to parenteral nutrition, significantly reduces complications and mortality. However, to date, there is no report regarding recovery of pancreatic function by oral nutrition at early times after AP. Moreover, it is unknown whether early feeding in AP improves histological alterations, and pancreatic exocrine and endocrine function.

### Research frontiers

Feeding *ad libitum* without any treatment delayed biochemical, histological and functional recovery from acute hemorrhagic pancreatitis. So, it is a hot research topic to find the most favorable strategy for the treatment of acute hemorrhagic pancreatitis to improve biochemical and histological alterations, and pancreatic secretory function.

## Innovations and breakthroughs

It is generally accepted that enteral nutrition may improve outcome in patients with AP if given early. However, feeding *ad libitum* without any treatment delayed biochemical, histological and functional recovery from acute hemorrhagic pancreatitis. Pancreatic rest made by blocking cholecystokinin (CCK)-1 receptor improved biochemical and histological alterations except pancreatic secretory function and HOMA- $\beta$ -cell score. Pancreatic stimulation caused by eliciting endogenous CCK release from an early stage after acute pancreatitis significantly improved biochemical and histological alterations, and recovered pancreatic insulin content and HOMA- $\beta$ -cell score, but slightly delayed the recovery of exocrine secretory function. The favorable treatment strategy for AP is to keep the pancreas at rest during the early stage, followed by pancreatic stimulation by promoting endogenous CCK release.

## Applications

These results suggest that high-protein meals should be avoided during the early stages after AP but protein meals may be important during later times to stimulate recovery of pancreatic function.

## Terminology

CCK is secreted by I cells of the upper small intestine. Its secretion is stimulated by the introduction of hydrochloric acid or fatty acids into the stomach or the duodenum. CCK stimulates the gallbladder to contract and release stored bile into the intestine. It also stimulates the secretion of pancreatic juice rich in digestive enzymes and may induce satiety. Two types of CCK receptors (type A, "alimentary," and type B, "brain") have been identified on a pharmacological basis. The CCK-A receptor was first characterized in pancreatic acini from rodents, whereas the CCK-B receptor was first found in the brain. Based on recommendations of the International Union of Pharmacology Committee regarding receptor nomenclature and drug classification, the CCK-A receptor has been renamed CCK1 receptor, and the CCK-B receptor has been renamed CCK2 receptor. CCK1 receptor binds and responds to sulfated CCK with a 500-1000-fold higher affinity or potency than sulfated gastrin or nonsulfated CCK. The CCK2 receptor binds and responds to gastrin or CCK with almost the same affinity or potency and discriminates poorly between sulfated and nonsulfated peptides. In the periphery, the CCK2 receptor is considered as the gastrin receptor.

## Peer-review

This is a very good manuscript that addresses an important problem, namely how to best manage patients with acute pancreatitis. There is no specific pharmacotherapy for this disease; thus, management of patients is critically important. The authors demonstrate in a rat model of acute pancreatitis that rest followed by stimulation of CCK secretion is the most effective protocol for recovery from this disease.

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## Basic Study

# Ezetimibe improves hepatic steatosis in relation to autophagy in obese and diabetic rats

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## Abstract

**AIM:** To investigate whether ezetimibe ameliorates hepatic steatosis and induces autophagy in a rat model of obesity and type 2 diabetes.

**METHODS:** Male age-matched lean control LETO and obese and diabetic OLETF rats were administered either PBS or ezetimibe (10 mg/kg per day) *via* stomach gavage for 20 wk. Changes in weight gain and energy intake were regularly monitored. Blood and liver tissue were harvested after overnight fasting at the end of study. Histological assessment was performed in liver tissue. The concentrations of glucose, insulin, triglycerides (TG), free fatty acids (FFA), and total cholesterol (TC) in blood and TG, FFA, and TG in liver

tissue were measured. mRNA and protein abundance involved in autophagy was analyzed in the liver. To investigate the effect of ezetimibe on autophagy and reduction in hepatic fat accumulation, human Huh7 hepatocytes were incubated with ezetimibe (10  $\mu$ mol/L) together with or without palmitic acid (PA, 0.5 mmol/L, 24 h). Transmission electron microscopy (TEM) was employed to demonstrate effect of ezetimibe on autophagy formation. Autophagic flux was measured with bafilomycin A1, an inhibitor of autophagy and following immunoblotting for autophagy-related protein expression.

**RESULTS:** In the OLETF rats that received ezetimibe (10 mg/kg per day), liver weight were significantly decreased by 20% compared to OLETF control rats without changes in food intake and body weight ( $P < 0.05$ ). Lipid parameters including TG, FFA, and TC in liver tissue of ezetimibe-administrated OLETF rats were dramatically decreased at least by 30% compared to OLETF controls ( $P < 0.01$ ). The serum glucose, insulin, HOMA-IR, and lipid profiles were also improved by ezetimibe ( $P < 0.05$ ). In addition, autophagy-related mRNA expression including ATG5, ATG6, and ATG7 and the protein level of microtubule-associated protein light chain 3 (LC3) were significantly increased in the liver in rats that received ezetimibe ( $P < 0.05$ ). Likewise, for hepatocytes cultured *in vitro*, ezetimibe treatment significantly decreased PA-induced fat accumulation and increased PA-reduced mRNA and protein expression involved in autophagy ( $P < 0.05$ ). Ezetimibe-increased autophagosomes was observed in TEM analysis. Immunoblotting analysis of autophagy formation with an inhibitor of autophagy demonstrated that ezetimibe-increased autophagy resulted from increased autophagic flux.

**CONCLUSION:** The present study demonstrates that ezetimibe-mediated improvement in hepatic steatosis might involve the induction of autophagy.

**Key words:** Autophagy; Ezetimibe; Hepatic steatosis; Nonalcoholic fatty liver disease

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**Core tip:** As an anti-hypercholesterolemia drug, ezetimibe is reported to improve metabolic disorders. Moreover, the hepatic expression of Niemann-Pick C1-like 1 protein, the target of ezetimibe, has led to increased interest in the effects, which have not been fully delineated, of ezetimibe on the liver. In the current study, ezetimibe treatment improved hepatic fat accumulation, which was accompanied by the induction of hepatic autophagy in obese and diabetic rats. In addition, *in vitro* hepatocytes treated with an inhibitor of autophagy showed that ezetimibe-induced autophagy resulted from an increase in autophagic flux. Therefore, ezetimibe-increased autophagy flux may play an important role in the improvement of hepatic steatosis.

Chang E, Kim L, Park SE, Rhee EJ, Lee WY, Oh KW, Park SW, Park CY. Ezetimibe improves hepatic steatosis in relation to autophagy in obese and diabetic rats. *World J Gastroenterol* 2015; 21(25): 7754-7763 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7754.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7754>

## INTRODUCTION

The increasing prevalence of nonalcoholic fatty liver disease (NAFLD) by up to 30% in Western countries has resulted in NAFLD becoming the most common feature of chronic liver disease<sup>[1-3]</sup>. Numerous studies have demonstrated a positive correlation between metabolic disorders such as insulin resistance and obesity and the progression of liver fibrosis, cirrhosis, and hepatocellular carcinoma<sup>[1,4]</sup>. Given the rapidly increasing prevalence of NAFLD and its positive relationship with metabolic syndrome, the prevention and attenuation of NAFLD is critical.

Ezetimibe decreases intestinal cholesterol incorporation by blocking Niemann-Pick C1-like 1 (NPC1L1) protein<sup>[5,6]</sup>. Given the association between dysregulated cholesterol metabolism and metabolic disorders, numerous human and animal studies have shown that the NPC1L1 inhibitor ezetimibe affects metabolic disorders including insulin resistance, type 2 diabetes, and atherosclerosis<sup>[7-11]</sup>. In addition to intestinal NPC1L1 expression, NPC1L1 is also highly abundant in the liver<sup>[5,12]</sup>. Consequently, fatty liver is improved by ezetimibe in obese subjects undergoing weight loss intervention<sup>[13]</sup> as well as in subjects with NAFLD<sup>[14]</sup> and nonalcoholic steatohepatitis (NASH) with dyslipidemia<sup>[15]</sup>. Ezetimibe administration ameliorates hepatic steatosis in diet-induced fatty liver animal models<sup>[16,17]</sup>, fatty liver Shionogi mice<sup>[18,19]</sup>, *db/db* mice<sup>[20]</sup>, and Zucker obese fatty rats<sup>[7,8]</sup>. Moreover, NPC1L1-ablated mice are protected from high fat-induced fatty liver<sup>[21]</sup>. Together, these data support the possibility that NPC1L1 inhibition might be an effective method for treating NAFLD. However, more studies investigating the molecular and intracellular mechanisms by which ezetimibe regulates hepatic lipid metabolism and improves NAFLD are necessary.

Autophagy is the process of intracellular degradation *via* the formation of double-membrane structures known as autophagosomes, which is followed by their transport to lysosomes, fusion with the lysosomes to form autolysosomes, and finally, degradation of the contents enclosed within the inner autophagosomal membrane<sup>[22]</sup>. The role of autophagy is to maintain cellular homeostasis by routine turnover of cytoplasmic components under various stress conditions such as starvation, virus infection, and endoplasmic reticulum (ER) stress<sup>[23-26]</sup>. However, recent studies have suggested a new function and role for autophagy in hepatic lipid storage *via* a process termed lipophagy. Hepatic lipid accumulation by lipid challenge inhibits



hepatic autophagy<sup>[27-29]</sup>, and inhibition of autophagy by genetic knockdown or pharmacological methods increases hepatocyte triglyceride (TG) and cholesterol levels<sup>[27,29]</sup>. Importantly, these results may suggest a new mechanism for the treatment of hepatic steatosis.

In the present study, we investigated the effects of ezetimibe on glycemic control, hepatic fat accumulation, and the induction of liver autophagy. In both a rat model of obesity and diabetes and palmitic acid (PA)-treated hepatocytes, ezetimibe attenuated hepatic fat accumulation concomitant with increased hepatic autophagy.

## MATERIALS AND METHODS

### Animals

All animal experiments were conducted following the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Experiments of Kangbuk Samsung Hospital, Sungkyunkwan University. Male OLETF ( $n = 11$ ) and age-matched LETO rats ( $n = 3$ ) were purchased from Otsuka Pharmaceuticals (Tokushima, Japan), and experiments were conducted in a specific pathogen-free facility with a 12 h light/dark cycle at Kangbuk Samsung Hospital, Sungkyunkwan University. The OLETF rat is a model that represents late-onset hyperglycemia and exhibits a chronic disease course, mild obesity and clinical onset of diabetes mellitus<sup>[30,31]</sup>. Animals had unrestricted access to water and food (PicoLab Rodent Diet 20 5053, Purina Mills, Richmond, IN, United States). At 12 wk of age, rats were randomized and treated with either PBS or ezetimibe (10 mg/kg per day) *via* a stomach gavage for 20 wk. At the end of the study, the rats were fasted overnight and anesthetized with intraperitoneal Zoletil/Rompun. Blood was collected from the abdominal aorta, and liver tissues were dissected, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until further analysis.

### Cell culture

Huh7 human hepatocytes (Korean Cell Line Bank, Seoul, South Korea) were cultured in high glucose DMEM (Gibco, Grand Island, NY, United States) containing 10% FBS (Gibco), 100 units/mL penicillin and 100  $\mu\text{g/mL}$  streptomycin (Gibco) at  $37^{\circ}\text{C}$  in a 95% air/5%  $\text{CO}_2$  atmosphere. Ezetimibe was provided by Merck Sharp and Dohme Corp. (Rahway, NJ, United States). Hepatocytes were treated with or without ezetimibe (10  $\mu\text{mol/L}$ , 1 h) and incubated with palmitic acid (PA, 0.5 mmol/L, 24 h; Sigma-Aldrich, St. Louis, MO, United States). Palmitic acid was prepared as previously described<sup>[32]</sup>.

### Measurement of metabolic parameters

Serum glucose and insulin were analyzed by enzymatic

assay (Sigma-Aldrich and Crystal Chem, Downers Grove, IL, United States). The blood and liver TG levels were also measured by enzymatic assay (Sigma-Aldrich). Commercial kits were employed for the measurement of free fatty acid (FFA; Wako Pure Chemical Industries, Osaka, Japan) and total cholesterol (TC; Cayman Chemical Com., Ann Arbor, MI, United States). The liver metabolic parameters were normalized to their respective protein concentrations. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: fasting glucose (mmol/L)  $\times$  fasting insulin (mU/L)/22.5.

### Histological Analysis and NAFLD activity score

Dissected liver tissues were fixed in 10% formalin buffer overnight. The tissues were then embedded in paraffin, sliced into 5- $\mu\text{m}$ -thick sections, and stained with hematoxylin and eosin (HE). Digital images were captured with an Olympus BX51 light microscope (magnification, 200  $\times$ , Tokyo, Japan). A pathologist blinded to the experimental conditions evaluated the NAFLD activity score (NAS). Three features of NAFLD, namely steatosis, lobular inflammation, and ballooning, were scored as described previously<sup>[33]</sup>. NAFLD activity score (NAS) was calculated as follows; steatosis (0-3), ballooning (0-2), and inflammation (0-3) were summed.

### RNA analysis

The total RNA was extracted using an RNeasy Mini Kit (Invitrogen, Carlsbad, CA, United States). A high-capacity cDNA Kit (Applied Biosystems, Foster City, CA, United States) was used for cDNA synthesis. Real-time quantitative PCR (RT-PCR) was performed with a Roche Lightcycler 480 (Roche, Mannheim, Germany) using Roche real-time PCR master mix and UPL. The primer sequences used are listed in Table 1. The PCR parameters were as follows: pre-denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 45 cycles of denaturation at  $95^{\circ}\text{C}$  for 10 s, and annealing/extension at  $60^{\circ}\text{C}$  for 20 s. Expression of each target gene was normalized to housekeeping gene (GAPDH or  $\beta$ -actin) and expressed as the fold change relative to the control treatment. CT values of GAPDH or  $\beta$ -actin were not statistically different among groups.

### Immunoblot analysis

Equal amounts of protein were loaded into each lane of a 4%-20% gradient SDS polyacrylamide gel, separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Marlborough, MA, United States). The membranes were probed with the following primary antibodies:  $\alpha$ -tubulin, ATG5, ATG6, LC3B, and GAPDH (Cell Signaling Technology, Danvers, MA, United States) followed by the appropriate secondary antibody. The immunoreactive bands were developed with the Amersham ECL plus system (Amersham-Pharmacia

**Table 1** Primers used for real-time quantitative-polymerase chain reaction

Organism	Gene	Forward primer	Reverse primer
Human	ATG5	CAACTGTGTTTCACGCTATATCAGG	CACTTTGTCAGTTACCAACGTCA
	ATG6	GGATGGTGTCCTCTCGCAGAT	TGGCACTTTCTGTGGACAT
	ATG7	CCGTGGAATTGATGGTATCTG	TCATCCGATCGTCACTGCT
	GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAATCC
Rat	$\beta$ -actin	CCTGTAIGCCTCTGGTCGTA	CCATCTCTTGCTCGAAGTCT
	ATG5	CTGTTTCGATCTTCTTGATCA	TCCTTTTCTGGAAAACCTCTTGAA
	ATG6	CAGGCGAAACCAGGAGAG	CGAGTTTCAATAAATGGCTCCT
	ATG7	TTCTTAGAAGATTGACTGGTCTTACA	TCACTCATGTCCAGATCTCA

ATG: Autophagy-related gene; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

**Table 2** Metabolic characteristics of the LETO control and control or ezetimibe-treated OLETF

	LETO control (n = 3)	OLETF control (n = 5)	OLETF Ezetimibe (n = 6)
Body weight (g)	524.00 $\pm$ 1.15 <sup>b</sup>	617.67 $\pm$ 23.29	642.29 $\pm$ 20.47
Daily food intake (g)	3.61 $\pm$ 0.12 <sup>b</sup>	5.06 $\pm$ 0.30	4.62 $\pm$ 0.13
Liver tissue weight (%BW)	2.52 $\pm$ 0.05 <sup>a</sup>	3.60 $\pm$ 0.29	2.90 $\pm$ 0.10 <sup>a</sup>
Serum concentration			
Glucose (mmol/L)	5.24 $\pm$ 0.01 <sup>b</sup>	10.23 $\pm$ 0.13	7.25 $\pm$ 0.08 <sup>b</sup>
Insulin (ng/mL)	0.10 $\pm$ 0.002 <sup>b</sup>	0.82 $\pm$ 0.08	0.29 $\pm$ 0.05 <sup>b</sup>
HOMA-IR	0.57 $\pm$ 0.01 <sup>b</sup>	3.84 $\pm$ 0.26	1.75 $\pm$ 0.04 <sup>b</sup>
TG (mmol/L)	4.56 $\pm$ 0.08 <sup>b</sup>	12.94 $\pm$ 0.94	9.83 $\pm$ 0.94 <sup>a</sup>
FFA (mmol/L)	0.37 $\pm$ 0.01 <sup>b</sup>	0.69 $\pm$ 0.02	0.51 $\pm$ 0.02 <sup>b</sup>
TC ( $\mu$ mol/L)	124.02 $\pm$ 4.34 <sup>b</sup>	466.22 $\pm$ 12.32	255.08 $\pm$ 5.10 <sup>b</sup>
Liver concentration			
TG (mmol/mg protein)	14.75 $\pm$ 0.72 <sup>b</sup>	22.72 $\pm$ 1.21	6.66 $\pm$ 1.01 <sup>b</sup>
FFA (nmol/mg protein)	9.93 $\pm$ 0.64 <sup>b</sup>	18.90 $\pm$ 1.35	13.10 $\pm$ 0.60 <sup>b</sup>
TC (nmol/mg protein)	4.74 $\pm$ 0.41 <sup>b</sup>	17.81 $\pm$ 0.98	12.66 $\pm$ 0.50 <sup>b</sup>

Data are expressed as the mean  $\pm$  SE. <sup>a</sup> $P$  < 0.05, <sup>b</sup> $P$  < 0.01 *vs* OLETF control. BW: Body weight; FFA: Free fatty acids; HOMA-IR: Homeostasis model assessment of insulin resistance; TC: Total cholesterol; TG: Triglycerides.

Biotech, Arlington Heights, IL, United States). Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, United States).

### Electron microscopy

After washing with PBS, samples were fixed with 2.5% glutaraldehyde plus paraformaldehyde in 0.1 mol/L PBS (pH 7.4) for 2 h and washed three times for 30 min in 0.1 mol/L PBS. Next, glutaraldehyde-fixed specimens were treated with 1% OsO<sub>4</sub> in 0.1 mol/L PBS for 2 h, dehydrated in increasing concentrations of ethanol (50%-100%), infiltrated with propylene oxide, and embedded in an EPON mixture. Polymerized sections were then cut, stained, and examined using transmission electron microscopy (JEM-1101, JEOL, Japan).

### Statistical analysis

The data are expressed as the mean  $\pm$  SE. Student's *t* test were employed for comparisons of two matched groups using PASW Statistics 18 (SPSS Inc., Chicago, IL, United States). Statistical significance was defined

as  $P$  < 0.05.

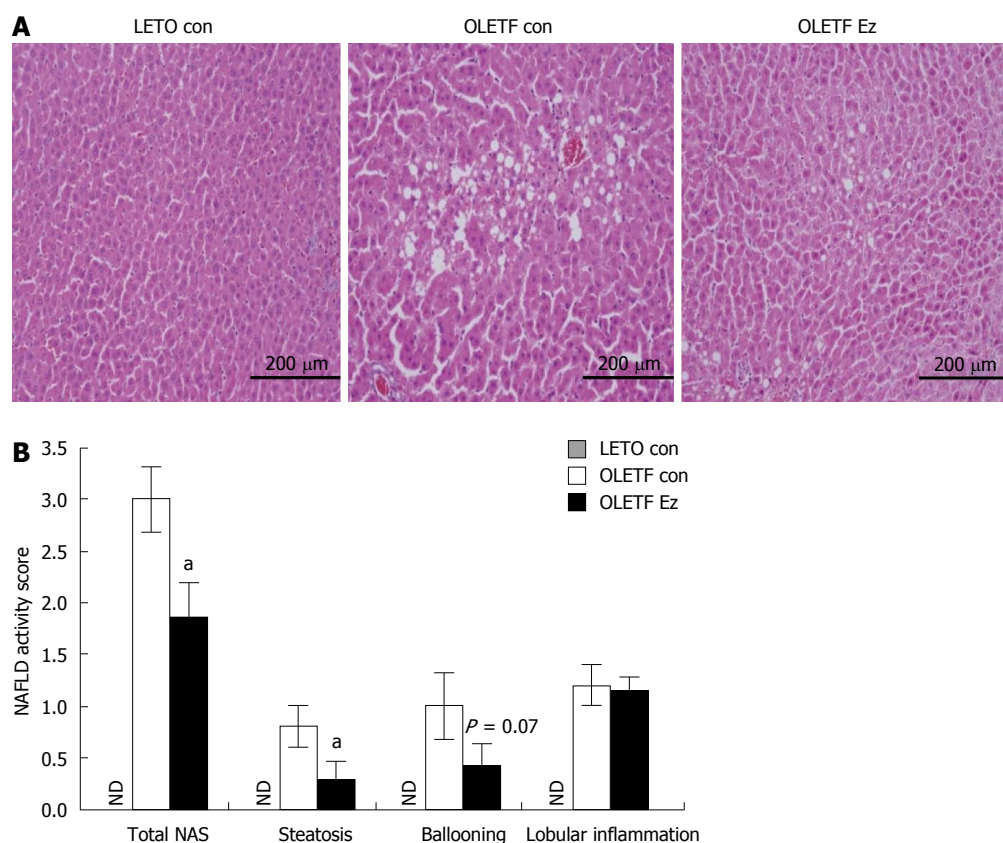
## RESULTS

### Ezetimibe affects hepatic steatosis without changing body weight and food intake in OLETF rats

As shown in Table 2, the body weight and daily food intake were not significantly different between control and ezetimibe-treated OLETF rats. Despite the lack of difference in body weight, the liver tissue weight was significantly decreased (20%) by ezetimibe in OLETF rats (Table 2). Likewise, blood and liver lipid levels including TG, FFA, and TC were significantly decreased in ezetimibe-treated OLETF rats (Table 2). Moreover, OLETF rats showed higher serum levels of glucose, insulin, HOMA-IR, TG, FFA, and TC than LETF animals, which were significantly reduced by ezetimibe (Table 2). In addition, histological analysis indicated that OLETF control rats showed larger lipid droplets in hepatocytes than age-matched LETO controls, which were attenuated by administration of ezetimibe (Figure 1A). Similar to these liver features, the NAFLD activity score (NAS) was also reduced by ezetimibe treatment (Figure 1B).

### Ezetimibe induces autophagy in OLETF liver tissue

To address whether ezetimibe administration alters the catabolic autophagy process, we first examined the mRNA and protein expression of autophagy-related genes (ATG). Among identified 30 ATG genes<sup>[34]</sup>, ATG5, ATG6, and ATG7 have been fully demonstrated using method of targeted deletion in animals and cells. In the process of autophagosome formation, ATG5 is conjugated and forms a complex with ATG12 and ATG16<sup>[35]</sup>. ATG6 and ATG7 are required for autophagy as a part of a lipid kinase complex or by specifically involvement in autophagosome formation<sup>[36,37]</sup>. In the present study, the hepatic mRNA expression of ATG5, ATG6, and ATG7 was significantly upregulated in ezetimibe-treated OLETF rats by at least 50% (Figure 2A), but no significant difference in protein levels was observed (Figure 2B and C). Microtubule-associated protein light chain 3 (LC3) is related to the extent of autophagosome formation<sup>[38]</sup>. Specifically, the level of LC3-II is closely associated with the number of



**Figure 1** Ezetimibe improves hepatic steatosis in OLETF rats. A: Representative HE liver sections (scale bar, 200  $\mu$ m; magnification  $\times$  200); B: NAFLD activity score. Data are expressed as the mean  $\pm$  SE. <sup>a</sup>P < 0.05 vs OLETF control. ND: Not detected in LETO control (LETO con); white bar: OLETF control (OLETF con); black bar: OLETF ezetimibe group (OLETF Ez); NAFLD: Nonalcoholic fatty liver disease.

autophagosomes present; thus, the ratio between LC3-I and LC3-II (LC3 conversion) can be used to measure the extent of autophagy<sup>[39]</sup>. In the current study, liver LC3 conversion (LC3-II/LC3-I) was decreased in OLETF controls compared to LETO controls, but ezetimibe administration significantly increased the relative ratio of LC3-II to LC3-I by 15% (Figure 2B and C).

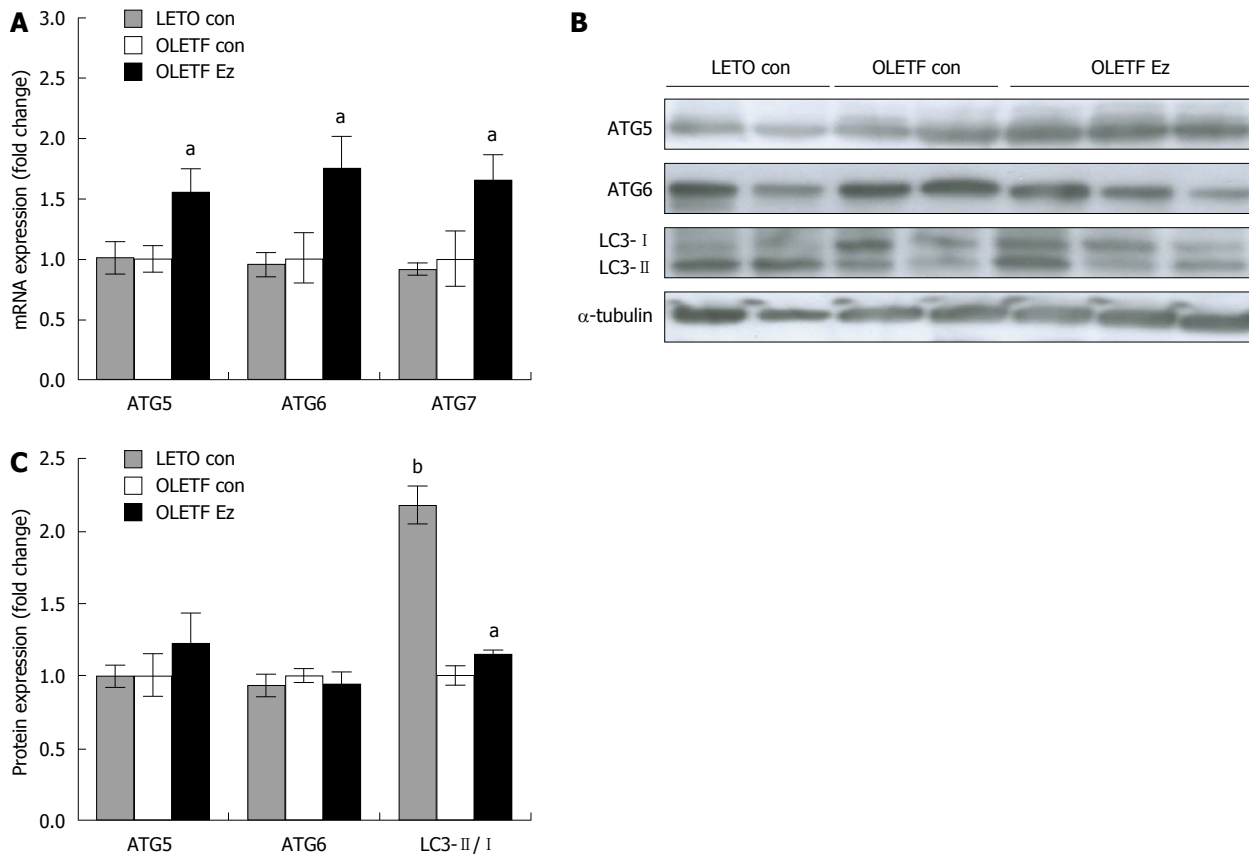
#### ***In vitro effects of ezetimibe on TG levels and autophagy in Huh7 hepatocytes***

Human huh7 hepatocytes were pretreated with ezetimibe (10  $\mu$ mol/L, 1 h) and incubated with PA (0.5 mmol/L, 24 h) to induce hepatic steatosis. As shown in Figure 3A, ezetimibe treatment significantly attenuated PA-increased TG levels, which was consistent with our animal study. PA treatment resulted in an approximately 20% decrease in mRNA expression of ATG5, ATG6, and ATG7, which had been increased by ezetimibe treatment (Figure 3B). In addition, ezetimibe treatment significantly increased the PA-induced reduction in LC3 protein abundance (Figure 3C and D). However, p62, specific substrates for autophagy turnover and degradation<sup>[40]</sup> was significantly elevated by PA which was attenuated by ezetimibe in PA-treated hepatocytes (Figure 3C and D). Transmission electron microscopy (TEM) was employed to investigate the

effects of ezetimibe on hepatocyte autophagosome formation. As shown in Figure 4A, PA plus ezetimibe-treated hepatocytes exhibited increased formation of autophagosomes compared to the PA-treated cells. To determine if ezetimibe increases autophagic flux, cells were co-treated with bafilomycin A1 (BAF), an inhibitor of autophagy that inhibits the vacuolar type H<sup>+</sup>-ATPase, and immunoblotting for LC3 was performed. The results shown in Figure 4B and C indicate that the combination of PA and ezetimibe in the presence of BAF increased the ratio of LC3-II to LC-I, suggesting that ezetimibe affects autophagic flux in PA-treated hepatocytes.

## **DISCUSSION**

Ezetimibe is a drug used to lower blood cholesterol levels by targeting NPC1L1<sup>[5,6]</sup>. As described in previous studies showing ezetimibe-mediated improvement in metabolic syndrome<sup>[7-11]</sup>, we found that chronic ezetimibe treatment improves glycemic control and leads to an increase in bioactive glucagon-like peptide-1 (GLP-1) and pancreatic  $\beta$ -cell mass in OLETF rats<sup>[41]</sup>. In addition, recent findings concerning hepatic NPC1L1 expression and its ability to mediate improvement in hepatic fat accumulation have attracted new research interest<sup>[42]</sup>. Previous studies have suggested that ezetimibe attenuates hepatic steatosis by decreasing



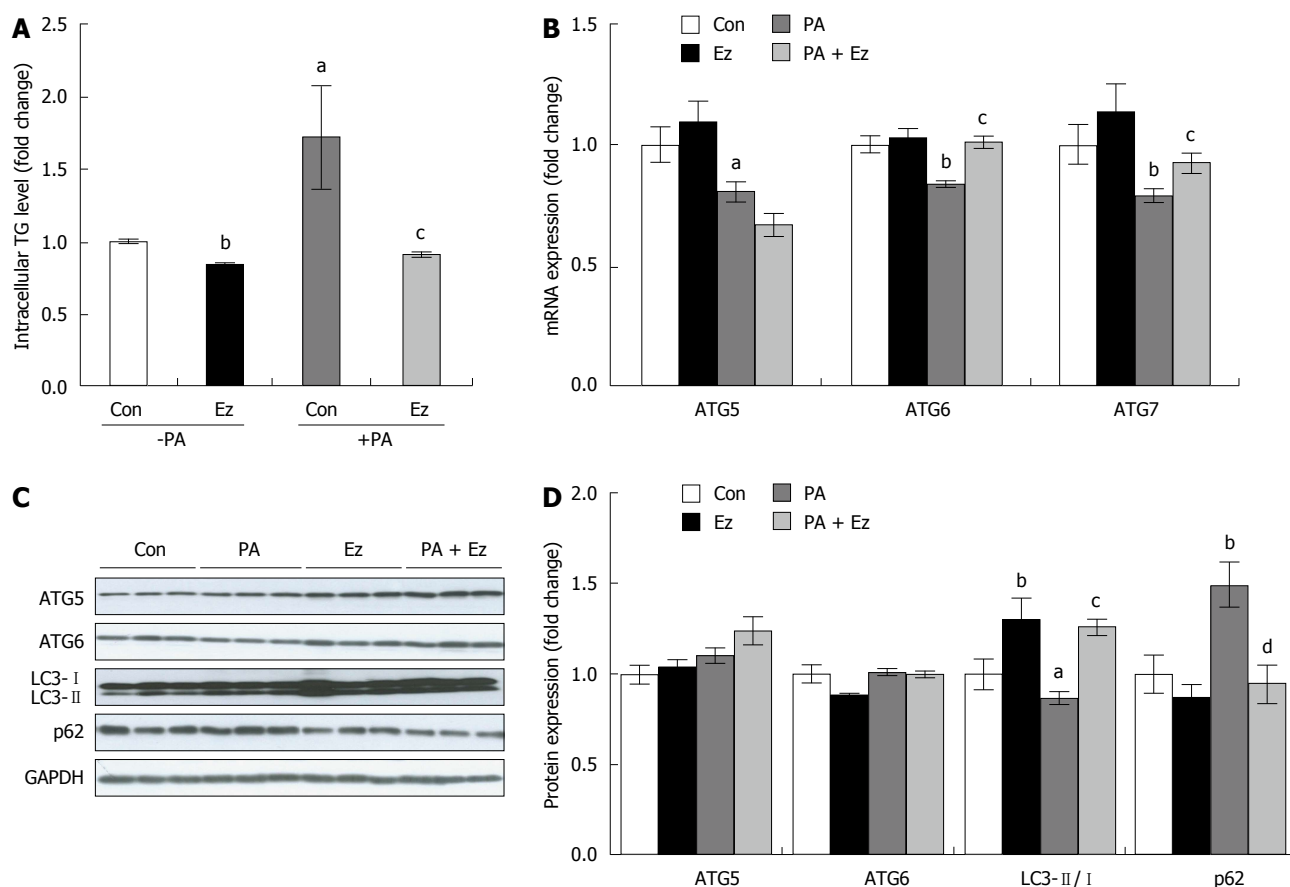
**Figure 2** Ezetimibe increases autophagy makers in OLETF liver tissue. mRNA level (A) and protein expression (B and C) was measured and expressed as the fold change compared to OLETF control. ATG 5 and 6 protein expression was normalized by  $\alpha$ -tubulin and LC3 protein abundance was expressed as the ratio between LC3-II (14 kDa) and LC3-I (16 kDa). Results are presented as mean  $\pm$  SE. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs OLETF control. ATG: Autophagy-related gene; LETO con: LETO control; OLETF con: OLETF control; OLETF Ez: OLETF ezetimibe.

hepatic oxidative stress and improving hepatic insulin sensitivity and lipid metabolism<sup>[7,8,16-20]</sup>. However, most studies used a mouse model in which hepatic NPC1L1 expression is undetectable. Therefore, these findings might be associated with the ability of ezetimibe to inhibit intestinal cholesterol uptake and the subsequent reduction in hepatic lipid trafficking, rather than a direct effect on liver and hepatic fat metabolism. In the current study, we investigated the role of ezetimibe in hepatic fat accumulation using both *in vivo* and *in vitro* models. Age-matched LETO rats were used to compare with OLETF rats. OLETF control rats showed bigger liver size, heavier body weight, and higher glucose and insulin levels than those of LETO controls. Ezetimibe administration in OLETF rats ( $n = 5$ ) significantly decreased liver weight and serum and liver lipid parameters including TG, FFA, and TC levels, compared to OLETF control animals ( $n = 6$ ). In addition to the improvement of hepatic fat accumulation, we also found that ezetimibe significantly decreased serum concentrations of glucose and insulin and HOMA-IR, an index of insulin resistance in OLETF rats, consistent with our previous study<sup>[41]</sup> and numerous other studies<sup>[7-9,13,17,20]</sup>. Moreover, *in vitro* hepatocytes, ezetimibe treatment significantly reduced PA-induced TG accumulation ( $P < 0.05$ ). According to the most

accepted hypothesis for hepatic steatosis, the multi-hits model, the accumulation of FFAs and TG in hepatocytes is the first hit which led up to subsequent hits to more advanced stages of liver injury<sup>[43]</sup>. Thus, ezetimibe-decreased concentrations of lipid parameters in the serum and liver might show the preventive effects of ezetimibe on the initial stage of NAFLD.

As a bulk degradation system, autophagy breaks down the plasma membrane and extracellular proteins in the lysosomal pathway *via* the formation of double-membrane structures known as autophagosomes<sup>[22]</sup>. This catabolic pathway has been described recently as a regulator of lipid storage and metabolism, suggesting that autophagy might be a potential therapeutic target for excessive fat accumulation<sup>[44]</sup>. Genetic and dietary animal models for obesity and hepatic steatosis decrease mRNA expression of autophagy-related genes and conditional ATG7-knockout mice improves hepatic lipid accumulation<sup>[27,45]</sup>. These animal studies reveal that autophagy play a major role in hepatic lipid metabolism. Moreover, deleted autophagy-related gene (ATG) leads to impaired insulin signaling and increased endoplasmic reticulum (ER) stress. Restoration of ATG in liver of this ATG deficient animals, decreases ER stress and improves hepatic insulin action<sup>[46]</sup>. It demonstrates that autophagy is involved in intracellular



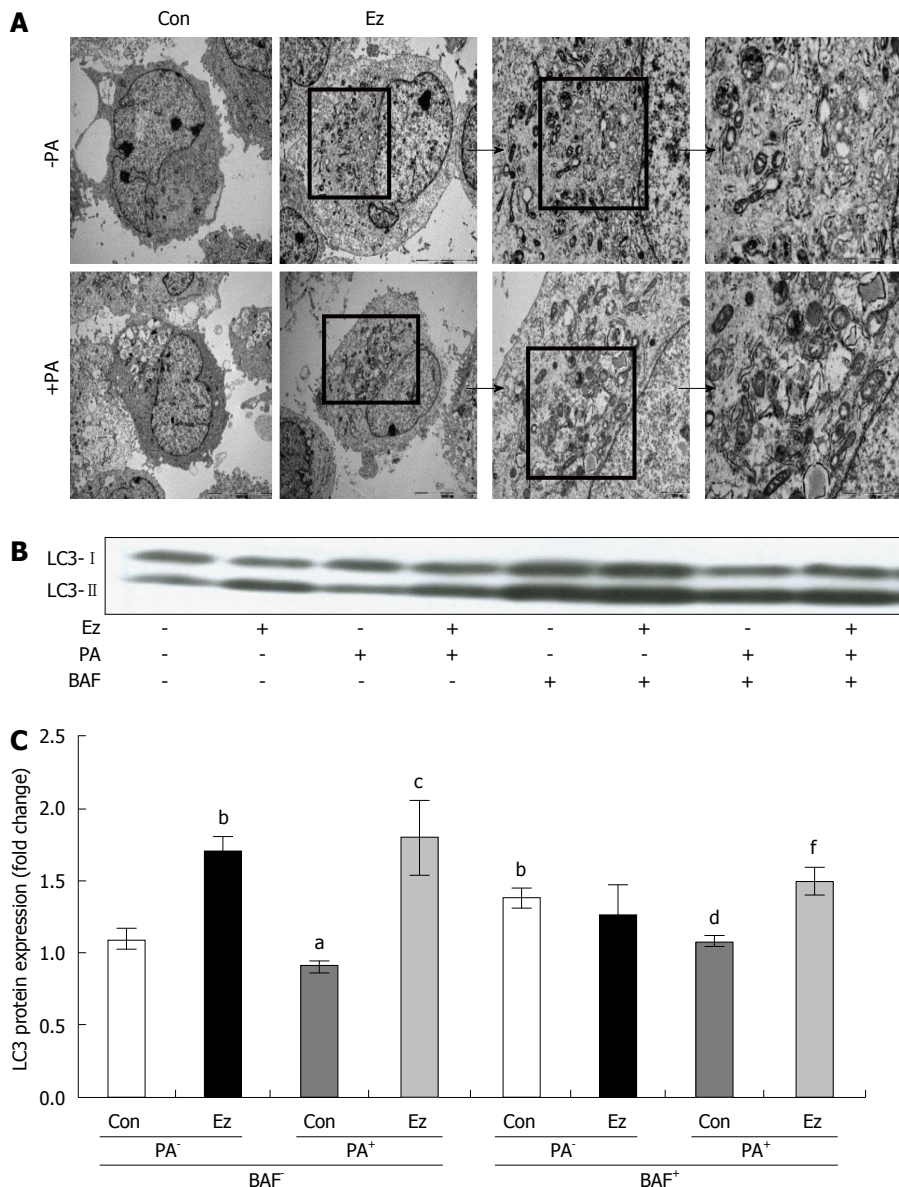


**Figure 3** Ezetimibe treatment attenuates triglycerides accumulation and induces autophagy in hepatocytes. Hepatic triglyceride concentration (A) and mRNA (B) and protein expression (C and D) involved in autophagy were expressed as the mean  $\pm$  SE. Experiments represent at least three independent experiments ( $n \geq 9$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs control; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , PA vs PA + Ez. ATG: Autophagy-related gene; Con: Control; Ez: Ezetimibe; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PA: Palmitic acid; TG: Triglycerides.

lipid storage, release of fatty acids from triglyceride within hepatocyte lipid droplets, lipotoxicity-induced ER stress, and hepatic insulin sensitivity. In addition, liver specific autophagy deficient mice show hepatocyte cell death and liver injury<sup>[47]</sup>. In autophagy deficient cells, hepatocyte survival is impaired and production of TNF $\alpha$  is increased, all of which leads to hepatocellular carcinoma<sup>[48]</sup>. Moreover, autophagy is associated with cell death by interacting with Fas-associated protein with death domain (FADD)<sup>[49]</sup>. It suggests that autophagy plays a critical role of tumor-suppression mechanism.

In the present study, ezetimibe administration decreased liver weight and lipid levels and improved serum metabolic parameters and histological features, in addition to increasing the expression of LC3-II, in a rat model of type 2 diabetes. The ezetimibe-induced autophagy that we observed was consistent with a recent study showing that ezetimibe plays a role in cholesterol homeostasis and liver degeneration in  $\alpha$ 1-antitrypsin deficiency<sup>[50]</sup>. To induce hepatic steatosis, we incubated Huh7 human hepatocytes with 0.5 mmol/L PA for 24 h. As shown in a previous study, PA treatment in hepatocytes significantly decreases autophagy, suggesting that hepatic autophagy is associated

with hepatic fat accumulation<sup>[29]</sup>. In the present study, ezetimibe treatment increased PA-decreased autophagosome formation in Huh7 hepatocytes as determined by TEM and Western blotting of LC3-II and p62 expression. In an autophagic flux assay designed to measure changes in LC3-II in the presence of BAF, an inhibitor of late-phase autophagy that prevents fusion between autophagosomes and lysosomes, ezetimibe treatment increased the level of LC3-II. Taken together, these data indicate that ezetimibe induces autophagy in PA-treated hepatocytes. Despite important findings illustrating the involvement of ezetimibe in autophagy formation and the improvement of hepatic fat accumulation, our study has limitations; gender bias, small sample size, and not to demonstrate direct ezetimibe action to autophagy. Like most previous other studies, the present study used only male animals to demonstrate the influence of ezetimibe on liver to prevent confounding factors such as reproductive cycles and hormone fluctuations. To know exactly how ezetimibe improves hepatic steatosis in women, further studies with female animal model are necessary to be executed. In addition, further investigation is also needed to determine whether ezetimibe as autophagy inducer is directly involved in the improvement of



**Figure 4 Ezetimibe increases autophagosome formation and autophagic flux in hepatocytes.** Electron microscopy of hepatocytes demonstrated ezetimibe-induced autophagy (A). Representative blot (B) and quantitative analysis (C) expressed as LC3-II (14 kDa)/LC3-I (16 kDa) followed by sample/control (mean  $\pm$  SE). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs control in the absence of PA and BAF; <sup>c</sup> $P < 0.05$ , PA vs PA + Ez in the absence of BAF; <sup>d</sup> $P < 0.01$ , vs control in the absence of PA but in the presence of BAF. <sup>f</sup> $P < 0.01$ , PA vs PA + Ez in the presence of BAF. BAF: Bafilomycin A1; Con: Control; Ez: Ezetimibe; PA: Palmitic acid.

hepatic steatosis by using liver-specific targeted ATG modification in animals with large sample number and precise methods such as electron microscopy, fluorescence microscopy, molecular assays, and use of chemical modulators.

In conclusion, ezetimibe treatment attenuates hepatic fat accumulation and improves hyperglycemia, which is accompanied by an increase in autophagy flux. To the best of our knowledge, this is the first study to show that ezetimibe induces autophagy in the liver of diabetic rats and PA-treated hepatocytes. Our findings suggest a possible target of ezetimibe action and the potential for the use of ezetimibe in the treatment of hepatic steatosis.

## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease representing fat accumulation with hepatocytes. NAFLD is strongly associated with other components of metabolic syndrome. Given the high prevalence of NAFLD and its positive correlation with metabolic syndrome, it is important to prevent fat accumulation in the liver. However, there is no satisfying therapeutic strategy for NAFLD.

### Research frontiers

Ezetimibe, a Niemann-Pick C1-Like 1 inhibitor has been used as an agent for hypercholesterolemia. In addition to the favorable effects of ezetimibe on lipid metabolism, numerous studies demonstrate that ezetimibe improves other metabolic disorders such as hepatic steatosis and diabetes. However, the mechanisms by which ezetimibe influences hepatic fat accumulation are still unclear.

## Innovations and breakthroughs

In this current study, ezetimibe administration improved glucose homeostasis and serum and hepatic lipid parameters, which is accompanied by increased autophagy-related gene and protein expression in liver of obese and diabetic rats. Authors also found that ezetimibe might affect autophagic flux in fatty acid-treated hepatocytes.

## Applications

The results in the present study suggest a possible target of ezetimibe action and potential use of ezetimibe in the treatment of NAFLD.

## Terminology

Autophagy is a cellular catabolic process by lysosome-dependent machinery, which role is to maintain cellular energy homeostasis. Ezetimibe is a lipid-lowering compound that selectively inhibits the intestinal cholesterol.

## Peer-review

The manuscript describes the effect of ezetimibe on hepatic steatosis in a rat model of obesity and type II diabetes. Major conclusion of the manuscript is the induction of autophagy in the liver by application of ezetimibe and, therefore, a reduction of hepatic steatosis. The idea of ezetimibe as inducer of autophagy in the liver/hepatocytes is in line with a previous report by a different group earlier this year. There are several points that need to be addressed.

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## Basic Study

# Overexpression of HMGB1 A-box reduced lipopolysaccharide-induced intestinal inflammation *via* HMGB1/TLR4 signaling *in vitro*

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## Abstract

**AIM:** To investigate the inhibitory effects and mechanism of high mobility group box (HMGB)1 A-box in lipopolysaccharide (LPS)-induced intestinal inflammation.

**METHODS:** Overexpression of HMGB1 A-box in human intestinal epithelial cell lines (SW480 cells) was achieved using the plasmid pEGFP-N1. HMGB1 A-box-overexpressing SW480 cells were stimulated with LPS and co-culturing with human monocyte-like cell lines (THP-1 cells) using a Transwell system, compared with another HMGB1 inhibitor ethyl pyruvate (EP). The mRNA and protein levels of HMGB1/toll-like receptor (TLR) 4 signaling pathways [including HMGB1, TLR4, myeloid differentiation factor88

(MYD88), Phosphorylated Nuclear Factor  $\kappa$ B (pNF- $\kappa$ B p65) in the stimulated cells were determined by real-time polymerase chain reaction and Western blotting. The levels of the proinflammatory mediators [including HMGB1, interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$ ] in the supernatants of the stimulated cells were determined by ELISA.

**RESULTS:** EP downregulated the mRNA and protein levels of HMGB1, inhibited the TLR4 signaling pathways (TLR4, MYD88 and pNF- $\kappa$ B p65) and reduced the secretion of proinflammatory mediators (HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) in the SW480 and THP-1 cells activated by LPS but not in the unstimulated cells. Activated by LPS, the overexpression of HMGB1 A-box in the SW480 cells also inhibited the HMGB1/TLR4 signaling pathways and reduced the secretion of these proinflammatory mediators in the THP-1 cells but not in the transfected and unstimulated cells.

**CONCLUSION:** HMGB1 A-box, not only EP, can reduce LPS-induced intestinal inflammation through inhibition of the HMGB1/TLR4 signaling pathways.

**Key words:** High mobility group box 1; Toll-like receptor 4; HMGB1 A-box; Ethyl pyruvate; Inflammatory bowel disease

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**Core tip:** We have provided the first report that high mobility group box (HMGB)1 A-box, not only ethyl pyruvate, can specifically inhibit HMGB1/toll-like receptor 4 signaling pathways and reduce lipopolysaccharide-induced intestinal inflammation. Our findings indicate the potential of the HMGB1 A-box as a novel approach in the treatment of inflammatory bowel disease.

Wang FC, Pei JX, Zhu J, Zhou NJ, Liu DS, Xiong HF, Liu XQ, Lin DJ, Xie Y. Overexpression of HMGB1 A-box reduced lipopolysaccharide-induced intestinal inflammation *via* HMGB1/TLR4 signaling *in vitro*. *World J Gastroenterol* 2015; 21(25): 7764-7776 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7764.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7764>

## INTRODUCTION

Inflammatory bowel disease (IBD) comprises the chronic relapsing inflammatory disorders Crohn's disease and ulcerative colitis. IBD is thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host<sup>[1]</sup>. Although the etiologies of IBD remain unclear, available evidence suggests that an abnormal immune response to microorganisms of the intestinal flora is responsible for the disease in genetically susceptible individuals. Therefore, this

abnormal immune response plays a major role in the pathogenesis of IBD<sup>[2]</sup>.

Intestinal epithelial cell lines constitutively express several members of a novel family of transmembrane receptors designated toll-like receptors (TLRs) that may serve as major links between the innate and adaptive mucosal immune responses. Within a susceptible individual, aberrant or dysfunctional TLR signaling may impair commensal-mucosal homeostasis, thereby contributing to the amplification and perpetuation of tissue injury, consequently leading to the chronic inflammation that occurs in IBD<sup>[3]</sup>. TLR4 is the primary receptor needed for the promotion of macrophage activation, cytokine release, and tissue damage. The presence of this receptor may indicate the anomalous regulation of innate immunity, and it contributes to the production of proinflammatory mediators and disease development<sup>[4]</sup>. TLR4 mediates the recognition of antigens in the intestinal lumen as lipopolysaccharides (LPS) due to the activation of NF- $\kappa$ B *via* myeloid differentiation factor (MYD)88, thereby increasing the production of proinflammatory cytokines, such as interleukin (IL)-1 $\beta$ , IL-6 and IL-8, and the susceptibility to invasion by pathogens in the lamina propria, perpetuating the inflammatory process<sup>[5]</sup>. Numerous reports have indicated that TLR4 plays a pivotal role in IBD, but the underlying mechanism remains to be elucidated.

Recent extensive studies have demonstrated that high mobility group box (HMGB)1 is a novel endogenous ligand for TLR4. HMGB1, which is an evolutionarily highly conserved and abundant nuclear protein, also functions within the cytoplasm and as an extracellular damage-associated molecular pattern (DAMP) molecule. Extracellular HMGB1 is the prototypic endogenous "danger signal" that triggers inflammation and immunity<sup>[6]</sup>. This protein is either actively secreted by monocytes/macrophages or passively released from necrotic cells from any tissue. It has recently been implicated in the pathogenesis of IBD. In IBD patients and mice with colitis, HMGB1 is secreted by inflamed intestinal tissues, and it is present at high levels in feces. The large quantities of HMGB1 in the gastrointestinal tract mediate inflammation and gastrointestinal barrier failure<sup>[7]</sup>. This protein is abundantly secreted by the inflamed intestinal tissues of pediatric patients with IBD. Once released, it behaves as a cytokine-like proinflammatory molecule by upregulating other proinflammatory mediators<sup>[8]</sup>. HMGB1 also alters intestinal epithelial cell permeability<sup>[9]</sup>. It has been implicated in the pathogenesis of diseases in which excessive inflammation plays a key role, such as IBD. Therefore, the targeting of the HMGB1/TLR4 signaling pathways may represent a novel approach for the treatment of IBD. A growing number of HMGB1 inhibitors, including neutralizing antibodies, endogenous hormones, medicinal-herb-derived small molecules and ethyl pyruvate (EP), have been developed. Studies have

shown that the neutralization of HMGB1 activity by the administration of anti-HMGB1 antibodies or EP attenuates colon injury, reduces weight loss, and improves colon scores in animal models of colitis<sup>[7]</sup>. Interestingly, recent studies have shown that HMGB1 A-box alone, as a natural antagonist of HMGB1, can competitively inhibit the binding of HMGB1 to its receptors and attenuate the proinflammatory effect of the full-length HMGB1 and the B-box peptide. The A-box is thus considered to be a specific blockade for endogenous HMGB1<sup>[10,11]</sup>. However, it is unknown whether the A-box can be used to treat IBD.

To investigate the effects of two HMGB1 inhibitors (HMGB1 A-box and EP) in IBD *in vitro*, we assumed that the targeting of the HMGB1/TLR4 signaling pathways would reveal whether the HMGB1 A-box represents a novel approach to the treatment of IBD. Therefore, in this study, we constructed human intestinal epithelial cell lines (SW480 cells) overexpressing HMGB1 A-box using gene transfection technology, and observed changes in the HMGB1/TLR4 signaling pathways in human monocyte-like cell lines (THP-1 cells) co-cultured with SW480 cells overexpressing HMGB1 A-box that were activated by LPS. We also investigated the effects of the specific inhibition of HMGB1 A-box on the HMGB1/TLR4 signaling pathways compared with those of EP. We aimed to provide theoretical and experimental evidence of HMGB1 A-box as a potential therapeutic target for IBD.

## MATERIALS AND METHODS

### Cell lines and reagents

The human intestinal epithelial cell line SW480 (ATCC NO.CCL-228) and human acute monocytic leukemia cell line THP-1 (ATCC NO.TIB-202) were obtained from the Gastroenterology Institute of Jiangxi Province. The cells were maintained in RPMI-1640 medium (HyClone, Logan, UT, United States) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone) at 37 °C and incubated in a humidified 5% (v/v) CO<sub>2</sub> incubator. Lipopolysaccharide (LPS) from *Escherichia coli* was used for the stimulation of the SW480 cells. SW480 cells were pretreated with EP (Sigma-Aldrich, St. Louis, MO, United States) for 1 h before LPS stimulation.

### Cell transfection

Overexpression of the truncated intracellular form of HMGB1 A-box in SW480 cells was achieved using the plasmid pEGFP-N1 (Generay, Shanghai, China). To eliminate endotoxin contamination, all plasmids were prepared using an Endo-free Plasmid Mini Kit II (Omega, San Carlos, CA, United States). Transient transfection was performed with FuGENE 6 Transfection Reagent (Promega, Sunnyvale, CA, United States). Overexpression of HMGB1 A-box was confirmed with dual-endonuclease digestion and sequencing.

### Transwell experiments

A Transwell system was used to prevent direct contact between SW480 cells and THP-1 cells. Our Transwell culture plates (Corning, Corning, NY, United States) had six wells composed of upper and lower chambers separated by polycarbonate membrane with a pore diameter of 0.4 mm. SW480 cells were prepared in the lower chamber and pretreated with EP (Sigma-Aldrich) for 1 h before LPS stimulation. THP-1 cells were loaded into the upper chamber.

### Real-time polymerase chain reaction

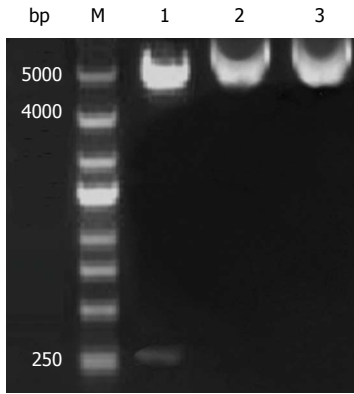
Total RNA was extracted from the cells using TRIzol reagent (Tiangen, Beijing, China). The obtained total RNA (500 ng to 1 µg) was reverse transcribed following the removal of genomic DNA using a PrimeScrip RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). Quantitative polymerase chain reaction (qPCR) amplification was performed using a thermal cycler (BioRad, Richmond, VA, United States) with SYBR Premix Ex Ta (Tli RNase H Plus). A total of 2 µL of cDNA was amplified. Relative expression levels were calculated and analyzed by the 2<sup>-ΔΔCt</sup> equation. The primer sequences used were as follows:

HMGB1 A-box forward: 5'-ACCCAGATGCTTCAGTCAACTT-3', reverse: 5'-CTCTTTCATAACGGGCCTTGT-3'; HMGB1 forward: 5'-GGAGATCCTAAGAAGCCGAGA-3', reverse: 5'-CATGGTCTTCCACCTCTCTGA-3'; TLR4 forward: 5'-AGGACTGGGTAAGGAATGAGC-3', reverse: 5'-ATCACCTTTCGGCTTTTATGG-3'; MYD88 forward: 5'-AAGAAAGAGTTCCTCCAGCATC-3', reverse: 5'-GCGAGTCCAGAACCAAGATTT-3'; and β-actin forward: 5'-TGACGTGGACATCCGCAAAG-3', reverse: 5'-TGACGTGGACATCCGCAAAG-3'.

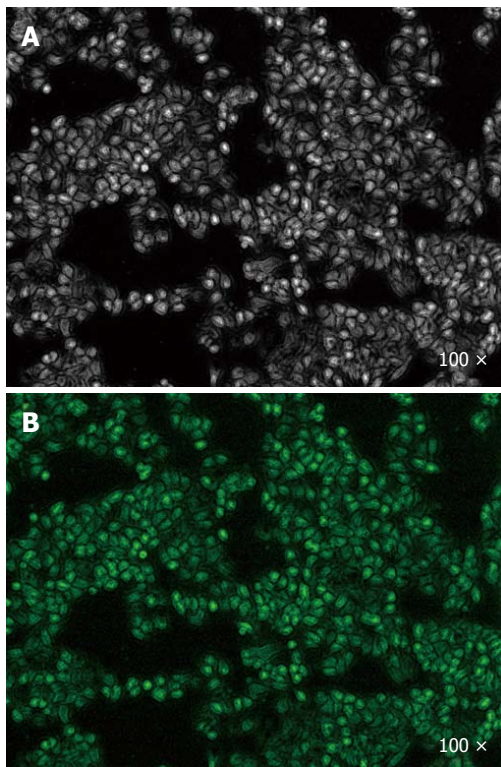
### Western blot analysis

Cells were subjected to cell lysis, and the total protein was extracted as previously described<sup>[8]</sup>. Protein concentrations were measured using the BCA protein assay (Generay, Shanghai, China). Lysates were separated using SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with a rabbit anti-HMGB1 antibody (ab18256, 1:1000; Abcam, Cambridge, MA, United States), mouse anti-TLR4 antibody (ab22048, 1:1000; Abcam), rabbit anti-MYD88 antibody (HFL-296) (Santa Cruz Biotechnology, Santa Cruz, CA, United States), rabbit anti-phospho-NF-κB p65 antibody (Cell Signaling Technology, Beverly, MA, United States) and rabbit anti-β-actin antibody (ab1801, 1:1000; Abcam). Goat anti-rabbit IgG-horseradish peroxidase (HRP) or donkey anti-mouse IgG-HRP (both 1:2000) served as secondary antibodies. Immunoreactive proteins were visualized, and band intensity was quantified using a ChemiDo MP System with Image La Software (BioRad, Richmond, VA, United States). The data were normalized to the β-actin levels.





**Figure 1** Restriction analysis of pEGFP-N1- high mobility group box 1 protein A-box. Lane M: marker; lane 1: pEGFP-N1- high mobility group box 1 protein (HMGB1) A-box digested by *XhoI* + *BamHI*; lane 2: pEGFP-N1-HMGB1 A-box digested by *XhoI*; and lane 3: pEGFP-N1-HMGB1 A-box digested by *BamHI*.



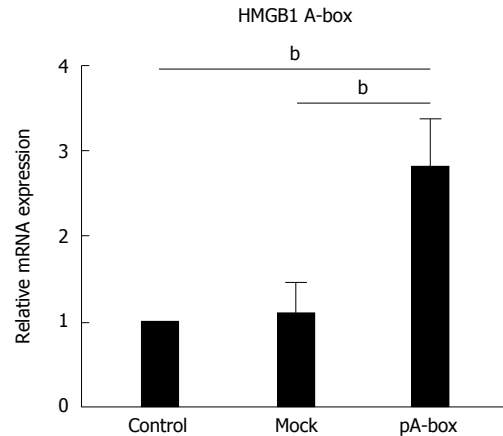
**Figure 2** SW480 cells at 48 h after transfection (magnification  $\times 100$ ). A: Bright-field images of cells (after 48 h); B: Green fluorescence of transfected cells (after 48 h).

#### ELISA for measuring IL-1 $\beta$ , IL-6, TNF- $\alpha$ and HMGB1

The concentrations of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and HMGB1 in the cell culture supernatants from the SW480 cells alone or those co-cultured with THP-1 were determined by ELISA using a commercial human multiplex kit (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) (Aushon Biosystems, Wuxi, China) or human HMGB1 kit (Uscn Life Science, Wuhan, China).

#### Statistical analysis

The data are expressed as the mean  $\pm$  SD of three independent experiments. The data were statistically



**Figure 3** Expression of HMGB1 protein pA-box mRNA in transfected SW480 cells. After SW480 cells were transfected for 48 h, HMGB1 pA-box mRNA levels were determined by real-time PCR. The expression of HMGB1 A-box mRNA in the pA-box-transfection SW480 cells was significantly upregulated compared with the control and mock-transfection groups. The data are expressed as the mean  $\pm$  SD and are derived from three independent experiments, which were each performed in duplicate. The means with asterisks above them are significantly different ( $^bP < 0.01$  vs control).

analyzed as indicated in the figure legends using GraphPad Prism version 5.0. Differences between any two groups were determined by the *t* test. Differences among multiple groups were determined by one-way ANOVA.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Construction of HMGB1 A-box-overexpressing eukaryotic plasmid cell lines and HMGB1 A-box identification

To construct the HMGB1 A-box recombinant expression plasmid and identify HMGB1 A-box, the pEGFP-N1-HMGB1 A-box recombinant plasmid was digested with *XhoI* and *BamHI*. After 2% agarose gel electrophoresis, the fragments in the agarose gel resulting from the enzymatic digestion were observed. Restriction analysis of pEGFP-N1-HMGB1 A-box is shown in Figure 1. The HMGB1 A-box fragment was 256 bp, consistent with the expected size. The inserted HMGB1 A-box fragments were consistent with published data (Gen-Bank Accession: NM\_002128.4).

To evaluate the expression of the HMGB1 A-box recombinant eukaryotic plasmid in SW480 cells, these cells were assessed by fluorescence microscopy (Olympus IX71, Tokyo, Japan). Those transfected with the HMGB1 A-box recombinant plasmid fluoresced green, and photographs were obtained using a digital camera. Green fluorescence of the transfected cells was observed at 48 h after transfection (Figure 2). The expression of HMGB1 A-box mRNA in the pA-box-transfected SW480 cells was higher than that in the control and mock-transfection groups ( $^bP < 0.01$ ) (Figure 3). These results suggested that the SW480 cells were successfully transfected.



### **Effects of overexpression of HMGB1 A-box and EP on HMGB1 and TLR4/LPS signaling pathways and secretion of cytokines in SW480 cells**

To elucidate the roles of HMGB1 and TLR4 in intestinal epithelial cells activated by LPS, we observed the effects of HMGB1 inhibitors (HMGB1 A-box and EP) on the HMGB1 and TLR4 signaling pathways in stimulated SW480 cells.

The HMGB1, TLR4, MYD88, pNF- $\kappa$ B p65 mRNA and protein levels in stimulated SW480 cells were determined by real-time PCR and western blotting (Figure 4A-H). There were no significant differences in the HMGB1/TLR4/MYD88/pNF- $\kappa$ B p65 mRNA and protein levels among the groups without the LPS treatment ( $P > 0.05$ ). In the LPS-treated groups, HMGB1/TLR4/MYD88/pNF- $\kappa$ B p65 mRNA and protein levels in the EP group were significantly lower than those in the control group, mock-transfection group and pA-box-transfection group ( $^aP < 0.05$  and  $^bP < 0.01$ ), but there were no significant differences detected among the control group, mock-transfection group and pA-box-transfection group ( $P > 0.05$ ). All LPS treatment groups except the EP group showed significantly higher levels of these mRNAs and proteins compared with the corresponding groups without LPS treatment ( $^aP < 0.05$ ,  $^bP < 0.01$ ).

The cytokine levels of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the supernatants of stimulated SW480 cells were determined by ELISA (Figure 5A-D). There were no significant differences in the levels of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  among the groups without LPS treatment ( $P > 0.05$ ). In the LPS treatment groups, the levels of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the EP group were significantly lower than those in the control group, mock-transfection group and pA-box-transfection group ( $^aP < 0.05$ ,  $^bP < 0.01$ ), but there were no significant differences among the control group, mock-transfection group and pA-box-transfection group ( $P > 0.05$ ). All LPS treatment groups except the EP group showed significantly higher levels of these mRNAs and proteins than the corresponding groups without the LPS treatment ( $^aP < 0.05$ ,  $^bP < 0.01$ ).

### **Effects of overexpression of HMGB1 A-box and EP on HMGB1 and TLR4/LPS signaling pathways and cytokine secretion in THP-1 cells**

To further elucidate the roles of HMGB1 and TLR4 in IBD, we observed the effects of HMGB1 inhibitors (HMGB1 A-box and EP) on the HMGB1 and TLR4 signaling pathways in THP-1 cells co-cultured with SW480 cells.

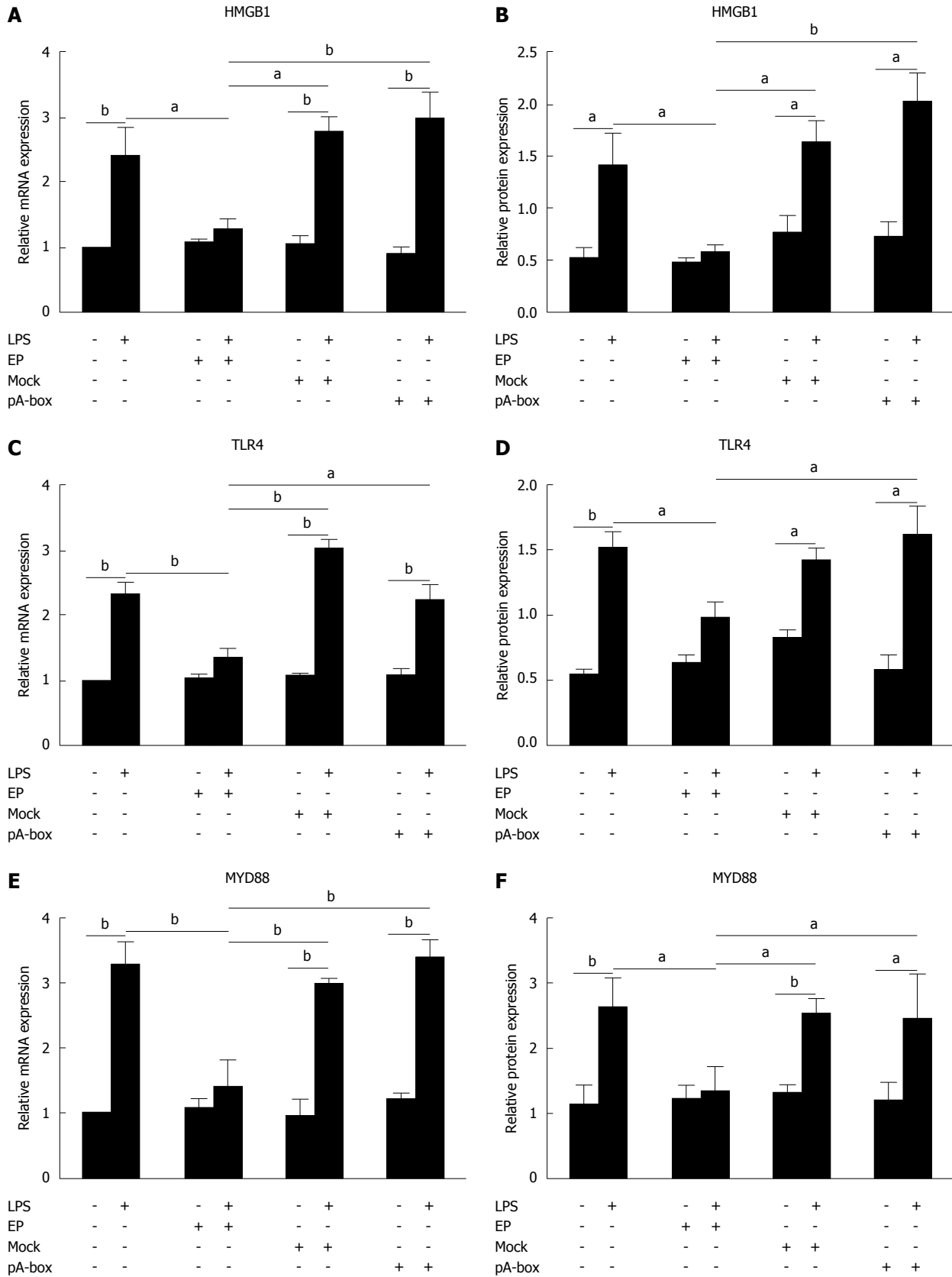
Following co-culturing with SW480 cells, the HMGB1, TLR4, MYD88, and pNF- $\kappa$ B p65 mRNA and protein levels in the THP-1 cells were determined by real-time PCR and western blotting (Figure 6A-H). There were no significant differences in the HMGB1/TLR4/MYD88/pNF- $\kappa$ B p65 mRNA and protein levels

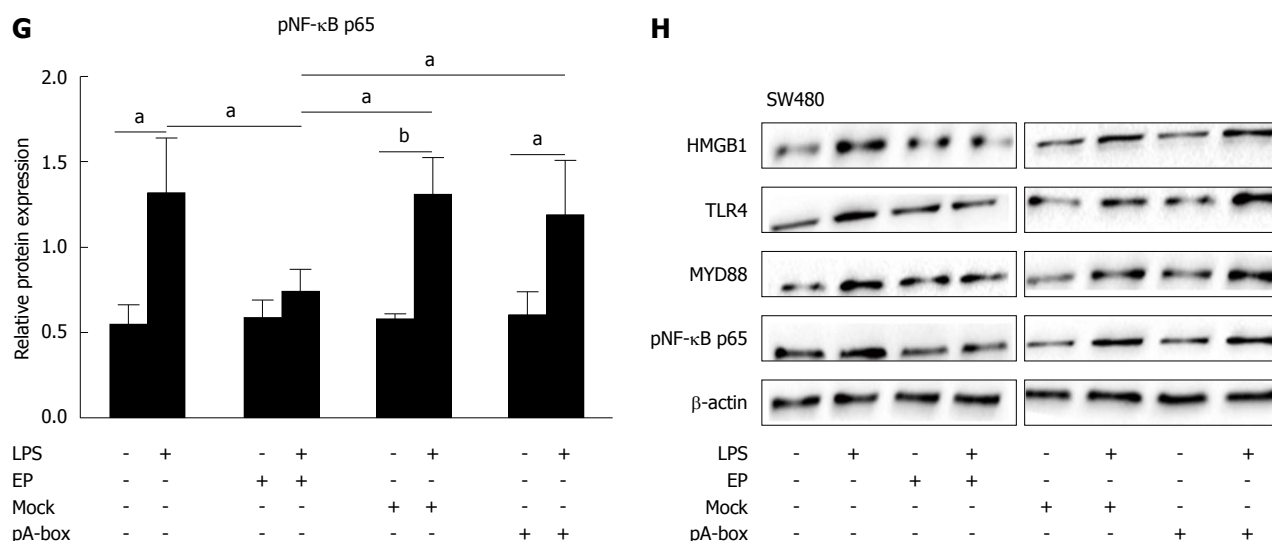
among the groups without LPS treatment ( $P > 0.05$ ). In the LPS treatment groups, the HMGB1/TLR4/MYD88/pNF- $\kappa$ B p65 mRNA and protein levels in the EP group and pA-box-transfection group were significantly lower than those in the control group and mock-transfection group ( $^aP < 0.05$ ,  $^bP < 0.01$ ). All LPS treatment groups except the EP group and pA-box-transfection group were significantly higher than the corresponding groups without LPS treatment ( $^aP < 0.05$ ,  $^bP < 0.01$ ).

Following co-culturing with SW480 cells, the cytokine levels of HMGB1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in the supernatants of the THP-1 cells were determined by ELISA (Figure 7A-D). There were no significant differences in the levels of HMGB1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 among the groups without LPS treatment ( $P > 0.05$ ). In the LPS treatment groups, the levels of HMGB1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in the EP and pA-box-transfection groups were significantly lower than those in the control and mock-transfection groups ( $^aP < 0.05$ ,  $^bP < 0.01$ ). All LPS treatment groups except the EP and pA-box-transfection groups showed significantly higher levels of these mRNAs and proteins compared with the corresponding groups without LPS treatment ( $^aP < 0.05$ ,  $^bP < 0.01$ ).

## **DISCUSSION**

The intestinal microbiota and gut immune system must communicate to maintain a balance between tolerance and activation. Although the immune system provides protection against pathogenic microbes, the human body is a host to trillions of microbes, symbionts, and mutualists; some of which are essential to human health<sup>[12]</sup>. The maintenance of immune tolerance and the inflammatory response depend on interactions between symbiotic bacteria, intestinal mucosa epithelial cells and immune cells<sup>[13]</sup>. If any of these components are disturbed, inflammation imbalances can occur that develop into inflammatory disease. TLRs can recognize some intrinsic molecules that are present in bacteria, viruses, epithelial cells and immune cells and play significant roles in maintaining symbiotic bacterial resistance and inducing the anti-inflammatory response to pathogens<sup>[14]</sup>. In IBD, the intestinal mucosa becomes intolerant of symbiotic bacteria and their products, leading to the excessive activation of the TLR signaling pathways, triggering an intracellular signaling cascade, producing chemokines, launching and controlling key transcription factors, and triggering and amplifying the inflammatory reaction<sup>[15]</sup>. TLR4 signaling pathways are activated by the recruitment of MYD88, resulting in the activation of phosphorylated IL-1-related kinases and tumor necrosis factor-associated receptor factor 6, further activating NF- $\kappa$ B transcription factors and releasing a series of inflammatory factors, such as interferon- $\gamma$ <sup>[3,16]</sup>. LPS is an outer membrane glycolipid of Gram-negative





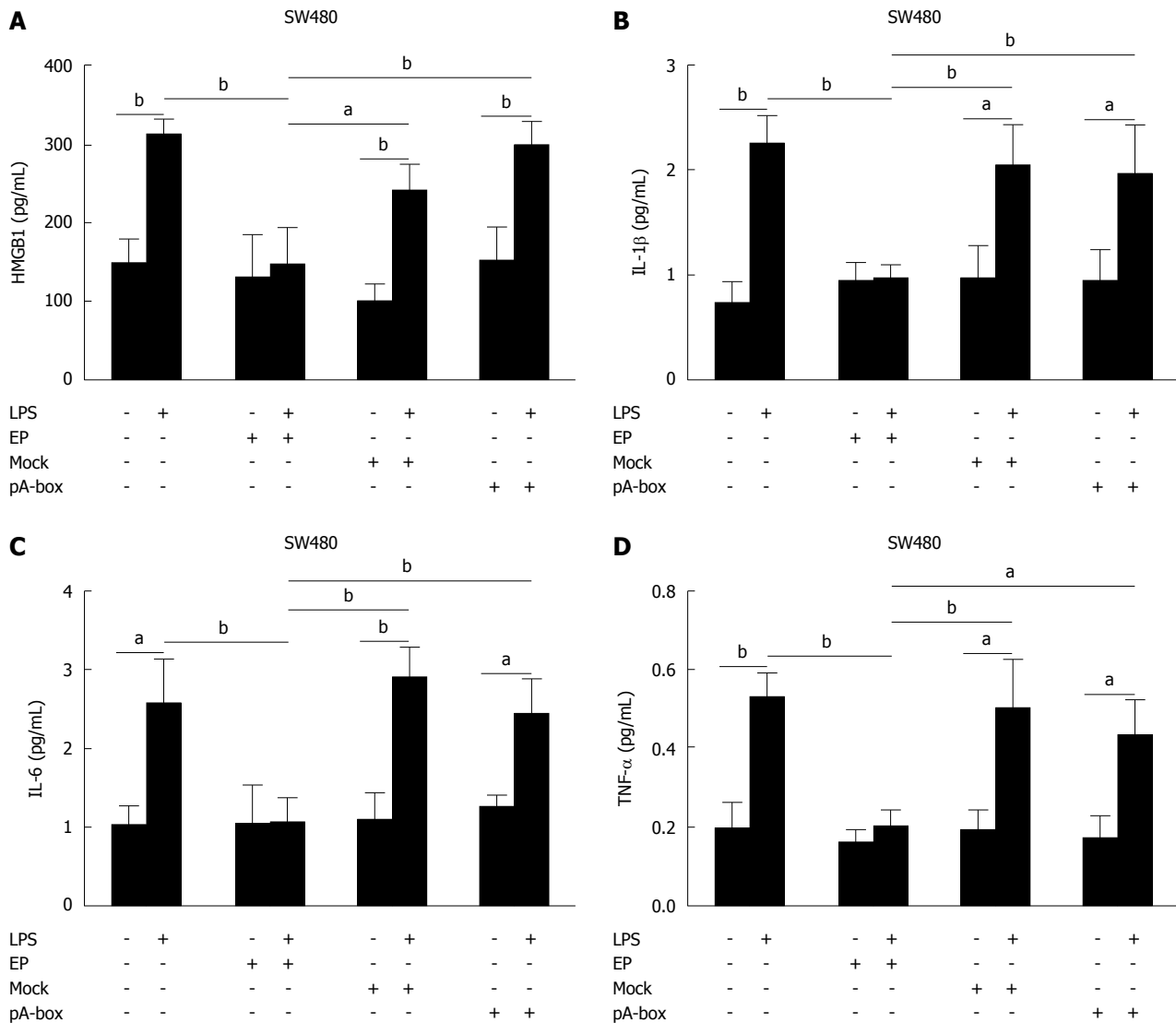
**Figure 4** Expression of the HMGB1 protein and toll-like receptor 4 signaling pathways in lipopolysaccharide-stimulated SW480 cells. After pretreatment with EP (5 mmol/L, 1 h), the SW480 cells were treated with LPS (1  $\mu$ g/mL, 24 h). The HMGB1 protein, TLR4, MYD88, and pNF- $\kappa$ B p65 mRNA and protein levels were determined by real-time PCR (A, C, E), densitometric quantification and representative western blotting with  $\beta$ -actin as the loading control (B, D, F, G, H). LPS increased the HMGB1, TLR4, MYD88, and pNF- $\kappa$ B p65 mRNA and protein levels in the LPS-stimulated SW480 cells compared with the LPS-untreated group, but EP downregulated these mRNA and protein levels in the LPS-stimulated SW480 cells. By contrast, HMGB1 A-box failed to downregulate the HMGB1, TLR4, MYD88, and pNF- $\kappa$ B p65 mRNA and protein levels compared with the mock-transfection group. The data are expressed as the mean  $\pm$  SD and are derived from three independent experiments, which were each performed in duplicate. The means with letters above them are significantly different (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control).

bacteria and a major ligand for TLR4. When there is an imbalance in intestinal mucosal immune responses in IBD, the LPS-induced excessive activation of TLR4 in the bowel mucosa triggers inflammation. In this study, after treating the SW480 and THP-1 cells for 24 h, LPS was able to upregulate the expression of TLR4 and the downstream molecules MYD88 and pNF- $\kappa$ B p65 and promote the secretion of proinflammatory cytokines, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , in cell culture supernatants. These findings suggest that LPS can produce proinflammatory factors and cause inflammation by activating the intracellular MYD88-dependent TLR4 signaling pathway.

HMGB1 is ubiquitously present in the nuclei of all mammalian cells, where it displays dual functions. Under normal conditions, HMGB1 binds to DNA and bends it to facilitate gene transcription. Under stress conditions, such as injury or infection, it is released and promotes inflammation, taking part in the pathogenesis of a variety of inflammatory diseases. During inflammatory reactions, the activation of monocytes/macrophages is the main source of HMGB1. When the production of exogenous bacterial endotoxins (e.g., LPS) or endogenous inflammatory cytokines is stimulated, HMGB1, which is mainly concentrated in the nucleus, is released from activated monocytes/macrophages in a time- and dose-dependent manner. This study found that LPS treatment not only upregulated HMGB1 expression and the secretion of human mononuclear cell THP-1, but also time- and dose-dependently increased HMGB1 expression and the secretion of intestinal epithelial SW480 cells. These findings showed that intestinal epithelial cells could also secrete HMGB1 following exposure to an

exogenous stimulus. It has been established that the excessive activation of the TLR signaling pathways in intestinal epithelial cells and inflammatory cells play an important role in the pathogenesis of IBD. LPS within the lumen is a major cause of the excessive activation of the TLR signaling pathways. This study showed that LPS not only activated these pathways, but also promoted the expression and release of HMGB1. HMGB1, which is an important DAMP, can bind to TLR4 and reactivate the TLR4 signaling pathways. Thus, HMGB1 is not only an endogenous ligand for TLR4, but also an amplifier of TLR-mediated inflammatory responses<sup>[17]</sup>. This function may be important for the persistent inflammation reaction that occurs in IBD. HMGB1 release is a dynamic process, involving exit from the nucleus into the cytoplasm, translocation from the cytosol into cytoplasmic organelles, and exocytosis<sup>[18]</sup>. In this study, after 24 h of LPS treatment, HMGB1 expression in the THP-1 and SW480 cells and HMGB1 secretion in the supernatants were markedly increased over time. In contrast with other proinflammatory cytokines, HMGB1 production and release continuously increased over 24 h, which may be related to the circulation cycles of the LPS/TLR4/HMGB1 signaling pathways.

EP, which is a stable fatty ester, is derived from the endogenous metabolite pyruvic acid. The pharmacological effects of EP include the downregulation of proinflammatory factors, the improvement of redox reaction-mediated cell and tissue damage, and the inhibition of apoptosis. EP protects inflammatory tissues from damage, such as fatal sepsis, systemic inflammation, uncontrolled hemorrhagic shock, and stroke<sup>[19-21]</sup>. Studies of various types of *in vivo* and *in*



**Figure 5** Levels of the inflammatory mediators HMGB1 protein, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the SW480 cell supernatant. After pretreatment with EP (5 mmol/L, 1 h), the SW480 cells were treated with LPS (1  $\mu$ g/mL, 24 h). The levels of the inflammatory mediators HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the supernatant of the SW480 cells were detected by ELISA (A-D). Compared with the LPS-untreated group, LPS increased the levels of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the LPS-stimulated SW480 cells, but EP downregulated the levels of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the SW480 cells activated by LPS. By contrast, HMGB1 A-box failed to decrease the secretion of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  compared with the mock-transfection group. The data are expressed as the mean  $\pm$  SD and are derived from three independent experiments, which were each performed in duplicate. The means with letters above them are significantly different (<sup>a</sup> $P$  < 0.05, <sup>b</sup> $P$  < 0.01 vs control).

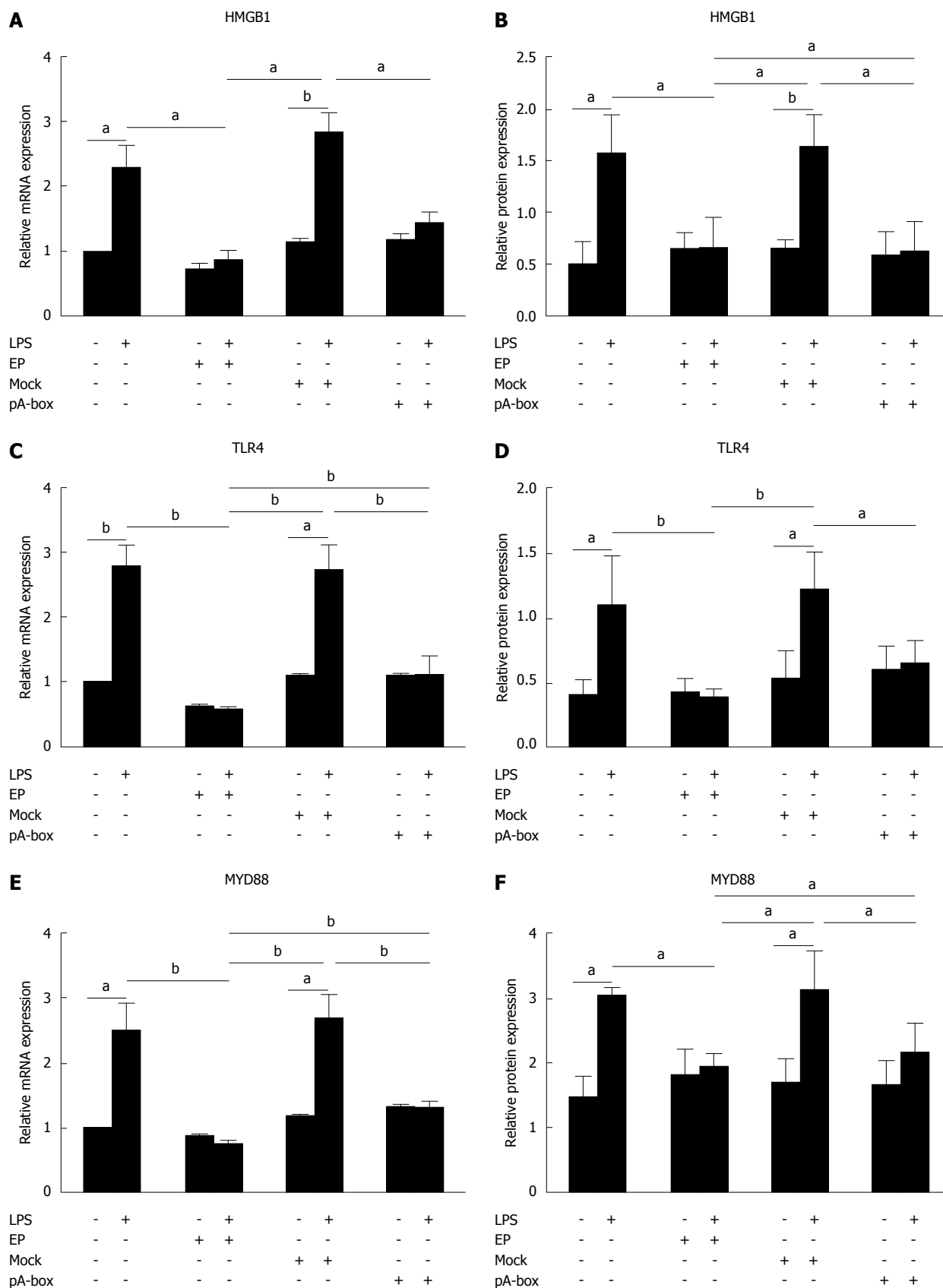
*vitro* models have proven that EP can downregulate the activation of the proinflammatory transcription factors NF- $\kappa$ B and reduce the expression of all types of proinflammatory factors<sup>[22,23]</sup>. In recent years, studies have shown that EP is an effective inhibitor of HMGB1 release.

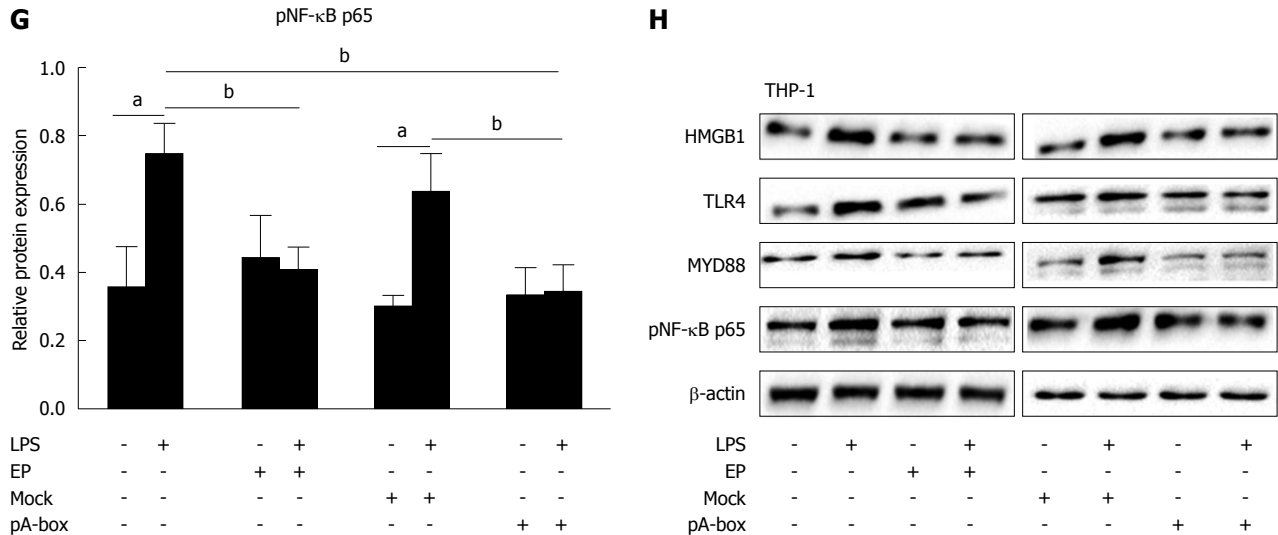
Our data revealed that EP inhibited the secretion and release of HMGB1 and the activation of the LPS/TLR4 signaling pathways as well as the release of inflammatory cytokines in the SW480 and THP-1 cells. Studies have found that in the traumatic brain injury (TBI) model, EP can inhibit the expression of HMGB1, TLR4, and NF- $\kappa$ B and the secretion of proinflammatory cytokines, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , in brain tissue following TBI, showing that EP can inhibit the HMGB1/TLR4/NF- $\kappa$ B signaling pathways<sup>[24]</sup>. It can effectively inhibit inducible nitric oxide synthase (iNOS)

expression and HMGB1 release in RAW264.7 cells following LPS stimulation<sup>[25]</sup>. EP also regulates HMGB1 release from macrophages following LPS stimulation by inhibiting the NF- $\kappa$ B and (or) p38 mitogen-activated protein kinase (MAPK) pathways<sup>[26]</sup>. In this study, in addition to its inhibition of the LPS-activated HMGB1/TLR4 signaling pathways, there may be other mechanisms by which EP inhibited the release of proinflammatory cytokines. Moreover, the group not receiving LPS treatment in this study exhibited a lack of effect of EP on HMGB1 expression and secretion, the activation of the HMGB1/TLR4 signaling pathways and the release of proinflammatory cytokines in the SW480 and THP-1 cells. These findings suggest that EP was inhibitory only under pathological and not physiological conditions.

The structure of HMGB1 is subdivided into two





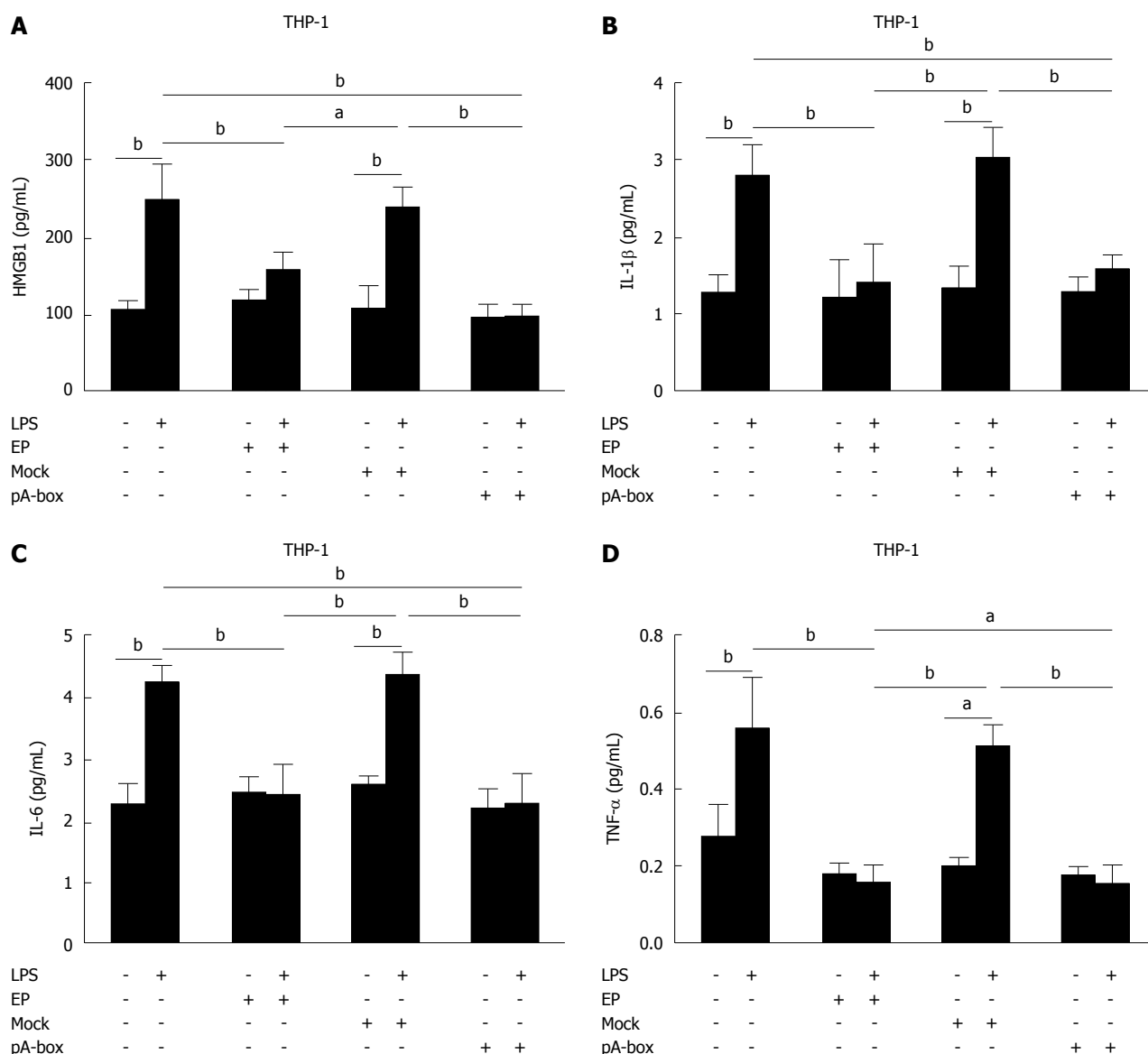


**Figure 6** Expression of the HMGB1 protein and TLR4 signaling pathways in THP-1 cells co-cultured with SW480 cells. After pretreatment with EP (5 mmol/L, 1 h), the SW480 cells were treated with 1 µg/mL LPS and co-cultured with THP-1 cells for 24 h. The HMGB1, TLR4, MYD88, pNF-κB p65 mRNA and protein levels were determined by real-time PCR (A, C, E), densitometric quantification and representative western blots, with β-actin as the loading control (B, D, F, G, H). Compared with the LPS-untreated group, LPS increased HMGB1, TLR4, MYD88, pNF-κB p65 mRNA and protein levels in the THP-1 cells, but EP downregulated these levels. Similarly, HMGB1 A-box also downregulated HMGB1, TLR4, MYD88, pNF-κB p65 mRNA and protein levels compared with the mock-transfected group. The data are expressed as the mean ± SD and are derived from three independent experiments, which were each performed in duplicate. The means with letters above them are significantly different (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control).

homologous HMG boxes, the A-box and B-box. As described previously, the truncation of HMGB1 into individual structural domains has revealed that the B box induces strong proinflammatory activities. Inversely, the A-box may act as an antagonist of HMGB1<sup>[27]</sup>. The A-box protein dose-dependently inhibits HMGB1-induced TNF and IL-1β release in macrophage cultures and is stimulated by HMGB1<sup>[28]</sup>. It is possible that these anti-inflammatory effects occur due to competition with receptors for HMGB1<sup>[29]</sup>. Animal experiments have shown that after the recombinant HMGB1 A-box protein is administered by intraperitoneal injection, a significant decrease in mortality occurs and the abnormal release of inflammatory cytokines is blocked compared with a control group<sup>[30]</sup>. After recombinant A-box protein is injected into mice with collagen-induced arthritis, its systemic administration significantly reduces the mean arthritis score, disease-induced weight loss, and the histological severity of arthritis<sup>[31,32]</sup>. Therefore, the A-box is considered a natural antagonist of HMGB1. Studies have reported that HMGB1 and the LPS/TLR4 signaling pathways have important associations with the pathogenesis of IBD<sup>[9,33,34]</sup> and that positive regulation may occur between them<sup>[35,36]</sup>. However, there are no reports of an influence of HMGB1 A-box overexpression on HMGB1 and the LPS/TLR4 signaling pathways.

Our data revealed that the overexpression of HMGB1 A-box had no effect on the HMGB1 or LPS/TLR4 signaling pathways in the SW480 cells themselves. However, using a Transwell co-culturing system, we further observed the effects of changes in the

above-mentioned molecules on HMGB1 A-box-overexpressing SW480 cells co-cultured with THP-1 cells. Following the LPS treatment of the THP-1 cells, we found that the expression and secretion of HMGB1, the expression of TLR4 and the downstream molecules MYD88 and pNF-κB p65, and the secretion of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, in the cell culture supernatants of the pA-box-transfection group were significantly lower compared with the control and mock-transfection groups. In addition, there were no significant differences compared with the corresponding LPS-untreated group in the THP-1 cells. These findings suggest that HMGB1 A-box-overexpressing SW480 cells can inhibit the secretion and release of HMGB1 and the activation of the HMGB1/TLR4 signaling pathways by LPS after co-culturing with THP-1 cells. Therefore, we hypothesize that HMGB1 A-box-overexpressing SW480 cells do not possess an autocrine function that suppresses the activation of their own HMGB1/TLR4 signaling pathways following LPS exposure, but they may have a paracrine function that inhibits the activation of the LPS/TLR4 signaling pathways and the proinflammatory activities of HMGB1 in THP-1 cells. This study also found that in the absence of LPS, HMGB1 A-box-overexpressing SW480 cells do not show alterations in the expression or secretion of HMGB1 or activation of the LPS/TLR4 signaling pathways after co-culturing with THP-1 cells. These results indicate that HMGB1 A-box antagonized HMGB1-induced inflammation only under pathological and not physiological conditions. Therefore, HMGB1 A-box may represent a new therapeutic target for the



**Figure 7** Levels of the inflammatory mediators high mobility group box 1 protein, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the supernatant of the THP-1 cells co-cultured with SW480 cells. After pretreatment with EP (5 mmol/L, 1 h), the SW480 cells were treated with 1  $\mu$ g/mL LPS and co-cultured with THP-1 cells for 24 h. The levels of the inflammatory mediators HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the supernatant of the THP-1 cells co-cultured with SW480 cells were detected by ELISA (A-D). Compared with the LPS-untreated group, LPS increased the levels of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the THP-1 cells, but EP downregulated these levels. Similarly, HMGB1 A-box also decreased the secretion of HMGB1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the THP-1 cells compared with the mock-transfection group. The data are expressed as the mean  $\pm$  SD and are derived from three independent experiments, which were each performed in duplicate. The means with letters above them are significantly different (\* $P$  < 0.05,  $^bP$  < 0.01 vs control).

treatment of IBD.

In conclusion, in the co-culture system of SW480 and THP-1 cells, LPS can not only activate HMGB1/TLR4 signaling pathways, but also promote the secretion of proinflammatory cytokines. EP, which is a potent inhibitor of HMGB1 release, can inhibit the HMGB1/TLR4 signaling pathways and the secretion of proinflammatory cytokines activated by LPS, but its mechanism remains unclear. Unlike EP, HMGB1 A-box, as a specific antagonist of HMGB1, can specifically inhibit the HMGB1/TLR4 signaling pathways and the secretion of LPS-activated proinflammatory cytokines. Taken together, HMGB1 A-box may represent a novel therapeutic HMGB1-targeting agent for IBD treatment.

## COMMENTS

### Background

Inflammatory bowel disease (IBD) is thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host. Although numerous reports have indicated that high mobility group box (HMGB)1/toll-like receptor (TLR)4 signaling pathways play an important role in IBD, but the possible regulatory mechanism remains to be elucidated yet.

### Research frontiers

The authors aimed to investigate the effects of two HMGB1 inhibitors (HMGB1 A-box and ethyl pyruvate) on the HMGB1/TLR-4 signaling pathway and the secretion of some proinflammatory cytokines in SW480 and THP-1 cells after activation by lipopolysaccharides (LPS).

### Innovations and breakthroughs

The authors have managed to describe theoretical and experimental evidence

of HMGB1 A-box as a potential inhibitor of HMGB1/TLR-4 signaling pathways convincingly.

## Applications

The authors hypothesize that HMGB1 A-box, as a natural antagonist of HMGB1, can competitively inhibit the binding of HMGB1 to its receptors and attenuate the proinflammatory effect. The authors have managed to describe theoretical and experimental evidence of HMGB1 A-box as a potential inhibitor of HMGB1/TLR-4 signaling pathways convincingly. Therefore it could be a possible therapeutic strategy for some patients with IBD.

## Terminology

HMGB1 is a non-histone nuclear protein that, depending on its location and post-translational modifications, has several functions. HMGB1 is ubiquitously expressed in nuclei, where it binds and stabilizes DNA. HMGB1 can be translocated from the nucleus to the cytoplasm and extracellular space. When released, it has been shown to act as a damage-associated molecular pattern (DAMP). HMGB1 is subdivided into A-box and B-box. A-box is the most used antagonist of HMGB1, which competes with receptors for binding with HMGB1.

## Peer-review

The purpose of the paper to investigate the effects of two HMGB1 inhibitors (HMGB1 A-box and ethyl pyruvate) on the HMGB1/TLR4 signaling pathway and the secretion of some proinflammatory cytokines in SW480 and THP-1 cells after activation by LPS, is well established and consecutively unfolded. The hypothesis of the potential role of the HMGB1 A-box as a novel approach to the treatment of IBD is presented in an interesting manner. They have determined HMGB1, TLR4 and its downstream signaling molecules MyD88 and NF- $\kappa$ B p65 protein and mRNA levels by RT-PCR, densitometry and Western blot, and also studied the cytokine levels in the culture supernatants. The authors conclude that HMGB1 A-box and ethyl pyruvate both inhibit HMGB1/TLR4 and downstream signaling molecules MyD88 as well as NF- $\kappa$ B p65 and the secretion of several important pro-inflammatory cytokines induced by LPS stimulation. In general, this study is methodologically well performed, the paper well written and has a clear message. The authors have used intestinal epithelial cell line to study the potential effect of above-mentioned compounds and to clarify some pathogenetic molecular mechanisms in IBD. Therefore, the objective of this study is relevant.

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## Basic Study

**Berberine inhibits hepatic gluconeogenesis *via* the LKB1-AMPK-TORC2 signaling pathway in streptozotocin-induced diabetic rats**

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**Author contributions:** Dong H, Lu FE and Yi P designed the research; Jiang SJ, Li JB, Xu LJ, Zou X and Wang KF performed the research; Dong H analyzed the data; Jiang SJ wrote the paper.

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**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at [pyi219@163.com](mailto:pyi219@163.com). Participants gave informed consent for data sharing. No additional data are available.

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**Abstract**

**AIM:** To investigate the molecular mechanisms of berberine inhibition of hepatic gluconeogenesis in a diabetic rat model.

**METHODS:** The 40 rats were randomly divided into five groups. One group was selected as the normal group. In the remaining groups ( $n = 8$  each), the rats were fed on a high-fat diet for 1 mo and received intravenous injection of streptozotocin for induction of the diabetic models. Berberine (156 mg/kg per day) (berberine group) or metformin (184 mg/kg per day) (metformin group) was intragastrically administered to the diabetic rats and 5-aminoimidazole-4-carboxamide- $\beta$ -D-ribofuranoside (AICAR) (0.5 mg/kg per day) (AICAR group) was subcutaneously injected to the diabetic rats for 12 wk. The remaining eight diabetic rats served as the model group. Fasting plasma glucose and insulin levels as well as lipid profile were tested.

The expressions of proteins were examined by western blotting. The nuclear translocation of CREB-regulated transcription co-activator (TORC)2 was observed by immunohistochemical staining.

**RESULTS:** Berberine improved impaired glucose tolerance and decreased plasma hyperlipidemia. Moreover, berberine decreased fasting plasma insulin and homeostasis model assessment of insulin resistance (HOMA-IR). Berberine upregulated protein expression of liver kinase (LK)B1, AMP-activated protein kinase (AMPK) and phosphorylated AMPK (p-AMPK). The level of phosphorylated TORC2 (p-TORC2) protein in the cytoplasm was higher in the berberine group than in the model group, and no significant difference in total TORC2 protein level was observed. Immunohistochemical staining revealed that more TORC2 was localized in the cytoplasm of the berberine group than in the model group. Moreover, berberine treatment downregulated protein expression of the key gluconeogenic enzymes (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) in the liver tissues.

**CONCLUSION:** Our findings revealed that berberine inhibited hepatic gluconeogenesis *via* the regulation of the LKB1-AMPK-TORC2 signaling pathway.

**Key words:** Berberine; Diabetes; AMPK; LKB1; Hepatic gluconeogenesis; TORC2

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**Core tip:** We showed that liver kinase (LK)B1 acts as the upstream regulator of AMP-activated protein kinase (AMPK) and participates in gluconeogenesis. AMPK phosphorylation triggers CREB-regulated transcription co-activator (TORC)2 phosphorylation, which results in the inhibition of the nuclear translocation of TORC2. Thus, gluconeogenesis is restrained. No previous studies have reported the molecular mechanisms of berberine reducing hyperglycemia *via* the inhibition of hepatic gluconeogenesis. We found that berberine upregulated protein expression of LKB1, AMPK, p-AMPK and p-TORC2. Moreover, we observed that berberine inhibited the translocation of TORC2 into the cell nucleus.

Jiang SJ, Dong H, Li JB, Xu LJ, Zou X, Wang KF, Lu FE, Yi P. Berberine inhibits hepatic gluconeogenesis *via* the LKB1-AMPK-TORC2 signaling pathway in streptozotocin-induced diabetic rats. *World J Gastroenterol* 2015; 21(25): 7777-7785 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7777.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7777>

## INTRODUCTION

The liver plays a crucial role in the maintenance of systemic glucose homeostasis. In the absorptive

state, the liver increases glucose uptake *via* the absorption of glucose by hepatocytes and subsequent transformation into glycogen and lipids. In the fasting state, hepatocytes provide glucose *via* glycogenolysis and gluconeogenesis to maintain glucose homeostasis. However, abnormal hepatic gluconeogenesis results in the elevation of glucose levels. Gluconeogenesis in the liver is regulated through the transcriptional modulation of gluconeogenic enzymes such as glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK)<sup>[1]</sup>.

AMP-activated protein kinase (AMPK) plays a vital role in gluconeogenesis in the liver. AMPK is a conserved sensor and regulator of cellular energy balance that is activated when the cellular AMP: ATP ratio exhibits a large increase due to conditions of nutrient deprivation or pathological stress<sup>[2]</sup>. Liver kinase (LK)B1 is a serine/threonine protein kinase that was originally identified as a tumor suppressor gene. The LKB1 mutation is responsible for the familial Peutz-Jeghers syndrome<sup>[3]</sup>. The deletion of hepatic LKB1 in adult mice results in the nearly complete loss of AMPK activity, which in turn, results in hyperglycemia due to increased gluconeogenic gene expression<sup>[4]</sup>. Previous research has indicated that LKB1 acts as the upstream regulator of AMPK and participates in gluconeogenesis. Koo *et al*<sup>[5]</sup> illustrated that CREB-regulated transcription co-activator (TORC)2 is a key regulator of glucose output that acts through the cAMP responsive element binding protein (CREB) and found that TORC2-deficient mice exhibit fasting hypoglycemia. Subsequently, CREB stimulates hepatic gluconeogenesis to drive the expression of the nuclear receptor coactivator peroxisome proliferator-activated receptor co-activator (PGC)-1 $\alpha$ <sup>[4,5]</sup>. PGC-1 $\alpha$  is a transcriptional coactivator of nuclear receptors and plays a vital role in activating the expression of the genes for key gluconeogenic enzymes such as PEPCK and G-6-P<sup>[6,7]</sup>. The research of Koo *et al*<sup>[5]</sup> showed that AMPK phosphorylation due to ATP depletion triggers TORC2 phosphorylation, which results in the inhibition of the nuclear translocation of TORC2; in turn, the cytoplasmic localization of TORC2 prevents its combination with CREB elements. Thus, gluconeogenesis is restrained. In the future, the LKB1-AMPK-TORC2 signaling pathway will probably be a target for the treatment of type 2 diabetes.

Berberine is an isoquinoline alkaloid extracted from *Rhizoma Coptidis*. The hypoglycemic effect of berberine was first identified in 1988 *via* the treatment of diarrhea in diabetic patients<sup>[8]</sup>. Since that time, many studies about the influence of berberine on hyperglycemia-reducing and insulin resistance-improving have been reported. Recently, berberine was proven to be capable of reducing hyperglycemia *via* the inhibition of hepatic gluconeogenesis<sup>[1,9,10]</sup>. Based on the inhibition of gluconeogenesis by the LKB1-AMPK-TORC2 signaling pathway, we hypothesized that berberine reduces hyperglycemia *via* the LKB1-AMPK-TORC2 signaling pathway to control gluconeogenesis.

## MATERIALS AND METHODS

### **Animal care and use statement**

Male Wistar rats, weighing 160 g, supplied by the Centers for Disease Control and Prevention (Wuhan, China) were fed adaptively for 1 wk in an ambient temperature of  $22 \pm 1^\circ\text{C}$  and on a 12-h light/dark cycle with free access to water and the standard rat diet (containing 35% flour, 20% soy meal, 20% corn meal, 15.5% bran, 0.5% bean oil, 5% fish meal, 2.5% bone meal, 1% dusty yeast, and 0.5% salt). All experimental procedures were performed in accordance with the guide principle for experimental animals (MSTPRC Directive of 1988, No. 88-2).

### **Chemicals and experimental drugs**

Streptozotocin (STZ) was produced by Sigma (St Louis, MO, United States). The assay kits used for blood lipid determinations were purchased from Jiancheng Bio-engineering Institute (Nanjing, China). Berberine was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Metformin was purchased from Shenzhen Vanda Pharmaceuticals (Shenzhen, China), and AICAR was procured from the Beyotime Institute of Biotechnology (Jiangsu, China).

### **Experimental design**

The rats were randomly assigned to a normal control group that received the standard rat diet (normal) or the remaining four groups that received a high-fat diet (containing 67.5% standard laboratory rat chow, 15% lard, 15% sugar, 2% cholesterol, and 0.5% bile salts) for 4 wk. Next, the rats received tail vein injections of STZ (30 mg/kg) dissolved in 0.05 mol/L sodium citrate (pH 4.5) after 12-h fast for induction of the diabetic models<sup>[11]</sup>. One week later, oral glucose tolerance test (OGTT) was performed. The 95% confidence intervals were calculated based on the plasma glucose levels of normal rats. The rats with diabetes (*i.e.*, rats with plasma glucose levels that were above the normal upper limit at two time points or 20% greater than the normal upper limit at one time point) were selected. Next, the diabetic rats were randomized into the following four groups ( $n = 8$  per group): an untreated diabetic group (model), a berberine-treated group (berberine), a metformin-treated group (metformin) and an AICAR-treated group (AICAR). Berberine (156 mg/kg per day) and metformin (184 mg/kg per day) were dissolved in sodium carboxymethylcellulose and intragastrically administered to the rats daily for 12 wk. AICAR (0.5 mg/kg per day) was dissolved in normal saline, and the rats in the AICAR-treated group were given daily subcutaneous injections of AICAR for 12 wk. The doses were adjusted according to the body weight, which was recorded once per week. The day before the rats were sacrificed, the rats were anesthetized with diethyl ether after fasting for 12 h, and orbital venous blood

was obtained. Next, the rats were given glucose by gavage (2 g/kg), and additional blood samples were collected at regular intervals ( $t = 60$  and  $120$  min) for glucose and insulin measurements. The rats were deeply anesthetized with pentobarbital in the fasting (12-h) condition. Blood samples were collected from the abdominal aorta and allowed to clot for 30 min at  $4^\circ\text{C}$ . After centrifuging at 3000 r/min for 15 min at  $4^\circ\text{C}$ , the serum was separated and stored at  $-80^\circ\text{C}$  until examination. The liver was removed and flushed with saline. Next the liver was collected and stored at  $-80^\circ\text{C}$  until use.

### **OGTT and fasting insulin**

Blood glucose levels were examined with the glucose oxidase method using a glucose monitor (LifeScan Milpitas, CA, United States). Serum fasting insulin concentrations were measured with radioimmunoassay.

### **Analysis of blood lipids**

The serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentrations were estimated *via* the oxidase method using commercial reagents.

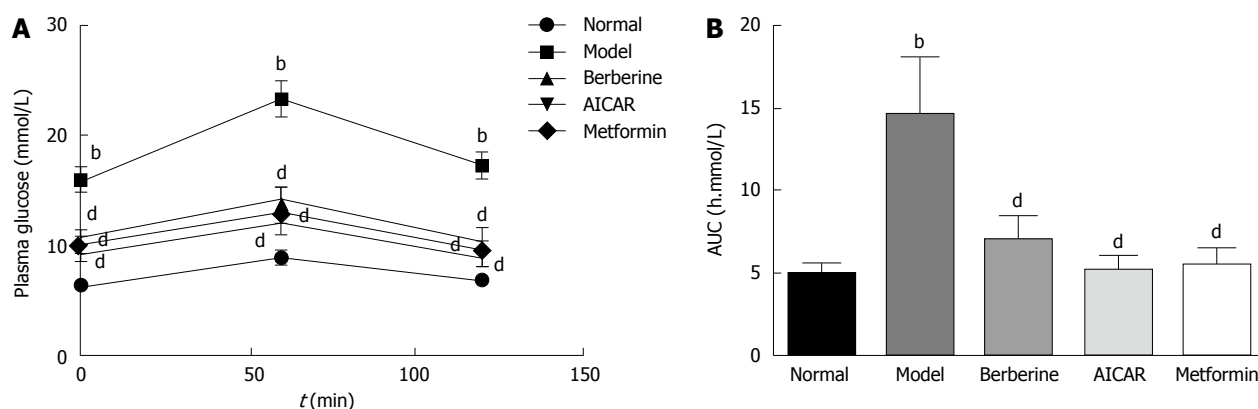
### **Western blot analysis**

Liver total protein was extracted, and the concentrations of total protein were measured by the BCA method. The liver extractions (100  $\mu\text{g}$ ) were mixed with sample buffer (25  $\mu\text{g}$ ), boiled for 10 min, and separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk dissolved in phosphate-buffered saline with Tween-20 (PBST) or 0.5% bovine serum albumin for 2 h at room temperature. The membranes were then washed in PBST and incubated overnight with primary antibodies (LKB1, AMPK, p-AMPK, TORC2, p-TORC2, G-6-P, PEPCK, and  $\beta$ -actin) at  $4^\circ\text{C}$ . After three washes in PBST, the membranes were incubated with the Dylight 800-labeled antibody to rabbit IgG (KPL, Hongkong, China) for 2 h. Immunoreactive proteins were visualized with a near-infrared double color laser imaging system (Odyssey, Lincoln, NE, United States). Quantity one 4.6.2 was used for assaying the protein quantification.

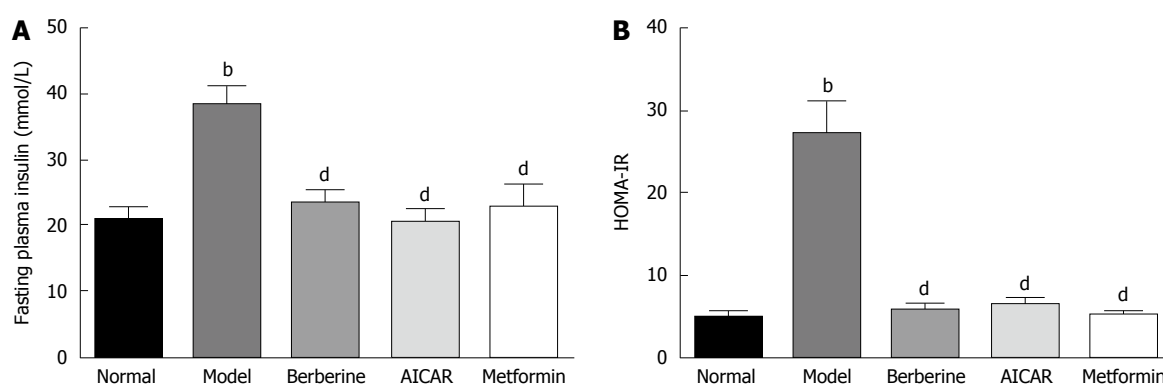
### **Immunohistochemical staining for TORC2**

The liver tissues were fixed with 4% paraformaldehyde for paraffin embedding. The paraffin-embedded sections were subjected to immunohistochemical staining for TORC2 in the liver. The tissue sections were incubated with rabbit anti-TORC2 primary antibody (1:50). After washing with PBST, the sections were incubated with secondary antibody, and the diaminobenzidine method was used. Next, the TORC2 protein expressions were observed under an optical microscope.





**Figure 1** Effects of berberine on plasma glucose levels in the oral glucose tolerance test and the areas under the curves for plasma glucose. <sup>b</sup> $P < 0.01$  vs the normal control group at the corresponding time point; <sup>d</sup> $P < 0.01$  vs the model group at the corresponding time point (by ANOVA). AUC: Areas under the curve.



**Figure 2** Effects of berberine on fasting plasma insulin level and homeostasis model assessment of insulin resistance in diabetic rats. Each bar represents the mean  $\pm$  SD ( $n = 8$ ). <sup>b</sup> $P < 0.01$  vs the normal control group; <sup>d</sup> $P < 0.01$  vs the model group (by ANOVA). HOMA-IR: Homeostasis model assessment of insulin resistance.

### Statistical analysis

The data are presented as the means  $\pm$  SD and were assayed with SPSS version 19.0 statistical software. All experience data were analyzed with one-way analysis of variance (ANOVA). Data with equal variances were evaluated with Tukey's test. A  $P$  value below 0.05 was considered significant. The statistical methods of the study were reviewed by Sheng Wei from the school of Public Health of Tongji Medical College.

## RESULTS

### Effect of berberine on glucose tolerance in type 2 diabetic rats

As shown in Figure 1A, the plasma glucose levels in the model group were significantly higher than those in the normal control group at 0, 1 and 2 h ( $P < 0.01$ ). Glucose tolerances were improved in the berberine, AICAR and metformin groups compared to the model group ( $P < 0.01$ ). In the berberine, AICAR and metformin groups, the areas under the curves (AUCs) constructed from the plasma glucose levels at the three time points were decreased by 52%, 64% and 62%, respectively, compared to the model group (Figure 1B).

### Effect of berberine on fasting plasma insulin and insulin resistance index

Blood insulin was monitored to assay pancreatic beta cell function. As shown in Figure 2, fasting insulin level was significantly higher in the model group than in the normal control group ( $P < 0.01$ ), and berberine significantly lowered fasting insulin level compared to the model group ( $P < 0.01$ ) (Figure 2A). Moreover, the fasting plasma insulin and homeostasis model assessment of insulin resistance (HOMA-IR) in the model group was higher than in the normal control group ( $P < 0.01$ ), and berberine notably decreased HOMA-IR compared to the model group ( $P < 0.01$ ) (Figure 2B).

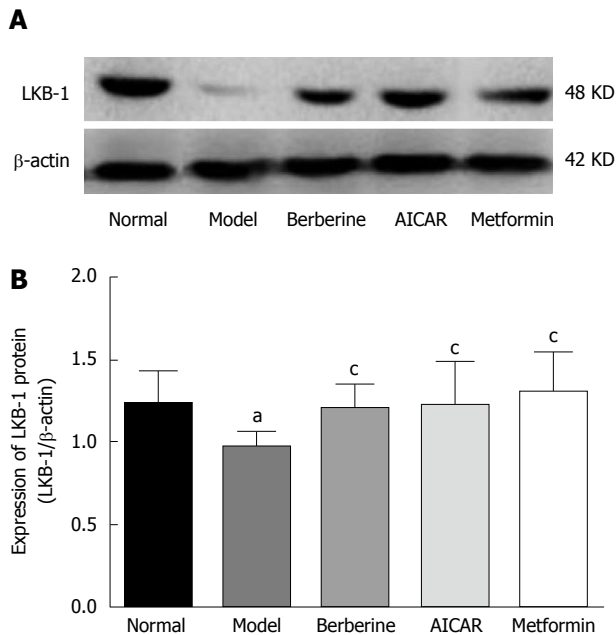
### Berberine improved hyperlipidemia in type 2 diabetic rats

As shown in Table 1, the model rats exhibited severe dyslipidemia. The serum TG, TC, and LDL-C levels were higher in the model group than in the normal control group ( $P < 0.01$ ). Treatments with berberine, AICAR and metformin markedly ameliorated the increases in the TG, TC and LDL-C levels in the diabetic rats compared to the model rats ( $P < 0.01$ ). The HDL-C levels of the model group were lower than those of

**Table 1** Effects of berberine on plasma lipid profiles of diabetic rats (mean  $\pm$  SD,  $n = 8$ )

Group	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
Normal	0.98 $\pm$ 0.15	3.74 $\pm$ 0.56	1.48 $\pm$ 0.18	2.68 $\pm$ 0.48
Model	2.7 $\pm$ 0.57 <sup>b</sup>	6.66 $\pm$ 1.14 <sup>b</sup>	4.26 $\pm$ 0.63 <sup>b</sup>	1.14 $\pm$ 0.15 <sup>b</sup>
Berberine	1.44 $\pm$ 0.23 <sup>d</sup>	4.88 $\pm$ 0.96 <sup>d</sup>	1.46 $\pm$ 0.32 <sup>d</sup>	2.12 $\pm$ 0.63 <sup>d</sup>
AICAR	1.28 $\pm$ 0.31 <sup>d</sup>	4.54 $\pm$ 0.55 <sup>d</sup>	1.82 $\pm$ 0.22 <sup>d</sup>	2.34 $\pm$ 0.40 <sup>d</sup>
Metformin	1.26 $\pm$ 0.37 <sup>d</sup>	4.72 $\pm$ 0.56 <sup>d</sup>	1.60 $\pm$ 0.27 <sup>d</sup>	2.52 $\pm$ 0.59 <sup>d</sup>

<sup>b</sup> $P < 0.01$  vs the normal control group, <sup>d</sup> $P < 0.01$  vs the model group (by ANOVA). TC: Total cholesterol; TG: Triglycerides; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol.



**Figure 3** Effect of berberine on hepatic LKB-1 protein expression. Western blot analyses of LKB-1 levels in liver tissues of normal control rats, model rats and diabetic rats treated with berberine, AICAR and metformin. A: Representative blots for each group are shown; B: Each bar is expressed as LKB-1/ $\beta$ -actin and represents the mean  $\pm$  SD ( $n = 8$ ). <sup>a</sup> $P < 0.05$  vs the normal control group; <sup>c</sup> $P < 0.05$  vs the model group (by ANOVA).

the normal control group, and the HDL-C levels of the treatment groups were increased compared to those of the model rats ( $P < 0.01$ ).

#### Berberine regulated expression of LKB1 protein in livers of type 2 diabetic rats

As shown in Figure 3, the expression of LKB1 protein in the model rats decreased compared to the normal control group ( $P < 0.05$ ). However, treatments of berberine, AICAR and metformin increased the expression of LKB1 protein compared to the model rats ( $P < 0.05$ ).

#### Berberine regulated expression of AMPK and p-AMPK proteins in the livers of type 2 diabetic rats

AMPK is an energy sensor, and phosphorylation of AMPK is increased when it is activated. As shown in Figure 4, the liver AMPK and P-AMPK protein levels were lower in the model group than in the normal control group, and berberine, AICAR and metformin

treatments considerably increased the expressions of AMPK and P-AMPK proteins compared to the model rats ( $P < 0.01$ ).

#### Berberine regulated TORC2 nuclear translocation in livers of type 2 diabetic rats

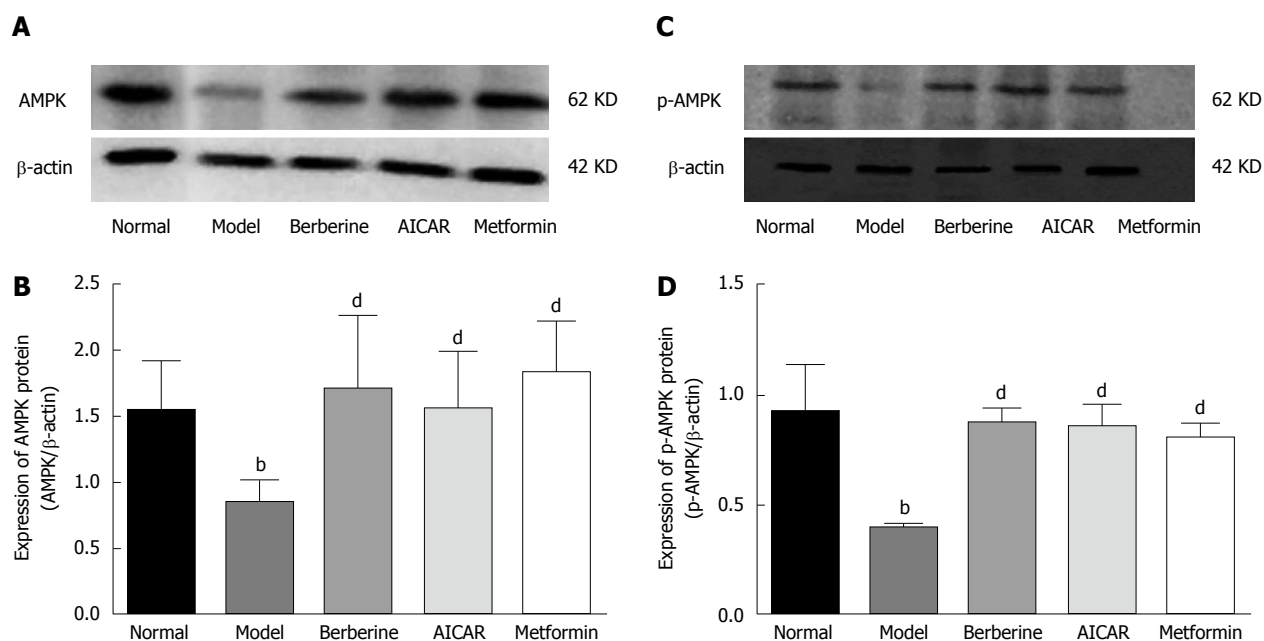
When TORC2 is phosphorylated in the liver, it is located in the cytoplasm and gluconeogenesis does not occur. As shown in Figure 5, the p-TORC2 levels of the model group was lower than that of the normal control group ( $P < 0.01$ ), and the p-TORC2 levels were significantly increased in groups treated with berberine, AICAR or metformin compared to the model group ( $P < 0.01$ ). However, there was no significant difference in the expression of total TORC2 protein across the five groups ( $P > 0.01$ ). As shown in Figure 6, we also verified that berberine inhibited TORC2 nuclear translocation in the liver tissues *via* immunohistochemical staining. The nuclear expression of TORC2 protein was obviously increased in the model group compared to the normal group; however, the treatments with berberine, AICAR and metformin inhibited the nuclear translocation of the TORC2 protein.

#### Berberine regulated expression of PEPCK and G-6-P proteins in livers of type 2 diabetic rats

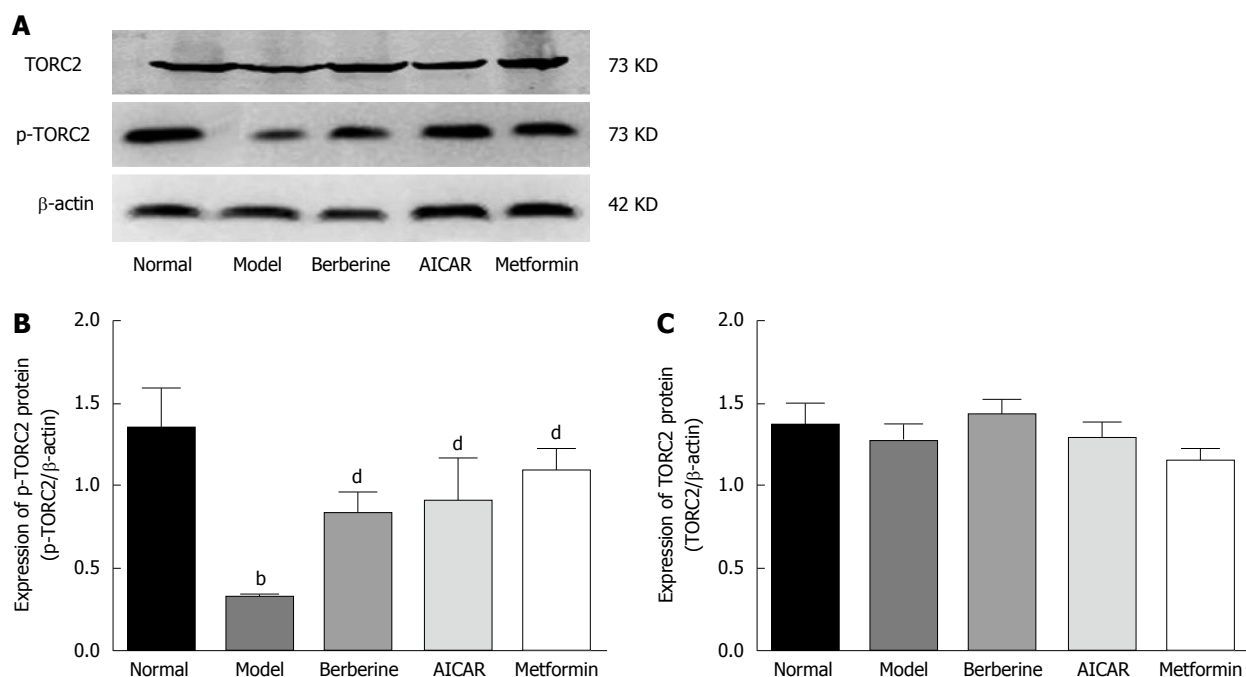
PEPCK and G-6-P are key gluconeogenesis enzymes and can affect plasma glucose. Expression of PEPCK and G-6-P proteins was increased in the model rats compared to the normal control group ( $P < 0.01$ ), and treatment with berberine, AICAR and metformin decreased expression of PEPCK and G-6-P protein compared to the model rats ( $P < 0.05$ ) (Figure 7).

## DISCUSSION

Berberine was first found to exhibit hypoglycemic actions in 1988, and numerous studies related to the ability of berberine to attenuate diabetes have been reported in the last 25 years. Previous evidence has shown that berberine can decrease blood glucose, regulate lipids, and improve insulin resistance *via* many different molecular mechanisms<sup>[12-14]</sup>; however, little research has focused on whether berberine inhibits hepatic gluconeogenesis *via* AMPK. Previous studies have illustrated that the regulation of gluconeogenesis



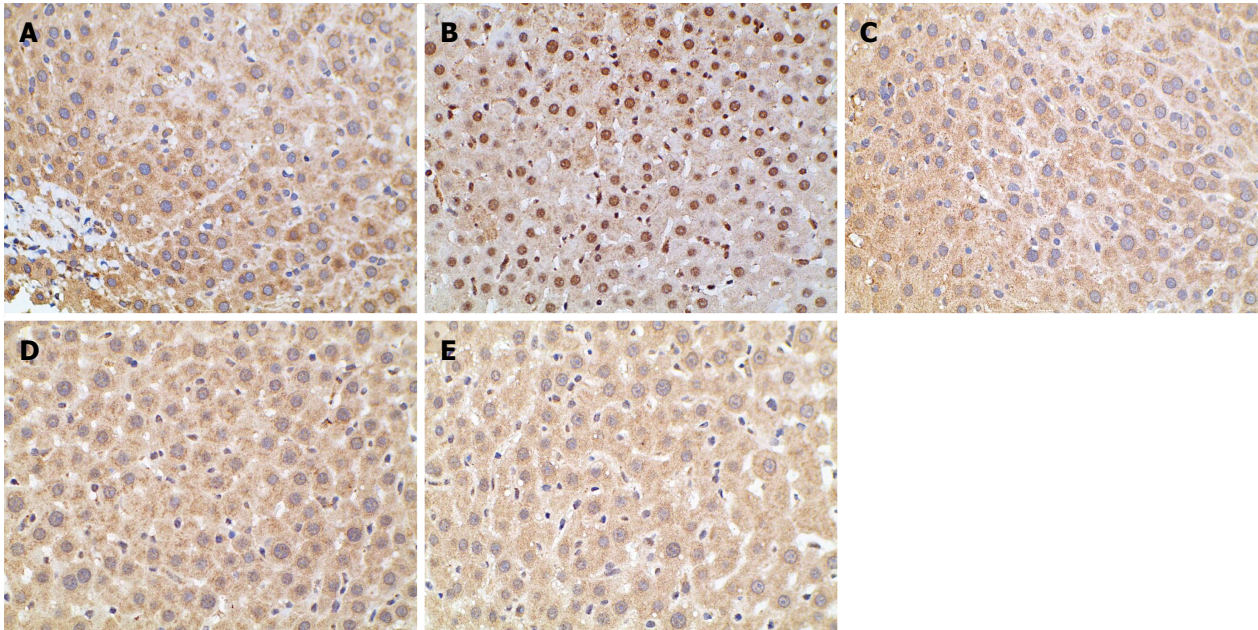
**Figure 4 Effect of berberine on hepatic AMPK and p-AMPK protein expression.** Western blot analyses of AMPK and p-AMPK protein in liver tissues of normal control rats, model rats and diabetic rats treated with berberine, AICAR or metformin. A, C: Representative blots for each group are shown; B: Each bar is expressed as AMPK/β-actin and represents the mean ± SD ( $n = 8$ ); D: Each bar is expressed as p-AMPK/β-actin and represents the mean ± SD ( $n = 8$ ). <sup>b</sup> $P < 0.01$  vs the normal control group; <sup>d</sup> $P < 0.01$  vs the model group (by ANOVA).



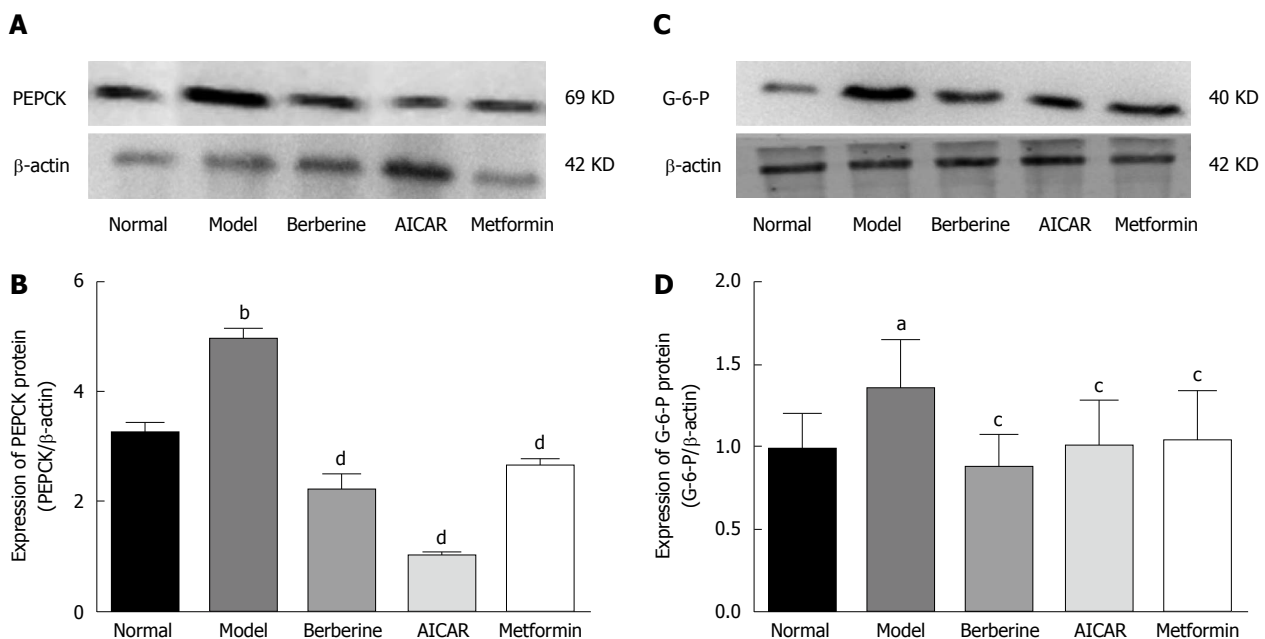
**Figure 5 Effect of berberine on hepatic p-TORC2 and total TORC2 protein expression.** Western blot analyses of p-TORC2 and TORC2 proteins from liver tissues of normal rats, model rats and diabetic rats treated with Berberine, AICAR and Metformin. A: Representative blots for each group are shown; B: Each bar is expressed as p-TORC2/β-actin and represents the mean ± SD ( $n = 8$ ); C: Each bar is expressed as total TORC2/β-actin and represents the mean ± SD ( $n = 8$ ). <sup>b</sup> $P < 0.01$  vs the normal group; <sup>d</sup> $P < 0.01$  vs the model group (by ANOVA). There was no significant difference in the expression of total TORC2 protein across the five groups.

is involved in the insulin signaling pathway. In re-feeding mice, insulin inhibits gluconeogenic gene expression *via* the promotion of the phosphorylation of TORC2<sup>[15]</sup>. In the models of insulin signaling deficiency, the expression of PGC-1 which plays a role in liver

gluconeogenesis is elevated. Thus, insulin is a primary suppressor of gluconeogenesis<sup>[16]</sup>. However, the current study revealed that glucose metabolism was regulated independently of insulin action. The loss of LKB1 in the mouse liver resulted in an increase in TORC2



**Figure 6** Immunohistochemical staining for TORC2 in the liver tissues. Optical microscopy image of TORC2 is shown in brown. The normal group (A) exhibited little TORC2 in the nuclei. However, more TORC2 was present in the nuclei of the model group (B). The groups treated with Berberine (C), AICAR (D) and Metformin (E) exhibited lower levels of TORC2 compared to the model group (magnification  $\times 400$ ).



**Figure 7** Berberine inhibited expression of key gluconeogenic enzyme proteins. Western blot analyses of PEPCK and G-6-P proteins in liver tissues of normal rats, model rats and diabetic rats treated with berberine, AICAR or metformin. A: PEPCK blots for each group are shown; C: G-6-P blots for each group are shown; B: Each bar is expressed as the total PEPCK/ $\beta$ -actin and represents the mean  $\pm$  SD ( $n = 8$ ); D: Each bar is expressed as the total G-6-P/ $\beta$ -actin and represents the mean  $\pm$  SD ( $n = 8$ ). <sup>b</sup> $P < 0.01$  vs normal control group; <sup>d</sup> $P < 0.01$  vs model group; <sup>a</sup> $P < 0.05$  vs normal control group; <sup>c</sup> $P < 0.05$  vs model group (by ANOVA).

gene expression and drove gluconeogenesis *via* the AMPK signaling pathway<sup>[4]</sup>. In a clinical trial, Keshavarz *et al.*<sup>[17]</sup> examined identification of single nucleotide polymorphisms in LKB1 and TOCR2 genes, and the results suggested a probable association between the LKB1-AMPK-TOCR2 signaling pathway and glucose homeostasis in the liver. These studies provided more insight to consider whether berberine suppresses

gluconeogenesis to attenuate hyperglycemia *via* the AMPK signaling pathway.

In this study, we showed that berberine restrained protein expression of the key gluconeogenic enzymes PEPCK and G-6-Pase in model rats (Figure 7). These results agree with those of previous reports<sup>[9,10]</sup>. Berberine inhibited PEPCK and G-6-Pase protein expression *via* the suppression of mitochondrial fun-



ction<sup>[10]</sup>. The glucose-lowering effect of berberine is related to the suppression of the expression of the key hepatic gluconeogenic enzymes PEPCK and G-6-Pase *via* the AMPK signaling pathway<sup>[9]</sup>. AMPK is a potential target for balancing glucose and lipid metabolism in the treatment of type 2 diabetes. Berberine treatment increases AMPK activity and contributes to the elevations in the level of AMPK phosphorylation in the liver<sup>[9,10,18,19]</sup>. In the present study, we examined the protein expression of AMPK and p-AMPK in the liver tissues (Figure 4). We observed that berberine increased the amount of total AMPK and phosphorylation of AMPK. Treatment with berberine restored the AMPK activity observed in the diabetic condition to the level observed in the non-diabetic condition (Figure 5). This increase in AMPK activity was accompanied by reductions in PEPCK and G-6-Pase expression. These results are consistent with previous data. The research of Shaw *et al.*<sup>[4]</sup> provided us with inspiration to explore further the hypoglycemic actions of berberine. In their study, LKB1 deletion in the liver led to a reduction in AMPK phosphorylation; thus, the activation of AMPK depends on LKB1. We considered whether LKB1 acts as a critical upstream target of AMPK when berberine treatment is accompanied by a change in AMPK. In our study, we measured the expression of LKB1 in the diabetic liver. Intriguingly, we found that LKB1 protein expression in treated groups was increased compared to the levels observed in the diabetic rats (Figure 3). Next, we sought to understand how AMPK affects the expression of the gluconeogenic enzymes PEPCK and G-6-Pase. Koo *et al.*<sup>[5]</sup> reported that the activation of AMPK promotes TOCR2 phosphorylation and blocks its nuclear accumulation. Consequently, gluconeogenic enzyme expression is interrupted<sup>[4,20]</sup>. In the current research, we detected no significant difference in the total amount of TOCR2 between the normal and diabetic rats, but TOCR2 phosphorylation in the cytoplasm was increased by the berberine treatment relative to model rats (Figure 5). Berberine treatment inhibited the translocation of TOCR2 into the cell nucleus, and the TORC2 nuclear accumulation observed in the berberine group was lower than that observed in the model group (Figure 6). Thus, the transcription of gluconeogenic genes was reduced, and the liver glucose output was decreased. In our study, we observed lower blood glucose levels in the treated group than in the model group (Figure 1). High blood glucose levels stimulate the pancreas to secrete insulin and result in hyperinsulinemia. Our results revealed that berberine treatment reduced fasting insulin level compared to those observed in the model group (Figure 2).

To research the therapeutic effects of berberine, we chose to use AICAR and metformin as positive control groups. Some studies have shown that AICAR and metformin are AMPK agonists, and that they inhibit gluconeogenesis to regulate glucose metabolism through the AMPK signaling pathway<sup>[4,21-23]</sup>. In our

research, we found no significant differences between these treatment groups.

In conclusion, our study revealed that berberine inhibited expression of the gluconeogenic proteins PEPCK and G-6-Pase in the liver. Consequently, reductions in blood glucose levels were accompanied by reductions in blood insulin levels reduction due to the inhibition of gluconeogenesis. Moreover, blood lipid levels simultaneously improved (Table 1). The mechanisms responsible for the effects of berberine treatment might be related to the suppression of gluconeogenesis through the LKB1-AMPK-TOCR2 signaling pathway.

## COMMENTS

### Background

Numerous studies related to the ability of berberine to attenuate diabetes have been reported. Previous evidence has shown that berberine can decrease blood glucose, regulate lipids, and improve insulin resistance *via* many different molecular mechanisms. However, little research has focused on whether berberine inhibits hepatic gluconeogenesis *via* AMP-activated protein kinase (AMPK).

### Research frontiers

Animal experiments showed that the loss of liver kinase (LK)B1 in the mouse liver resulted in an increase in CREB-regulated transcription co-activator (TORC)2 gene expression and drove gluconeogenesis *via* the AMPK signaling pathway. Moreover, a clinical trial suggested a probable association between the LKB1-AMPK-TOCR2 signaling pathway and glucose homeostasis in the liver. Recently, berberine was proven to be capable of reducing hyperglycemia *via* the inhibition of hepatic gluconeogenesis. Therefore, we hypothesized that berberine reduces hyperglycemia *via* the LKB1-AMPK-TORC2 signaling pathway to control gluconeogenesis.

### Innovations and breakthroughs

This is the first study to show that berberine reduces hyperglycemia *via* the LKB1-AMPK-TORC2 signaling pathway to control gluconeogenesis.

### Applications

In the future, the LKB1-AMPK-TORC2 signaling pathway will probably be a target for berberine treating type 2 diabetes.

### Terminology

Hepatic gluconeogenesis is strongly stimulated in the fasting state and converts glycogen into glucose to increase glucose output. AMPK is a conserved sensor and regulator of cellular energy balance that is activated when the cellular AMP:ATP ratio exhibits a large increase.

### Peer-review

In this paper, the authors identified the association between the LKB1-AMPK-TOCR2 signaling pathway and glucose homeostasis in the liver. At the time, this study proved the molecular mechanisms of berberine inhibiting hepatic gluconeogenesis. The research is important for further research of berberine.

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## Case Control Study

# ***NOD2/CARD15* gene mutations in North Algerian patients with inflammatory bowel disease**

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**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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**Data sharing statement:** Technical appendix, statistical code, and dataset are available from the corresponding author, Mesbah-Amroun H, at [amrounhamida@yahoo.com](mailto:amrounhamida@yahoo.com) or [hamroun@usthb.dz](mailto:hamroun@usthb.dz). Consent was not obtained by participants for data sharing but the presented data are anonymized and risk of identification is low.

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## Abstract

**AIM:** To analyse allelic frequency of *NOD2* gene variants

and to assess their correlation with inflammatory bowel disease (IBD) in Algeria.

**METHODS:** We studied 132 unrelated patients diagnosed with IBD, 86 with Crohn's disease (CD) and 46 with ulcerative colitis (UC). Data was prospectively collected between January 2011 and December 2013. The demographic and clinical characteristics were recorded for all the patients. A group of 114 healthy unrelated individuals were selected as controls. All groups studied originated from different regions of North Algeria and confirmed the Algerian origin of their parents and grandparents. Informed and written consent was obtained from each of the participants. All individuals were genotyped for the three CD-associated *NOD2* variants (p.Arg<sup>702</sup>Trp, p.Gly908Arg and p.Leu<sup>1007</sup>fsinsC mutations) using the polymerase chain reaction-restriction fragment length polymorphism method. Allele and genotype frequencies in patients and control subjects were compared by  $\chi^2$  test and Fisher's exact test where appropriate. Odds ratios (OR) and 95% confidence intervals (95%CI) were also estimated. Association analyses were performed to study the influence of these variants on IBD and on clinical phenotypes.

**RESULTS:** The p.Arg<sup>702</sup>Trp mutation showed the highest frequency in CD patients (8%) compared to UC patients (2%) ( $P = 0.09$ , OR = 3.67, 95%CI: 0.48-4.87) and controls (5%) ( $P = 0.4$ , OR = 1.47, 95%CI: 0.65-3.31). In CD patients allelic frequencies of p.Gly908Arg and p.Leu<sup>1007</sup>fsinsC variants compared to HC were 3% *vs* 2% ( $P = 0.5$ , OR = 1.67, 95%CI: 0.44-6.34); 2% *vs* 1% ( $P = 0.4$ , OR = 2.69, 95%CI: 0.48-14.87 respectively). In UC patients, allelic frequencies of p.Gly908Arg and p.Leu<sup>1007</sup>fsinsC variants compared to HC were 1% *vs* 2% ( $P = 1$ , OR = 1.62, 95%CI: 0.17-4.74) and 2% *vs* 1% ( $P = 0.32$ , OR = 0.39, 95%CI: 0.05-2.87). The total frequency of the mutated *NOD2* chromosomes was higher in CD (13%), than in HC (8%) and UC (5%). In addition, *NOD2* variants were linked to a particular clinical sub-phenotype in CD in this Algerian cohort. As expected, the three *NOD2* variants showed a significant association with CD but did not reach statistical significance, despite the fact that the allele frequency of *NOD2* variants was in the range found in most of the European populations. This might be due to the non-exposure of the *NOD2* carriers to environmental factors, required for the expression of the disease.

**CONCLUSION:** Further analyses are necessary to study genetic and environmental factors in IBD in the Algerian population, using larger patient groups.

**Key words:** Algeria; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; *NOD2* mutations; Polymerase chain reaction-restriction fragment length polymorphism method

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**Core tip:** We evaluated allelic frequency of *NOD2* variants among 132 inflammatory bowel disease (IBD) patients and 114 unrelated healthy subjects from Algeria. Despite the fact that the frequency of *NOD2* mutant alleles is in the range found in most of the European populations, we failed to demonstrate the association of these *NOD2* variants with IBD susceptibility. This might be due to the non exposure of the *NOD2* carriers to environmental factors, required for the expression of the disease. We can expect in the coming years to see an increased incidence of IBD associated with the spread of Western lifestyle in this region.

Boukercha A, Mesbah-Amroun H, Bouzidi A, Saoula H, Nakkemouche M, Roy M, Hugot JP, Touil-Boukoffa C. *NOD2/CARD15* gene mutations in North Algerian patients with inflammatory bowel disease. *World J Gastroenterol* 2015; 21(25): 7786-7794 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7786.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7786>

## INTRODUCTION

The nucleotide-binding oligomerization domain containing 2 gene (*NOD2*) is 3.1 kb in size and is located on chromosome 16q12.1<sup>[1-3]</sup>. Consisting of 12 exons and 11 introns, *NOD2* encodes a protein expressed mainly in monocytes, dendritic cells, enterocytes and Paneth cells. *NOD2* has an important role in immune system function<sup>[4]</sup>. In response to bacterial infection, *NOD2* acts as an intracellular bacterial receptor in monocytes and activates nuclear factor kappa B (NF- $\kappa$ B) specifically after recognition of the bacterial cell wall component, muramyl dipeptide (MDP)<sup>[5]</sup> and leads to activation of the inflammatory response<sup>[6,7]</sup>. *NOD2* mutations are associated with Crohn's disease (CD) as well as other disorders, including Blau syndrome<sup>[8,9]</sup> and bipolar disorder<sup>[10]</sup>. *NOD2* mutations have also been associated with a higher incidence of specific types of cancer in affected patients<sup>[11]</sup>.

More than 40 non-conservative mutations were identified on the *NOD2* gene<sup>[12]</sup>. The most common are two missense mutations, p.Arg<sup>702</sup>Trp (SNP8, C/T) in exon 4, leading to substitution of arginine in position 702 by tryptophan, p.Gly908Arg (SNP12, G/C) in exon 8, leading to substitution of glycine in position 908 by arginine and an insertion mutation of a C in exon 11, a frame shift resulting in a truncated *NOD2* protein at position 1007, p-Leu<sup>1007</sup>fsinsC (SNP13, insC). Independent groups have reported the association of these three mutations with increased susceptibility to CD<sup>[12-14]</sup>. These mutations affect the structure of



either the carboxy-terminal leucine-rich repeat (LRR) domain of the protein or the adjacent region. The activating functions of NF- $\kappa$ B are regulated by the LRR domain, which has an inhibitory role and acts as an intracellular receptor for components of microbial pathogens<sup>[12]</sup>. In particular, these *NOD2* mutations are associated with the phenotypes of CD that involve the ileum, and with fibrostenosing disease<sup>[15]</sup>. In Caucasian populations, the contribution of the *NOD2* gene mutations to CD has been studied. The risk of CD has been evaluated to be 1.5-3-fold for heterozygous carriers and 10-44-fold for homozygous/compound heterozygous carriers<sup>[15-19]</sup>. However, the background prevalence of *NOD2* mutations depends on ethnicity. In Asian populations such as Chinese, Korean and Japanese, the three previously described major variants of the *NOD2* gene were not found in CD patients and controls<sup>[20,21]</sup>, indicating that although ethnically divergent populations may present identical phenotypes, they do not necessarily share the same set of predisposing genes. In African populations, very few studies have been performed to investigate the influence of genetic factors in the development of CD. Gasche *et al.*<sup>[22]</sup>, screened the three *NOD2* SNPs in a collection of 1064 DNA samples from 52 worldwide populations, including seven sub-Saharan populations and one North African population composed of 30 Algerian Mozabites. They likewise found no *NOD2* mutations in African populations except for a single positive case of p.Arg<sup>702</sup>Trp mutation in the Algerian Mozabite population. They concluded that the three CD-associated single nucleotide polymorphisms (SNPs) were almost exclusively found in Europe and are absent in native populations from Africa. In North African populations, two previous genotyping analyses in Morocco and Tunisia<sup>[23,24]</sup> revealed that the *NOD2* allele's frequencies are very low when compared to the frequencies seen in Caucasians of European origin. More recently, the contribution of *NOD2* polymorphisms to CD has been studied for the first time in an Algerian population<sup>[25]</sup> and a strong association between CD and *NOD2* variants was reported. However, p.Leu<sup>1007</sup>fsinsC variant was not investigated in this study even it showed the strongest association with CD in previous studies<sup>[15,17]</sup>. The objectives of the present study were thus to determine allelic frequency of the three major *NOD2* gene variants and to investigate whether they determined specific phenotypes of IBD among North Caucasian Algerians.

## MATERIALS AND METHODS

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the ethics committee of the Agence Thématique de la Recherche Scientifique en Santé, (ATRSS, ex ANDRS). All patients signed an informed consent form for this investigation.

### Patients with IBD

A total of 132 unrelated patients diagnosed with IBD (86 Crohn's disease (CD), 46 ulcerative colitis (UC)) reported in this study were recruited from Maillot Hospital, Algiers, Algeria, between January 2011 and December 2013 and represented an independent cohort not studied before in any IBD genetic studies. Diagnosis of IBD was based on standard clinical, radiological, endoscopic and histological criteria. The following demographic and clinical characteristics were recorded for all the patients: geographical origin, gender, age, age at diagnosis, disease location, presence of extra intestinal manifestations, surgery and familial or sporadic disease (familial disease was considered if one first or second degree relative had IBD). In addition, we carried out an evaluation of the incidence of IBD in our monocentric study, since no published epidemiological data on IBD in Algeria are available. Data were obtained from the registry of IBD of the Department of Gastroenterology at the Maillot University Hospital of Algiers. We have considered all patients permanent residents of Algiers, consulted for the first time between January 1988 and December 2008, for symptoms consistent with a diagnosis of IBD.

### Healthy control subjects

One hundred and fourteen healthy unrelated individuals (mainly students, blood donors and hospital employees) with a mean age of  $25.21 \pm 8.66$  were selected as controls on the basis of a lack of personal or family history of IBD or any other autoimmune or immune diseases.

### Statistical analysis

A comparison of genotype frequencies between different groups was evaluated using the  $\chi^2$  test or Fisher's exact test. Odds ratios (OR) were noted with a 95%CI. Expected and observed heterozygosity and Hardy-Weinberg equilibrium (HWE) were calculated. Variables were considered to indicate a statistically significant difference for a *P* value less or equal to 0.05. We used GraphPad prism version 6.01 (GraphPad Software, San Diego, California, United States).

### DNA extraction

DNA was extracted from blood samples by the phenol-chloroform method according to standard protocols<sup>[26]</sup>.

We screened samples for the presence of p.Arg<sup>702</sup>Trp, p.Gly908Arg and p.Leu<sup>1007</sup>fsinsC mutations of the *NOD2* gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primers and PCR reactions were previously described<sup>[27]</sup>. Primers used were synthesized by Invitrogen (Invitrogen, Life Technologies, Carlsbad, California, United States) and PCR reactions were carried out using a T100 thermal cycler (BIO-RAD, Richmond CA, United States). PCR products were analysed on 1% agarose gels and stained with 1 mg/mL ethidium bromide. The digestion reaction

**Table 1** Demographic, clinical data and genotype-phenotype correlations in Crohn's disease patients *n* (%)

Variables	All subjects	Non carriers	Carriers	P value
HC	114	102 (89.47)	12 (10.52)	0.120
CD patients	86	70 (81.39)	16 (18.6)	0.103
Sex (F/M)	46/40	40/30	6/10	0.155
Age at diagnosis	25.28 ± 14.85	25.70 ± 15.55	22.12 ± 10.66	
A1	28 (32.5)	22	6	0.809
A2	43 (50)	35	8	
A3	15 (17.5)	13	2	
Location				
L1: Ileum	25 (29)	23	2	0.020
L2: Ileo-colon	49 (57)	35	14	
L3: Colon	12 (14)	12	0	
Resective	37 (43)	31	6	0.620
Surgery				
EIM	24 (28)	18	6	0.343
Positive family history for CD	23 (27)	17	6	0.281

A1 < 16, A2 = 16-40, A3 > 40 years according Montreal classification. CD: Crohn's disease; HC: Healthy controls; EIM: Extra intestinal manifestations.

**Table 2** Demographic, clinical data and genotype-phenotype correlations in ulcerative colitis patients *n* (%)

Variables	All subjects	Non carriers	Carriers	P value
HC	114	102 (89.47)	12 (10.52)	0.120
UC patients	46	42 (91.3)	4 (8.7)	0.726
Sex (F/M)	30/16	27/15	3/1	0.667
Age at diagnosis	36.86 ± 12.30	36.92 ± 11.34	36.25 ± 22.60	
A1	1 (2.1)	1	0	0.916
A2	31 (67.4)	28	3	
A3	14 (30.5)	13	1	
Location				
E1: proctitis	9 (20)	9	0	0.115
E2: distal colitis	19 (41.5)	16	3	
E3: extensive colitis	18 (39)	17	1	
Resection	4 (9)	3	1	0.225
Surgery				
EIM	11 (24)	11	0	0.240
Positive family history for UC	11 (24)	10	1	0.957

A1 < 16, A2 = 16-40, A3 40 years according Montreal classification. UC: Ulcerative colitis; HC: Healthy controls; EIM: Extra intestinal manifestations.

and restriction enzyme digestion conditions were taken from the manuals included with the restriction enzymes MspI, HhaI purchased from Promega (Promega, Madison, Wisconsin, United States) and NlaIV purchased from New England Biolabs (New England Biolabs, Massachusetts, United States). Digestion products were loaded on polyacrylamide gel electrophoresed under 120 volts for 60 min, coloured with 1 mg/mL ethidium bromide. Images were captured with a Gel Doc EZ

imager analyser (BIO-RAD, Richmond CA, United States).

## RESULTS

In our study, we have analysed two series of data. We have obtained data retrospectively from the Department of Gastroenterology's register. A group of 770 IBD patients resident in Algiers, were consulted between January 1988 and December 2008. Based on these data, we evaluated the incidence rate for IBD. It ranges from 0.5 to 2 cases/10<sup>5</sup> per year, with an average rate of 1.2. The second series of data was obtained prospectively between January 2011 and December 2013. Our cohort was composed of 132 IBD patients (86 CD and 46 UC). The mean age of the patients at diagnosis was 25.28 ± 14.85 and 36.86 ± 12.30 years for CD and UC, respectively. The clinical characteristics and phenotype data are reported in Tables 1 and 2. Family history in a first- or second-degree relative was found in 27% and 24% of the CD and UC patients studied, respectively, which is higher than reported before in CD patients in the same population<sup>[25]</sup>. There was a slight female gender predominance in IBD patients (57%). PCR-RFLP was employed to detect p.Arg<sup>702</sup>Trp, p.Gly908Arg and p.Leu<sup>1007</sup>fsinsC mutations of the *NOD2* gene in 132 IBD patients and 114 healthy control subjects. Allele frequencies of mutant alleles of each *NOD2* variant are shown in Table 3. The p.Arg<sup>702</sup>Trp mutation showed the highest frequency in CD patients (8%) compared to UC patients (2%) ( $P = 0.09$ , OR = 3.67, 95%CI: 0.48-4.87) but its frequency was also high in controls (5%) ( $P = 0.4$ , OR = 1.47, 95%CI: 0.65-3.31). Likewise, p.Gly<sup>908</sup>Arg and p.Leu<sup>1007</sup>fsinsC mutations showed similar frequency in CD patients and in controls (3% vs 2%,  $P = 0.5$ , OR = 1.67, 95%CI: 0.44-6.34; 2% vs 1%,  $P = 0.4$ , OR = 2.69, 95%CI: 0.48-14.87, respectively). In UC patients, allelic frequencies of p.Gly<sup>908</sup>Arg and p.Leu<sup>1007</sup>fsinsC variants compared to HC were 1% vs 2%, ( $P = 1$ , OR = 1.62, 95%CI: 0.17-4.74) and 2% vs 1% ( $P = 0.32$ , OR = 0.39, 95%CI: 0.05-2.87). As expected, the total frequency of the mutated *NOD2* chromosomes was higher in CD (13%), than in HC (8%) and UC (5%). However, this difference was not statistically significant (CD vs HC,  $P = 0.12$ , OR = 1.71, 95%CI: 0.88-3.3; UC vs HC,  $P = 0.63$ , OR = 0.67, 95%CI: 0.24-1.86). In addition, the comparison of the total allelic frequencies between CD and UC patients did not show any statistical difference ( $P = 0.08$ , OR = 0.39, 95%CI: 0.14-1.07).

When considering the carriers of at least one copy of mutant alleles in any variant and the carriers of two copies (Table 4), a similar result was observed. 18.6% of CD patients and 8.7% of UC patients carried at least one mutant allele in any of the three considered variants, vs 10.52% of controls ( $P = 0.103$ ,  $P = 0.726$ , respectively). Two copies of mutant alleles were

**Table 3** *NOD2* Allele frequencies in Crohn's disease, ulcerative colitis and healthy control groups

Polymorphisms		Allele frequency (%)				<i>P</i> value <sup>1</sup>	OR (95%CI)	<i>P</i> value <sup>2</sup>	OR (95%CI)	<i>P</i> value <sup>3</sup>	OR (95%CI)	<i>P</i> value <sup>4</sup>	OR (95%CI)
		CD	UC	HC	IBD								
		( <i>n</i> = 86)	( <i>n</i> = 46)	( <i>n</i> = 114)	( <i>n</i> = 132)								
p.Arg <sup>702</sup> Trp (C/T)	C	92	98	95	94	0.09	3.67	0.40	1.47	1.00	0.92	0.36	2.5
	T	8	2	5	6		0.48-4.87		0.65-3.31		0.42-2.01		0.54-11.4
p.Gly <sup>908</sup> Arg (G/C)	G	97	99	98	98	0.66	2.72	0.50	1.67	0.75	0.76	1.00	1.62
	C	3	1	2	2		0.31-23.69		0.44-6.34		0.21-2.75		0.17-14.74
p.Leu <sup>1007</sup> fsinsC (WT/insC)	WT	98	98	99	98	1.00	1.07	0.40	2.69	0.29	0.38	0.32	0.39
	insC	2	2	1	2		0.19-5.9		0.48-14.87		0.07-1.9		0.05-2.87
Total frequency (%)		13	5	8	10	0.08	0.39	0.12	1.71	0.43	1.32	0.63	0.67
							0.14-1.07		0.88-3.3		0.71- 2.48		0.24-1.86

<sup>1</sup>The *P* value for CD patients *vs* UC patients; <sup>2</sup>The *P* value for CD patients *vs* HC group; <sup>3</sup>The *P* value for IBD patients *vs* HC group; <sup>4</sup>The *P* value for UC patients *vs* HC group. (%) represents allele frequency. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease (CD + UC patients); HC: Healthy controls; WT: Wild type allele; insC: Insertion mutation of a C.

**Table 4** Number of different genotypes observed in Crohn's disease/ulcerative colitis/healthy controls groups

Variant	p.Arg <sup>702</sup> Trp	p.Gly <sup>908</sup> Arg	p.Leu <sup>1007</sup> fsinsC	WT
p.Arg <sup>702</sup> Trp	2/0/4	1/0/1	2/1/1	6/1/2
p.Gly <sup>908</sup> Arg		0/0/0	1/0/0	3/1/3
p.Leu <sup>1007</sup> fsinsC			0/0/0	1/1/1
WT				70/42/102

In each cell of the table, the genotype is obtained by combining the two genetic variants indicated in the corresponding row and column. No patients homozygous for *NOD2* gene p.Gly908Arg and p.Leu1007fsinsC mutations were found in this study. CD: Crohn's disease; UC: Ulcerative colitis; HC: Healthy controls; WT: Wild type.

**Table 5** Hardy-Weinberg equilibrium and the *P* value

Groups	Genotypes Frequencies	<i>NOD2</i> polymorphisms											
		p.Arg <sup>702</sup> Trp				p.Gly <sup>908</sup> Arg				p.Leu <sup>1007</sup> fsinsC			
		CC	CT	TT	<i>P</i> value	GG	GC	CC	<i>P</i> value	WT/WT	insC/WT	insC/insC	<i>P</i> value
HC	Observed	92.98	3.51	3.51	0	96.49	3.51	0	0.8488	98.25	1.75	0	0.9247
	Expected	89.75	9.97	0.28		96.52	3.45	0.03	NS	98.25	1.74	0	NS
CD	Observed	87.21	10.46	2.33	0.0117	94.19	5.81	0	0.7813	95.35	4.65	0	0.8252
	Expected	86.92	12.62	0.46		94.27	5.64	0.08	NS	95.41	4.54	0.05	NS
IBD	Observed	90.15	8.33	1.52	0.0106	95.45	4.55	0	0.7893	95.45	4.55	0	0.7893
	Expected	88.96	10.72	0.32		95.51	4.44	0.05	NS	95.51	4.44	0.05	NS

CD: Crohn's disease; HC: Healthy controls; IBD: Inflammatory bowel disease; NS: Non-significant *P* value; WT: Wild type; insC: Insertion mutation of a C.

carried by 7% of the CD subjects (either homozygous for one variant or compound heterozygous), *vs* 5.26% in the control group (*P* = 0.613). Only a single subject was compound heterozygous for p.Arg<sup>702</sup>Trp and p.Leu<sup>1007</sup>fsinsC variant in the UC group (2.17%) (*P* = 0.397). No subjects were homozygous for p.Gly908Arg or p.Leu<sup>1007</sup>fsinsC variants in the whole sample of CD. This reveals the risk conferred by the possession of more than one of these mutations.

When analysing the genotype data, the HWE test *P* value was not significant for any of the SNPs except for p.Arg<sup>702</sup>Trp (Table 5). The observed excess of

p.Arg<sup>702</sup>Trp homozygous genotypes in the HC sample was significant and could be due to consanguinity.

No significant associations were found with *NOD2* mutations for family history, presence of extra intestinal manifestations or surgery, even when p.Arg<sup>702</sup>Trp/p.Gly908Arg/p.Leu<sup>1007</sup>fsinsC polymorphisms were considered together or separately or when only p.Gly908Arg/p.Leu<sup>1007</sup>fsinsC were considered.

The stratification of CD according to age at diagnosis showed that CD patients carrying one or two copies of any rare variant had a mean age at diagnosis lower than non-carrier CD patients. However, this

**Table 6** Comparative study of *NOD2* allele frequencies in North African populations

Population	Number		Allele frequency (%)							
			p.Arg <sup>702</sup> Trp		p.Gly <sup>908</sup> Arg		p.Leu <sup>1007</sup> fsinsC		Total allele	
	CD	HC	CD	HC	CD	HC	CD	HC	CD	HC
North Algeria (this study)	86	114	8	5	3	2	2	1	13	8
Algeria <sup>[25]</sup>	204	201	5	0.50	3	0.5	ND	ND	ND	ND
Morocco <sup>[23]</sup>	101	107	0.49	0.46	6.43	2.8	0.9	0	8	3.2
Tunisia <sup>[24]</sup>	130	90	2	0.60	5	3	1	0	8	3.6

CD: Crohn's disease; HC: Healthy controls; ND: Not determined.

association was not significant ( $P = 0.809$ ).

One positive association was shown between *NOD2* mutations and location of disease. In the CD group, 59% of patients had combined small bowel and colonic involvement, 30% had isolated small bowel involvement and 11% had an isolated colonic disease. *NOD2* carriers with any risk allele showed ileal or ileocolonic CD and none of them had an isolated colonic CD. There were significant differences between allele frequencies and location of disease ( $P = 0.020$ ). In our study, *NOD2* alleles were associated with a particular CD sub-phenotype. This result is in agreement with previous studies in which ileal disease was found associated with *NOD2* variants<sup>[15]</sup>.

In the UC group, no significant associations were found among the *NOD2* mutations and age at diagnosis, presence of extra intestinal manifestations, surgery or family history. When considering extent of disease, 9 (20%) patients had proctitis, 19 (41.5%) distal colitis and 18 (39%) extensive colitis. Interestingly, none of the UC patients with *NOD2* risk alleles had E1 disease location, suggesting a potential association between *NOD2* mutations carrier status and disease severity. However, the frequency of *NOD2* mutations did not achieve statistical significance ( $P = 0.115$ ), probably because of the small number of patients in this sub-group (only 4).

## DISCUSSION

Allele frequency of the three major *NOD2* gene variants was assessed in this analysis. As expected, the total allele frequency was higher in CD patients than in UC patients and HC. The frequencies of the three *NOD2* variants range from 4% to 5%, 1% to 2% and 2% to 3% for p.Arg<sup>702</sup>Trp, p.Gly<sup>908</sup>Arg and p.Leu<sup>1007</sup>fsinsC, respectively, in healthy Caucasian populations<sup>[28]</sup>. The corresponding frequencies among Caucasian CD patients range from 9% to 13%, 3% to 6% and 7% to 16%, respectively<sup>[29]</sup>. In UC patients, the frequencies of these three variants are lower, ranging from 4% to 6%, 2% and 2.5% to 3.3%<sup>[30-32]</sup>. In our cohort of IBD and controls, the observed frequency of *NOD2* mutant alleles is in agreement with a high Caucasian component of the targeted population and is in the range found in most European

populations, except for p.Leu<sup>1007</sup>fsinsC frequency which was lower. Much lower frequency was also found for the frameshift mutation in neighbouring populations from Morocco and Tunisia, with similar sample size (Table 6). Interestingly, another study in a North Tunisian population has shown that p.Leu<sup>1007</sup>fsinsC mutation was strongly associated with susceptibility to CD with an allelic frequency value of 15%<sup>[33]</sup>, indicating that significant differences might be obtained for the same population.

The p.Arg<sup>702</sup>Trp allelic frequency obtained on 114 North Algerian healthy controls is likewise significantly different from that recently determined on 201 controls ( $P = 0.0002$ , OR = 11.11)<sup>[25]</sup>. This divergence indicates that the control samples used in the two studies are significantly different. Previous studies described the population of North Africa as extremely heterogeneous, composed of a mosaic of sub-populations with significantly different genetic structures and with high endogamy rates<sup>[33,34]</sup>. This is confirmed by the Hardy-Weinberg analyses performed in our study. Thus, recruitment from different geographic origins in Algeria may lead to different *NOD2* frequencies. Taken together, all these data from the Algerian population suggested that genetic variations of *NOD2* gene are not homogeneously distributed.

The correlation of *NOD2* genotypes with phenotypic expression of IBD was assessed in this analysis. IBD is a heterogeneous disorder characterized by the presence of different clinical sub-phenotypes. In our study, we have established different sub-groups, after the stratification of IBD patients according to demographic and phenotypic data. In CD patients, no significant association was found between *NOD2* variants and phenotypic data (Tables 1 and 2). However, this association was significant with disease location, as described previously. In the UC group, our data suggests that *NOD2* mutations appear to be associated with a more aggressive course of UC. Similar findings have been recently reported on UC in the Portuguese population<sup>[35]</sup>. To our knowledge, this study is the first one performed to investigate the influence of genetic factors in the development of UC in the Algerian population. The small number of UC patients with *NOD2* risk alleles is the limitation in our study and a larger sample is certainly needed to clarify



the role of *NOD2* gene variants in phenotype severity of UC in Algeria.

Finally, our results clearly show an influence of *NOD2* gene variants on specific CD clinical sub-groups. Since only 18.6% of CD patients and 8.69% of UC patients carry at least one mutation in the *NOD2* gene, it is possible that other polymorphisms in the *NOD2* gene or in other genes, in combination with environmental factors, are involved in IBD susceptibility. Indeed, despite the fact that the observed frequency of *NOD2* mutant alleles is in the range found in most European populations, the association of these polymorphisms on IBD susceptibility in our studied population could not be demonstrated. This might be due not only to the statistical test power failure but also to an environmental effect. *NOD2* carriers do not express the disease, because they are not exposed to environmental factors required for the expression of the disease and are probably protected because they lead a lifestyle that is not at risk. In these conditions, the OR values attributed to the *NOD2* mutations are proportional to the population exposure. Our study showed that the incidence rate for IBD increased from 0.5 to 2 cases/10<sup>5</sup> per year, between 1988 and 2008. This incidence rate is lower than that of other population groups and is certainly underestimated because it is evaluated only in a monocentric study. However, it is interesting to note that over these two decades the population in Algeria became predominantly urban. Thus, the increased incidence of IBD observed in the targeted population might be associated with the change in lifestyle. This is in agreement with a previous study demonstrating that urbanization of society is an important risk factor for the development of IBD. Soon *et al.*<sup>[36]</sup>, demonstrated that living in an urban society was positively associated with the development of IBD. Since change in lifestyle is relatively recent in Algeria, an increased incidence in IBD associated with the spread of Western lifestyle in this region can be expected in the coming years.

In conclusion, the data we obtained are relevant to estimate the *NOD2* gene variants in the Algerian population. Our study confirmed that the *NOD2* gene is significantly associated with a specific clinical sub-phenotype in CD. The results obtained up to now in Algeria have shown that the *NOD2* gene is involved in IBD susceptibility and have suggested a heterogeneous distribution of *NOD2* mutations across Algerian populations. Further analyses are necessary to study genetic and environmental factors in IBD in the Algerian population, using larger patient groups.

## COMMENTS

### Background

Very few studies have been performed to investigate the influence of genetic factors in the development of Crohn's disease (CD) in Algeria.

### Research frontiers

The authors have explored *NOD2/CARD15* gene mutations in North Algerian patients with inflammatory bowel disease (IBD).

### Innovations and breakthroughs

This is a novel study in which we determine, for the first time, the allelic frequency of the three major *NOD2* gene variants among the North Caucasian Algerians and investigate whether they determine specific phenotypes of IBD. The authors clearly showed an influence of *NOD2* gene variants on specific CD clinical sub-groups. The authors also proposed that an increased incidence of IBD observed in the targeted population might be associated with the change in lifestyle.

### Applications

Investigating other genetic determinants, but also environmental factors, in IBD patients could help us to understand the pathogenic pathway of disease both in CD and ulcerative colitis (UC).

### Peer-review

This is an original work in which a genetic study has revealed the importance of the environment on the expression of IBD. The format of the manuscript was slightly revised.

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## Retrospective Cohort Study

# Clinical predictors of thiopurine-related adverse events in Crohn's disease

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## Abstract

**AIM:** To determine the incidence and predictors of thiopurine-related adverse events.

**METHODS:** Subjects with Crohn's disease who were followed in the Alberta Inflammatory Bowel Disease Consortium patient database registry were identified. Retrospective chart review was conducted between August 5<sup>th</sup>, 2010 and June 1<sup>st</sup>, 2012. We collected data on: age at diagnosis; sex; disease location and behaviour at time of prescribing thiopurine; perianal fistulising disease at or prior to thiopurine prescription; smoking status at time of thiopurine prescription, use of corticosteroid within 6 mo of diagnosis; dosage, age at onset, and cessation of 5-aminosalicylic acid (5-ASA); anti-tumour necrosis factor medication exposure and intestinal resection before thiopurine prescription. The primary outcome of interest was the first adverse event that led to discontinuation of the first thiopurine medication used. Logistic regression models were used to associate clinical characteristics with outcomes after adjusting for potential confounders. Risk estimates were presented as odds ratios (OR) with 95% CI. Effect modification by age and sex were explored.

**RESULTS:** Our cohort had a median follow-up duration of 5.8 years [interquartile range (IQR 25<sup>th</sup>-75<sup>th</sup>) 2.7-9.1]. Thiopurine therapy was discontinued in 31.3% of patients because of: hypersensitivity reactions (7.1%), acute pancreatitis (6.2%), gastrointestinal intolerance (5.4%), leucopenia (3.7%), hepatotoxicity (3.4%), infection (1.1%) and other reasons (4.3%). A higher incidence of thiopurine withdrawal was observed in patients over the age of 40 (39.4%,  $P = 0.007$ ). A sex-by-age interaction ( $P = 0.04$ ) was observed. Females older than 40 years of age had an increased risk of thiopurine discontinuation due to an adverse event (age above 40 *vs* age below 40, adjusted OR = 2.8; 95%CI: 1.4-5.6). In contrast, age did not influence thiopurine withdrawal in males (age above 40 *vs* below 40, adjusted OR = 0.9; 95%CI: 0.4-2.1). Other clinical variables (disease location and phenotype, perianal disease, smoking history, history of intestinal resection and prior 5-ASA or corticosteroid use) were not associated with an increased risk an adverse event leading to therapy cessation.

**CONCLUSION:** Thiopurine withdrawal due to adverse events is commoner in women over the age of 40 at prescription. These findings need to be replicated in other cohorts.

**Key words:** Thiopurines; Azathioprine; Mercaptopurine; Adverse events

**Core tip:** In Crohn's disease, adverse events to thiopurines are a common occurrence leading to discontinuation of therapy in 1 in 3 patients in this referral centre cohort. Adverse events leading to discontinuation of the drug were significantly more common in female patients over the age of 40 years at drug prescription. These findings should be replicated in other centres, in other clinical indications for thiopurine use and correlated to thiopurine 6-methyltransferase genotype, activity and thiopurine metabolites.

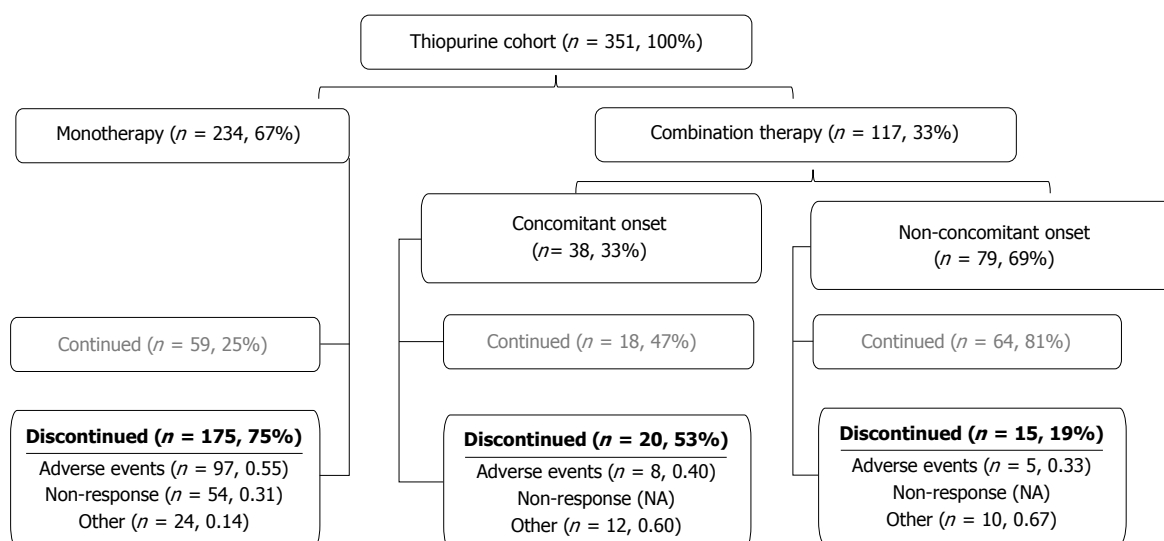
Moran GW, Dubeau MF, Kaplan GG, Yang H, Eksteen B, Ghosh S, Panaccione R. Clinical predictors of thiopurine-related adverse events in Crohn's disease. *World J Gastroenterol* 2015; 21(25): 7795-7804 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7795.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7795>

## INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by chronic and relapsing intestinal inflammation that can affect any segment of the gastrointestinal tract. The aetiology of CD is multifactorial consisting of an interplay of altered immune responses to environmental stimuli such as the gut microbiota in genetically predisposed individuals<sup>[1-3]</sup>. Most patients with CD require chronic immune-suppressing medications to control their disease, and when these drugs fail intestinal resections are required<sup>[4]</sup>.

Thiopurine analogs consist of mercaptopurine (MP) and its pro-drug azathioprine (AZA). Thiopurines have been shown to reduce corticosteroid use and maintain remission in patients with CD<sup>[5,6]</sup>, but this evidence has been questioned by more recent data from two randomised controlled trials<sup>[7,8]</sup> in CD patients with early disease, precluding a widespread usage of thiopurines in all patients with early CD. Treatment paradigms have evolved in the last decade with the introduction of anti-tumour necrosis factor (TNF) therapy<sup>[9-11]</sup>. Emerging evidence suggests that the combination of thiopurines with anti-TNF therapy may be associated with greater efficacy for moderate to severe CD when compared to monotherapy with anti-TNF agents<sup>[12-14]</sup>. However, balancing efficacy against adverse events associated with immunosuppressive medications remains a persistent challenge in IBD management.

Adverse events lead to discontinuation of thiopurines in 9%-25% of cases<sup>[15,16]</sup>. In a previous meta-analysis reporting on studies between 1966 to 1994, thiopurine withdrawal due to an adverse event was



**Figure 1 Patient disposition.** Disposition of the whole study cohort. Drug disposition is described in the materials and methods section. Thiopurines were discontinued due to adverse events, non-response or other non-clinical reasons.

described in 8.9% of the cases<sup>[17]</sup>. Reported rates have varied over the years but large referral-centre studies have shown higher discontinuation rates than previously reported (11%<sup>[18]</sup>, 15%<sup>[19]</sup>, 18.3%<sup>[20]</sup>, 39%<sup>[21]</sup>, 28%<sup>[22]</sup>, 31%<sup>[23]</sup>, 25.9%<sup>[24]</sup>, 27.4%<sup>[25]</sup>). Thiopurine discontinuation and adverse events in the era of anti-TNF therapy has not been well described. Further, clinical variables that predict adverse events when prescribing thiopurines are not available. Genetic polymorphisms of the thiopurine 6-methyltransferase (TPMT) have been shown to correlate with subnormal enzyme activity and myelotoxicity. The effect of TPMT polymorphism on gastrointestinal toxicity is still unclear with an earlier study<sup>[26]</sup> showing a dis-concordance between TPMT heterozygosity and gastrointestinal intolerances. More recent data however has shown a significant association<sup>[27]</sup> between TPMT polymorphism and gastrointestinal intolerances. It is unclear what is the effect of gender on TPMT activity, with some studies showing an increased activity in males<sup>[28-31]</sup>, decreased activity in females<sup>[32]</sup> or no effect of gender at all<sup>[33]</sup>. Patient age<sup>[30,31]</sup>, has no effect on TPMT activity but combination treatment with 5-ASA therapy might increase 6-thioguanine nucleotide levels due to a negative effect of 5-ASA therapy on TPMT activity. Earlier reports had shown no effect of 5-ASA co-administration<sup>[30,34,35]</sup> on TPMT activity but more recent data has indicated 5-ASA therapy increases 6-thioguanine nucleotide levels<sup>[36,37]</sup>. Due to inconsistent data, identifying a clinical phenotype associated with thiopurine intolerance may facilitate in the decision-making process when prescribing a thiopurine and thus enable improved patient experiences and outcomes.

Thus, we evaluated thiopurine discontinuation due to adverse events in a cohort of CD patients and investigated clinical characteristics associated with adverse events.

## MATERIALS AND METHODS

### Study population

Subjects with CD who were followed in the Alberta Inflammatory Bowel Disease (IBD) Consortium patient database registry were identified<sup>[38]</sup>. Retrospective chart review was conducted between August 5<sup>th</sup>, 2010 and June 1<sup>st</sup>, 2012. We identified all patients with CD in our registry who had a current or previous prescription of a thiopurine agent (AZA or MP). Patients with no history of thiopurine therapy or with a diagnosis of ulcerative colitis, IBD unspecified, or microscopic colitis at time of chart review were not included in this study. We identified 366 CD patients with a current or prior prescription of a thiopurine. The clinical scenarios that patients with CD were prescribed a thiopurine are described in Figure 1. Fifteen subjects were excluded because the reason for withdrawal was unavailable ( $n = 12$ ) or the status of thiopurine prescription was not obvious from the chart review ( $n = 3$ ).

### Outcomes

The primary outcome of interest was the first adverse event that led to discontinuation of the first thiopurine medication (AZA or MP) used. The diagnosis of the adverse event was based on the clinical opinion of the prescribing gastroenterologist in conjunction with investigations (e.g., elevated lipase for pancreatitis) when available. Adverse events were defined as: acute pancreatitis as defined by relevant clinical symptoms (e.g., epigastric pain) and serum lipase > 3 times above the reference range; leucopenia defined as a white blood cell count of less than 3500/mL; gastrointestinal intolerance (GI) as defined by a gastroenterologist recording symptoms of nausea, vomiting or non-specific abdominal pain in the absence of any other cause; hepatotoxicity as defined

by a rise in either (1) alanine transaminase levels more than three times of upper limit of normal; (2) alkaline phosphatase levels more than twice upper limit of normal; or (3) total bilirubin level more than twice upper limit of normal (not including Gilbert's syndrome) when associated with increased alanine transaminase or alkaline phosphatase<sup>[39,40]</sup>; infection and hypersensitivity reactions including arthralgias, myalgias, rash, fever and flu-like reaction alone or in combination as diagnosed by a gastroenterologist<sup>[41]</sup>. Uncommon adverse events (e.g., alopecia, photosensitivity, skin cancer) were grouped as other. Data on the date of the adverse event and if the adverse event led to drug withdrawal were recorded.

### Variables

Information extracted using a comprehensive chart and electronic health record review included demographic data, laboratory studies, microbiology results, diagnostic imaging, operative and pathology reports, dictation notes, discharge summaries, and medication profiles. Data extraction was conducted independently by two trained clinicians (GM and MFD). Data extracted included: age at diagnosis (A1  $\leq$  16 years, A2 17-40 years, A3  $>$  40 years); sex; location of disease (L1 ileal disease, L2 colonic disease, L3 ileocolonic disease and L4 upper gastrointestinal disease) by Montreal Classification<sup>[42]</sup> at time of prescribing thiopurine; disease behaviour by Montreal Classification<sup>[42]</sup> at thiopurine prescription (B1 inflammatory, B2 fibrostenotic, B3 penetrating disease); perianal fistulising disease at or prior to thiopurine prescription; smoking status at time of thiopurine prescription, which was classified as current smoking, past history of smoking, never smoker, or unknown; use of corticosteroid within 6 mo of diagnosis; dosage, age at onset, and cessation of 5-aminosalicylic acid (5-ASA); thiopurines (AZA and MP) and anti-TNF (infliximab and adalimumab); combination therapy; and intestinal resection before thiopurine prescription. Based on their thiopurine exposure, patients were categorized into three groups; (1) thiopurine monotherapy, defined as patients naive to anti-TNF therapy or suffering an adverse event that led to discontinuation of the thiopurine prior to exposure to anti-TNF therapy; (2) non-concomitant onset combination therapy defined as anti-TNF therapy started more than 3 mo after thiopurine onset; and (3) concomitant onset combination therapy defined as concomitant prescription of thiopurine and anti-TNF within 3 mo. In the last two groups, the adverse event occurred while the patient was being exposed to combination therapy.

### Statistical analysis

The primary outcome was cessation of the first thiopurine used (AZA or MP) due to an adverse event. An adverse event was defined as one or more of the following: acute pancreatitis; leucopenia; GI; hepatotoxicity;

infection; and hypersensitivity reactions<sup>[41]</sup>. The frequency distribution for categorical variables and median with interquartile range (IQR) for continuous variables were calculated, and their comparisons were based on the Fisher exact test and Wilcoxon rank-sum test, respectively. Logistic regression was performed to evaluate associations between clinical factors and discontinuing a thiopurine for an adverse event. Risk estimates were presented as OR with 95%CI. The clinical factors that were a priori included into the model included: disease phenotype at the onset of thiopurine as described by age, location, behavior (B), and perianal fistulising disease; intestinal resection prior to thiopurine prescription, history of smoking, corticosteroids within 6 mo of diagnosis, and drug utilization patterns prior to thiopurine prescription for 5-ASA and anti-TNF. An interaction term between age (defined as  $\leq$  or  $>$  40 years) and sex was modeled to assess for effect modification. All statistical analyses were conducted using SAS version 9.2 (SAS Institute, Inc, Cary, NC). *P* values  $<$  0.05 were considered to be statistically significant.

## RESULTS

The study consisted of 351 subjects with a median follow-up duration of 5.8 years (IQR 25<sup>th</sup>-75<sup>th</sup>, 2.7-9.1]. Two patients were first initiated on MP while 349 patients were first treated with AZA. Most ( $n = 234$ , 66.7%) patients received thiopurine monotherapy, with the rest were exposed to anti-TNF therapy. The median dose of AZA prescribed was 2.1 mg/kg (IQR 25<sup>th</sup>-75<sup>th</sup>, 1.9-2.4 mg/kg). The median dose of MP prescribed was 1.2 mg/kg (IQR, 25<sup>th</sup>-75<sup>th</sup> 0.9-1.3 mg/kg). Drug disposition of the whole study group is described in Figure 1. Patient characteristics stratified by adverse events are shown in Table 1.

### Adverse events

Adverse events leading to thiopurine discontinuation occurred in 110 patients out of the total cohort of 351 and were distributed as follows (Table 2): hypersensitivity reactions ( $n = 25$ , 7.1%); acute pancreatitis ( $n = 22$ , 6.2%); GI toxicity ( $n = 19$ , 5.4%); leucopenia ( $n = 13$ , 3.7%); hepatotoxicity ( $n = 12$ , 3.4%) and infection ( $n = 4$ , 1.1%). Fifteen patients (4.3%) stopped medication for other adverse events (Table 2). Multiple adverse events were not recorded in a single patient as first adverse event leading to therapy cessation was the primary end point of this study. The four infections described were two intra-abdominal abscesses, one case of molluscum contagiosum and one case of pulmonary coccidioidomycosis. These patients were not leukopenic. Details regarding the types of adverse events were missing in the medical charts of four patients (3.6%).

The median time from initiation to cessation of therapy for patients with a hypersensitivity reaction,

**Table 1 Cohort demographics *n* (%)**

Variables	Total ( <i>n</i> = 351)	Discontinued due to adverse event ( <i>n</i> = 110)	Continued therapy ( <i>n</i> = 241)	<i>P</i> value
Gender				0.36
Female	185 (52.7)	62 (56.3)	123 (51.1)	
Male	166 (47.3)	48 (43.6)	118 (48.9)	
Age at diagnosis (A) (yr)				0.05
< 17	56 (16.0)	13 (11.8)	43 (17.8)	
17-40	233 (66.4)	70 (63.3)	163 (67.6)	
> 40	62 (17.7)	27 (24.5)	35 (14.5)	
Age at thiopurine (yr)				0.007
< 17	21 (6.5)	5 (4.5)	16 (6.6)	
17-40	195 (60.0)	44 (40.0)	151 (62.7)	
> 40	109 (33.5)	43 (39.1)	66 (27.4)	
L4 (upper gastrointestinal disease)				0.52
Yes	27 (7.7)	10 (9.1)	17 (7.1)	
No	324 (92.3)	100 (90.1)	224 (92.9)	
Behaviour (B) (%)				0.91
B1 (inflammatory)	183 (56.0)	55 (50.0)	128 (53.1)	
B2 (fibrostenotic)	62 (19.0)	20 (18.2)	42 (17.4)	
B3 (penetrating)	82 (25.1)	26 (23.6)	56 (23.2)	
Perianal disease before thiopurine				0.69
Yes	82 (23.4)	24 (21.8)	58 (24.1)	
No	269 (76.6)	86 (78.2)	183 (75.9)	
Corticosteroid at diagnosis				0.35
Yes	142 (52.8)	39 (35.5)	103 (42.7)	
No	127 (47.2)	42 (38.2)	85 (35.3)	
Pre-thiopurine intestinal resection				0.81
Yes	140 (39.9)	45 (40.9)	95 (39.4)	
No	211 (60.1)	65 (59.1)	146 (60.6)	
Disease duration before thiopurines (yr)				0.22
< 1	100 (30.8)	22 (20.0)	78 (32.4)	
1-5	81 (24.9)	22 (20.0)	59 (24.5)	
5-10	63 (19.4)	23 (20.9)	40 (16.6)	
> 10	81 (24.9)	25 (22.7)	56 (23.2)	
5-ASA exposure before thiopurine	66 (24.8)	18 (16.4)	48 (19.9)	0.47
Anti-TNF $\alpha$ exposure <sup>1</sup>				< 0.0001
Thiopurine monotherapy	234 (66.7)	97 (88.2)	137 (56.9)	
Non-con. Comb. therapy	79 (22.5)	5 (4.5)	74 (30.7)	
Con. Comb therapy	38 (10.8)	8 (7.3)	30 (12.4)	
Smoking history				0.12
Never	180 (51.3)	48 (43.6)	132 (54.8)	
History of smoking	126 (35.9)	49 (44.5)	77 (32.0)	
Unknown	45 (12.8)	13 (11.8)	32 (13.2)	

<sup>1</sup>Thiopurine monotherapy, defined as patients naive to anti-TNF therapy or suffering an adverse event that led to discontinuation of the thiopurine prior to exposure to anti-TNF therapy; non-concomitant onset combination therapy defined as anti-TNF therapy started more than 3 mo after thiopurine onset; concomitant onset combination therapy defined as concomitant prescription of thiopurine and anti-TNF within 3 mo. Clinical characteristics of the 351 CD patients treated with a thiopurine stratified by an adverse event requiring withdrawal of thiopurines. Anti-TNF: Anti-tumour necrosis factor; ASA: Aminosalicylic acid; CD: Crohn's disease.

acute pancreatitis, and gastrointestinal intolerance were of 31.0 (IQR 29.0, 65.0), 29.0 (IQR 14.5, 30.0) and 17.0 (IQR 7.0, 26.0) d respectively. In contrast, leucopenia resulted in drug cessation after a median of 347.5 d (IQR 159.0, 866.0) ( $P < 0.0001$ ). Moreover, median AZA dosages were lower in patients who had to discontinue drug therapy due to leucopenia (1.6 mg/kg, IQR 0.8, 2.3) and hepatotoxicity (1.3 mg/kg, IQR 0.8, 2.0) ( $P = 0.04$ ).

### Clinical predictors of adverse events

Patients over the age of 40 when they started a thiopurine were more likely to discontinue the drug ( $P = 0.007$ ) as compared to patients under the age of 40 (Table 1). In the multivariate analysis, effect modification was identified by age at thiopurine prescription for sex ( $P < 0.05$ , Wald test). In patients over 40 years of age at thiopurine prescription, females had a 4.0-fold (adjusted OR = 4.0, 95%CI: 1.9-8.3) increased risk of discontinuing therapy due to an adverse event than females under the age of 40. In contrast, age did not influence thiopurine withdrawal (adjusted OR = 1.31, 95%CI: 0.59-2.9) in males (Table 3).

In the stratified multivariate analysis by sex, female gender with an age of over 40 at thiopurine prescription was associated with a significantly increased risk of thiopurine discontinuation due to an adverse event (adjusted OR = 2.8, 95%CI: 1.4-5.6). This risk was not seen in male patients (adjusted OR = 0.9, 95%CI: 0.4-2.1). The other modeled clinical factors (smoking history, pre-thiopurine intestinal resection, disease behaviour and location, perianal disease and previous corticosteroid or 5-ASA use) were not associated with thiopurine discontinuation secondary to an adverse event (Table 4).

## DISCUSSION

Thiopurine antimetabolite drugs are effective therapy in IBD. Thiopurines are commonly used first line immunosuppressive therapy in subjects with moderate-severe CD. Thiopurines are also prescribed in combination with anti-TNF therapy to optimize effectiveness<sup>[42]</sup>. However, widespread use of thiopurines is hampered by potential adverse events that can lead to drug cessation. Our study highlights that intolerance to thiopurines is prevalent; particularly, among women with CD who are over the age of 40. This study demonstrates real-life clinical practice that suggests that combination strategies with anti-TNF therapy and long-term thiopurine monotherapy are therapeutic aims that might not be easy to achieve for patients with CD.

Nearly two-thirds of patients discontinued thiopurine therapy after a median follow-up of 5.8 years. This finding was comparable to prior studies<sup>[15,43]</sup>. However, a prospective cohort study of 394 patients exposed to thiopurine therapy, reported a lower frequency of



**Table 2** Adverse events *n* (%)

Variables	Hypersensitivity	Pancreatitis	GI	Leucopenia	Hepatotoxicity	Infection
patients	25 (7.1)	22 (6.2)	19 (5.4)	13 (3.7)	12 (3.4)	4 (1.1)
Gender						
Males	16 (64.0)	9 (40.9)	7 (36.8)	4 (30.8)	4 (33.3)	2 (50.0)
Females	9 (36.0)	13 (59.1)	12 (63.2)	9 (69.2)	8 (66.7)	2 (50.0)
Age at thiopurine (yr)	37.6 (32.3, 47.9)	43.9 (32.2, 48.8)	26.5 (22.8, 47.1)	29.6 (23.6, 47.7)	49 (41.8, 57.9)	24.3 (19.3, 27.1)
Time from prescription to withdrawal of thiopurine (d)	31 (29.0, 65.0)	29 (14.5, 30.0)	17 (7.0, 26.0)	347.5 (159.0, 866.0)	51 (30.0, 70.0)	1907 (603.0, 2718.0)
AZA dose (mg/kg)	2.2 (1.6, 2.4)	2.3 (2.0, 2.4)	2 (1.7, 2.2)	1.6 (0.8, 2.3)	1.3 (0.8, 2.0)	2.1 (2.0, 3.1)

Main reasons leading to discontinuation of thiopurine therapy in 351 patients due to an adverse event. No multiple reasons were recorded. Data are given as median and IQR unless otherwise stated. Other (*n* = 15, 13.6%) causes for discontinuation were alopecia (*n* = 3), photosensitivity (*n* = 1), basal cell carcinoma (*n* = 2), mood disturbance (*n* = 1), syncopal episodes (*n* = 1), fatigue (*n* = 1), headache (*n* = 1), eye problems (*n* = 1) and unknown (*n* = 4). AZA: Azathioprine; IQR: Interquartile range; GI: Gastrointestinal intolerance.

**Table 3** Multivariate analysis

Adjust variables	Crude analysis	Adjusted analysis
<b>Total (<i>n</i> = 351)</b>		
<b>Age starting thiopurine older than 40 vs 40 or younger</b>	<b>OR (95%CI)</b>	
Female	3.6 (1.9-7.1)	4.0 (1.9-8.3)
Male	1.2 (0.6-2.6)	1.3 (0.6-3.0)

Predictors of thiopurine-related adverse events as determined by multivariate analysis. A significant age-by-gender interaction was observed in both crude and adjusted analyses, *P*-value = 0.03 and 0.04 respectively.

withdrawals (16%) from thiopurines due to adverse events<sup>[44]</sup>.

Leucopenia led to treatment cessation in 13 out of the 351 subjects with a median duration of treatment before discontinuation of 348 d. Thiopurine-induced myelotoxicity has a cumulative incidence of 7% with an incident rate of 3% per patient per year<sup>[43]</sup>. Our study was limited because of the lack of TPMT genotype and activity from our study population. However, TPMT polymorphisms explain leukopenia in only a quarter of cases<sup>[45]</sup>.

The mean overall prevalence of thiopurine-induced liver toxicity was 3.4% in our study, which was similar to the prevalence of 3.4% described in a systematic review including 3485 patients<sup>[46]</sup>. Only 4 infectious events were described in this cohort of 351 patients on thiopurines. Although this study was not designed to assess the risk of infectious adverse events in CD patients exposed to thiopurines, our data is in line with previous studies<sup>[47]</sup> and is similar to previous findings in the TREAT registry that did not identify and increased incidence of sepsis in subjects on immunomodulators<sup>[48]</sup>.

Pancreatitis is an idiosyncratic reaction. Pancreatitis was reported in 22 out of the 351 subjects (6.3%) which is slightly higher than the incident rate reported elsewhere (1.4%-3.3%)<sup>[49-51]</sup>. As expected from rarity of the reported incidence of lymphomatous adverse events in thiopurine-exposed subjects (0.9/1000 patient-years in the literature<sup>[52]</sup>), no cases were

reported in our cohort.

We have shown after adjusting for different factors that females over the age of 40 years are at a higher risk of adverse events secondary to thiopurines as compared to women prescribed the drug below the age of 40 years. In contrast, age did not modify this association among men. This is novel finding has a potential clinical implication, if further research validates this finding in other cohorts. The higher risk of toxicity in older women may alter the decision to prescribe a thiopurine when compared against a different treatment option. Disease phenotype, smoking history, surgery or previous corticosteroid or 5-ASA usage were not found to be significantly associated with adverse events. Similar findings have been previously described in a small IBD Spanish cohort<sup>[53]</sup>. These findings could possibly be explained by recent data showing a significantly lower TPMT activity in females as compared to males<sup>[29-31,54]</sup>.

We showed that patients exposed to an anti-TNF had significantly less episodes of drug cessation due to an adverse event. This variable was not included in our multivariate analysis as we did not feel this would be clinically useful. We would not advocate initiating anti-TNF therapy in CD patients to decrease the incidence of thiopurine discontinuation due to adverse events. Moreover, we feel that persistence with a thiopurine in patients on combination therapy is an indication of disease severity in an otherwise sick CD population with already a number of disease-related symptoms.

There are several strengths to this study. This is a large referral centre that follows a large cohort of CD patients. Fine phenotyping was conducted by a select group of trained physicians who performed random audits for quality assurance. Limitations to the study should be noted. This is a retrospective study that relied on a chart review process and thus, some clinical data were not comprehensively captured. Cessation of thiopurine therapy due to an adverse event was left at the discretion of the primary caring physician. As this was the primary inclusion criteria, this inherent variability might explain some of the noted differences in the adverse events incidence data.

Table 4 Stratified multivariate analysis

Adjusted variables	All cohort ( <i>n</i> = 351)	Adjusted OR (95%CI)	
		Female ( <i>n</i> = 185)	Male ( <i>n</i> = 166)
Age at Thiopurine prescription > 40 (sex-by-age interaction, <i>P</i> = 0.04)	2.4 (1.4-4.2)	2.8 (1.4-5.6)	0.9 (0.4-2.1)
Female <i>vs</i> male (referent)	1.2 (0.7-2.0)	NA	NA
Smoking history (current/past)	1.5 (0.9-2.5)	1.5 (0.8-3.1)	1.6 (0.8-3.6)
Pre-thiopurine intestinal resection <i>vs</i> no resection (referent)	0.86 (0.43-1.71)	0.7 (0.3-1.8)	1.0 (0.4-2.8)
L1 <i>vs</i> L2 (referent)	1.4 (0.6-3.2)	1.2 (0.4-3.6)	3.1 (0.9-10.4)
L3 <i>vs</i> L2 (referent)	1.5 (0.7-3.0)	1.1 (0.4-2.7)	2.1 (0.7-6.3)
B2 <i>vs</i> B1 (referent)	1 (0.5-2.2)	1.0 (0.3-2.8)	0.7 (0.2-2.4)
B3 <i>vs</i> B1 (referent)	1.1 (0.5-2.5)	1.0 (0.3-2.8)	0.8 (0.3-2.5)
Pre-thiopurine perianal disease <i>vs</i> no perianal disease (referent)	0.7 (0.4-1.3)	1.1 (0.5-2.7)	0.7 (0.3-1.8)
5-ASA at time of thiopurine <i>vs</i> past or never (referent)	0.8 (0.4-1.5)	0.7 (0.3-1.8)	1.0 (0.4-2.4)
Corticosteroid at diagnosis <i>vs</i> no exposure at diagnosis (referent)	0.9 (0.5-1.6)	0.7 (0.3-1.5)	0.9 (0.4-2.2)
L4 <i>vs</i> L2 (referent)	1.8 (0.7-4.3)	3.4 (0.9-12.2)	0.9 (0.3-3.1)

Predictors of thiopurine discontinuation in all cohort and as determined by this stratified multivariate analysis by sex. Demographic data suggests a higher incidence of adverse events in female patients over the age of 40 years. An age-by-gender interaction is seen as described in this multivariate analysis of thiopurine-exposed patients in female subjects over the age of 40 years. ASA: Aminosalicic acid; NA: Not available.

Patients were followed in a referral centre and thus our study population may have been skewed towards CD patients with a more complicated disease course. Also, data on TPMT genotype, activity and thiopurine metabolites were not available to explain these clinical findings in our study population. Finally, our results are exclusive to CD. We aimed to carry these analyses in a CD cohort in order to allow the multivariate analysis to identify a clinically useful phenotype in this population significantly related to a thiopurine adverse event. Entering other clinical parameters related to other inflammatory bowel diseases might have produced a more composite but less clinically meaningful outcome.

Thiopurines are effective therapy in certain CD phenotypes. Significantly more adverse events have been noted in female patients over the age of 40 years. Although our findings should not preclude this group from the thiopurine class of drugs, clinicians should be aware of the possible increased risk of toxicity in this patient cohort. Further work is needed to validate our findings in different patient populations and to try and explain the aetiology of this novel finding.

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## COMMENTS

### Background

Thiopurine medication is effective therapy in the management of inflammatory bowel disease. Their clinical effectiveness is hampered by the incidence of related adverse events leading to drug discontinuation. A large recent retrospective Spanish cohort study indicates that a quarter of patients suffer an adverse event when exposed to thiopurine therapy leading to discontinuation of therapy in 17%. Multiple variables have been associated with the onset of adverse events including age, gender, the type of inflammatory bowel disease,

co-administration with 5-aminosalicylic acid and thiopurine 6-methyltransferase activity (TPMT). Although a higher TPMT activity is noticed in infants and young children, this is unaffected by age in adulthood. Gender does seem to have an effect on thiopurine metabolism with some reports showing a disparity in TPMT activity in between gender with a lower TPMT activity being described in women. These findings are not universal with some reports finding no difference in thiopurine metabolism between males and females. Co-administration of thiopurine and 5-aminosalicylic acid therapy does increase the incidence of adverse events due to a negative effect of 5-aminosalicylic acid therapy on TPMT activity with a consequent rise of 6-thioguanine levels leading to adverse events. Despite these observations, measurements of TPMT activity and thiopurine metabolites are not routinely carried out in most healthcare systems. Most regions in Canada do not support these expensive tests. Similarly in the United Kingdom, despite TPMT measurement prior to therapy initiation is endorsed by the British Society of Gastroenterology, a substantial number of clinical commissioning groups do not financially support this test. Similar limitations are seen across other parts of the world. Moreover, in most cases adverse thiopurine-related adverse events are not explained by TPMT deficiencies. Identifying a clinical phenotype that could potentially predict adverse events to thiopurine in a real-life practice would be inexpensive and clinically useful.

### Research frontiers

Thiopurine-related adverse events are common. Some may be explained by TPMT deficiency, though in most cases (including myelosuppression), it is clinically impossible to predict which patients will be intolerant to this medication. The current research hotspot is to identify a clinical phenotype associated with increased adverse event. This would be clinically useful as it would inform decision-making when starting immunosuppressive therapy.

### Innovations and breakthroughs

To this date, the authors try and predict adverse events to thiopurine therapy by measuring TPMT activity prior to first prescription. Measuring thiopurine metabolites may be useful to try and optimise therapy and decrease adverse events. It is not clinically possible as yet to predict other adverse events. Furthermore, these expensive tests are not routinely available in large parts of the world. The authors hereby describe a clinical phenotype that significantly predicts an increased risk to discontinuation of thiopurine therapy due to an adverse event. This is a clinically useful finding that will improve decision-making when prescribing immunosuppressive therapy.

### Applications

Patients of a female gender prescribed a thiopurine at any age over 40 years are at an increased risk to discontinuation of therapy due to an adverse event.

### Terminology

TPMT is an enzyme involved in the breakdown of azathioprine/mercaptopurine to the active metabolite 6-thioguanine. A low TPMT activity is associated with increased levels of 6-thioguanine which enhances the clinical efficacy of any prescribed dose but also leads to a higher incidence of myelosuppression.

# Peer-review

This is a retrospective study on chart review dealing with adverse events among patients with Crohn's disease followed in the Alberta inflammatory bowel disease Consortium patient database. The manuscript reveals frequencies of a number of adverse events observed in a referral centre cohort including that of women older than 40 years have an increased risk for adverse events and discontinuation of thiopurine therapy.

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## Retrospective Study

# Gastric cancer in women: A regional health-center seven year retrospective study

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**Author contributions:** Suryawala K and Soliman D contributed equally as first authors; Becker F and Alexander J contributed equally as senior authors; Suryawala K and Soliman D collected the data and conducted most of the analysis; Mutyala M and Nageeb S contributed to the data analysis and interpretation; Boktor M, Seth A, Aravantagi A, Sheth A, Morris J, Jordan P and Manas K were attending physicians and contributed to the study design; Cvek U and Trutschl M performed the statistical analysis; Becker F and Alexander J planned and supervised the study, analyzed the data and wrote the manuscript; Suryawala K and Soliman D contributed equally as first authors, and Becker F and Alexander J contributed equally as senior authors.

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proposed activity is not research involving human subjects as defined by DHHS and FDA regulations.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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**Data sharing statement:** Combined demographic data, statistical codes and datasets are available upon request from the corresponding author at [jalexa@lsuhsc.edu](mailto:jalexa@lsuhsc.edu). Consent was not obtained from the study participants because the presented data are retrospective, de-identified, and anonymized; therefore, the risk of identification is low.

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## Abstract

**AIM:** To investigate whether regional geography influences ethnic and gender trends for the development of gastric cancer (GC).

**METHODS:** This retrospective analysis of the INVISION patient database at Louisiana State University Health Sciences Center-Shreveport (LSUHSC-S), a southern United States regional hospital, was performed from 2005-2011. Using the international statistical classification of diseases 9 (ICD-9), inpatient, day surgery outpatient, and emergency outpatient diagnosis codes entered into medical records were used to identify GC patients. For each study year, the patients were evaluated for age, ethnicity, and gender, and each patient was counted only once throughout the study. Subsequent patient encounters were counted as visits and separated by inpatient and clinic visits. Complex or severe disease may require more frequent and intensive clinical management; therefore, we evaluated annual clinic visits as "surrogate markers" of disease severity. Finally, we studied the primary diagnosis for *Helicobacter pylori* (*H. pylori*) infection (ICD-9 code 41.86) as an additional factor that might increase the risk of GC.

**RESULTS:** A total of 285 patients were diagnosed with GC at LSUHSC-S between 2005 and 2011. African Americans (181 patients, 89 males and 92 females, 63.5% of total patients) had significantly higher frequencies of GC diagnosis compared with non-Hispanic whites (104 patients, 54 males and 50 females, 36.5% of total patients), at a ratio of 1.74 ( $P = 0.002$ ). Within each ethnic group, men and women were diagnosed at approximately equal annual rates. Our findings differed significantly from United States national trends, which found that African American females and white females had lower risks for GC than their corresponding male counterparts. The United States national trend between 2005 and 2011 showed that African Americans males had a higher incidence of GC, with an annual mean (per 100000) of  $16.31 \pm 0.76$  compared with white males ( $9 \pm 0.1$ ,  $P < 0.001$ ), African American females ( $8.7 \pm 0.34$ ,  $P < 0.001$ ) and white females ( $4.05 \pm 0.07$ ,  $P < 0.001$ ). Among the GC patients, the number of clinic visits was highest among African American males ( $195.1 \pm 28.1$ ), who had significantly more clinic visits than African Americans females ( $123 \pm 13.02$ ,  $P < 0.05$ ), white males ( $41.57 \pm 4.74$ ,  $P < 0.001$ ) and white females ( $35 \pm 8.9$ ,  $P < 0.001$ ). Similar trends were found for inpatient visits, with an annual mean of  $11.43 \pm 1.5$  for

African American males, followed by African American females ( $7.29 \pm 1.36$ ), white males ( $2.57 \pm 0.69$ ) and white females ( $1.57 \pm 0.612$ ). African American males had significantly more inpatient visits than white males ( $P < 0.001$ ), and African American females had more inpatient visits than white females ( $P < 0.01$ ). African American patients showed the highest frequency of *H. pylori* positive status, with approximately 72% vs 28% for the white patients.

**CONCLUSION:** Increase in GC diagnoses among women at LSUHSC-S is significantly higher than United States national averages, suggesting local geographic and socioeconomic influences may alter GC disease course.

**Key words:** Gender; *Helicobacter pylori*; Ethnicity; Risk factors; Health disparities

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**Core tip:** Gastric cancer (GC) remains a leading cause of morbidity and mortality. Nationally, AAs reportedly develop GC at twice the rate of Caucasians. Male gender is a significant risk factor for GC development in the United States with a nearly 2:1 male to female dominance. However, at Louisiana State University Health Sciences Center-Shreveport, the annual rates of GC diagnosis among women in either ethnic grouping were statistically indistinguishable from that of their male counterparts. This result indicates that regional geography and socioeconomic factors may contribute to the ethnicity and gender differences observed in patients with GC. Therefore, additional GC surveillance for women, particularly African American females, may improve patient outcomes.

Suryawala K, Soliman D, Mutyala M, Nageeb S, Boktor M, Seth A, Aravantagi A, Sheth A, Morris J, Jordan P, Manas K, Cvek U, Trutschl M, Becker F, Alexander J. Gastric cancer in women: A regional health-center seven year retrospective study. *World J Gastroenterol* 2015; 21(25): 7805-7813 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7805.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7805>

## INTRODUCTION

Over the past 10 years, there have been significant declines in the incidence and mortality of gastric cancer (GC) in the United States, most likely due to earlier detection and treatment of *Helicobacter pylori* (*H. pylori*) infection, as well as decreased consumption of smoked foods<sup>[1,2]</sup>. However, GC remains a leading cause of morbidity and mortality worldwide; it is ranked as the fourth most common type of cancer and the second most common cause of cancer-related deaths globally<sup>[3,4]</sup>. The highest incidence rates of GC are now

reported in Japan and developing regions of China, the Middle East, Central America and South America<sup>[5]</sup>. Despite the observed decline in GC incidence in the United States, it is estimated that in 2014, there will be 22220 new cases of gastric malignancy, with 13730 (61.8%) of those cases in males and 8490 (38.2%) in females. The estimated number of total deaths among males and females will be 10990, with 6720 (61.1%) in males and 4270 (38.9%) in females<sup>[6]</sup>.

Numerous risk factors have been implicated in the development of GC, including *H. pylori* infection, obesity, smoking, diet, atrophic gastritis, ethnicity, gender and age<sup>[7-12]</sup>. Recent studies have indicated that male gender is the most significant risk factor for the development of gastric malignancy in the United States, with a nearly 2:1 male to female dominance<sup>[3,13]</sup>. Therefore, male gender appears to represent a major predictor of GC. Additionally, in a study of the survival rates of metastatic GC patients, Yang *et al.*<sup>[14]</sup> showed that male patients had lower survival rates compared to female patients.

In terms of ethnicity, African Americans (AAs) reportedly develop GC at twice the rate of Caucasians<sup>[14]</sup>. AAs also had 2 - 6 × greater seropositivity for eight *H. pylori* infection markers, including the cytotoxin-associated gene A (CagA) and Vacuolating cytotoxin A (VacA) virulence factors, suggesting greater symptomatology during *H. pylori* infection<sup>[15]</sup>. These observations show that ethnicity, gender, and *H. pylori* infection status may cooperate in increasing the risk of GC.

However, studies on ethnicity and gender influences on epidemiology can be confounded by uneven sampling, as well as geographic and socioeconomic factors. For example, in a previous study at LSUHSC-S on inflammatory bowel diseases (IBD), we reported that AAs were diagnosed with IBD at nearly equivalent annual rates as whites; these values exceeded national averages<sup>[16]</sup>. Because AAs compose 54.7% of the population in the city of Shreveport and 38.9% of the population in the Shreveport-Bossier Metropolitan area, compared to only 13.2% of the national population (as of 2013), studies on racial contributions to disease onset and progression at LSUHSC-S may reveal important trends that are not readily observed in large, homogeneous demographic studies<sup>[17,18]</sup>. Therefore, to better evaluate racial and gender influences on GC diagnoses in a patient population with an equivalent representation of AAs and whites, from 2005 to 2011, this study examined annual GC diagnoses as a function of gender, ethnicity, age and *H. pylori* infection status at LSUHSC-S, a tertiary care public health hospital. Lastly, clinic and inpatient were evaluated as possible "surrogate markers" of disease severity<sup>[18]</sup>.

We found that at LSUHSC-S, the annual GC diagnosis rates among women in either racial group (non-Hispanic Caucasians and African-American) were statistically indistinguishable from those of their male counterparts (in each respective ethnic group), unlike

United States national averages (*i.e.*, males in each ethnic group had a significantly higher incidence of GC). Therefore, regional geography and socioeconomic factors may contribute to the ethnicity and gender differences observed in GC, and additional GC surveillance for women, particularly African American females, may improve their outcomes.

## MATERIALS AND METHODS

This retrospective analysis included de-identified patients in the INVISION database at LSUHSC-S from 2005 to 2011. Inpatient, day-surgery outpatient, and emergency outpatient diagnostic codes were extracted from medical records, and all codes represented conclusive diagnoses. Individuals with a primary diagnosis of GC were selected using the international statistical classification of diseases 9 (ICD-9) code 151.0-151.9, encoding for malignant neoplasm of the stomach at an unspecified site. *H. pylori* infected individuals were identified using the ICD-9 code 41.86 [primary diagnosis code for *H. pylori* infection, diagnosed by tissue biopsies with subsequent testing for Campylobacter-like organism (CLO), immunohistochemical detection, or urea breath, stool, urine, saliva or serum antigen testing]. For each study year, de-identified patient ages, ethnicity, and gender were evaluated, and each patient was counted once in the study. Subsequent individual de-identified patient encounters were counted as visits and were further separated by hospital visits and clinic visits. This study identified 285 patients (Table 1) and evaluated the following factors: (1) the number of new GC cases diagnosed in each ethnic [AAs and non-Hispanic Whites (Ws)] and gender [female (F)] and male (M)] grouping each year; (2) the number of annual clinic visits per group; (3) the number of annual hospitalizations per group; and (4) the annual numbers of *H. pylori* diagnoses for each group. Patient gender and ethnicity were self-identified. According to the United State Census Bureau report from 2010, Hispanics account for 4.7% of the total Louisianan population, however, they account for only for 2.5% of the total Shreveport population (vs 41.2% Ws, 54.7% AAs and 1.3% Asians in Shreveport). From the 3 million patients investigated at LSUHSC-S over the seven-year study period, only 2.5% were races other than AAs and Ws, which allowed us to identify only 3 Hispanic patients who matched our previously described inclusion criteria for GC (see above). Therefore, we excluded all 3 Hispanic patients diagnosed with gastric cancer from our study protocol because it was not possible to accurately compare them to Ws and AAs.

Statistical group analysis (see biostatistics statement) comparisons were performed using Instat TM software GraphPad 3.06 (GraphPad Software Inc., La Jolla, CA, United States). All comparisons were performed using one-way ANOVAs, with Tukey-Kramer Multiple comparisons test or Student-Newman-



**Table 1** A total of 285 patients were diagnosed with gastric cancer between 2005 and 2011

Year/group	WMs	WFs	AAMs	AAFs	Total
2005	5	10	10	16	41
2006	5	11	12	10	37
2007	5	3	9	10	27
2008	9	7	13	13	42
2009	8	8	19	12	47
2010	15	4	14	15	48
2011	7	7	12	16	42
Total	54	50	89	92	284

Patient numbers are presented per year and grouped by sex and ethnicity. WMs: Non-Hispanic white males; WFs: Non-Hispanic white females; AAMs: African American males; AAFs: African American females.

Keuls multiple comparisons test (*H. pylori* diagnosis). Comparisons were considered statistically significant at  $P < 0.05$ . All statistical data are expressed as the mean  $\pm$  SE.

### Statistical analysis

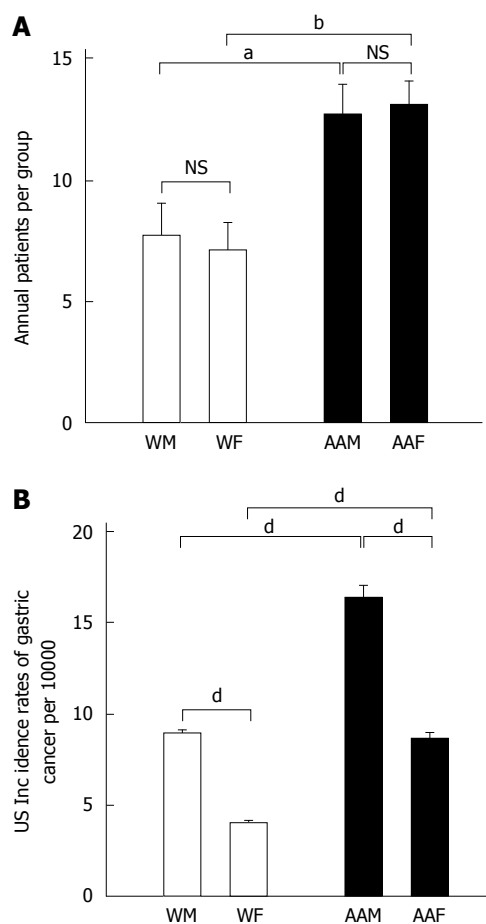
The statistical methods of this study were reviewed by Urska Cvek, Sc.D., MBA, Professor of Computer Science, Director, Laboratory for Advanced Biomed. Inf. and Marjan Trutschl, Sc.D., Abe Sadoff Distinguished Chair in Bioinformatics, Director, Laboratory for Advanced Biomed. Inf. Professor of Computer Science.

## RESULTS

Patient data were collected to calculate the annual number of GC cases diagnosed in different population groups. During the study period (2005 to 2011), we identified 285 patients who were diagnosed with GC at LSUHSC-S. We found a total of 181 AA (63.5% of the total number of patients, 89 males and 92 females) and 104 W (36.5% of total patients, 54 males and 50 females) patients who were newly diagnosed with GC. While there was a significant difference between AA and W ethnicities in terms of the annual number of individuals diagnosed with GC, gender did not influence the frequency of GC diagnoses within the racial groups. That is, women in both racial groups were diagnosed at approximately equal proportions as their male counterparts.

### Local trends in GC-influence of ethnicity and gender

As a group, AAs had significantly more annual diagnoses of GC than whites ( $P < 0.0002$ ), a ratio of 1:1.74 (Ws:AAs). AAMs and African American females (AAFs) at LSUHSC-S had nearly equal annual proportions of GC (Figure 1A). AAFs had  $13.14 \pm 0.99$  annual GC diagnoses, which was not significantly different from AAMs, who had  $12.71 \pm 1.23$  annual diagnoses. AAFs had significantly higher annual rates of GC diagnoses than either WMs ( $7.71 \pm 1.39$ ,  $P < 0.05$ ) or WFs ( $7.14 \pm 1.1$ ,  $P < 0.01$ ), who developed GC at equivalent rates.



**Figure 1** Gastric cancer at Louisiana State University Health Sciences Center-Shreveport: Influence of ethnicity and gender. A: The average individual number of patients (per group) treated annually for gastric cancer is highest in AAs. There were significantly more AAFs than WFs ( $^bP < 0.01$ ), and AAMs had more annual gastric cancer diagnoses than WMs ( $^bP < 0.05$ ). The number of WMs was not significantly different from that of WFs; and the number of AAMs was not significantly different from that of AAFs; B: National trends in gastric cancer - Influence of ethnicity and gender. AAMs had more annual gastric cancer diagnoses than AAFs ( $^dP < 0.001$ ) and WMs ( $^dP < 0.001$ ). AAFs had more annual gastric cancer diagnoses than WFs ( $^dP < 0.001$ ). WMs had more annual gastric cancer diagnoses than WFs ( $^dP < 0.001$ ). WMs: Non-Hispanic white males; WFs: Non-Hispanic white females; AAMs: African American males; AAFs: African American females; NS: Not significant.

### National trends in GC-influence of ethnicity and gender

We used the United States national trends for GC incidence from 2005 to 2011, as shown in (Figure 1B), as a scale for our results, which reflect diagnoses per 100000 individuals. The national GC incidence (2005 to 2011) was greater among AAMs (mean of  $16.3 \pm 0.76$ ) than AAFs ( $8.67 \pm 0.34$ ,  $P < 0.001$ ), WMs ( $9 \pm 0.1$ ,  $P < 0.001$ ) and WFs ( $4.05 \pm 0.07$ ,  $P < 0.001$ ). Furthermore, WMs had a higher GC incidence than WFs ( $P < 0.001$ ). AAFs also showed a statistically significant higher proportion of GC diagnoses than WFs ( $P < 0.001$ ).

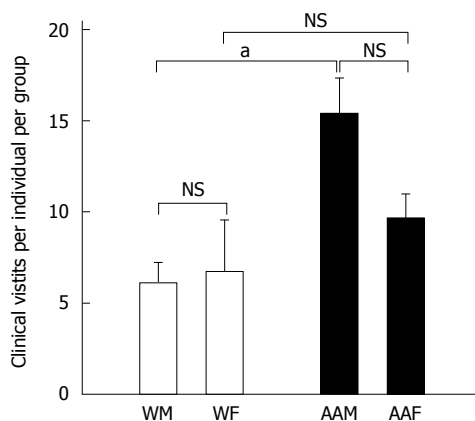
### Clinic visits as "surrogate markers" of disease activity

There were 2763 total clinic visits at LSUHSC-S for patients who received a primary GC diagnosis from 2005 to 2011. There were 181 AA patients, repre-

**Table 2** A total of 2763 clinic visits for patients with the primary diagnosis of gastric cancer were identified between 2005 and 2011

Year/group	WMs	WFs	AAMs	AAFs	Total
2005	53	10	60	108	231
2006	48	23	222	148	441
2007	22	67	181	106	376
2008	29	66	201	78	374
2009	51	41	298	148	538
2010	52	17	162	98	329
2011	36	21	242	175	474
Total	291	245	1366	861	2763

The patient numbers are presented per year and grouped by sex and ethnicity. WMs: Non-Hispanic white males; WFs: Non-Hispanic white females; AAMs: African American males; AAFs: African American females.

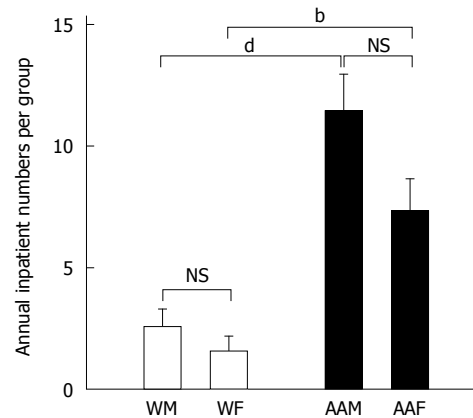


**Figure 2** Annual gastric cancer patients' clinic visits per person. AAMs had a higher annual average of clinic visits than WMs ( $^aP < 0.05$ ). The number of AAMs was not significantly different from that of AAFs, and the number of WFs was not significantly different from that of WMs. WMs: Non-Hispanic white males; AAMs: African American males; AAFs: African American females; NS: Not significant.

senting 63.5% of the total number of patients studied, while 104 patients were Ws, representing the remaining 36.5% (Table 2). AAMs had the greatest number of clinic visits (annual mean of  $195.1 \pm 28.1$ ), and they had significantly more clinic visits than AAFs ( $123 \pm 13.02$ ,  $P < 0.05$ ), WMs ( $41.57 \pm 4.74$ ,  $P < 0.001$ ) and WFs ( $35 \pm 8.9$ ,  $P < 0.001$ ), corresponding to an average of  $394.7 \pm 38.2$  visits annually. AAMs had a significantly greater number of annual clinic visits ( $15.36 \pm 1.94$ ) than WMs ( $6.12 \pm 1.11$ ,  $P < 0.05$ ) or WFs ( $6.75 \pm 2.8$ ,  $P < 0.05$ ) (Figure 2). Although not significant, AAMs also had more annual clinic visits per individual than AAFs ( $9.71 \pm 1.27$ ). Because AAMs had the highest number of annual clinic visits as a group (approximately 2.54  $\times$  more visits than WMs, 2.27  $\times$  more visits than WFs and 1.58  $\times$  more visits than AAFs), they may either seek or require more medical attention to manage their disease.

#### Inpatient visits as "surrogate markers" of disease activity

Similar trends were also found when examining in-

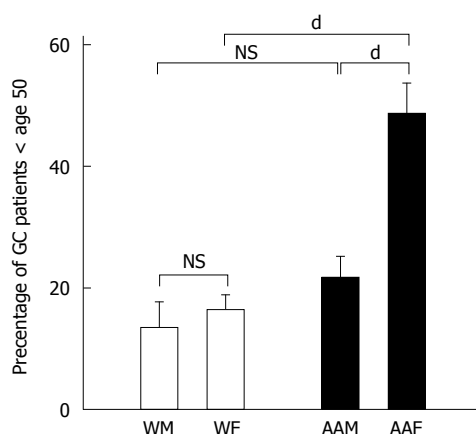


**Figure 3** Annual gastric cancer inpatient visits per patient visits. AAMs had more annual inpatient visits than WMs ( $^dP < 0.001$ ). AAFs had more annual inpatient visits than WFs ( $^bP < 0.01$ ). The number of AAM visits was not significantly different from the number of AAF visits; and the number of WF visits was not significantly different from the number of WM visits. WFs: Non-Hispanic white females; AAMs: African American males; AAFs: African American females.

patient visits for patients with a primary diagnosis of GC at LSUHSC-S between 2005 and 2011 (Figure 3). AAMs again had the highest number of annual inpatient visits ( $11.43 \pm 1.5$  visits per year), followed by AAFs, who had  $7.29 \pm 1.36$  visits per year. WMs averaged  $2.57 \pm 0.69$  visits per year, and WFs averaged  $1.57 \pm 0.612$  visits per year. AAMs were again noted to have statistically more inpatient visits compared to WMs and WFs (both  $P < 0.001$ ), however, there was no statistical significance between AAMs and AAFs. These findings in AAMs may indicate a more complex course of management that requires more frequent hospitalization.

#### Age at diagnosis in different ethnic groups at LSUHSC-S

In addition to evaluating the influence of ethnicity and gender on GC, we also studied the influence of age on the frequency with which GC is diagnosed. We divided GC patients into individuals younger than 50 years of age (Figure 4) and individuals 50 years of age or older at the time of diagnosis. Although the average age of diagnosis for gastric cancer is 69, we divided the age groups at 50 years because of variations in trends that were observed. During the 7-year study period, among the 285 patients studied, 71 patients were younger than 50 years of age, and 214 patients were 50 years of age or older. The annual mean percentages of patients younger than 50 years of age diagnosed with GC were  $48.46\% \pm 5.2\%$  for AAFs,  $21.6\% \pm 3.6\%$  for AAMs,  $16.44\% \pm 2.5\%$  for WFs and  $13.48\% \pm 4.2\%$  for WMs. For the patients younger than 50 years of age, AAFs had significantly more GC diagnoses than AAMs ( $P < 0.001$ ), WFs ( $P < 0.001$ ) and WMs ( $P < 0.001$ ). Our data revealed that AAFs showed an apparent earlier onset of GC compared to other groups. In contrast, the annual mean percentages for GC patients (primarily diagnosed at 50 years of age



**Figure 4 Gastric cancer patients younger than 50 years of age.** AAFs had more gastric cancer diagnoses than AAMs ( $^dP < 0.001$ ), WFs ( $^dP < 0.001$ ) and WMs ( $^dP < 0.001$ ). AAMs: African American males; AAFs: African American females; GC: Gastric cancer.

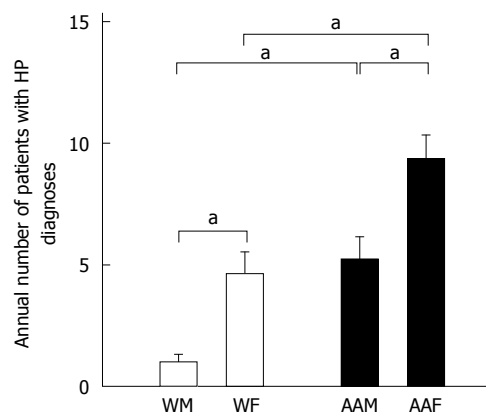
and older) were comparable between genders within each ethnic group, with  $35.1\% \pm 3.1\%$  for AAMs,  $27.35\% \pm 2.2\%$  for AAFs,  $17.8\% \pm 3.34\%$  for WFs and  $19.8\% \pm 3.5\%$  for WMs. AAMs had significantly more GC diagnoses than both WMs ( $P < 0.01$ ) and WFs ( $P < 0.01$ ).

#### *H. pylori* infection as a “surrogate marker” for GC risk

We also used the primary diagnosis of *H. pylori* infection as a “surrogate marker” of additional risk for GC (Figure 5). Strikingly, AAFs had significantly more *H. pylori* infection primary diagnoses (65) compared to other groups (36 for AAMs, 32 for WFs, and 7 for WMs). AAFs had more annual *H. pylori* infection diagnoses ( $9.29 \pm 1$ ) than both AAMs ( $5.14 \pm 1.01$ ,  $P < 0.05$ ) and WFs ( $4.6 \pm 0.9$ ,  $P < 0.05$ ). WMs had only  $1 \pm 0.31$  *H. pylori* infections diagnoses annually, which were significantly fewer, compared to AAMs ( $P < 0.05$ ). Therefore, because *H. pylori* infection is known to be a risk factor for GC, it is possible that AAFs have some increased risk because of their higher proportion of *H. pylori* infection diagnoses compared with other groups. It is unclear whether the relatively higher proportion of *H. pylori* diagnoses in WFs relative to WMs may help to explain the higher apparent increased risk of GC diagnosis in WFs in our study.

## DISCUSSION

In this retrospective study of GC patients at LSUHSC-S, there was a significant difference in the number of GC diagnoses between African American and white patients. Accordingly Horner *et al.*<sup>[19]</sup> reported that in the United States, AAs develop GC at approximately twice the rate of white population. Previous studies have identified numerous risk factors, which might be involved in the increased incidence of gastric malignancy in AA populations, including heritable risk, as well as socioeconomic status, dietary and smoking



**Figure 5 Annual *Helicobacter pylori* infection diagnoses.** AAFs had more annual *H. pylori* infection diagnoses than either AAMs ( $^aP < 0.05$ ) or WFs ( $^aP < 0.05$ ). AAMs had more annual *H. pylori* diagnoses than WMs ( $^aP < 0.05$ ). WFs had more annual *H. pylori* diagnoses than WMs ( $^aP < 0.05$ ). *Helicobacter pylori*: *H. pylori*.

habits, access to health care, local environmental and geographic influences, and regional *H. pylori* infection epidemiology<sup>[20,21]</sup>. Our results parallel reported United States national trends in terms of the overall influence of ethnicity, and they appear to be consistent with AA ancestry as a significant risk for the development of GC. However, when ethnicity is considered as a risk factor for the development of GC, it is absolutely important and necessary to state that the observed trends in AAs and Ws are more likely to reflect socioeconomic disproportions (imbalances in work, wealth, income, education, housing and standard of living) rather than biological differences associated with the respective ethnicity. Although recent evidence in the state of Louisiana has linked AA related polymorphisms in the IL-1b gene (particularly the IL1B+3954T allele) with pre-cancerous atrophic gastritis and advanced gastric premalignant lesions, their roles in the epidemiology of GC are only now emerging, and they may still not fully explain the substantial differences in incidences among different ethnic groups within our region compared to United States national trends<sup>[22,23]</sup>. In comparison, socioeconomic factors are well established in the development of GC and are extensively considered in the following discussion. As described above, additional factors that are believed to be more relevant than the respective ethnic group are barriers to sufficient cancer prevention, early detection, and treatment services, all of which are most likely to significantly impact the reported data in our study.

In the United States, AAs still have significantly lower incomes compared to whites, and socioeconomic disparities remain an important potential factor contributing to their higher proportions of observed GC diagnoses<sup>[24]</sup>. Low socioeconomic status may also represent an important obstacle towards seeking medical care for AAs. The affordable CARE ACT of 2014 may improve public access to healthcare in the future. However, our study is less likely to be influenced

by this bias, as LSUHSC-S provides equal access to healthcare regardless of the ability to pay.

A meta-analysis by Wang *et al.*<sup>[2]</sup> showed that *H. pylori* infection is strongly associated with early GC. Although, the prevalence of *H. pylori* infection in the United States is approximately 30%, AA populations have a higher prevalence that approaches 50%-60%<sup>[25]</sup>. Interestingly, AAFs accounted for 48% of the total *H. pylori* infection-diagnosed patients compared to 24% for AAMs, 23% for WFs and 5% for WMs. AAFs had statistically higher proportions of *H. pylori* infection diagnoses than AAMs ( $P < 0.05$ ) or WFs ( $P < 0.05$ ). Similarly, Graham and Malaty found that the prevalence of *H. pylori* infection was higher in AAs (70%) compared to Ws (34%)<sup>[21]</sup>. This percentage did not change after adjusting the data for gender, and it remained closely correlated with low socioeconomic standards<sup>[20]</sup>. Similarly, in our study, AAs represented  $69.6\% \pm 6.1\%$  of the total *H. pylori* infected individuals compared to  $26.3\% \pm 6.1\%$  for whites. Between 2005 and 2011, AAFs had the highest proportion of *H. pylori* infections annually. WFs had a higher number of *H. pylori* infections diagnoses than WMs, although this finding was not statistically significant. This may help to explain the high number of GC diagnoses observed in WFs in our area, which is significantly higher than the United States national average.

In our study, females (of both ethnicities) appear to have almost equal diagnoses as their corresponding male counterparts and, thus, have higher risks for stomach cancer than what was expected based on the United States national average. Importantly, the numbers of AAFs with GC in our study appears to occur at approximately twice the proportion of the national average. Furthermore, WFs in our study also showed a higher proportion of GC diagnoses than what would be anticipated from the reported United States national averages. Several possible reasons might contribute to these findings. At LSUHSC-S, AAFs were found to have the highest proportion of GC in our study, nearly the same as AAMs. AAFs had the highest mean percentage of GC ( $32.6\% \pm 2.1\%$ ), followed by AAMs ( $31.2\% \pm 1.9\%$ ), WMs ( $18.6\% \pm 2.4\%$ ) and WFs ( $17.6\% \pm 2.7\%$ ). By comparison, AAFs nationally had a lower GC incidence compared to AAMs, and the higher proportion of male cases was also found in whites. In 2009, in a national examination of new GC cases (per 100000 in the United States), AAMs and AAFs had incidence rates of 15.1 and 8.8, respectively, compared to 8.7 and 3.7 for WMs and WFs, respectively<sup>[19]</sup>. The United States national incidence of GC in AAFs was noted to be approximately half of that in AAMs. In our study, however, AAFs were found to have strikingly higher proportions, nearly double that which was anticipated from national averages and nearly equal to that of AAM. AAFs may be at higher risk of having more advanced GC and having poor prognoses at the time of diagnosis due to poor surveillance. Early onset GC in AAFs in our

region could reflect an amalgamation of several risk factors. Combined with other factors, including lack of medical care access and higher incidence of *H. pylori* infections, AAFs may represent the group with the highest combined risks. The high proportion of AAFs diagnosed with *H. pylori* infections might be related to the apparent early onset of GC in AAFs observed in our study. WFs in our study also showed a higher risk for GC than that which would be anticipated based on United States national averages, but AAFs had the highest overall proportions, which could reflect lower socioeconomic, educational, occupational and medical care access for women of both ethnicities in this lower income southern United States region.

Because of the high proportions of AAs (58.4%) and women (61.8%) in our study, our data may have more accurate samplings of these groups, and our data support regional differences in the GC risk for women. In addition, because our study involved the population in North Louisiana, we were able to determine different trends in AAs and Ws, thereby eliminating the common problem of an unequal distribution of these ethnic groups within mixed populations. This biasing factor might lead to underestimated risk stratifications within diverse and uneven distributed ethnic societies. Thus, our regional study demonstrates gastric cancer trends within a nearly equally distributed population of African Americans and Whites and could lead to more specific risk stratification for even small ethnic groups in global populations.

It is unclear exactly what accounts for the higher proportions of GC among women of both ethnicities at LSUHSC-S. Relatively lower socioeconomic status, geographic location as well as high rates of *H. pylori*, and possibly high body mass index may influence the development of GC, but these factors cannot fully explain the observed increased incidence of GC in females of both ethnicities.

In conclusion, Over the past three decades, there has been a significant decline in both the incidence and mortality of GC within the United States, where the incidence rates decreased by 1.7% and 0.8% for men and women, respectively, between 1992 and 2010<sup>[26,27]</sup>. In this study, however, our data suggest that women in the Northwest Louisiana metropolitan/Shreveport area may be at significantly higher risk, as demonstrated by the increased diagnoses of GC among women of both ethnicities, as well as in the AA populations, compared to United States national averages. The apparent earlier onset of GC seen in AAFs should also be studied more extensively.

Although male gender is a well-established risk factor for GC (and demonstrated in United States national studies), in our region, women have higher overall rates of GC, equal to those of men. While there is a high frequency of *H. pylori* infections in AAFs at our institution, which might help to explain the equivalent numbers of annual male and female GC patients in our AA population, *H. pylori* infection is (comparatively)



less frequently observed in whites. Therefore, the factors responsible for the increased GC proportion observed in WFs remain unclear.

Our study population evaluated patients in North Louisiana; therefore, we were able to determine different trends in AAs and Ws, thereby eliminating the common problem of an unequal distribution of these ethnic groups within mixed populations. This biasing factor might lead to underestimated risk stratifications within diverse and uneven distributed ethnical societies. Thus, our regional study, demonstrates gastric cancer trends within a nearly equally distributed African American and White population, and it could lead to more specific risk stratification of even small ethnic groups in global populations.

Thus, it is imperative that greater efforts and resources be directed towards improving the healthcare of those individuals at higher risk, including earlier and more advanced screening, smoking education, diet, and weight control, successful eradication of *H. pylori* infections and availability of medical care. These risk factors are known to confer risk for GC. Guidelines for screening "at risk" groups should be changed to include younger individuals, particularly AAFs. More in depth studies should be performed to investigate interactions between the synergistic risk factors that lead to these findings.

## COMMENTS

### Background

Although no longer the leading cause of worldwide cancer deaths, gastric cancer (GC) remains one of the most frequently diagnosed malignancies, with more than 20000 annual diagnoses in the United States. Because gastric cancer can be influenced by diverse socioeconomic, ethnic background and lifestyle choices, the authors investigated whether regional geographic sampling at a free-care hospital serving primarily economically disadvantaged patients might differ from nationally reported GC statistics.

### Research frontiers

The current research hotspot indicates that regional demographics can differ substantially from cumulative national data, and note that assumptions about GC incidence and risk may indicate the need to consider how different geographic locations affect risk stratification.

### Innovations and breakthroughs

Previous studies of GC risk factors in the United States have suggested increased diagnoses among males and in white (Caucasian) ethnic groupings. Based on cumulative national studies, increased surveillance in these groups might marginalize evaluations in females and in other ethnic groupings, leading to delays in diagnosis and treatment, particularly when regional risks deviate from nationwide findings.

### Applications

This study showed that data collected over seven years at a southern US regional hospital failed to confirm that male gender and white ethnic groups were at greater risk for GC. Our data suggest that African Americans and females may benefit from at least equal surveillance; the current study found equal risk among genders and ethnic divisions.

### Terminology

In this study, ethnic groupings are self-identified affiliations with either "white" (Caucasian), which is defined as persons of European, North African, or southwest Asian ancestry or "African American", which is defined as United States residents with total or partial ancestry deriving from the native populations of Sub-Saharan Africa.

## Peer-review

In this descriptive/comparative health disparity study, the authors analyzed GC diagnoses (in terms of gender and ethnicity) at a southern regional hospital. The results are interesting and suggest that regional studies may reveal important health care risks that can guide surveillance policies for GC.

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## Retrospective Study

# miR-122 negatively correlates with liver fibrosis as detected by histology and FibroScan

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**Author contributions:** Halász T and Schaff Z designed the study; Halász T, Horváth G, Pár G, Werling K, Kiss A and Schaff Z chose, diagnosed, or examined the patients and the histologic samples; Lendvai G performed the data acquisition; Halász T, Kiss A, Schaff Z and Lendvai G analyzed and interpreted the data and drafted the paper; all authors critically reviewed and approved the manuscript.

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**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrolment.

**Conflict-of-interest statement:** The authors have nothing to declare.

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## Abstract

**AIM:** To investigate whether expression of selected miRNAs obtained from fibrotic liver biopsies correlate with fibrosis stage.

**METHODS:** Altogether, 52 patients were enrolled in the study representing various etiologic backgrounds of fibrosis: 24 cases with chronic hepatitis infections (types B, C), 19 with autoimmune liver diseases (autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, overlapping syndrome cases), and 9 of mixed etiology (alcoholic and nonalcoholic steatosis, cryptogenic cases). Severity of fibrosis was determined by both histologic staging using the METAVIR scoring system and noninvasive transient elastography. Following RNA

isolation, expression levels of miR-21, miR-122, miR-214, miR-221, miR-222, and miR-224 were determined using TaqMan MicroRNA Assays applying miR-140 as the reference. Selection of miRNAs was based on their characteristic up- or downregulation observed in hepatocellular carcinoma. Relative expression of miRNAs was correlated with fibrosis stage and liver stiffness (LS) value measured by transient elastography, as well as with serum alanine aminotransferase (ALT) level.

**RESULTS:** The expression of individual miRNAs showed deregulated patterns in stages F1-F4 as compared with stage F0, but only the reduced level of miR-122 in stage F4 was statistically significant ( $P < 0.04$ ). When analyzing miRNA expression in relation to fibrosis, levels of miR-122 and miR-221 showed negative correlations with fibrosis stage, and miR-122 was found to correlate negatively and miR-224 positively with LS values (all  $P < 0.05$ ). ALT levels displayed a positive correlation with miR-21 ( $P < 0.04$ ). Negative correlations were observed in the fibrosis samples of mixed etiology between miR-122 and fibrosis stage and LS values ( $P < 0.05$ ), and in the samples of chronic viral hepatitis, between miR-221 and fibrosis stage ( $P < 0.01$ ), whereas miR-21 showed positive correlation with ALT values in the samples of autoimmune liver diseases ( $P < 0.03$ ). The results also revealed a strong correlation between fibrosis stage and LS values ( $P < 0.01$ ) when etiology of fibrosis was not taken into account.

**CONCLUSION:** Reduced expression of miR-122 in advanced fibrosis and its correlation with fibrosis stage and LS values seem to be characteristic of hepatic fibrosis of various etiologies.

**Key words:** Expression; FibroScan; Liver fibrosis; METAVIR; microRNA; miR-122

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**Core tip:** In this study, the expression of selected miRNAs was determined in fibrotic liver tissues of various etiologic backgrounds and was correlated with fibrosis stage (METAVIR scores) and liver stiffness as measured by transient elastography. In advanced fibrosis, the level of miR-122 was reduced and showed negative correlations with fibrosis stage and liver stiffness values, indicating that it could be a useful molecule to assess severity of fibrosis regardless of etiology.

Halász T, Horváth G, Pár G, Werling K, Kiss A, Schaff Z, Lendvai G. miR-122 negatively correlates with liver fibrosis as detected by histology and FibroScan. *World J Gastroenterol* 2015; 21(25): 7814-7823 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7814.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7814>

## INTRODUCTION

Hepatic fibrosis develops as a wound-healing response of the liver to cellular injury, reflecting the balance between liver repair and scar formation<sup>[1]</sup>. Upon injury, the activated hepatic stellate cells (HSC) undergo transition into proliferative, profibrogenic, contractile myofibroblasts, which are responsible for the excess deposition of the extracellular matrix (ECM)<sup>[2,3]</sup>. The eventual structural abnormalities, which result from the histologic rearrangement of various types of collagens, proteoglycans, and structural glycoproteins and the excess deposition of ECM<sup>[2,4-7]</sup> cause increased liver stiffness (LS). When injury persists, fibrosis may advance into cirrhosis-the most severe stage, which may then further progress into hepatocellular carcinoma (HCC)<sup>[1,2,8]</sup>. Liver fibrosis is caused by diverse etiologies that include alcoholic and nonalcoholic steatohepatitis, chronic viral hepatitis, autoimmune disorders, and toxins<sup>[1,4,9]</sup>.

Liver biopsy is the gold standard method for the identification of hepatic fibrosis. As this procedure is invasive, painful, and carries the risk of complications<sup>[10-12]</sup>, other alternatives have been developed, such as noninvasive transient elastography (TE), serum-based aspartate aminotransferase-to-platelet ratio index, and Fibrotest<sup>[13,14]</sup>. TE is an ultrasound-based examination method that measures the fibrosis-related rigidity of the liver tissue, with the velocity of the shear wave being directly related to LS, expressed as the LS value<sup>[15]</sup>. Using this method, the progress of fibrosis and early asymptomatic cirrhosis can be assessed with high sensitivity and specificity. However, not all information needed for the diagnosis of fibrosis can be obtained from TE examination, such as the histologic liver conditions, including necroinflammation.

MicroRNAs (miRNA) are short regulating RNA molecules that interfere with gene expression at the posttranscriptional level by way of inducing translational arrest, which in turn leads to reduced or prevented protein synthesis<sup>[3]</sup>. As a result of this negative modulating function, miRNAs fine-tune the expression of genes involved predominantly in normal cellular processes, such as development, differentiation, and proliferation<sup>[16]</sup>. Deregulated miRNA expression in comparison to normal state has been found in many disorders, including liver diseases<sup>[17,18]</sup>. In hepatic fibrosis, the members of the miR-27, miR-29, and miR-19 families have been reported to show altered expression<sup>[5,7,8,19]</sup>. These miRNAs either hinder the expression of various ECM components (miR-29) or regulate the signal transduction pathways connected to fibrosis (miR-29)<sup>[6,8,20]</sup> or the resting state of HSCs (miR-27)<sup>[21]</sup>.

It has been suggested that an imbalance in the normal miRNA pattern can be measured long before the onset of a disease<sup>[22]</sup>. Therefore, in the present



**Table 1** Clinicopathologic data of patients with liver fibrosis of various etiologies

Etiology	No. of cases	Etiology subgroups (n)	Fibrosis stage <sup>2</sup> (n)	LS level (kPa)	ALT level (U/L)	HAI
Autoimmune	19	AIH (8) <sup>1</sup>	F0 (1) <sup>1</sup>	6.1	452	5
		PBC (6)	F1 (3)	5.3-7.6	20-904	0-4
		PSC (2)	F2 (4)	5.1-8.8	20-368	0-6
		AIH/PBC (2)	F3 (9)	5.5-17.1	17-558	0-12
		AIH/PSC (1)	F4 (2)	20.6 - 45.7	26-83	0-4
Chronic viral	24	HCV (22)	F0 (3)	4.6-5.3	14-125	3-6
		HBV (2)	F1 (4)	3.8-6.8	12-35	2-6
			F2 (4)	5.4-7.6	20-88	3-4
			F3 (11)	5.6-20.4	12-257	0-8
			F4 (2)	18.0-26.3	60-108	0-10
Mixed etiology	9	ALD (1)	F0 (1)	4.9	71	0
		NAFLD (2)	F1 (2)	3.7-4.1	252-272	0
		Cryptogenic (6)	F2 (3)	5.3-11.9	13-101	0
			F3 (1)	45	12	0
			F4 (2)	75	23-32	0
Total	52	-	F0 (5)	4.6-6.1	14-452	0-6
			F1 (9)	3.7-7.6	12-904	0-6
			F2 (11)	5.1-11.9	13-368	0-6
			F3 (21)	5.5-45.0	12-558	0-12
			F4 (6)	18.0-75.0	23-108	0-10

<sup>1</sup>Number of patients included in the subgroup; <sup>2</sup>METAVIR. LS: Liver stiffness; ALT: Alanine aminotransferase; HAI: Histologic activity index; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease.

study, the relative expression levels of selected miRNAs that are characteristically up- or downregulated in HCC in comparison to non-tumorous liver tissue<sup>[23]</sup> were determined in hepatic fibrosis samples of various etiologies. We wished to find out the degree to which these levels were altered in fibrosis and how they correlated with fibrosis stage (analyzed by histology and METAVIR scoring system) and LS values (measured by TE), as well as with serum alanine aminotransferase (ALT) levels.

## MATERIALS AND METHODS

### Patients characteristics

Biopsy samples of 52 patients were selected from the archives of the First Department of Internal Medicine at the University of Pécs, the Hepatology Center Buda in Budapest and the Second Department of Pathology at the Semmelweis University, Budapest. Selection was based on two criteria: diagnosis of histologically confirmed chronic, diffuse liver disease and LS measurement with intervals no longer than 3 mo between the two examinations. Permission for the retrospective analysis of the samples was obtained from the local Ethical Committee (45727-2/2013/EKU) based on the ethical guidelines of the 1975 Declaration of Helsinki. Patients were aged between 15 and 67 years with an average of 45.18 years. The female/male ratio was 35/17. ALT serum values were also recorded at the time of liver biopsy.

The cases were selected to represent the diverse etiology of fibrosis: 24 cases of chronic viral hepatitis, including 22 hepatitis C virus (HCV) and 2 hepatitis

B virus (HBV) infections; 19 autoimmune cases, including 8 autoimmune hepatitis (AIH), 6 primary biliary cirrhosis (PBC), 2 primary sclerosing cholangitis (PSC), and 3 overlapping syndrome cases (AIH/PBC and AIH/PSC); 9 cases of mixed etiology, including 1 case of alcoholic and 2 of nonalcoholic liver diseases (ALD and NAFLD, respectively) and 6 cryptogenic cases (Table 1). Accordingly, three sample groups were formed for analysis: autoimmune (AIH, PBC, PSC, and overlaps), chronic viral hepatitis (HCV, HBV), and mixed etiology (ALD, NAFLD, cryptogenic).

### Histology

Biopsy samples were processed according to routine pathology procedures. In brief, the small, 1-3-cm long samples were submerged in 10% neutral buffered formalin (in PBS, pH 7.0) and fixed for 24 h at room temperature. Following dehydration in a series of ethanols and xylene, the formalin-fixed samples were embedded in paraffin (FFPE samples). These samples were cut into 3-4-μm thick sections and stained with hematoxylin-eosin and picrosirius red to highlight the connective tissue.

### Determination of fibrosis

Histologic staging and TE examination were applied to determine the severity of fibrosis. Histologic staging was performed by two pathologists using the METAVIR scoring system from stages F0 to F4, with stage F0 indicating no fibrosis and stage F4 representing cirrhosis<sup>[24]</sup>. The noninvasive TE was carried out using FibroScan 502 (Echosens, Paris, France), with low LS values reflecting no or mild fibrosis and high LS values

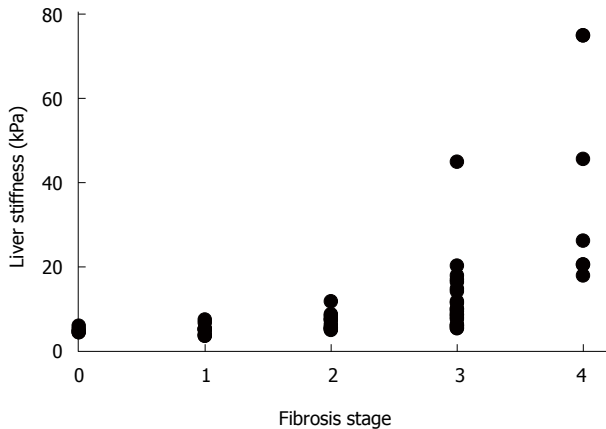


Figure 1 Correlation between fibrosis stage and liver stiffness measured by transient elastography.  $r = 0.8$ ;  $P < 0.01$ .

implying advanced fibrosis or cirrhosis. The elapsed time between date of histologic sampling and date of LS measurement was a maximum of 3 mo, with an average of 1.5 mo.

#### RNA isolation

RNA was isolated from several 3–4- $\mu$ m thick sections using the RNeasy FFPE Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions with modifications for copurification of miRNAs<sup>[25]</sup>. Traces of genomic DNA were eliminated using Turbo DNase digestion (Ambion, Austin, TX, United States).

#### Reverse transcription and quantitative PCR

Expression of individual miRNAs was determined using the following TaqMan MicroRNA Assays (Life Technologies of Thermo Fisher Scientific Inc., Waltham, MA, United States): miR-21 (ID: 000397), miR-122 (ID: 002245), miR-140 (ID: 000462), miR-214 (ID: 002306), miR-221 (ID: 000524), miR-222 (ID: 002276), and miR-224 (ID: 002099). Reverse transcription (RT) and quantitative (q)PCR were performed according to the manufacturer's instructions. Briefly, RT reaction was carried out using the TaqMan MicroRNA Reverse Transcription Kit in a final volume of 7.5  $\mu$ L containing 10 ng total RNA. The qPCR was performed using TaqMan Universal PCR Master Mix No AmpErase UNG in a final volume of 10  $\mu$ L containing 0.65  $\mu$ L RT product. The amplification reaction was run in triplicate on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems of Thermo Fisher Scientific Inc.). Relative expression was calculated by the  $2^{-\Delta\Delta C_q}$  formula, applying miR-140 as the most stable reference determined by the NormFinder application<sup>[26]</sup> and normalized to the median  $\Delta C_q$  value of F0 liver samples.

#### Statistical analysis

The differences between fibrosis stages F0–F4 were analyzed with a nonparametric Kruskal-Wallis analysis

of variance and median test using STATISTICA software, version 9.1 (StatSoft Inc., Tulsa, OK, United States). Correlation analyses between miRNA expression and fibrosis stage, LS values, and ALT levels were performed with a nonparametric Spearman rank order correlation using GraphPad PRISM software, version 5.01 (GraphPad Software Inc, La Jolla, CA, United States). A  $P$  value of 0.05 was set as the threshold for statistical significance. The statistical methods of this study were reviewed by Istvan Kenessey from the Second Department of Pathology, Semmelweis University.

## RESULTS

#### Determination of fibrosis

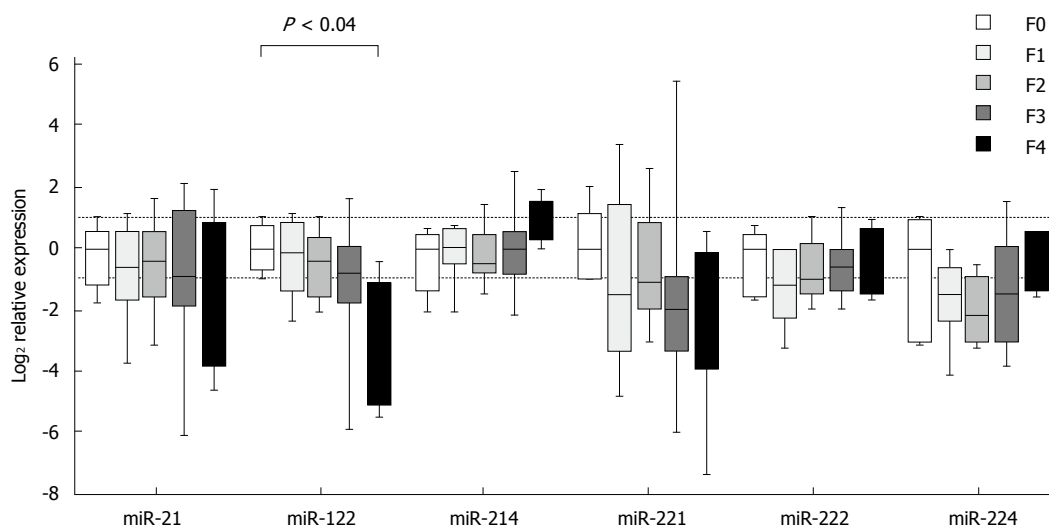
The METAVIR scoring system allowed an unambiguous determination of fibrosis stages in each tissue sample. In contrast, the noninvasive LS measurement showed a wide range of values and when matched with the corresponding METAVIR stages, an overlap between the neighboring ranges was observable (Table 1). This was predominantly manifested in cases of LS value ranges that corresponded to fibrosis stages F0–F3. In addition, the LS value ranges showed slight variances between the various etiology groups. Yet, a highly significant correlation was found between the gradually increasing LS values and fibrosis stage ( $r = 0.8$ ;  $P < 0.01$ ), as presented in Figure 1.

#### Expression of individual miRNAs in relation to METAVIR stage

Expression of individual miRNAs showed deregulated patterns in stages F1–F4 in comparison to stage F0, but the observed differences, except for one case, did not reach the set threshold for statistical significance. The exception was miR-122, in which case the expression in stage F4 was decreased as compared with stage F0 ( $P < 0.04$ ) (Figure 2). The expression differences were close to reaching a statistical significance in two cases: miR-122 between stages F1 and F4 ( $P = 0.06$ ) and miR-214 between stages F2 and F4 ( $P = 0.07$ ). When looking at the expressional patterns of individual miRNAs, in general, the levels were lower in stages F1–F4 in comparison to F0, showing an increasing tendency in case of miR-214 from F2 to F4, miR-222 from F1 to F4 and miR-224 from F2 to F4 (Figure 2). Nevertheless, the differences in the three etiologic groups did not reach the set threshold for statistical significance.

#### Correlation of miRNA expression with fibrosis and ALT values

In relation to miRNA expression and fibrosis, miR-122 and miR-221 showed a negative correlation with METAVIR stage ( $P < 0.01$  and  $P < 0.03$ , respectively) (Figure 3A), and miR-122 was found to correlate negatively ( $P < 0.01$ ) and miR-224 positively ( $P < 0.04$ ) with LS values (Figure 3B). Furthermore, a positive



**Figure 2** Relative miRNA expression in relation to fibrosis stage, detected in the biopsy samples of various etiologic backgrounds. The level of miR-122 is reduced in stage F4 as compared with stage F0 ( $P < 0.04$ ), analyzed using a nonparametric Kruskal-Wallis analysis of variance and median test. The upper dotted line indicates twofold elevation in expression; the lower dotted line signifies a one-half reduction of expression.

correlation of miR-21 was found between miRNA expression and ALT levels ( $P < 0.04$ ) (Figure 3C). A summary of the correlation analyses is provided in Table 2. With respect to etiology, miR-122 expression correlated negatively with fibrosis stage and LS values ( $P < 0.02$  and  $P < 0.05$ , respectively) in the mixed etiology group, and miR-221 level showed a negative correlation with fibrosis stage ( $P < 0.01$ ) in the chronic viral hepatitis group, whereas a positive correlation between miR-21 and ALT values ( $P < 0.03$ ) was found in the autoimmune group.

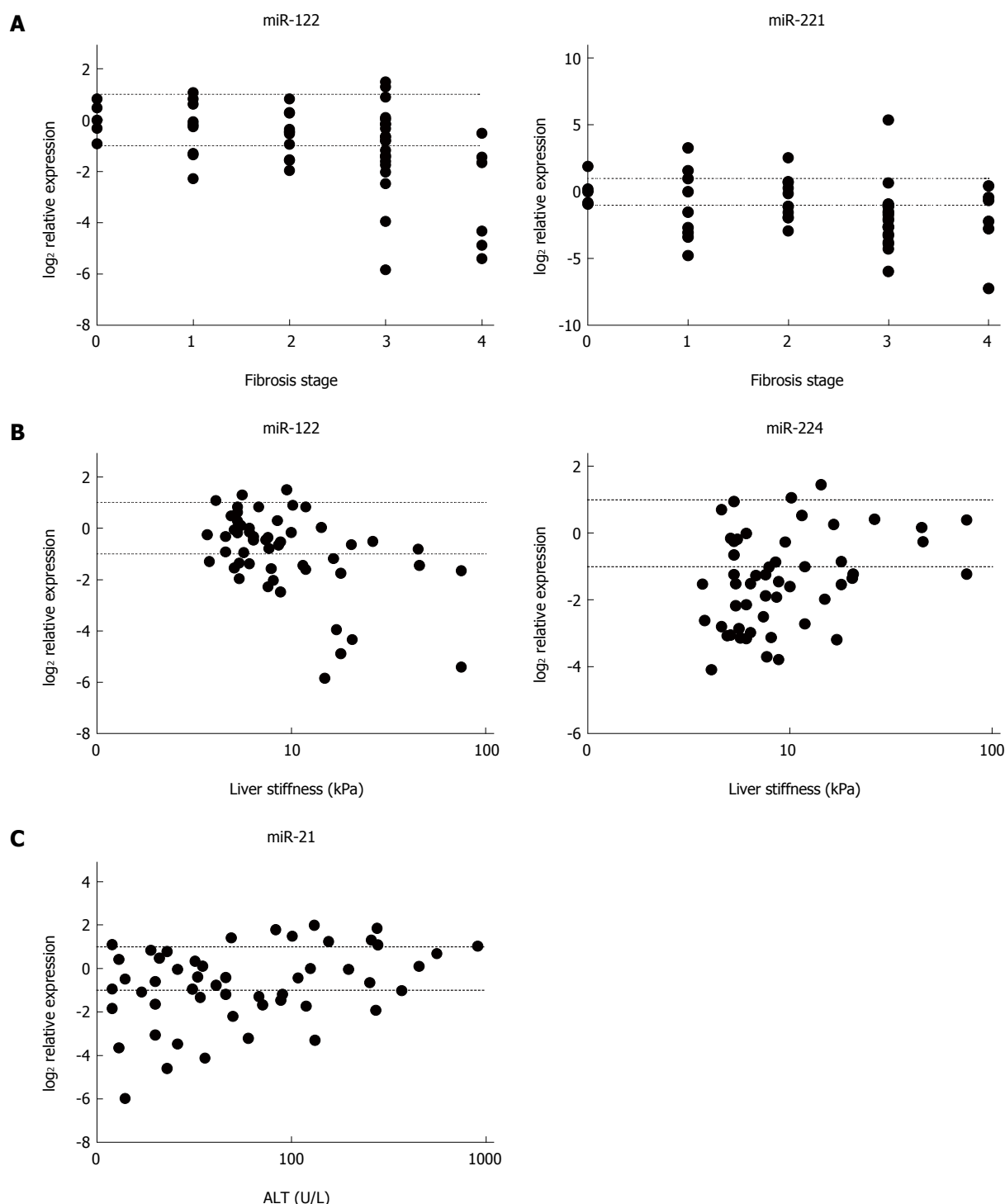
## DISCUSSION

The diagnosis of liver fibrosis and the decision on therapy are important factors in the treatment of chronic liver diseases, with liver biopsy being the widely used procedure for the accurate determination of fibrosis. Owing to certain limitations of liver biopsy, such as possibility of serious complications, contradictions, sampling as well as intra- and inter-observer errors<sup>[12]</sup>, noninvasive approaches, such as TE, are available as alternatives. However, the diagnostic accuracy of TE is not entirely precise over the measuring range. Although the measured values increase with fibrosis stage, the method gives excellent results predominantly from advanced fibrosis (F2) to early and symptom-free cirrhosis (F4), and performs with limitations when early stage fibrosis (F0-F1) is to be determined, and when having to differentiate between F2/F3 stadium or in case of obese patients<sup>[27]</sup>. Yet, a highly positive correlation between fibrosis stage and LS values was found in the present study, which is supportive of the positive finding reported earlier<sup>[28]</sup>.

There is a general need to find indicators at the molecular level to help predict disease progression. For example, hepatic cirrhosis is characterized by

an increased proliferation rate that correlates with a higher tendency to develop HCC<sup>[29]</sup>. The miRNAs are foreseen to be such indicators based on their altered expression found in liver diseases, fibrosis<sup>[7,17]</sup>, and liver carcinogenesis<sup>[23,30]</sup>. In addition, deregulated expression of miRNAs may be present long before the onset of a disease<sup>[22]</sup>. As alterations of miRNA expression in relation to fibrosis stage have predominantly been studied in chronic HCV-infected samples, we aimed, in the present study, to investigate the expression of fibrosis- and hepatocarcinogenesis-related miRNAs in hepatic fibrosis samples of various etiologies, and to correlate the found expression levels with the severity of fibrosis and serum ALT levels.

Our results reveal a reduced level of miR-122 in stage F4 fibrosis as compared with stage F0, and miR-122 showed a negative correlation with fibrosis stage in fibrotic liver samples and, intriguingly, also with LS values. These findings are supported by reports of a negative correlation between miR-122 and fibrosis stage in chronic HCV infection, HCV-based HCC, and cirrhosis<sup>[31,32]</sup>, and also by observations of a decreased level of miR-122 in NAFLD<sup>[33,34]</sup> and in HCC studies<sup>[23]</sup>. miR-122 is a liver-characteristic miRNA that composes about 70% of the total miRNAs found in normal hepatocytes<sup>[35]</sup>, most probably due to the fact that it positively regulates the accumulation of cholesterol and triglycerides and the metabolism of fatty acids<sup>[16]</sup>. Thus, a decreased level of miR-122 in fibrotic liver biopsies may be interpreted as the result of compromised normal hepatocytic activity or as the eliminated suppressive function of miR-122 that hinders fibrogenesis. Namely, miR-122 has been found to suppress the proliferation of HSCs, resulting in decreased maturation of collagen by downregulating the expression of P4HA1, a key enzyme in collagen maturation<sup>[36]</sup>. miR-122 may also impede carcinogenesis<sup>[37]</sup>, as expression of proteins involved



**Figure 3** Correlation of miRNA expression with fibrosis stage, liver stiffness (measured by transient elastography), and alanine aminotransferase levels. A: The negative correlation of miR-122 ( $r = -0.4$ ;  $P < 0.01$ ) and miR-221 ( $r = -0.3$ ;  $P < 0.03$ ) with fibrosis stage; B: The negative correlation of miR-122 ( $r = -0.4$ ;  $P < 0.01$ ) and positive correlation of miR-224 ( $r = 0.3$ ;  $P < 0.04$ ) with liver stiffness; C: The positive correlation of miR-21 ( $r = 0.3$ ;  $P < 0.04$ ) with serum alanine aminotransferase (ALT) levels. The upper dotted lines indicate twofold elevation in expression; the lower dotted lines signify a one-half reduction of expression.

in the cell cycle, differentiation, and proliferation is downregulated by miR-122<sup>[38]</sup>, and loss of miR-122 in HCC is a frequent finding, which correlates with migration, invasion and *in vivo* tumorigenesis<sup>[39]</sup>. In association with HCV, miR-122 has been found to protect viral RNA from exonuclease degradation by binding at two positions near the 5' end of the RNA

molecule. However, the capacity of this protection seems to be independent of the promotion of HCV infectivity, indicating that miR-122 has other unknown functions in the viral life cycle<sup>[40]</sup>. Taken together, downregulation of miR-122 seems to be both a sensitive sign of hepatic injury and a possible step on the path toward liver cancer.



**Table 2** Correlation of miRNA levels with fibrosis stage, liver stiffness, and alanine aminotransferase values

miRNA	All samples ( <i>n</i> = 52)	Etiology groups		
		Autoimmune ( <i>n</i> = 19)	Viral ( <i>n</i> = 24)	Mixed ( <i>n</i> = 9)
Fibrosis stage				
miR-122	<i>r</i> = -0.4 <i>P</i> < 0.01	<i>r</i> = -0.3 <i>P</i> < 0.20	<i>r</i> = -0.3 <i>P</i> < 0.10	<i>r</i> = -0.8 <i>P</i> < 0.02
miR-221	<i>r</i> = -0.3 <i>P</i> < 0.03	<i>r</i> = -0.3 <i>P</i> < 0.20	<i>r</i> = -0.5 <i>P</i> < 0.01	<i>r</i> = -0.2 <i>P</i> < 0.40
Liver stiffness				
miR-122	<i>r</i> = -0.4, <i>P</i> < 0.01	<i>r</i> = -0.4 <i>P</i> < 0.09	<i>r</i> = -0.2 <i>P</i> < 0.30	<i>r</i> = -0.7 <i>P</i> < 0.05
miR-224	<i>r</i> = 0.3 <i>P</i> < 0.04	<i>r</i> = 0.3 <i>P</i> < 0.20	<i>r</i> = 0.1 <i>P</i> < 0.70	<i>r</i> = 0.6 <i>P</i> < 0.07
Alanine aminotransferase				
miR-21	<i>r</i> = 0.3 <i>P</i> < 0.04	<i>r</i> = 0.5 <i>P</i> < 0.03	<i>r</i> = 0.4 <i>P</i> < 0.09	<i>r</i> = -0.4 <i>P</i> < 0.30

Increased levels of miR-221, miR-224, and miR-21 have been reported in HCC as these oncomiRs inhibit expression of tumor suppressor genes<sup>[23]</sup>. For example, downregulation of P27 and P57, as targets of miR-221 and key regulators of cell cycle progression, has been found to promote cancer cell proliferation<sup>[30,41]</sup>. Moreover, miR-221 is observed to be increased in early preneoplastic stage, such as cirrhosis and steatosis or steatohepatitis<sup>[42-44]</sup>, and is also found to be overexpressed in a mouse liver-regeneration model, in which the proliferation of hepatocytes is accelerated by miR-221 *in vitro* and *in vivo* in the presence of epidermal or hepatocytes growth factors (EGF or HGF), thereby facilitating a rapid S-phase entry of hepatocytes<sup>[45]</sup>. In HCV and nonalcoholic steatohepatitis biopsies, miR-221 is observed to increase with fibrosis stage and to correlate positively with expression levels of  $\alpha 1$  chain of collagen type I<sup>[46]</sup>. In contrast, statistical difference in miR-221 expression was not found in the present study; moreover, fibrosis stage showed a negative correlation with miR-221 expression in our samples and the samples of the chronic viral hepatitis group. An explanation for this could be that the representations of the F0-F4 cases, as well as the statistical methods used, were different; furthermore, the extent of regeneration was possibly different in the analyzed samples. Another reason could be the DNA methylation status of the miR-221 locus, as hypomethylation of this locus was found in HCC that contributed to the overexpression of miR-221<sup>[47]</sup>.

miR-224 has been described to promote proliferation, migration, and invasion in HCC by the activation of AKT signaling; thus, miR-224 has been suggested to play a role in liver carcinogenesis and progression<sup>[48]</sup>. With respect to fibrosis stage, the present study did not reveal any differences or correlations. However, LS values positively correlated with miR-224 expression, suggesting that a gradual increase in miR-224 level may occur in liver tissues prior to malignant transformation.

Indeed, miR-224 expression has been found to correlate with fibrosis stage in chronic hepatitis C<sup>[49]</sup>, and elevated levels of miR-224 have also been observed in chronic hepatitis C samples with steatosis and HCV-negative steatotic liver biopsies<sup>[50]</sup>.

It has been reported that miR-21 reduces the expression of fibrogenesis-related tumor suppressor genes, such as SMAD-7, the negative regulator of transforming growth factor- $\beta$  signaling<sup>[8,31]</sup>, and proliferation-related PTEN, an inhibitor of the AKT pathway<sup>[51]</sup>. Positive correlation between miR-21 and fibrosis stage is reported in chronic HCV-infected patients and in a CCl<sub>4</sub> mouse fibrosis model<sup>[31]</sup>, and stimulation of the fibrogenic effect by miR-21 is also found in a HSC cell line<sup>[51]</sup>. In contrast, we did not find any difference or correlation between miR-21 and fibrosis stage or LS values in the present study, but positive correlation of miR-21 with serum ALT values was clearly visible. In our chronic viral hepatitis group, this correlation did not reach the set significance level, which is in partial agreement with data reported in chronic hepatitis C patients<sup>[31]</sup>. In addition, ALT levels have been found to correlate with serum miR-21 levels of chronic HCV patients, suggesting that miR-21 is an indicator of the extent of necroinflammation in the liver<sup>[52]</sup>.

In the present study, the expression of several hepatocarcinogenesis-related miRNAs was assayed in fibrotic liver biopsy samples of various etiologies and correlated with fibrosis stage (measured by METAVIR) and, to the best of our knowledge, for the first time, with LS values (measured by TE). Reduced miR-122 expression was found in advanced fibrosis as compared with stage F0 and a negative correlation was observed not only with fibrosis stage, but with LS values as well. In addition, we detected a positive correlation between miR-224 and LS values, indicating the role of this oncomiR in advanced fibrosis, indicating a link between fibrosis and HCC. Nevertheless, an ideal staging tool should be able to discriminate not only between mild and advanced stages of fibrosis, but also between intermediate stages of fibrosis. Although this is a reasonable demand, the biologic variances may result in overlaps between the observed intermediate values, especially if the intermediate ranges are small. The main focus of the present study was to analyze fibrotic samples of different etiologic backgrounds. The limitation of our analysis is the small sample size in the various etiologic groups. Therefore, further studies are warranted in order to reveal whether the observed miRNA correlations are also characteristic of the various etiology groups or whether these relationships are only summed characteristics of the fibrosis samples by reason of the various etiologies. In conclusion, the observed negative correlation between fibrosis stage and LS values in case of miR-122 indicates that this molecule could be useful in assessing the severity of fibrosis regardless of etiology.

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## COMMENTS

### Background

Hepatic fibrosis is a wound-healing response of the liver to cellular injury that is characterized by deposition of collagen fibers and contributes to the deterioration of normal liver function. When injury persists, fibrosis may advance into cirrhosis (the most severe stage of fibrosis) and further into hepatocellular carcinoma. Assessment of the stage of fibrosis is important for diagnosis and is predominantly based on liver biopsy. Owing to some limitations, other alternatives have been developed such as transient elastography.

### Research frontiers

The regulatory role of microRNAs (miRNA) is to fine-tune the expression of genes involved in normal cellular processes, such as development, differentiation, and proliferation. For this reason, it may not be surprising that altered miRNA expression can be found in cancers and in several other pathologies, including liver diseases. Moreover, it has been suggested that an imbalance in the normal miRNA pattern is measurable long before the onset of a disease, indicating that miRNAs may be useful molecules in diagnostics.

### Innovations and breakthroughs

There is a need to find indicators that will help predict the progression of a disease. As miRNAs have been reported to show altered expression in fibrosis and have also been suggested to play role in liver carcinogenesis, these molecules may be useful candidates. miRNA expression in relation to fibrosis stage has predominantly been investigated in chronic hepatitis C virus-infected samples. Therefore, in the current study, the authors investigated miRNA expression in samples of diverse etiologies, including autoimmune and chronic viral hepatitis and alcoholic and nonalcoholic liver diseases to find out whether miRNA levels become altered in fibrosis and whether they could be correlated with fibrosis stage and liver stiffness values measured by transient elastography.

### Applications

The reduced expression of miR-122 observed in stage F4 as compared with stage F0 and the negative correlation of miR-122 levels with fibrosis stage and liver stiffness suggest that miR-122 could be a useful molecule to assess fibrosis regardless of etiology. However, further studies are warranted with larger sample sizes. Furthermore, the staging tool should also be able to discriminate between intermediate stages of fibrosis, although biologic variances themselves may result in overlaps between the intermediate values.

### Terminology

miRNAs are regulating molecules that interfere with gene expression upon binding to the untranslated regions of mRNAs and induce translational arrest, the result being reduced or prevented protein synthesis. Transient elastography is an ultrasound-based examination method for the noninvasive assessment of liver stiffness in fibrosis, with the value increasing with the advancement of fibrosis.

### Peer-review

The novelty of the study is the correlation analysis of transient elastography and miRNA expression in samples obtained from chronic liver disease of various etiologies. Although the study examined a limited sample size in each subgroup of fibrosis-related liver diseases, the presented results are in agreement with recent reports that support a strong correlation between decrease of miR-122 levels and severity of fibrosis or increase in liver stiffness. The most significant finding of the study is that miR-122 levels are altered independent of the etiologic cause of liver damage. The presented evidences support the usefulness of miR-122 as an indicator of fibrosis progression.

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## Retrospective Study

# Gd-EOB-DTPA-enhanced magnetic resonance imaging for bile duct intraductal papillary mucinous neoplasms

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## Abstract

**AIM:** To investigate gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) of intraductal papillary mucinous neoplasms of the bile duct (IPMN-B).

**METHODS:** The imaging findings of five cases of IPMN-B which were pathologically confirmed at our hospital between March 2012 and May 2013 were retrospectively analyzed. Three of these cases were diagnosed by duodenal endoscopy and biopsy pathology, and two cases were diagnosed by surgical pathology. All five patients underwent enhanced and non-enhanced computed tomography (CT), magnetic resonance cholangiopancreatography, and Gd-EOB-DTPA-enhanced MRI; one case underwent both Gd-EOB-DTPA-enhanced MRI and positron emission tomography-CT. The clinical data and imaging results for these cases were compared and are presented.

**RESULTS:** Conventional imaging showed diffuse

dilatation of bile ducts and multiple intraductal polypoid and papillary neoplasms or serrated changes along the bile ducts. In two cases, Gd-EOB-DTPA-enhanced MRI revealed dilated biliary ducts and intraductal tumors, as well as filling defects caused by mucin in the dilated bile ducts in the hepatobiliary phase. Gd-EOB-DTPA-enhanced MRI in one case clearly showed a low-signal tumor in the hepatobiliary phase, similar to what was seen by positron emission tomography-CT. In two patients, routine inspection was unable to discern whether the lesions were inflammation or tumors. However, Gd-EOB-DTPA-enhanced MRI revealed a pattern of gradual enhancement during the hepatobiliary phase, and the signal intensity of the lesions was lower than the surrounding liver parenchyma, suggesting tissue inflammation in both cases, which were confirmed by surgical pathology.

**CONCLUSION:** Gd-EOB-DTPA-enhanced MRI reveals the intraductal mucin component of IPMN-B in some cases and the extent of tumor infiltration beyond the bile ducts in invasive cases.

**Key words:** Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid; Magnetic resonance imaging; Magnetic resonance cholangiopancreatography; Multidetector computed tomography; Bile duct neoplasms

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**Core tip:** Gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) can be used to demonstrate the filling defects due to mucin secreted by intraductal papillary mucinous neoplasms of the bile duct (IPMN-B) and to display the extent of tumor infiltration beyond bile ducts in cases with invasive IPMN-B. It also has the potential to differentiate tumor tissue from inflammatory lesions. Therefore, Gd-EOB-DTPA-enhanced MRI may improve the clinical management of IPMN-B.

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## INTRODUCTION

Intraductal papillary mucinous neoplasms of the bile duct (IPMN-B) are a subtype of intraductal papillary neoplasms of the bile duct with macroscopically visible mucin secretion<sup>[1-7]</sup>. The clinical manifestations, histopathologic features, and immunohistochemical and

biologic behaviors of this bile duct subtype are similar to IPMNs of the pancreas<sup>[1-6]</sup>. IPMN-B originates from biliary epithelial cells, and can be pathologically defined as papillary adenoma, papillomatosis, carcinoma *in situ*, or invasive adenocarcinoma. Imaging findings of IPMN-B include: papillary or polypoid growth of the tumor along the bile duct or serrated inner lining of the bile duct; expansive and significant dilation of the bile duct upstream and downstream of the tumor; or aneurysmal dilation of the bile duct at the site of the tumor<sup>[8-10]</sup>. However, confirmation of a diagnosis requires the use of endoscopic retrograde cholangiopancreatography (ERCP) to determine the presence of mucin secreted by the tumor<sup>[4]</sup>. In addition, malignant IPMN-Bs can invade the liver and form a mass, although conventional ultrasonography (US), computed tomography (CT), and dynamic enhancement magnetic resonance scans cannot define the scope of tumor invasion, or distinguish the accompanying inflammation from the tumor<sup>[11,12]</sup>.

Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is a double-specific contrast agent that provides enhancement effects similar to Gd-DTPA in dynamic enhanced scans. With Gd-EOB-DTPA, 50% of the contrast injection is absorbed by hepatocytes and drained *via* the bile duct in the hepatobiliary phase. Although this contrast agent has been used for the diagnosis of hepatogenic tumor lesions<sup>[11]</sup>, only two reports (four cases) describe the use of Gd-EOB-DTPA-enhanced magnetic resonance imaging (MRI) for the diagnosis of IPMN-B<sup>[13,14]</sup>. In those reports, Gd-EOB-DTPA-enhanced MRI not only revealed the dilated bile duct and the enhanced tumor tissues within it, but also confirmed that the filling defect of the bile duct at the hepatobiliary phase was mucus secreted by the tumors<sup>[13,14]</sup>, thus demonstrating its unique value for tumor diagnosis. The present study describes the application of Gd-EOB-DTPA-enhanced MRI in five cases of IPMN-B. In addition to displaying the features of IPMN-Bs, this method reveals the extent of invasion into the extrahepatic bile duct in malignant cases. Furthermore, Gd-EOB-DTPA-enhanced MRI can discern tumor tissue from surrounding inflammation, which has not previously been reported.

## MATERIALS AND METHODS

### Study subjects

Five cases of IPMN-B in our hospital during the period from March 2012 to May 2013 were retrospectively analyzed (Table 1). Two cases were confirmed by surgical pathology. Three cases underwent thick-needle biopsy, and 3-5 pieces of cord-like tissue were obtained for each case; diagnoses were confirmed by two senior pathologists.

**Table 1** Clinical data of five patients with intraductal papillary mucinous neoplasms of the bile duct

No.	Sex	Age (yr)	History and symptoms	Laboratory findings (normal range)	Imaging						
					US	CT + C	Gd-DTPA MRI	MRCP	ERCP	PET-CT	Gd-EOB-DTPA-enhanced MRI
1	Female	59	Recurrent upper abdominal pain with nausea and vomiting for 6 mo	No significant abnormalities	A	A	NA	A	A	NA	A
2	Male	52	Physical examination revealed liver tumor	CEA: 7.5 ng/mL (0.0-8.0)	A	A	NA	A	A	NA	A
3	Female	72	Jaundice	CEA: 9.8 ng/mL CA199: 715.1 U/mL (0.0-37.0) Total bilirubin: 611 mmol/L (0-21)	A	A	NA	A	A	NA	A
4	Male	66	Jaundice	CEA: 44.0 ng/mL CA199: > 12000 U/mL Total bilirubin: 424 mmol/L	A	A	NA	A	NA	NA	A
5	Male	56	8-yr history of liver contusion; liver pain for 6 mo	CA199: 217.3 U/mL CRP: 34.5 mg/L (0.0-8.0)	A	A	A	A	NA	A	A

A: Applied; CA: Cancer antigen; CEA: Carcinoembryonic antigen; CRP: C-reactive protein; CT + C: Contrast enhanced-CT; ERCP: Endoscopic retrograde cholangiopancreatography; Gd-DTPA MRI: Gadolinium-diethylenetriamine pentaacetic acid magnetic resonance imaging; Gd-EOB-DTPA-enhanced MRI: Gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid-enhanced magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; NA: Not applied; PET: Positron emission tomography; US: Ultrasound.

### Hepatic CT examinations

CT images were acquired by a 16-slice CT scanner (Aquilion; Toshiba, Tokyo, Japan) using the following scan parameters: 120 kV; 250 mA; reconstruction thickness, 5 mm; layer spacing, 5 mm. CT scans were obtained before and after intravenous administration of 80-100 mL contrast agent (300 mg I/mL Ultravist; Bayer Healthcare, Berlin, Germany) at a speed of 3.0 mL/s. CT scan delays after the injection of contrast agent were 25-30 s, 65 s, and 120 s for the arterial, portal venous, and delayed phases, respectively.

### Hepatic MRI examinations

MRI was performed using a 3.0 T scanner (Signa HDxt 3.0 T; GE Healthcare, Little Chalfont, United Kingdom) with a corresponding eight-channel phase-array abdomen coil system. MRI sequences included axial T2- and diffusion-weighted imaging, magnetic resonance cholangiopancreatography (MRCP) and fat suppression T1-weighted three-dimensional liver acquisition with volume acceleration (LAVA)-enhanced imaging. The parameters for MRI sequences used in this study are listed in Table 2.

The LAVA-enhanced MRI was conducted *via* bolus i.v. injection of Gd-DTPA (Magnevist; Schering, Berlin, Germany) at 0.1 mmol/kg body weight and a speed of 2 mL/s. Pre-contrast and dynamic contrast-enhanced multi-phase MRI scans were acquired. The MRI scan delays were 25-30 s, 85 s, and 180 s for the arterial, portal venous, and delayed phases, respectively. The same sequence and parameters were used for dynamic Gd-EOB-DTPA-enhanced MRI following a bolus i.v. injection of EOB (Primovist; Bayer

Healthcare) at 0.025 mmol/kg body weight and a speed of 2 mL/s. Fat suppression T1-weighted three-dimensional LAVA was performed 20 min after contrast injection and the images of the initial hepatobiliary phase were obtained, and repeated after 42-52 min for the delayed scans of the hepatobiliary phase. The image of the hepatobiliary phase was visualized with maximum intensity projections or multiple planar reconstructions to observe the filling condition of the intra- and extrahepatic bile ducts<sup>[11,12,15]</sup>.

### US and PET-CT

All US studies were performed using a color ultrasound (Sequoia 512; Siemens Medical Solutions, Munich, Germany) with a 1.0- to 4.0-MHz convex probe. One patient received a whole-body PET/CT scan performed on an integrated PET/CT scanner (Biograph 16; Siemens Medical Solutions) after the injection of 350 MBq 18F-FDG.

## RESULTS

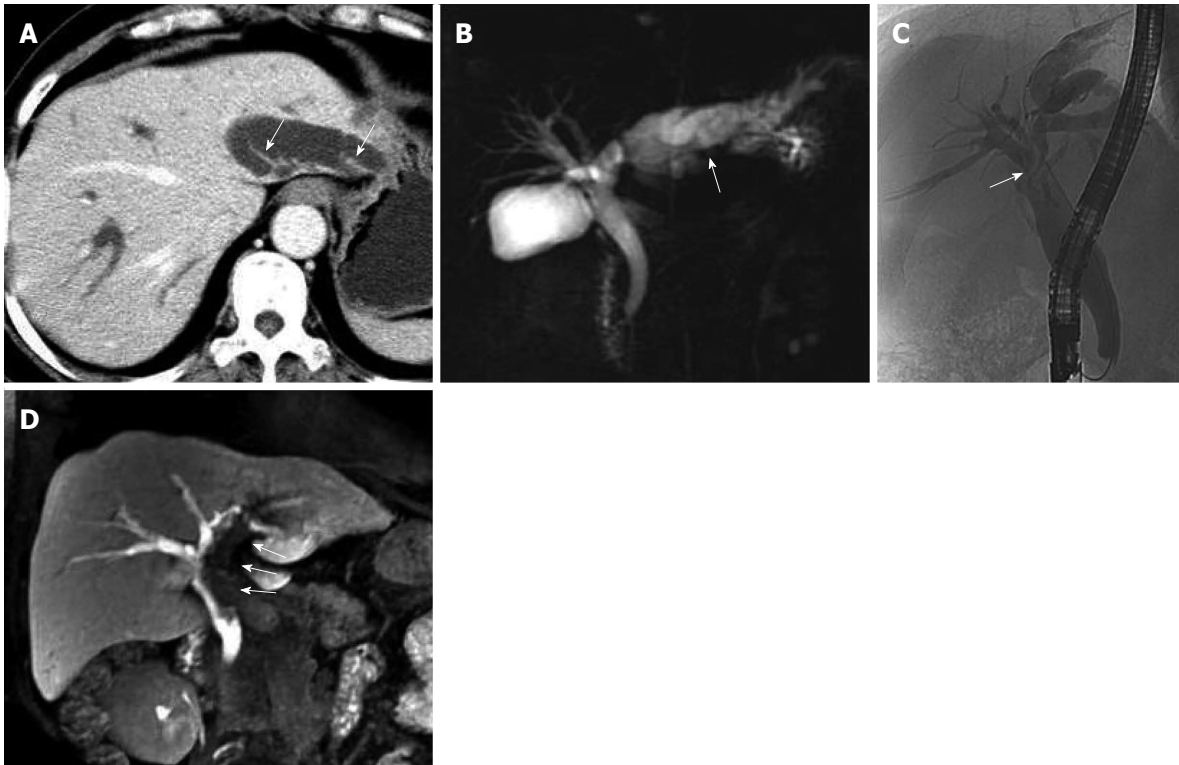
### General imaging findings

US, CT/MRI, and MRCP depicted extensive intra- and extrahepatic bile duct dilatation in cases 1-4, including upstream and downstream of the tumor and at the segmental bile duct (Figures 1, 2, 3 and 4). In contrast-enhanced CT and MRI examinations, multiple polypoid or papillary tumors which were distributed along the bile ducts showed mild to medium enhancement. In case 2, partial right intrahepatic bile ducts were seen as aneurysm-like dilatations (Figure 2). In case 4, a slightly higher-signal nodule was also

**Table 2** Parameters for magnetic resonance imaging sequences

Sequence	TR (ms)	TE (ms)	Section thickness (mm)	Gap (mm)	Matrix size (pixels)	Flip angle (degrees)	Field of view (cm)
T2WI	6000-10000	91	6	2	320 × 224	90	40 × 32
DWI (b = 1000)	8000	65	6	2	128 × 128	90	40 × 32
MRCP	6000-10000	800	2.4	0	384 × 224	90	40 × 32
Gd-DTPA-enhanced MRI	2.84	1.34	5	0	384 × 256	10	40 × 32
Gd-EOB-DTPA-enhanced MRI	2.84	1.34	5	0	384 × 256	10	40 × 32

DWI: Diffusion-weighted imaging; DTPA: Diethylenetriamine pentaacetic acid; EOB: Ethoxybenzyl; Gd: Gadolinium; MRCP: Magnetic resonance cholangiopancreatography; MRI: Magnetic resonance imaging; T2WI: T2-weighted imaging; TE: Echo time; TR: Repetition time.



**Figure 1** Fifty-nine-year-old female patient with intraductal papillary mucinous neoplasm of the bile duct (case 1). A: Axial contrast-enhanced computed tomography in the portal phase; B: Magnetic resonance cholangiopancreatography showed extensive intra- and extrahepatic bile duct dilatation and multiple hydra-like protrusions (arrows) in the markedly dilated left intrahepatic bile duct (arrow); C: Endoscopic retrograde cholangiopancreatography showed extensive intra- and extrahepatic bile duct dilatation, particularly in the left intrahepatic and common bile ducts. Bile ducts in the liver hilar appeared more transparent due to the local aggregation of mucin (arrow); D: Multiple planar reconstruction of gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid-enhanced magnetic resonance imaging in the hepatobiliary phase showed dilatation of the bile duct and filling defects in the left intrahepatic and common bile ducts, with a cup-shaped contrast filling edge (arrows). The filling defects were caused by mucus retention, which was confirmed by endoscopy.

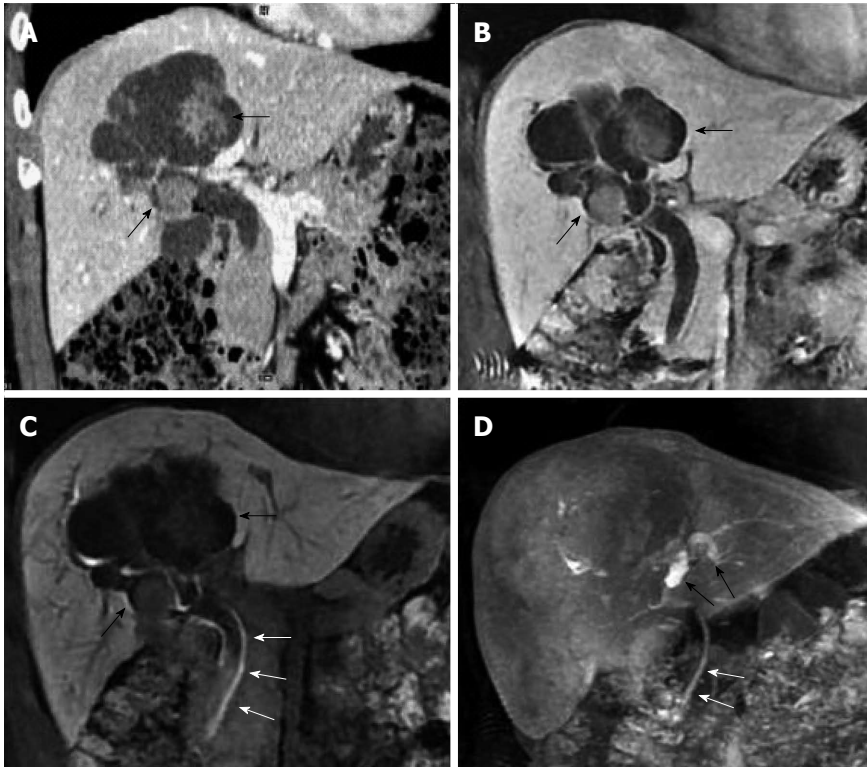
seen at the edge of the dilated left intrahepatic bile ducts in T2- and diffusion-weighted imaging, which was enhanced slightly during all three phases of the contrast-enhanced CT scan (Figure 4). In this case, the tumor lesions were considered to penetrate into the liver *via* the bile duct wall. In case 5, CT/MRI scans depicted right hepatic atrophy surrounding the right dilated intrahepatic bile duct (Figure 5). Enhanced CT and Gd-DTPA-enhanced MRI in both the arterial and portal venous phases depicted marked, although ill-defined, patchy enhancement around the dilated bile duct, which was isodense in the delayed phase. Positron emission tomography (PET)-CT in case 5 revealed a mass of 6.5 cm × 6.0 cm with high uptake

of fluorodeoxyglucose (FDG) around the dilated bile duct in the posterior segment of the liver (the maximum standard uptake value was 6.5), indicating a malignant tumor (Figure 5). Therefore, the tumor in this patient was considered to be an invasive IPMN-B.

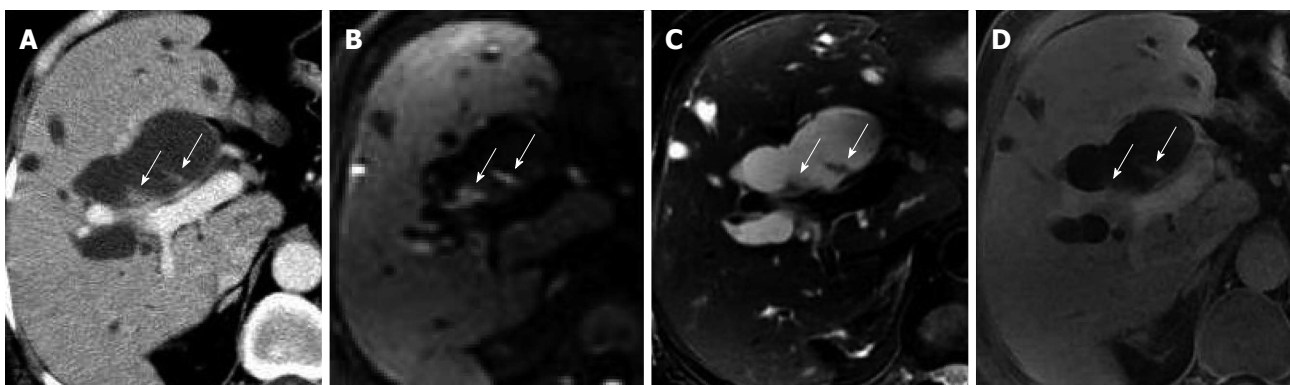
#### **Gd-EOB-DTPA-enhanced MRI**

Dilated bile ducts and tumors on the bile duct walls in the five cases were also observed with plain MRI and dynamic triple-enhanced EOB scans. In addition, some specific signs were seen in ≥ 20 min delayed scans, namely the hepatobiliary phase. Cases 1 and 2 showed irregular columnar filling defects, and the edge of the contrast reagent appeared in a cupped or half-





**Figure 2** Fifty-two-year-old male patient with cystic intraductal papillary mucinous neoplasm of the bile duct (case 2). A: Coronal image of contrast-enhanced computed tomography; B: Gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid-enhanced magnetic resonance imaging (Gd-EOB-DTPA-enhanced MRI) in the portal venous phase showed extensive biliary and aneurysmal dilatations of the right intrahepatic bile duct, with multiple significantly enhanced masses within the lumen (black arrows); C: Gd-EOB-DTPA-enhanced MRI also showed a contrast-filling defect within the common bile duct (white arrows), suggesting the presence of mucin; D: Multiple planar reconstruction of images acquired in the hepatobiliary phase showed a dilated left intrahepatic bile duct with good contrast filling (black arrows). The contrast filling was incomplete in the right intrahepatic and common bile ducts (white arrows).

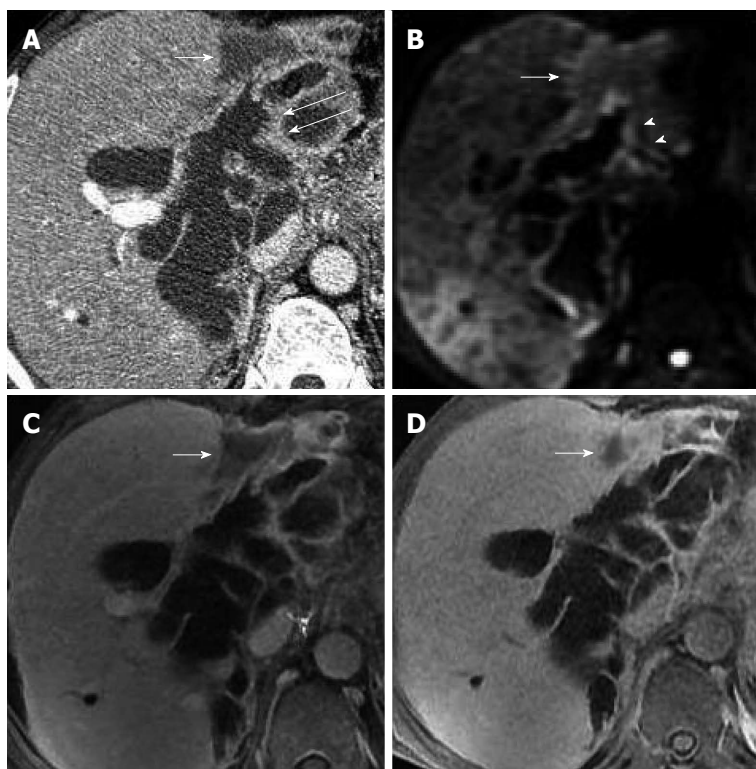


**Figure 3** Seventy-two-year-old female patient with malignant intraductal papillary mucinous neoplasm of the bile duct (case 3). A: Axial image of enhanced computed tomography in the portal venous phase showed extensive intra- and extrahepatic bile duct dilatation with multiple hydra-like protrusions (arrows); B: Dilation appeared hyperintense (arrows) with diffusion-weighted imaging; C: Hypointense (arrows) with T2-weighted imaging; D: Gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid-enhanced magnetic resonance imaging in the hepatobiliary phase revealed no hyperintense bile with distribution of the contrast agents in the dilated bile duct, indicating a large amount of mucin retention, which was confirmed by endoscopy.

ring distribution (Figures 1 and 2).

Cholangiectasis was apparent in cases 3 and 4, and a high signal of contrast filling was not seen in the dilated bile ducts in the hepatobiliary phase (Figures 3 and 4). Total bilirubin levels were markedly elevated in these two cases (Table 1). Routine inspection showed an abnormal signal and low-strengthened focal area in the edge of the left intrahepatic bile duct in case 4,

and the edge of the lesion was enhanced in the 20-min delayed EOB-enhanced MRI. In the 45-min delayed scan, most of the area showed isointensity, with the exception of the low signal in the lesion center, which is a pattern of gradual enhancement (Figure 4). These findings suggest decreased hepatic uptake of contrast agent, indicative of inflammation. No signs of tumor invasion were seen in the EOB-enhanced scans of



**Figure 4** Sixty-six-year-old male patient with malignant intraductal papillary mucinous neoplasm of the bile duct (case 4). A: Axial image of contrast-enhanced computed tomography in the portal venous phase showed a patchy hypointense area abutting the dilated bile ducts in the front edge of the left liver lobe (white arrow), and multiple small nodules distributed along the bile duct wall within the lumen (white long arrows); B: These nodules were hyperintense (white arrowheads) with diffusion-weighted imaging; C: Gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid-enhanced magnetic resonance imaging (Gd-EOB-DTPA-enhanced MRI) in the portal venous phase also showed a patchy hypointense area abutting the dilated bile ducts (white arrow); D: The patch (white arrow) appeared smaller with Gd-EOB-DTPA-enhanced MRI in the hepatobiliary phase, due to the gradual filling of contrast agent, which suggests the presence of functional liver cells. The patchy area was thought to be an inflammatory change secondary to the tumor.

cases 1-4.

Although there was no high signal of contrast filling in the dilated bile duct on the inferior right liver in case 5, the 20-min delayed scan showed a well-defined massive (6.5 cm × 6.0 cm) lesion with low-signal intensity around the dilated bile duct, indicating a lack of EOB uptake in the adjacent region of the liver (Figure 5). This was in accordance with the tumor extent seen on PET-CT. The signal in the liver parenchyma in front of the tumor was slightly lower than the normal liver tissue, but higher than the mass tissue in the hepatobiliary phase of EOB-enhanced MRI. A low signal vessel with normal direction can be seen within it, suggesting that this area is not tumor tissue, but inflamed liver tissue with reduced uptake ability for the contrast agent (Figure 5).

#### Pathology and treatment

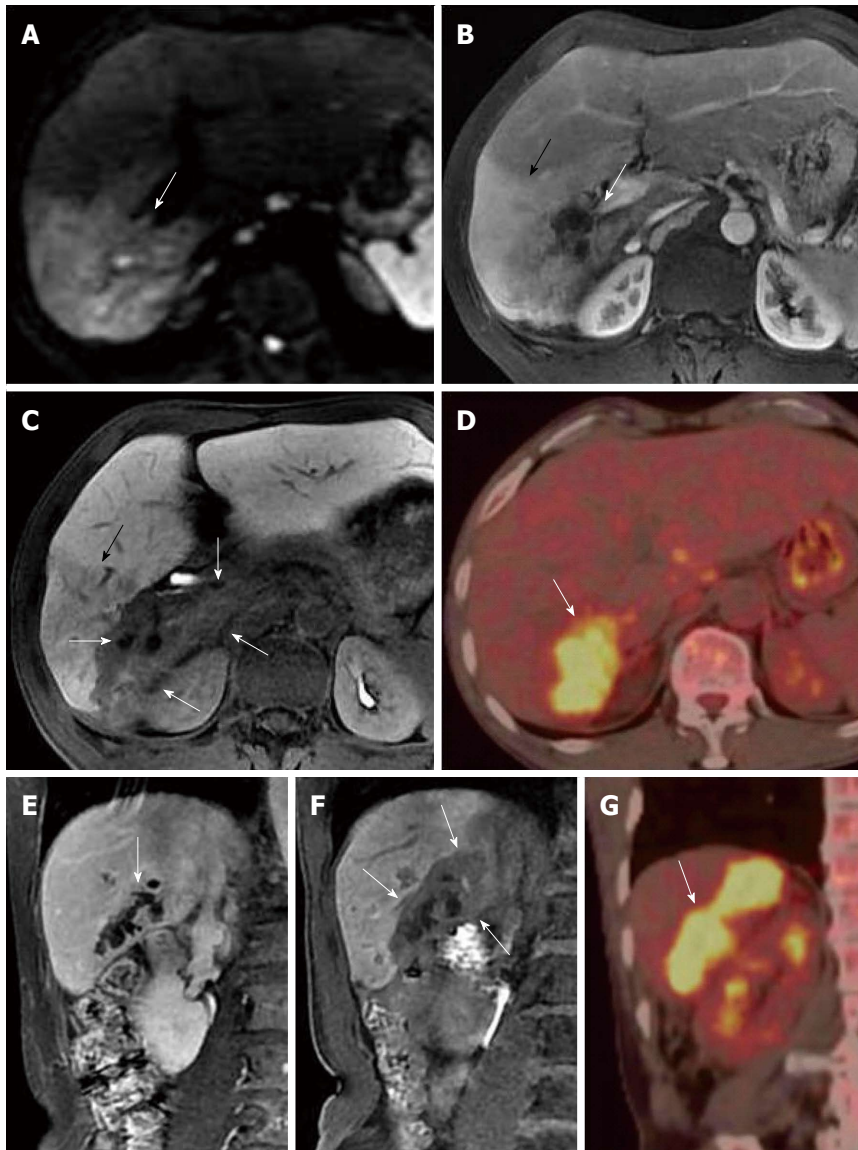
Cases 1-3 underwent duodenal endoscopy to drain mucus from the duodenal papilla within 3 d. In case 4, EOB-enhanced MRI showed tumor distribution in the left and right hepatic ducts, but no signs of extra-biliary tumor invasion, therefore, biliary tumor enucleation and T tube drainage were performed. During the procedure, the dilated bile duct was filled with mucus, and multiple nodular or papillary tumors

were observed in the bile duct.

Microscopic examination of the biopsies from cases 1-4 showed cuboidal or columnar epithelial cells around the fibers and vessels growing within the bile duct, with no tumor invasion beyond the bile duct. Immunohistochemical results were: mucin (MUC)1<sup>+</sup>, MUC2<sup>+</sup>, MUC5AC<sup>+</sup>, MUC6<sup>+</sup>, and p53<sup>+</sup>.

Based on the findings from imaging, endoscopy, and biopsy or surgical pathology, cases 1 and 3 were pathologically diagnosed as papillary adenoma, and cases 2 and 4, which showed obvious tumor nuclear atypia in the focal lesions, were diagnosed as papillary adenoma accompanied by focal cancer. In case 4, lesions beyond the left dilated bile duct were removed, and the pathology showed infiltration of inflammatory cells without tumor cells, thus, these were diagnosed as inflammatory lesions. Lesions in cases 1 and 2 were confined to the left or right lobe, suggesting lobe resection, although the patients refused surgery. Case 3 involved the left and right intrahepatic bile ducts and lobe resection could not be performed. Hence, cases 1-3 underwent endoscopic nasobiliary drainage and/or percutaneous transhepatic cholangial drainage to alleviate the symptoms.

Case 5 underwent a right hepatic resection. The EOB-enhanced MRI indicated that the anterior right



**Figure 5** Fifty-six-year-old male patient with infiltrative intraductal papillary mucinous neoplasm of the bile duct (case 5). A: Diffusion-weighted imaging showed a hyperintense area in segment VI of the liver (white arrow); B: Gadolinium (Gd)-diethylenetriamine-pentaacetic acid (DTPA)-enhanced magnetic resonance imaging (MRI) in the portal venous phase showed marked enhancement in the anterior subsegment (black arrow) and a slightly hypointense area in the posterior subsegment of segment VI (white arrow); C: Gd-ethoxybenzyl (EOB)-DTPA-enhanced MRI in the hepatobiliary phase revealed bile duct dilatation in the posterior subsegment of segment VI without contrast filling. The hypointense area around the dilated bile duct indicated tumor infiltration to the surrounding tissue (white arrows). The anterior subsegment of segment VI with a slightly decreased low signal intensity suggested an inflammatory change (black arrow); D: Positron emission tomography-computed tomography (CT) confirmed the tumor in the posterior subsegment of segment VI with substantial uptake of fluorodeoxyglucose (white arrow), suggesting active tumor metabolism; E: Coronal Gd-DTPA-enhanced MRI in the equilibrium phase showed serrated changes in the bile duct wall of the posterior subsegment of segment VI, and the extra-biliary tumor infiltration was not well identified (white arrow); F: Gd-EOB-DTPA-enhanced MRI in the hepatobiliary phase showed bile duct dilatation in the posterior subsegment of segment VI without contrast filling. The tumor infiltration surrounding the dilated bile duct was hypointense and well defined (white arrows); G: Fluorodeoxyglucose uptake by the tumor (white arrow) was also shown in the posterior subsegment of segment VI during positron emission tomography-CT.

liver was inflamed, so the middle hepatic vein was completely retained. During surgery, a mass (6.5 cm × 6.5 cm × 6.0 cm) was seen in the posterior right liver, showing extra-biliary infiltration with dilated bile ducts filling with mucus. Postoperative pathology showed cuboidal or columnar epithelial cells in the tumor tissues growing around the fibers and vessels inside the bile duct. The tumor cells had obvious

nuclear atypia, which invaded outward to the bile duct wall. Immunohistochemistry showed that the cells were MUC1<sup>+</sup>, MUC2<sup>+</sup>, MUC5AC<sup>+</sup>, MUC6<sup>-</sup>, and p53<sup>+</sup>, confirming the diagnosis of infiltrating intraductal papillary mucinous adenocarcinoma of the bile duct. The right anterior liver parenchyma showed interstitial fibrous tissue hyperplasia with chronic inflammatory cell infiltration, which was diagnosed as chronic



inflammation with fibrous tissue hyperplasia.

## DISCUSSION

Surgery is the preferred treatment for IPMN-B. Partial hepatectomy can be performed for localized tumors, whereas bilateral bile duct involvement may require liver transplantation, duodenal papilla dissection, endoscopic nasobiliary drainage, and/or percutaneous transhepatic cholangial drainage<sup>[16-19]</sup>. Biliary tumor enucleation can be performed for tumors that do not invade into the bile duct<sup>[6,20-23]</sup>. Thus, accurate preoperative diagnosis, with determination of the extent of tumor involvement, is crucial for treatment decisions.

Reports by Lim *et al.*<sup>[8-10]</sup> describe the typical imaging findings of IPMN-B. The disease can be clearly diagnosed when imaging findings are indicative of IPMN-B, and the presence of mucin within the dilated bile duct is confirmed. Although duodenal endoscopy, ERCP, biliary tract endoscopy, and endoscopic US can be used to clearly identify the tumor and mucus<sup>[13,24-27]</sup>, these are invasive examinations, the success of which is associated with the proficiency of the operator, and are accompanied by a risk of pancreatitis. Moreover, it is difficult to evaluate upstream of the bile duct obstruction and extra-biliary conditions with these methods<sup>[28-30]</sup>.

Takanami *et al.*<sup>[13]</sup> and Oki *et al.*<sup>[14]</sup> used EOB-enhanced MRI to study four patients with IPMN-B, and found that Gd-EOB-DTPA-enhanced MRI shows the dilated bile duct and tumor, but also the bile duct filling defects caused by mucin retention. Therefore, Gd-EOB-DTPA-enhanced MRI is an alternative to ERCP for the diagnosis of IPMN-B. Indeed, the performance of EOB-enhanced MRI in cases 1 and 2 in the present study is in agreement with the previous reports<sup>[13,14]</sup>. Although biliary sludge and stones can also appear as filling defects in EOB-enhanced MRI, they show a low signal in T2-weighted images that can be differentiated from mucus, as shown in cases 1 and 2. Although the cystic appearance of the IPMN-B in case 2 is similar to cystadenoma or cystadenocarcinoma, these cystic lesions are not connected with the bile duct. Moreover, contrast agent was seen in both the cystic lesion and the bile duct in case 2 during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI.

A large amount of mucin in the bile duct was confirmed during ERCP/operation in cases 3 and 4, which resulted in marked bile duct dilatation and obvious impaired liver function (indicated by elevated total bilirubin levels). Furthermore, liver cell uptake and secretion of EOB into the bile duct was obviously decreased, with none observed in the hepatobiliary phase in the 42-52-min delayed scan. Mucin within the bile duct can be distinguished from bile based on the similarity with signals in the downstream bile duct, lobe, or segmental bile duct where the tumor is not located.

Case 4 showed a long T2-signal nodule with hypo-enhancement at the edge of the left intrahepatic bile duct, which was considered to be focal. However, EOB-enhanced imaging during the hepatobiliary phase showed gradual enhancement of the lesion area, consistent with chronic inflammation. Therefore, the patient underwent biliary tumor enucleation and T tube drainage, a procedure that could accelerate the disease if the tumor had invaded tissue outside of the bile duct. Therefore, when multifocal IPMN-B occurs in intrahepatic bile ducts, EOB-enhanced MRI in the hepatobiliary phase can be used to select the appropriate treatment.

Importantly, case 5 describes imaging results from an invasive IPMN-B, which cannot be discerned with conventional-enhanced CT or Gd-DTPA-enhanced MRI<sup>[1,6,16]</sup>. With similar cases, it is difficult to judge the extent of tumor invasion into the extrahepatic bile duct by routine inspection, as demonstrated in this patient. The extent of the infringement was clear by PET-CT examination, and the FDG uptake suggested that the tumor was malignant, although it is difficult to distinguish this tumor type from cholangiocarcinoma and other malignant liver tumors. Takanami *et al.*<sup>[31]</sup> also reported PET-CT manifestations of invasive IPMN-B, observed as high intake of FDG. Despite its advantage, PET-CT should be used very carefully due to the relatively high cost and risk of radioactive damage to patients. Case 5 further demonstrates that normal liver cells uptake Gd-EOB-DTPA, whereas tumor cells do not, thus more clearly defining the extent of liver invasion. Moreover, case 5 shows that the signal intensity of the right anterior liver in the hepatobiliary phase is intermediate between normal liver parenchyma and tumor, representing the nature of inflammatory tissue with decreased liver function (EOB uptake). In this case, the tumor, inflammation, and normal liver tissues were clearly demarcated, thus we decided to retain the middle hepatic vein during the right liver resection, and were confident in the negative cut edge of the tumor.

In conclusion, patients with suspected IPMN-B based on imaging findings would benefit from EOB-enhanced MRI for determination of the presence of mucus and extent of extra-biliary invasion.

## COMMENTS

### Background

Reports and awareness of intraductal papillary mucinous neoplasm of the bile duct (IPMN-B) are increasing. However, difficulties remain with noninvasive methods for diagnosis and determining the extent of the tumor.

### Research frontiers

This study reports the use of a novel imaging technique, gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI), for diagnosing IPMN-B.

### Innovations and breakthroughs

This study shows that Gd-EOB-DTPA-enhanced MRI can reveal bile duct filling defects due to mucin, which is highly suggestive of a diagnosis for IPMN-B. Compared with positron emission tomography-computed tomography, Gd-EOB-



DTPA-enhanced MRI can be used to differentiate inflammatory lesions from tumor tissue. Therefore, Gd-EOB-DTPA-enhanced MRI facilitates accurate diagnosis and proper management of IPMN-B.

### Applications

EOB-enhanced MRI is a useful, noninvasive method to detect mucus and assess the extent of extra-biliary invasion in patients whose imaging findings indicate IPMN-B.

### Terminology

Gd-EOB-DTPA is a double-specific contrast agent that is absorbed by hepatocytes and drained *via* the bile duct in the hepatobiliary phase, and can be used to detect filling defects due to mucus. It can also be used to distinguish between tumor cells, which do not take up the contrast agent, and inflammatory cells, which show reduced signal intensity compared to normal liver tissue.

### Peer-review

This is an interesting case series of patients with IPMN-B, in which Gd-EOB-DTPA-enhanced MRI was applied for better evaluation of the lesion and differentiation between tumor invasion and inflammation.

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## Retrospective Study

# When and why a colonoscopist should discontinue colonoscopy by himself?

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**Author contributions:** Gan T and Yang JL contributed equally to this work; Wu JC, Wang YP and Yang L designed the research; Gan T, Yang JL and Wu JC performed the research; Gan T, Yang JL and Yang L analyzed the data; and Gan T and Yang JL wrote the paper.

**Ethics approval:** The study was reviewed and approved by the Huaxi Hospital Institutional Review Board.

**Informed consent statement:** All study participants provided informed written consent prior to their colonoscopy examination.

**Conflict-of-interest statement:** No conflict-of-interest exists.

**Data sharing statement:** No additional data are available.

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## Abstract

**AIM:** To investigate when and why a colonoscopist should discontinue incomplete colonoscopy by himself.

**METHODS:** In this cross-sectional study, 517 difficult colonoscope insertions (Grade C, Kudo's difficulty classification) screened from 37800 colonoscopy insertions were collected from April 2004 to June 2014 by three 4<sup>th</sup>-level (Kudo's classification) colonoscopists. The following common factors for the incomplete insertion were excluded: structural obstruction of the colon or rectum, insufficient colon cleansing, discontinuation due to patient's discomfort or pain, severe colon disease with a perforation risk (*e.g.*, severe ischemic colonopathy). All the excluded patients were re-scheduled if permission was obtained from the patients whose intubation had failed. If the repeat intubations were still a failure because of the difficult operative techniques, those patients were also included in this study. The patient's age, sex, anesthesia and colonoscope type were recorded before colonoscopy. During the colonoscopic examination, the influencing factors of fixation, tortuosity, laxity and redundancy of the colon were assessed, and the insertion time (> 10 min or ≤ 10 min) were registered. The insertion time was analyzed by *t*-test, and other factors were analyzed by univariate and multivariate logistic regression.

**RESULTS:** Three hundred and twenty-two (62.3%) of the 517 insertions were complete in the colonoscope insertion into the ileocecum, but 195 (37.7%) failed in the insertion. Fixation, tortuosity, laxity or redundancy occurred during the colonoscopic examination. Multivariate logistic regression analysis revealed that fixation (OR = 0.06, 95%CI: 0.03-0.16, *P* < 0.001) and tortuosity (OR = 0.04, 95%CI: 0.02-0.08, *P* < 0.001) were significantly related to the insertion into the ileocecum in the left hemicolon; multivariate logistic regression analysis also revealed that fixation (OR = 0.16, 95%CI: 0.06-0.39, *P* < 0.001), tortuosity (OR

0.23, 95%CI: 0.13-0.43,  $P < 0.001$ ), redundancy (OR = 0.12, 95%CI: 0.05-0.26,  $P < 0.001$ ) and sex (OR = 0.35, 95%CI: 0.20-0.63,  $P < 0.001$ ) were significantly related to the insertion into the ileocecum in the right hemicolon. Prolonged insertion time ( $> 10$  min) was an unfavorable factor for the insertion into the ileocecum.

**CONCLUSION:** Colonoscopy should be discontinued if freedom of the colonoscope body's insertion and rotation is completely lost, and the insertion time is prolonged over 30 min.

**Key words:** Colonoscopy; Colonoscopy insertion; Insertion technique

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**Core tip:** This original article investigated when and why a colonoscopist should discontinue incomplete colonoscopy by himself. If freedom of the colonoscope body's insertion and rotation is lost because of unfavorable factors, such as fixation, tortuosity, laxity, and redundancy occurring in the colon, and the insertion time is prolonged  $> 30$  min after repeated attempts by the 4<sup>th</sup>-level colonoscopists, we suggest the colonoscopy should be discontinued by the colonoscopist.

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## INTRODUCTION

Because of the advances of colonoscopic techniques<sup>[1,2]</sup> and the improvements in the design and construction of the colonoscope<sup>[3,4]</sup>, colonoscopy has not been a difficult procedure for most colonoscopists; even the colonoscope insertion into the ileocecum has not been as tough a task as it used to be. However, there are still some difficulties in the insertion of the colonoscope. According to the reports in the medical literature, some factors caused incomplete colonoscopy, e.g., the patient's age, sex, obesity, preoperative bowel preparation, previous abdominal surgery, and constipation<sup>[5-7]</sup>. But all these factors can only roughly predict that these patients are difficult to intubate to the ileocecum, and even highly-skilled 4<sup>th</sup>-level colonoscopists (Kudo's classification<sup>[3]</sup>, Table 1) were unable to guarantee a 100% success rate when ileocecal intubation was performed. Therefore, to determine when and understand why this kind of colonoscopic intubation should be discontinued is very important to colonoscopists. The present study was designed to investigate this problem.

## MATERIALS AND METHODS

From April 2004 to June 2014, a total of 37800 colonoscopy insertions were performed by three 4<sup>th</sup>-level expert colonoscopists at our endoscopic center, who had performed  $> 10000$  on average and whose completion rate of the insertion into the ileocecum was 95%-98%. In order to explore the extraordinary factors for incomplete colonoscopy insertion into the ileocecum, we excluded the following commonly-encountered factors for incomplete colonoscopy insertion into the ileocecum: structural obstruction of the colon or the rectum; insufficient colon cleansing; discontinuation due to patient's discomfort or pain; severe colon disease with a perforation risk (e.g., severe ischemic colonopathy). All the excluded patients were re-scheduled if permission was obtained from the patients whose intubation had failed. If the repeat intubations were still a failure because of the difficult operative techniques, those patients were also included in this study. Thus, 517 patients were included, who underwent the most difficult colonoscopic procedures (Grade C, Kudo's difficulty classification<sup>[3]</sup>, Table 2) by three 4<sup>th</sup>-level expert colonoscopists. Of the 517 patients, 322 (62.3%) completed the colonoscopy insertion into the ileocecum, but 195 (37.7%) had incomplete colonoscopy insertion into the ileocecum, among whom 81 (41.5%) had an insertion only reaching the right hemicolon and 114 (58.5%) only reaching the left hemicolon. Therefore, the colonoscopists had to discontinue the colonoscopy insertion into the ileocecum. This cross-sectional study investigated the influencing factors for the colonoscopists discontinuing the colonoscopic examination, and discussed when and why the colonoscopists should discontinue the colonoscopic examination.

Complete colonoscopy insertion into the ileocecum was judged by the colonoscopist, who could successfully observe the ileocecal valve, appendiceal orifice, or terminal ileum. If any doubt existed, the colonoscopist considered the insertion an incomplete insertion into the ileocecum<sup>[8]</sup>. The time required for the colonoscopy to reach the proximal end of the colon was defined as the insertion time<sup>[8]</sup>.

The data collected in a retrospective manner and taken from the computer graphic database included the following preoperative indexes: the patient's age; sex; type of anesthesia for colonoscopy; type of colonoscope (variable-stiffness or not) and the following intraoperative indexes: fixation; tortuosity; laxity; and redundancy occurring during colonoscopic examination. Fixation was defined as resistance during the insertion or the pull back of the colonoscope body without loop formation, which meant the body was 40 cm into the colon cavity, after the colonoscope head passed through the descending sigmoid flexure, or 60 cm near the hepatic flexure by the removal of



**Table 1 Kudo's colonoscopist level classification**

Colonoscopist level	Presentation of correlative level
I	Mostly beginners, able to push forward the colonoscope body in the colon cavity; unable to use the method of the colon axis constriction to shorten the length of the colon
II	Able to push the colonoscope through the descending sigmoid flexure by $\alpha$ -loop or N-loop, and pull back the colonoscope to set free the loop when the colonoscope head reaches the transverse colon; then, use the method mentioned above
III	Able to control the colonoscope passing through the descending sigmoid flexure by $\alpha$ -loop or N-loop, and pull back the colonoscope to set free the loop when it reaches the descending colon; then, use the above-mentioned method
IV	Able to control the colonoscope and keep the colon axis constriction from rectosigmoid flexure, pushing the colonoscope passing through the descending sigmoid flexure without loop formation

the colon loop. Fixation was caused by abdominal surgical adhesions<sup>[9]</sup>, peritonitis, abdominopelvic cancer, abdominopelvic radiotherapy, or intestinal adhesions of unknown origin. Tortuosity was defined as sharp turns<sup>[9]</sup> or convolutions of the colon during the insertion of the colonoscope body without loop formation, which often occurred in the physiological flexures, *e.g.*, the descending sigmoid flexure and the rectosigmoid flexure. Laxity was defined as no or low resistance during the insertion of the colonoscope; even if the loop formation was felt by the colonoscopists, the colonoscope body was still easy to insert, but the head could not go deep into the colon cavity. Redundancy was defined as tedium of the colon during the insertion of the colonoscope without resistance or loop formation, but the colon could not be shortened because of the loss of the hooked points by the physiological flexure.

General anesthesia: sulfentanyl 0.1  $\mu$ g/kg was given *via* a slow intravenous injection (iv); then, midazolam 1 mg iv after the nasal oxygen inhalation. If the patient had stable vital signs with no bucking or body movements, propofol 1.5-2.5 mg/kg *iv* could be given at a rate of 2 mL/10-20 s. The patient's spontaneous respiration should be kept, and all the body muscles should be relaxed without lash reflex<sup>[10]</sup>. If the patient was restless, propofol 0.5-1 mg/kg was given, maintaining the patient in a painless state until the end of the examination.

Types of colonoscopes: variable-stiffness colonoscopes<sup>[11,12]</sup> including CF 240AH and CF 240AI (Olympus Optical Co. Ltd., Tokyo, Japan); invariable-stiffness colonoscopes including CF 240, CF 240I (Olympus Optical Co. Ltd., Tokyo, Japan), and Fujinon EC-410D (Fujinon Optical Co. Ltd., Tokyo, Japan).

Statistical analysis was performed using SPSS (version 13.0). *t*-test, Univariate and multivariate logistic regression analyses were performed, and a *P* value < 0.05 was considered statistically significant.

**Table 2 Kudo's classification of colonoscopy difficulty**

Difficulty classification	Presentation of correlative pattern
Grade A	Relatively short sigmoid colon, easy to be shortened. Applied to most young and middle-aged men. 2-3 min taken to reach the cecum
Grade B	Lengthy sigmoid colon and relatively tortuous descending sigmoid flexure, easier to form a loop
Grade C	Sigmoid colon with local or partial adhesion after abdominal disease, surgery, or unusually long sigmoid colon, with or without obvious tortuous descending sigmoid flexure, easier to form a loop

## RESULTS

### General conditions of patients

Of the 517 patients undergoing colonoscopic examination, 322 ( $\geq 60$  years in 153; < 60 years in 169; 136 male, 186 female; 201 given anesthesia, 121 given no anesthesia; 171 using variable-stiffness colonoscope, 151 using invariable-stiffness colonoscope) had a complete insertion into the ileocecum, and the other 195 had an incomplete insertion into the ileocecum, among whom 81 ( $\geq 60$  in 41, < 60 in 40; 57 male, 24 female; 53 given anesthesia, 28 given no anesthesia; 46 using variable-stiffness colonoscope, 35 using invariable-stiffness colonoscope) had the colonoscope reaching the right hemicolon, and the other 114 ( $\geq 60$  in 42, < 60 in 72; 60 male, 54 female; 73 given anesthesia, 38 given no anesthesia; 53 using variable-stiffness colonoscope, 61 using invariable-stiffness colonoscope) had the colonoscope reaching the left hemicolon.

### Incompletion rates and reasons for colonoscopy being discontinued by colonoscopists

Of the 195 incomplete colonoscopy insertions, 81 (41.5%) were discontinued by the colonoscopists when the endoscope reached the right hemicolon (ascending colon, hepatic flexure, transverse colon) and the other 114 (58.5%) were discontinued when the endoscope reached the left hemicolon (between rectosigmoid flexure and splenic flexure). The most common sites where the endoscope insertions were discontinued were the hepatic flexure in the right hemicolon (45 cases, 23.1%) and the descending sigmoid flexure (40 cases, 20.5%) in the left hemicolon. The reasons for discontinuation by the colonoscopists were fixation, tortuosity, laxity, and redundancy occurring in the left hemicolon and the right hemicolon (Table 3).

### Comparisons of influencing factors between difficult colonoscopy insertion into the left hemicolon and difficult but complete colonoscopy insertion into ileocecum

Among the 322 difficult insertions into the ileocecum, 223 (69.3%) encountered fixation, 144 (44.7%) encountered tortuosity, 93 (28.9%) encountered laxity, and 25 (7.8%) encountered redundancy.

**Table 3** Sites for colonoscopy discontinuation and reasons for discontinuation *n* (%)

Site	Value	Fixation	Tortuosity	Laxity	Redundance
Right hemicolon					
Ascending colon	10 (5.1)	9	4	3	3
Hepatic flexure	45 (23.1)	41	30	5	12
Transverse colon	26 (13.3)	22	15	2	7
Left hemicolon					
Splenic flexure	9 (4.6)	8	8	2	0
Descending colon	14 (7.2)	13	11	4	1
Descending sigmoid flexure	40 (20.5)	35	39	6	2
Sigmoid colon	29 (14.9)	27	26	2	1
Rectosigmoid flexure	22 (11.3)	22	19	0	0
Total	195 (100)	177	152	24	26

**Table 4** Factors related to sites for colonoscope reaching left hemicolon and right hemicolon

	Left hemicolon			Right hemicolon		
	OR	95%CI	P value	OR	95%CI	P value
Preoperative variable						
Sex (female <i>vs</i> male)	0.69	0.41-1.17	0.17	0.35	0.20-0.63	< 0.001
Age (> <i>vs</i> ≤ 60 yr)	1.63	0.95-2.80	0.08	0.88	0.50-1.52	0.64
Anesthesia (yes <i>vs</i> no)	0.74	0.43-1.30	0.30	0.98	0.55-1.75	0.93
Type of colonoscope (yes <i>vs</i> no)	0.88	0.50-1.53	0.64	1.11	0.62-2.00	0.72
Intraoperative variable						
Fixation (yes <i>vs</i> no)	0.06	0.03-0.16	< 0.001	0.16	0.06-0.39	< 0.001
Tortuosity (yes <i>vs</i> no)	0.04	0.02-0.08	< 0.001	0.23	0.13-0.43	< 0.001
Laxity (yes <i>vs</i> no)	0.56	0.24-1.33	0.19	1.16	0.50-2.71	0.73
Redundancy (yes <i>vs</i> no)	0.80	0.21-3.10	0.75	0.12	0.05-0.26	< 0.001

Among the 114 difficult insertions that only reached the left hemicolon, 105 (92.1%) encountered fixation, 103 (90.4%) encountered tortuosity, 14 (12.3%) encountered laxity, and 4 (3.5%) encountered redundancy.

Univariate analysis revealed that there were significant differences in fixation and tortuosity between the above two conditions ( $P < 0.001$ ). They could be used as an indicator for colonoscopy, and they were the inverse factors significantly related to the colonoscope insertion into the ileocecum. However, difficulties of insertion into the ileocecum were not associated with the patient's age, sex, anesthesia use, colonoscope type or the colon influencing factors, *e.g.*, laxity and redundancy.

Multivariate logistic regression analysis also revealed that fixation (OR = 0.06, 95%CI: 0.03-0.16,  $P < 0.001$ ) and tortuosity (OR = 0.04, 95%CI: 0.02-0.08,  $P < 0.001$ ) were independent inverse factors significantly related to the insertion into the ileocecum, but the other indexes, *e.g.*, age, sex, laxity, and redundancy, were not that kind of factor ( $P > 0.05$ ) (Table 4).

#### **Comparisons of influencing factors between difficult colonoscope insertion into the right hemicolon and difficult but complete colonoscope insertion into the ileocecum**

Among the 81 difficult insertions reaching the right hemicolon, 72 (88.9%) encountered fixation,

49 (60.5%) encountered tortuosity, 10 (12.3%) encountered laxity, and 22 (27.2%) encountered redundancy.

Univariate analysis revealed that there were significant differences in fixation, tortuosity and redundancy between the above two conditions ( $P < 0.001$ ). They could be used as an indicator for colonoscopy, and they were the inverse factors significantly related to the colonoscope insertion into the ileocecum. The insertions only reaching the right hemicolon in the male patients were significantly greater in frequency than those in the female patients ( $P < 0.001$ ). However, difficulties of insertion into the ileocecum were not associated with the patient's age, anesthesia use, colonoscope type or colon influencing factors, *e.g.*, laxity.

Multivariate logistic regression analysis also revealed that fixation (OR = 0.16, 95%CI: 0.06-0.39,  $P < 0.001$ ), tortuosity (OR = 0.23: 95%CI: 0.13-0.43,  $P < 0.001$ ), redundancy (OR = 0.12, 95%CI: 0.05-0.26,  $P < 0.001$ ), and sex (OR = 0.35, 95%CI: 0.20-0.63,  $P < 0.001$ ) were independent inverse factors significantly related to insertion into the ileocecum, but the other indexes, *e.g.*, age, laxity, and redundancy were not that kind of factor ( $P > 0.05$ ) (Table 4). There were no colonoscopy-related complications during or within 7 d of the procedure.

#### **Factors influencing insertion time in left hemicolon**

In the 322 difficult colonoscope insertions into the

**Table 5** Factors related to insertion time (> 10 min) in left hemicolon and right hemicolon

	Left hemicolon			Right hemicolon		
	OR	95%CI	P value	OR	95%CI	P value
Preoperative variable						
Sex (female <i>vs</i> male)	1.30	0.86-1.96	0.22	1.47	0.96-2.26	0.08
Age (> 60 yr <i>vs</i> ≤ 60 yr)	1.18	0.78-1.79	0.43	1.35	0.88-2.06	0.17
Anesthesia (yes <i>vs</i> no)	0.68	0.44-1.03	0.07	0.84	0.54-1.30	0.43
Type of colonoscope (yes <i>vs</i> no)	0.68	0.45-1.04	0.08	0.61	0.39-0.94	0.02
Intraoperative variable						
Fixation (yes <i>vs</i> no)	0.99	0.55-1.79	0.98	1.92	1.04-3.52	0.04
Tortuosity (yes <i>vs</i> no)	1.80	1.14-2.86	0.01	2.40	1.51-3.82	< 0.001
Laxity (yes <i>vs</i> no)	1.62	0.92-2.85	0.10	1.53	0.87-2.70	0.14
Redundancy (yes <i>vs</i> no)	2.44	1.09-5.44	0.03	4.65	2.30-9.39	< 0.001

ileocecum, the insertion mean time was  $9.6 \pm 4.4$  min (range: 2.9-44.4 min); in the 114 difficult colonoscopy insertions only reaching the left hemicolon, the insertion mean time was  $9.4 \pm 7.2$  min (range: 1.4-42.5 min). No significant difference was found in the insertion mean time between the above two conditions ( $P > 0.2$ ), but based on the univariate analysis, a significant difference was still found in tortuosity ( $P < 0.05$ ) and redundancy ( $P < 0.05$ ) between the above two conditions. They could be used as an indicator for colonoscopy, and they were significant factors related to the insertion time (> 10 min) in the patients whose colonoscopy only reached the left hemicolon. However, the time for the colonoscopy insertion into the ileocecum was not related to the patient's age, anesthesia use, colonoscopy type or the colon influencing factors, *e.g.*, fixation and laxity.

Multivariate logistic regression analysis also revealed that tortuosity (OR = 1.80, 95%CI: 1.14-2.86,  $P = 0.01$ ) and redundancy (OR = 2.44, 95%CI: 1.09-5.44,  $P = 0.03$ ) were significant factors related to the insertion time (>10 min) but the other indexes, *e.g.*, age, sex, fixation, and laxity were not that kind of factors ( $P > 0.05$ ) (Table 5).

#### Factors influencing the insertion time in the right hemicolon

In the 81 difficult colonoscopy insertions only reaching the right hemicolon, the insertion mean time was  $17.6 \pm 7.8$  min (range: 5.1-48.7 min) but the insertion mean time for the colonoscopy insertions into the ileocecum was  $9.6 \pm 4.4$  min (range: 2.9-44.4 min). There was a significant difference in the insertion mean time between the above two conditions ( $P < 0.01$ ).

Univariate analysis revealed that fixation ( $P < 0.05$ ), tortuosity ( $P < 0.01$ ) and redundancy ( $P < 0.01$ ), which could be used as an indicator for colonoscopy, were significant factors related to the insertion time (> 10 min) in patients whose colonoscopy insertion only reached the right hemicolon, but the type of colonoscopes were inverse factors significantly related to the insertion time (> 10 min) in patients whose

colonoscopy insertion only reached the right hemicolon ( $P < 0.05$ ). However, the time for the insertion into the ileocecum was not associated with the patient's age, sex, anesthesia use or the colon influencing factor, *e.g.*, laxity.

Multivariate logistic regression analysis also revealed that fixation (OR = 1.92; 95%CI: 1.04-3.52,  $P = 0.04$ ), tortuosity (OR = 2.40, 95%CI: 1.51-3.82,  $P < 0.001$ ), and redundancy (OR = 4.65, 95%CI: 2.30-9.39,  $P < 0.001$ ) were significant factors related to the insertion time (> 10 min), but the types of colonoscopes were independent inverse factors significantly related to the insertion time (> 10 min). However, the other indexes, *e.g.*, age, sex, anesthesia use, and laxity were not related to insertion time ( $P > 0.05$ ) (Table 5).

The reasons for discontinuation of colonoscopy insertions by the colonoscopists were fixation (72, 36.9%; 105, 53.8%), tortuosity (49, 25.1%; 103, 52.8%), laxity (10, 5.0%; 14, 7.2%), and redundancy (22, 11.3%; 4, 2.1%) in the right hemicolon and the left hemicolon, respectively.

## DISCUSSION

In the field of digestive endoscopy, colonoscopy is a challenging procedure only secondary to small intestine endoscopy in the toughness degree. So, a safe and efficient colonoscopy insertion into the ileocecum is a fundamental task for all the colonoscopists<sup>[8]</sup>. The success in colonoscopy chiefly depends on the following three factors: patients, colonoscopists, and types of colonoscopes. Among these three factors, the colonoscopists are the most important factor, whose operative skills play a decisive role in successful colonoscopy. The colonoscopists are usually confronted by the following four difficulties: fixation, tortuosity, laxity, and redundancy occurring in the colon, which lead to the loss of freedom of the colonoscopy body's insertion and rotation. If the colonoscopists could not overcome one or more difficulties, successful colonoscopy insertion into the ileocecum would be an arduous task. According to Kudo's classification criterion<sup>[3]</sup>, the 4<sup>th</sup>-

level (top level) colonoscopist can smoothly insert the colonoscope into the ileocecum within 3-5 min though faced by the Grade C (the most difficult grade) patient without any obvious looping formation, achieving a 95%-98% success rate of insertion into the ileocecum.

To solve the problem of the remaining (2%-5%) incomplete colonoscope insertions into the ileocecum, so many pre-examination evaluation indexes were put forward in the medical literature<sup>[5,13-15]</sup>, *e.g.*, the patient's body posture, age, sex, body mass index, constipation, and previous abdominal surgery.

The results of the study revealed that the influencing factors for the colonoscope insertion only reaching the left hemicolon were fixation and tortuosity occurring in the colon, and the influencing factors for the insertion only reaching the right hemicolon were fixation, tortuosity, and redundancy. The results also revealed that male patients were more likely to have their insertion only reaching the right hemicolon than female patients. This finding could be explained by the male patients being more likely to have redundancy in the colon. The other factors were not so closely correlated with difficult insertion into the ileocecum.

No statistically significant difference was found in the insertion mean time between the insertions only reaching the left hemicolon and the insertions completely into the ileocecum ( $P > 0.2$ ); however, a significant difference was found in the insertion mean time between the insertions only reaching the right hemicolon and the insertions completely into the ileocecum ( $P < 0.01$ ).

Compared with the difficult insertions into the ileocecum, the insertions only reaching the left hemicolon encountered the same difficult factors (fixation, tortuosity), so there is no difference in the overall time expenditure for a 4<sup>th</sup>-level expert colonoscopist.

As for the insertion reaching the right hemicolon, the main difficulty was usually due to redundancy in the right hemicolon. The colonoscopists would not easily discontinue the insertion but attempted to use posture change, abdominal compression, hooking the fold for removal of the colon loops to shorten the colon and inserting the colonoscope into the ileocecum. When they still failed and finally discontinued the insertion, much more time was used. This kind of discontinuation was relatively great in proportion in clinical practice.

In the colonoscope insertions reaching the left hemicolon and the right hemicolon, the factors unfavorably influencing the insertion mean time ( $> 10$  min) were different in the following two conditions: tortuosity and redundancy often occurred in the left hemicolon, but fixation, tortuosity, and redundancy often occurred in the right hemicolon. The variable-stiffness colonoscope was a favorable factor for reducing the mean insertion time, but only favorable factor for the colonoscope insertion reaching the right hemicolon.

Evaluation on discontinuation of the colonoscope insertion during colonoscopic examination was made in this study. According to the Kudo's Classification criterion<sup>[3]</sup>, discontinuation of the most difficult insertions usually occurred in the Grade C patients. The result analysis revealed that fixation and tortuosity were the coexistent difficulty factors for influencing the insertion into the ileocecum in both the left hemicolon and the right hemicolon. Thus, during the colonoscopic examination, the following three indexes for judging whether the colonoscope insertion should be discontinued should be considered.

First, the colonoscope insertion time was the most important index for the most difficult colonoscopy. Once the insertion time was prolonged  $> 10$  min in the colonoscopy performed by the 4<sup>th</sup>-level colonoscopist, this colonoscopy should be considered the most difficult one. At this time, the colonoscopist should determine which influencing factor (fixation, tortuosity, laxity or redundancy) had caused the prolongation of the insertion time, and should determine what position the colonoscope head reached, and what counter-measures should be taken in the next management procedures.

Second, if the colon axis constriction<sup>[3]</sup> could not be achieved or maintained because of the following one or more influencing factors: fixation, tortuosity, laxity, and redundancy, the colonoscopist should use such assisting techniques as posture change, abdominal compression, and hooking the fold for removal of the colon loops after insertion with the loop, to decrease the degrees of the descending sigmoid flexure and/or hepatic flexure to shorten the length of the colon cavity. If those attempts were still a failure, these kind of patients were considered relatively difficult for colonoscopic examination, and colonoscopic examination should be discontinued.

Third, though the colon axis constriction could be achieved after the above-mentioned efforts<sup>[3]</sup>, *i.e.*, the colonoscope body was 40 cm in the colon cavity after the colonoscope passed through the descending sigmoid flexure, or 60 cm near the hepatic flexure, the freedom of the colonoscope body could still not be obtained<sup>[3]</sup>, and paradoxical movements (the head of the colonoscope back off) happened when the colonoscope was further inserted into the colon cavity. Even if the assisting techniques (posture change, abdominal compression, the hooking of the fold for shortening the colon) were attempted, the colonoscope insertion was still a failure. These kinds of patients were considered relatively difficult for colonoscopic examination, and the examination should be discontinued.

#### **Further measures for difficult insertion that may be discontinued**

In management of those difficult colonoscope insertions, 4<sup>th</sup>-level colonoscopists would often choose



to challenge the limits of the colonoscopic operation technology rather than discontinue colonoscopic examination at once. They would attempt to break through the key difficult points, such as the redundant and fixed right hemicolon, and the tortuous and fixed descending sigmoid flexure. Some of the 4<sup>th</sup>-level colonoscopists who had a different management style would be asked to continue the operation<sup>[16]</sup> with professional nurses who were good at the assisting techniques of abdominal compression for the patient<sup>[17,18]</sup>. They succeeded in their attempts to insert the colonoscope into the ileocecum. But how to explain and copy those individualized technical characteristics of those colonoscopists and nurses is still quite difficult.

In addition, some other assisting techniques have been used as further measures, *e.g.*, changing the type of colonoscope<sup>[16]</sup>, using spiral overtube<sup>[19]</sup> or cap-assisted colonoscopy<sup>[20]</sup>, water infusion colonoscopy<sup>[21,22]</sup>, and magnetic endoscopic imaging<sup>[23,24]</sup>, which can be attempted even though no sufficient evidence has been found to verify those techniques used to increase the success rate of colonoscopy insertion into the ileocecum in those difficult patients.

In short, the reasons for discontinuation of colonoscopy insertion into the ileocecum are as follows: in those difficult patients, if the above-mentioned management techniques fail in breaking through those difficult key points after repeated attempts by the expert colonoscopists, the freedom of the colonoscope body in insertion and rotation is completely lost, the colon axis constriction cannot be achieved or maintained after repeated attempts, and the colonoscopy insertion should be discontinued.

Considering the mean time of discontinuation in the left hemicolon (about 10 min) and right hemicolon (about 20 min), the time for the discontinuation of the colonoscopy insertion into the ileocecum is suggested as follows: the total insertion time is prolonged > 30 min after the repeated attempts by the 4<sup>th</sup>-level colonoscopists then the colonoscopy insertion should be discontinued.

The patients whose colonoscopy insertions are discontinued can still use some other instruments and techniques, *e.g.*, virtual colonoscopy<sup>[25,26]</sup>, single<sup>[27]</sup> or double-balloon<sup>[28]</sup> enteroscopy, or colon capsule endoscopy<sup>[29,30]</sup> if their clinical and economic conditions permit.

## COMMENTS

### Background

Colonoscopy is not a difficult procedure for most of the colonoscopists now. However, there are still some difficulties in the insertion of the colonoscope; even highly-skilled colonoscopists are unable to guarantee a 100% success rate when the ileocecal intubation is performed. Therefore, to determine when and recognize why this kind of colonoscopic intubation should be discontinued is very important for the colonoscopists.

### Research frontiers

Many research indexes, such as age, sex and abdominal surgery have been introduced to predict the success rate of ileocecal intubation. In this paper, four

indexes, *i.e.*, fixation, tortuosity, laxity and redundancy were introduced to solve this problem.

### Innovations and breakthroughs

The new research indexes, *i.e.*, fixation, tortuosity, laxity and redundancy could only be obtained by the colonoscopists during the examination. These indexes are precise and direct in clarifying the difficulty of colonoscopy and helping the colonoscopists to decide whether they should continue or discontinue the examination at the proper time.

### Applications

If freedom of the colonoscope body during the insertion and rotation is completely lost because of fixation, tortuosity, laxity and/or redundancy during the examination, and the insertion time is prolonged > 30 min, the colonoscopy should be discontinued.

### Terminology

Colonoscopy is an important commonly-used examination for colonic diseases, such as carcinoma of the colon, polyps of the colon, ulcerative colitis and Crohn's disease. To prevent diagnosis of colonic diseases from failing, the principal task for the colonoscopists to finish is that they should try their best to insert the colonoscope into the ileocecum. Freedom of the colonoscope body during the insertion and rotation will decide its success in the insertion into the ileocecum.

### Peer-review

This is an interesting and generally well written paper. Please recommend language polishing to benefit the wider readership.

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## Observational Study

# Seroepidemiology of hepatitis B virus infection and impact of vaccination

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## Abstract

**AIM:** To investigate hepatitis B virus (HBV) prevalence in the general population in China.

**METHODS:** A total of 148931 individuals were investigated by multistage random sampling in Eastern China. Data were collected on demographics and hepatitis B vaccination history, and serum was tested for hepatitis B surface antigen (HBsAg) by ELISA.

**RESULTS:** A total of 11469 participants (7.70%, 95%CI: 7.57%-7.84%) were positive for HBsAg. HBsAg prevalence was 0.77% among children < 5 years old but increased progressively from adolescents

(1.40%-2.55%) to adults (5.69%-11.22%). A decrease in HBsAg prevalence was strongly associated with vaccination and familial history of HBV among both children and adult groups. Meanwhile, HBsAg risk in adults was associated with invasive testing and sharing needles. The HBV immunization rate among participants aged < 20 years was 93.30% (95%CI: 93.01%-93.58%). Significant difference in HBsAg prevalence appeared between vaccinated and unvaccinated participants (3.59% *vs* 10.22%).

**CONCLUSION:** Although the national goal of HBsAg prevalence < 1% among children < 5 years old has been reached, immunization programs should be maintained to prevent resurgence.

**Key words:** Epidemiological study; Familial history; Hepatitis B surface antigen; Immunization; General population

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**Core tip:** A total of 148931 individuals were investigated in Eastern China to evaluate the impact of hepatitis B vaccination since 1992. 7.70% were positive for hepatitis B surface antigen (HBsAg) which has not achieved the national goal for the whole population in decreasing the prevalence of HBsAg to < 7%. Prevalence was 0.77% among children aged < 5 years and the rate of hepatitis B virus immunization among teenagers aged < 20 years was 93.30%, which have reached the national goals of < 1% and > 90%, respectively.

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## INTRODUCTION

Due to its global prevalence and potential for adverse consequences such as cirrhosis and hepatocellular carcinoma, chronic hepatitis B virus (HBV) infection has become a serious public health problem<sup>[1,2]</sup>. The WHO estimates there are approximately 2 billion people infected by HBV worldwide, and 240 million of them have chronic infection. Each year an estimated 600000 people die from liver diseases caused by HBV infection<sup>[3]</sup>. The distribution of HBV infection is uneven, and sub-Saharan Africa, Asia, and the Pacific Islands, where HBV is most commonly spread from mother to child at birth or from person to person in early childhood, have the most significant burden. In China, a highly endemic area, the national prevalence

for hepatitis B surface antigen (HBsAg) positivity is 7.20%<sup>[2,4]</sup>.

The Centers for Disease Control and Prevention (CDC) reported that the incidence of newly acquired HBV infection in China has declined steadily since the 1990s by virtue of a number of public health interventions, such as the screening of pregnant women and the vaccination of infants and adolescents, the promotion of safe injection practices, and the development of health education in general<sup>[4,5]</sup>. Yet, HBV infection prevalence is still high among Chinese high-risk populations such as drug users (ranging from 8.8% to 51.6%) and men who have sex with men (MSM) (ranging from 6.5% to 10.3%)<sup>[6-10]</sup>. Moreover, few studies have evaluated the impact of the hepatitis B vaccination program since 1992 in a large general population, and studies on the relationship between socioeconomic status or familial history and the HBV carrier state are scant.

In this study, we planned a community-based epidemiological and serological study to identify simultaneously the prevalence of HBsAg among all age groups, to determine the potential risk factors for infection, and to evaluate the impact of the hepatitis B vaccination program in Eastern China. These findings are hoped eventually to guide the development, adaptation, and evaluation of prevention strategies for HBV.

## MATERIALS AND METHODS

This study was approved by the Institutional Review Board of Nanjing Medical University (Nanjing, China). All participants provided written informed consent to be interviewed. Planning for this study was started in January 2011 and data analysis was completed in December 2012. All field work was conducted from September 2011 to July 2012.

### Participates

The target population was local residents of all age groups living in Jiangsu provinces, Eastern China (Supplemental Figure 1). Participants were selected as representative of the population of Eastern China, in that there was no statistically significant difference between participants in this study and the whole of Eastern China in demographics, the situation of the population or economic conditions. All local residents who had resided in Jiangsu for > 6 mo at the time of the survey were selected. A list of residents was obtained from the Residents' Committee.

### Sampling method

The sample size was calculated as following:

$$n = p(1 - p)/(d/z_{\alpha})^2 = z_{\alpha}^2 \times p(1 - p)/d^2$$

where  $p$  means the estimated prevalence rate,  $d$  represented power and was calculated by  $0.1 \times p$  and  $z_{\alpha}$  was the statistics of  $\alpha$ . For this study,  $\alpha$  was set 0.05.



Considering the variance of hepatitis C virus prevalence in different age groups in the survey (0.5% for age < 5 years, 1.5% for age 5-19 years, and 8% for age > 19 years), the desired sample size was 110466, and included 79600 people age < 5 years, 26266 people age 5-19 years, and 4600 people age > 19 years.

To represent the population of the whole province, a multistage sampling method was applied. First, the whole province was divided into three major groups (south, middle and north) determined by regional distinctions in earnings, education level, awareness of health care among the inhabitants, and health care system. One county was randomly selected from each major regional group: Zhangjiagang, Danyang and Taixing were chosen to represent south, middle and north sites, respectively. There were 887 communities in these three counties, and 20 communities were randomly chosen in each county. Finally, all local residents from those 60 communities were targeted for participation in this study by cluster sampling.

### Investigation

Before the survey was administered, community doctors issued a notice of physical examination to each household in the list of residents. The letter introduced the survey objective, examination items, and matters needing attention. Local residents willing to participate arrived at the survey spots in the community hospital at the appointed time. In each community hospital, there was a survey location and a team of 20-25 trained staff, including physicians, nurses, and community doctors, to carry out the investigation. A structured questionnaire was used by trained staff to ask basic information such as name, gender, birth date, education, occupation, marital status, smoking, drinking, history of hypertension and diabetes, immunization and familial history of HBV, and possible risk factors. All investigations were conducted through face to face interviews. Information on children under 15 years old was provided by their parents. Educational level was defined by academic certificate. Persons who had frequent contact with the public, such as an employee in a hotel, restaurant, barbershop, and market, were defined as public service workers. Children under 17 years old established their immunization status from the record of the immunization certificate kept by the parents. If they had no information about hepatitis B immunization, it was necessary to review the village doctor's registry; if these sources were not available, the status was recorded as unvaccinated. In cases in which adults had no immunization records, immunization information for adults was from memory recall (vaccinated, unvaccinated, or unknown).

### Serological testing

Blood samples collected from each study participant

included 5 mL for the population aged > 2 years and 2 mL for children aged ≤ 2 years. Blood samples were collected without anticoagulant and were separated by centrifugation at 1800 × *g* for 10 min and room temperature. Serum samples were stored at -70 °C. These procedures were completed within 6 h of sample collection. All samples were collected and frozen according to standardized procedures and tested in a central laboratory. HBsAg was detected by EILSA (Kehua Bio-Engineering Co. Ltd., Shanghai, China). Each reaction plate included two negative controls, three positive controls, and one blank control. More than 10% of the samples were randomly selected for repeated assays, and the results were 97% concordant.

### Statistical analysis

Differences in demographic characteristics between HBsAg-positive and -negative groups were calculated using the Student *t* test or one-way analysis of variance (for continuous variables) and the  $\chi^2$  test (for categorical variables). The associations of potential risk factors with HBV infection risk were estimated by computing the ORs and their 95% CIs from both univariate and multivariate logistic regression analyses. The Cochran-Armitage test was used for trend analysis. All statistical analyses were performed with SAS 9.1.3 software (SAS Institute, Cary, NC, United States), and *P* < 0.05 in a two-sided test was considered statistically significant.

## RESULTS

### Basic characteristics

The characteristics of the study population are shown in Table 1. Among the 148931 participants, 0.79% were aged 1-4 years, 18.83% aged 5-19 years, and 80.38% ≥ 20 years. The male to female ratio was 0.82:1. Among those aged ≥ 20 years, 88.88% were married, 57.27% were illiterate or had a primary school diploma, 33.72% were agricultural workers, and 72.80% and 79.75% never smoked and drank, respectively. The proportion of those adults without a history of hypertension and diabetes were 82.97% and 97.49%, respectively. In all age groups, subjects with a familial history of HBV and those reporting any hepatitis B vaccination were 8.23% and 34.28%.

### Prevalence of HBsAg in the survey

Among the 148931 participants studied, 11469 were HBsAg positive, while the overall prevalence of HBsAg in this study population was 7.70% (95%CI: 7.57%-7.84%). There was a great variation in HBsAg positivity among different age and gender groups. The prevalence was low in children (0.70%-0.77%), but increased progressively from adolescents (1.40%-2.55%) to adults aged ≥ 20 years (5.69%-11.22%) (Figure 1). Meanwhile, men had a higher prevalence of HBV infection than women in all age strata except the 5-9

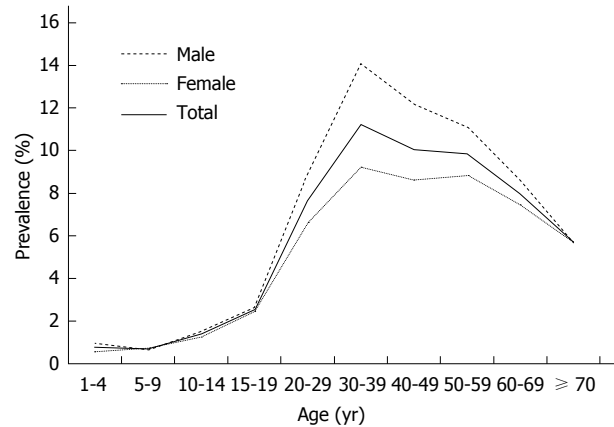
**Table 1** Characteristics of the study population *n* (%)

Variables		Value
Age (yr)	1-4	1175 (0.79)
	5-19	28046 (18.83)
	≥ 20	119710 (80.38)
Sex	Male	67319 (45.20)
	Female	81612 (54.80)
Marital status (≥ 20 yr)	Unmarried	6614 (5.53)
	Married	106395 (88.88)
	Divorced	6264 (5.23)
	Unknown	437 (0.37)
Education (≥ 20 yr)	Illiterate or primary school	68552 (57.27)
	Middle school	43460 (36.31)
	College school	7097 (5.93)
	Unknown	599 (0.50)
Occupation (≥ 20 yr)	Agricultural worker	40363 (33.72)
	Worker	40530 (33.86)
	Student	1248 (1.04)
	Civil servant	5129 (4.28)
	Public service worker	5222 (4.36)
	Unemployed	21763 (18.18)
	Others	4883 (4.08)
	Unknown	572 (0.48)
	Never	87144 (72.80)
Smoking status (≥ 20 yr)	Current	27024 (22.57)
	Past	2731 (2.28)
	Unknown	2811 (2.35)
	Never	95470 (79.75)
Drinking status (≥ 20 yr)	Frequently	5900 (4.93)
	Every day	2132 (1.78)
	Unknown	16208 (13.54)
	< 18.5	4238 (3.54)
BMI (kg/m <sup>2</sup> ) (≥ 20 yr)	18.5-24	59117 (49.38)
	24-28	41559 (34.72)
	≥ 28	14796 (12.36)
	No	99327 (82.97)
History of hypertension (≥ 20 yr)	Yes	20383 (17.03)
	No	116701 (97.49)
History of diabetes (≥ 20 yr)	Yes	3009 (2.51)
	No	132997 (91.06)
	Yes	12016 (8.23)
Familial history of HBV	Unknown	1034 (0.71)
	No	75868 (51.77)
	Yes	50233 (34.28)
Immunization history of HBV	Unknown	20444 (13.95)
	No	75868 (51.77)
	Yes	50233 (34.28)

BMI: Body mass index.

years age group.

The prevalence of HBsAg stratified by other demographic and selected variables in this study population is described in Table 2. Significant differences were observed in marital status, education level, occupation, immunization and familial history of HBV about the prevalence of HBsAg. Among participants aged ≥ 20 years, the HBsAg prevalence among married participants (9.41%, 95%CI: 9.24%-9.59%) was higher than among the unmarried (7.36%, 95%CI: 6.75%-8.02%) or divorced (7.28%, 95%CI: 6.65%-7.95%) participants. HBsAg prevalence increased with decreasing education level. Participants who were illiterate or had primary school diplomas had the highest HBsAg prevalence (9.82%, 95%CI: 9.60%-10.05%), followed by middle school (8.35%, 95%CI: 8.09%-8.62%) and college

**Figure 1** Age, gender-specific prevalence of hepatitis B surface antigen in China.

school groups (8.05%, 95%CI: 7.42%-8.70%). HBsAg prevalence was significantly lower among students (6.81%, 95%CI: 5.48%-8.35%) and unemployed groups (6.74%, 95%CI: 6.41%-7.08%). The prevalence of HBsAg was higher in participants who had no history of hepatitis B vaccine, while it was lower in participants without a family history of HBV ( $P < 0.05$  for both comparisons).

### Risk factors

The frequency of various risk factors associated with HBV infection and the calculated crude OR estimated by univariate and multivariate analysis are shown in Tables 3 and 4. Among the population aged 1-19 years, age, sex, hepatitis B immunization, and familial history of HBV were correlated with HBsAg positivity. However, after adjusting for those variables, older age (OR = 4.12, 95%CI: 1.93-8.75 for 15-19 years vs 1-4 years group) and familial history of HBV (OR = 2.0, 95%CI: 1.88-2.13) were associated with an increased risk of HBV infection. Participants with a history of immunization had a 29% decrease in the risk of HBV infection.

Among the population aged ≥ 20 years, univariate logistic regression showed that age, sex, hepatitis B immunization, familial history of HBV, invasive testing, and sharing needles were correlated with HBsAg positivity. After adjusting for those variables, older age (OR = 1.18, 95%CI: 1.08-1.30 for 30-39 years vs 20-29 years group), familial history of HBV (OR = 1.93, 95%CI: 1.83-2.05), invasive testing (OR = 1.18, 95%CI: 1.11-1.26), and sharing needles (OR = 1.44, 95%CI: 1.12-1.86) were associated with an increased risk of HBV infection. However, female gender and a history of HBV immunization had a significant decrease in the risk of HBV infection (OR = 0.72, 0.52 for gender and immunization, respectively).

### Relationship between familial history of HBV and prevalence of HBsAg

Among the 148931 participants studied, 12016

**Table 2** Prevalence of hepatitis B surface antigen by other demographic and selected variables in Eastern China

Variables	HBsAg negative ( <i>n</i> = 137462)	HBsAg positive ( <i>n</i> = 11469)	Prevalence (%; 95%CI)	<i>P</i> value
Marital status ( $\geq 20$ yr)				< 0.001
Unmarried	6127	487	7.36 (6.75-8.02)	
Married	96383	10012	9.41 (9.24-9.59)	
Divorced	5808	456	7.28 (6.65-7.95)	
Education ( $\geq 20$ yr)				< 0.001
Illiterate or primary school	61819	6733	9.82 (9.60-10.05)	
Middle school	39830	3630	8.35 (8.09-8.62)	
College school	6526	571	8.05 (7.42-8.70)	
Occupation ( $\geq 20$ yr)				< 0.001
Agricultural worker	36186	4177	10.35 (10.05-10.65)	
Worker	36717	3813	9.41 (9.13-9.70)	
Student	1163	85	6.81 (5.48-8.35)	
Civil servant	4672	457	8.91 (8.14-9.72)	
Public service worker	4749	473	9.06 (8.29-9.87)	
Unemployed	20297	1466	6.74 (6.41-7.08)	
Others	4411	472	9.67 (8.85-10.53)	
Immunization history of HBV				< 0.001
No	68111	7757	10.22 (10.01-10.44)	
Yes	48430	1803	3.59 (3.43-3.76)	
Familial history of HBV				< 0.001
No	123472	9525	7.16 (7.02-7.30)	
Yes	10319	1697	14.12 (13.50-14.76)	

HBsAg: Hepatitis B surface antigen.

**Table 3** Risk factors associated with hepatitis B surface antigen positivity among population aged 1-19 years

Variables	uaj. OR (95%CI)	<i>P</i> value	aj. OR (95%CI)	<i>P</i> value
Age group (yr)				
5-9 <i>vs</i> 1-4	0.91 (0.45-1.86)	0.805	1.14 (0.52-2.54)	0.740
10-14 <i>vs</i> 1-4	1.84 (0.93-3.63)	0.078	1.18 (0.86-3.21)	0.095
15-19 <i>vs</i> 1-4	3.39 (1.74-6.60)	< 0.001	4.12 (1.93-8.75)	< 0.001
Sex (female <i>vs</i> male)	0.89 (0.74-1.07)	0.218	-	-
Immunization history of HBV (yes <i>vs</i> no)	0.50 (0.35-0.70)	< 0.001	0.71 (0.49-0.98)	0.028
Familial history of HBV (yes <i>vs</i> no)	2.52 (1.83-3.49)	< 0.001	2.00 (1.88-2.13)	< 0.001

aj. OR: Logistic regression analyses adjusted for age group, familial history of HBV, and immunization history of HBV; uaj.: Unadjusted.

**Table 4** Risk factors associated with hepatitis B surface antigen positivity among population aged > 19 years

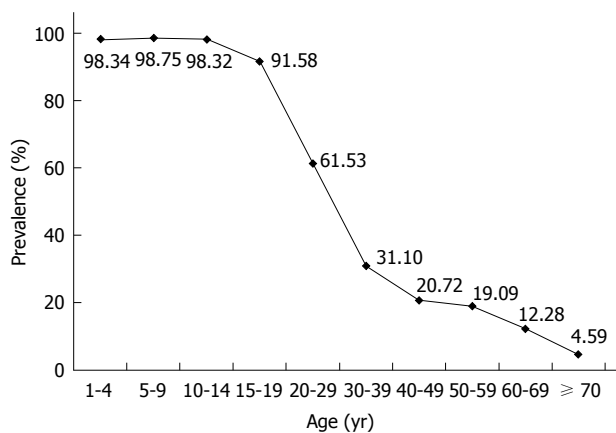
Variables	uaj. OR (95%CI)	<i>P</i> value	aj. OR (95%CI)	<i>P</i> value
Age group (yr)				
30-39 <i>vs</i> 20-29	1.52 (1.41-1.65)	< 0.001	1.18 (1.08-1.30)	< 0.001
40-49 <i>vs</i> 20-29	1.35 (1.25-1.45)	< 0.001	0.97 (0.88-1.06)	0.494
50-59 <i>vs</i> 20-29	1.32 (1.22-1.42)	< 0.001	0.87 (0.79-1.02)	0.108
60-69 <i>vs</i> 20-29	1.04 (0.96-1.13)	0.339	0.84 (0.57-1.02)	0.212
$\geq 70$ <i>vs</i> 20-29	0.92 (0.85-1.10)	0.962	0.81 (0.69-1.02)	0.478
Sex (female <i>vs</i> male)	0.74 (0.71-0.77)	< 0.001	0.72 (0.69-0.75)	< 0.001
Familial history of HBV (yes <i>vs</i> no)	1.90 (1.80-2.01)	< 0.001	1.93 (1.83-2.05)	< 0.001
Immunization history of HBV (yes <i>vs</i> no)	0.56 (0.53-0.59)	< 0.001	0.52 (0.49-0.56)	< 0.001
Hospitalization (yes <i>vs</i> no)	0.95 (0.92-1.04)	0.227	-	-
Surgery (yes <i>vs</i> no)	0.92 (0.85-1.03)	0.312	-	-
Blood transfusion (yes <i>vs</i> no)	1.00 (0.88-1.13)	0.966	-	-
Blood donation (yes <i>vs</i> no)	0.88 (0.75-1.12)	0.215	-	-
Invasive testing (yes <i>vs</i> no)	1.44 (1.36-1.53)	< 0.001	1.18 (1.11-1.26)	< 0.001
Hemodialysis (yes <i>vs</i> no)	0.56 (0.20-1.53)	0.255	-	-
Acupuncture (yes <i>vs</i> no)	0.94 (0.87-1.09)	0.612	-	-
Dental therapy (yes <i>vs</i> no)	0.88 (0.82-1.01)	0.061	-	-
Tattoos (yes <i>vs</i> no)	1.24 (0.84-1.82)	0.280	-	-
Piercing (yes <i>vs</i> no)	0.97 (0.92-1.03)	0.388	-	-
Sharing needles (yes <i>vs</i> no)	1.40 (1.09-1.80)	0.008	1.44 (1.12-1.86)	0.005
Sharing razors (yes <i>vs</i> no)	0.90 (0.81-1.02)	0.478	-	-
Sharing toothbrush (yes <i>vs</i> no)	1.04 (0.76-1.41)	0.814	-	-

aj. OR: Logistic regression analyses adjusted for age group, sex, familial history of HBV, immunization history of HBV, invasive testing, and sharing needles.

**Table 5** Cumulative effects of familial history of hepatitis B virus on hepatitis B virus infection

Variables	HBsAg negative	HBsAg positive	OR (95%CI)	P value
Mother	1031	317	3.57 (3.12-4.08)	< 0.001
Father	2488	432	1.95 (1.75-2.17)	< 0.001
Spouse ( $\geq 20$ yr)	3363	531	1.02 (1.00-1.04)	0.041
Offspring ( $\geq 20$ yr)	1817	283	1.61 (1.41-1.83)	< 0.001
Siblings	1059	353	3.10 (2.73-3.51)	< 0.001
Other families	1061	130	1.57 (1.30-1.89)	< 0.001
Combined familial history of HBV and HBV infection				
0	123472 (92.29)	9525 (84.88)	1	-
1	9838 (7.36)	1562 (13.95)	1.93 (1.82-2.05)	< 0.001
2	462 (0.34)	121 (1.05)	3.19 (2.60-3.92)	< 0.001
3-6	19 (0.01)	14 (0.12)	9.82 (4.80-20.11)	< 0.001
Trend				< 0.001 <sup>1</sup>
0	123472 (92.29)	9525 (84.88)	1	-
1-6	10319 (7.71)	1697 (15.12)	2.00 (1.89-2.12)	< 0.001

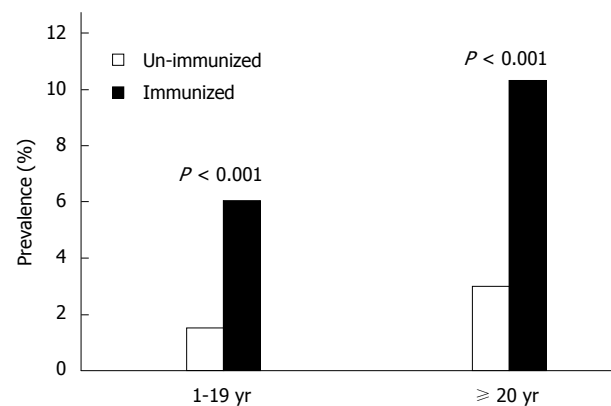
<sup>1</sup>The *P* value of Cochran-Armitage's trend test. Logistic regression analyses adjusted for age, sex, and immunization history of HBV.

**Figure 2** Age-specific prevalence of hepatitis B immunization in China.

(8.28%, 95%CI: 8.14%-8.43%) had a familial history of HBV. When different classes of relatives were analyzed separately, HBV infection risks showed a statistically significant increase for mothers, fathers, spouses, offspring and siblings (Table 5). In particular, mothers and siblings who were HBsAg seropositive significantly increased risk by 25.7% and 21.0%, respectively. We evaluated the combined effects by adding up the number of families with HBsAg seropositivity. The ORs for risk of HBV infection increased concomitantly with the number of HBsAg-seropositive family members (Table 5). Participants with 3-6 family members who were HBsAg seropositive were significantly associated with HBV infection (OR = 9.82, 95%CI: 4.80-20.11), compared with participants without a familial history of HBV.

#### Relationship between hepatitis B immunization and prevalence of HBsAg

Among the 126101 participants effectively surveyed about hepatitis B immunization, 50233 (39.84%, 95%CI: 35.57%-40.11%) had a history of HBV immunization. There was a significant decreasing trend

**Figure 3** Age- and hepatitis B immunization-specific prevalence of hepatitis B surface antigen in China.

in vaccination prevalence associated with increasing age (Figure 2). There was great variation in immunization prevalence among different age groups, ranging from 98.75% to 4.59%. The rate among children aged 1-19 years born after the hepatitis B vaccine was recommended for routine childhood immunization was 93.30% (95%CI: 93.01%-93.58%). Among all hepatitis-B-vaccinated persons in this study, the main methods of immunization were planned immunization and collective organization vaccination (44.84% and 41.10%, respectively). The main reason for lack of immunization was that participants had little information about hepatitis B vaccination (80.60%).

The prevalence of HBsAg among vaccinated persons was only 3.59% (95%CI: 3.56%-3.90%), compared to 10.22% (95%CI: 10.15%-11.63%) among unvaccinated persons (*P* < 0.001). This difference was proportionally greater in adult groups (Figure 3).

## DISCUSSION

We report here a community-based epidemiological study of HBV infection in a large sample from an Eastern



Chinese population. Unlike the previous population-based epidemiological studies that were mainly from Europe and North America, the prevalence of HBV infection and characterization of prevailing hepatitis B immunization in the same population provide new insight into the contribution of HBV to the causation of liver disease in China, and this knowledge may also be relevant to other countries; particularly those with similar levels of endemicity<sup>[11-16]</sup>. Earlier studies of HBV prevalence mostly focused on high-risk populations such as drug users, paid blood donors, and MSM in China and other countries<sup>[6-10,17,18]</sup>. However, owing to low numbers of children and elderly in these groups investigated, their results cannot be extrapolated to the general population.

Without a strict standard of sampling procedures, prior community-based studies on HBV prevalence in Eastern China could not eliminate bias. Compared with these prior studies, this study involved a greater number of participants<sup>[19-21]</sup>. Moreover, the present investigation used a multistage sampling procedure. Zhangjiagang, Danyang and Taixing were chosen to represent southern, middle and northern sites of Jiangsu province, Eastern China, respectively. This aimed to eliminate bias from local social, economic or cultural factors that might be associated with the prevalence of HBV. Twenty communities were randomly chosen in each county. Finally, all local residents in those 60 communities were targeted for participation by cluster sampling. Therefore, the data in this study with a rigorous standard of sampling procedures and a large sample size represents the general population in Jiangsu effectively.

The prevalence of HBsAg (7.70%, 95%CI: 7.57%-7.84%) in the present survey was close to that of the whole country (7.20%) and southeast area (7.90%)<sup>[4,19]</sup>. The age-specific prevalence of HBV in the study was low in children (0.70%-0.77%) but increased progressively from adolescents (1.40%-2.55%) to adults aged  $\geq 20$  years (5.69%-11.22%). This suggests a cumulative increase of incidence of infection, probably as a result of the sporadic transmission of infection persisting in the community through the years. In this survey, men had a higher prevalence of HBV infection than women. This may be because drug users are more likely to be male, which may lead the prevalence among males to be higher, or because cultural factors induce women to focus on their health status and pay more attention to personal hygiene in China, which may effectively decrease the risk of HBV infection.

Our multivariate analysis revealed that among adults aged  $\geq 20$  years, invasive testing and sharing needles increased the risk of HBV infection. One reason might be that the general population in that period had less education on health care, and a lack of knowledge about modes of viral transmission also

increased the risk of infection. Another reason might be the scarcity of systematically trained physicians. Without high standards for medical training, most medical staff may not be able to understand HBV and sterilization procedures well. However, the most influential factor associated with HBV was a familial history of HBV and hepatitis B vaccination in both children and adults groups. One of the reasons for this might be mother-to-child vertical transmission, which is the main route of chronic HBV infection in China<sup>[22]</sup>. Meanwhile, studies also indicate that chronic infection with HBV may closely related to the transmission of HBV between siblings during childhood in certain areas<sup>[23,24]</sup>.

After the hepatitis B vaccine was recommended for routine infant immunizations in 1992, and especially after it was fully integrated into routine infant immunizations in 2002, newborn children had less HBV infection and lower HBsAg prevalence<sup>[25]</sup>. The decline of HBsAg will eventually decrease the prevalence of HBsAg and the future incidence of cirrhosis and hepatocellular carcinoma<sup>[3,26,27]</sup>. One cohort study has estimated that on a global scale, the vaccine has prevented 16-20 million HBV carriers and 2.8-3.5 million future HBV infections and deaths<sup>[28]</sup>. Most of the unimmunized population that we studied lacked knowledge about the hepatitis B vaccine. Therefore, health education of the general older population is important and may prove useful in preventive interventions in China.

In a large country like China, having population-based data on HBV prevalence will be informative. At present, China has successfully decreased the number of HBsAg carriers and reached the national goal of reducing HBsAg prevalence to  $< 1\%$  among children under 5 years, and the hepatitis B immunization rate to  $> 90\%$  among teenagers,  $< 20$  years after integrating hepatitis B vaccine into routine immunization programs. However, the national goal for the whole population of decreasing the prevalence of HBsAg to  $< 7\%$  has not yet been achieved. Therefore, free immunization of infants should be maintained, expanded vaccination is needed for adults, especially, in those aged  $\geq 30$  years, and health education should be further strengthened to limit the spread of HBV infection.

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## COMMENTS

**Background**

Hepatitis B virus (HBV) poses a serious global health problem because of its adverse clinical outcomes, such as cirrhosis and hepatocellular carcinoma. The WHO estimates there are approximately 2 billion people infected by HBV worldwide, and 240 million of them have chronic infection. In China, a highly endemic area, the national prevalence for hepatitis B surface antigen (HBsAg) positivity is 7.20%.

**Research frontiers**

Many studies have suggested that the incidence of newly acquired HBV infection in China has declined steadily since the 1990s by virtue of a number of public health interventions, such as the screening of pregnant women and the vaccination of infants and adolescents, the promotion of safe injection practices, and the development of health education in general. Yet HBV infection prevalence is still high among Chinese high-risk populations such as drug users and men who have sex with men.

**Innovations and breakthroughs**

The present study aimed to evaluate the impact of the hepatitis B vaccination program since 1992 in a large general population, and to clarify the relationship between socioeconomic status or familial history and the HBV carrier state.

**Applications**

7.70% were positive for HBsAg which has not achieved the national goal for the whole population in decreasing the prevalence of HBsAg to < 7%. The prevalence was just 0.77% among children aged < 5 years and the rate of HBV immunization among teenagers < 20 years was 93.30%, which have reached the national goal of < 1% and > 90%, respectively.

**Peer-review**

It is important to know impact of the hepatitis B vaccination program. The study is innovative in nature. The original study conducted on large groups of participants is very valuable.

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## Prospective Study

# Tissue resonance interaction accurately detects colon lesions: A double-blind pilot study

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**Ethics approval:** The study protocol was reviewed and approved by the Local Ethics Committee, more specifically by the "Comitato di Bioetica dell'Azienda Sanitaria Locale (A.S.L.) n° 1 di Sassari".

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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## Abstract

**AIM:** To investigated the performance of the tissue resonance interaction method (TRIM) for the non-invasive detection of colon lesions.

**METHODS:** We performed a prospective single-center blinded pilot study of consecutive adults undergoing colonoscopy at the University Hospital in Sassari, Italy. Before patients underwent colonoscopy, they were examined by the TRIMprobe which detects differences in electromagnetic properties between pathological and normal tissues. All patients had completed the polyethylene glycol-containing bowel prep for the colonoscopy procedure before being screened. During the procedure the subjects remained fully dressed. A hand-held probe was moved over the abdomen and variations in electromagnetic signals were recorded for 3 spectral lines (462-465 MHz, 930 MHz, and 1395 MHz). A single investigator, blind to any clinical information, performed the test using the TRIMprob system. Abnormal signals were identified and recorded



as malignant or benign (adenoma or hyperplastic polyps). Findings were compared with those from colonoscopy with histologic confirmation. Statistical analysis was performed by  $\chi^2$  test.

**RESULTS:** A total of 305 consecutive patients fulfilling the inclusion criteria were enrolled over a period of 12 months. The most frequent indication for colonoscopy was abdominal pain (33%). The TRIMprob was well accepted by all patients; none spontaneously complained about the procedure, and no adverse effects were observed. TRIM proved inaccurate for polyp detection in patients with inflammatory bowel disease (IBD) and they were excluded leaving 281 subjects (mean age  $59 \pm 13$  years; 107 males). The TRIM detected and accurately characterized all 12 adenocarcinomas and 135/137 polyps (98.5%) including 64 adenomatous (100%) found. The method identified cancers and polyps with 98.7% sensitivity, 96.2% specificity, and 97.5% diagnostic accuracy, compared to colonoscopy and histology analyses. The positive predictive value was 96.7% and the negative predictive value 98.4%. Among the 281 non-IBD subjects, there were 7 cases with discordant results (2.5%) between TRIMprob and the reference standard including 5 false positive results (1.8%) and 2 false negative (0.7%) results. The main limitation of the TRIMprob system is the need for trained operators.

**CONCLUSION:** The study confirmed that TRIM provides rapid, accurate, convenient and noninvasive means to identify individuals most likely to benefit from colonoscopy.

**Key words:** Colon cancer screening; Bioscanner; Non-invasive diagnosis; Electromagnetic; Resonance

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**Core tip:** In this study we evaluated the potential role of a non-invasive method: the tissue resonance interaction method or TRIMprob, for enriching the population for colonoscopy with patients most likely to benefit. The apparatus was initially developed by the Italian scientist Clarbruno Vedruccio for military purpose and is another example of technology originally developed for military purpose adapted for medical use. The method is designed to detect differences in electromagnetic properties of pathologic and normal tissues and is currently being used in cancer detection in a number of other organs. The sensitivity, specificity and accuracy of the TRIMprob for detecting and correctly identifying colon cancer or polyps compared to endoscopy with histological examination were greater than 95%.

Dore MP, Tufano MO, Pes GM, Cuccu M, Farina V, Manca A, Graham DY. Tissue resonance interaction accurately detects colon lesions: A double-blind pilot study. *World J Gastroenterol*

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## INTRODUCTION

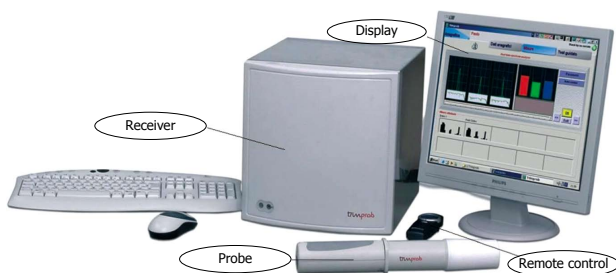
Colon rectal cancer (CRC) is a common disease worldwide and is associated with a high morbidity and mortality<sup>[1]</sup>. Despite improvement in cancer detection and treatment, the American Cancer Society estimated that more than 50 thousand of Americans would die of CRC in 2013<sup>[2]</sup>. Clinical outcomes in CRC are closely related to the stage of the disease at presentation. Current colon cancer prevention programs are predicated on identifying and removing premalignant and malignant lesions curable by local resection. Population screening with fecal occult blood testing, endoscopy, or radiology are currently being used in an attempt to reduce both the incidence and mortality from CRC<sup>[3]</sup>.

Colonoscopy is currently the single best diagnostic test since it can identify, biopsy, and remove lesions throughout the large bowel<sup>[4]</sup>. Because most CRC are thought to develop from adenomas, the detection and removal of premalignant polyps has the potential, and has been proven, to reduce deaths from CRC. Despite major programs for CRC screening in many countries, approximately 50% of the cases of colon cancer are still diagnosed at a late stage<sup>[5]</sup>, and test utilization is largely influenced by demographic and social-cultural factors<sup>[6]</sup>.

Ongoing research to identify methods to enrich the proportion of patients with positive findings among the population undergoing colonoscopy have included development of improved methods for detection of fecal occult bleeding and fecal DNA testing<sup>[7]</sup>. A potential alternate approach, as described here, is based on an electronic device employing frequency-selective (resonant) absorption of electromagnetic waves capable of detecting biological anomalies in tissue *in vivo* such as inflammation, fibrosis, and malignant solid tumors developed by the Italian physicist, Clarbruno Vedruccio<sup>[8,9]</sup>. This technology uses a non-linear radiofrequency oscillator probe emitting electromagnetic waves at 462-465, 930, and 1395 MHz, plus harmonics as previously described<sup>[10,11]</sup>. This non-linear resonance interaction provides a selective characterization, that can be likened to an "electronic biopsy" of the tissues. The biophysical mechanisms responsible for differences in electromagnetic waves absorption have not yet been entirely elucidated. In the case of cancer, Pokorný *et al*<sup>[12]</sup> proposed that the effect is related to a mitochondrial malfunction (the Warburg effect) associated with increased damping of microtubule-based cellular elasto-electrical vibration states<sup>[13,14]</sup>. The principle of detection lies in the resonance between the coupled active nonlinear oscillator (the probe) and



**Figure 1** Colon area is scanned with the TRIMprob while the patient remains normally dressed and standing approximately 2 m in front of the receiver placed at the level of abdominal wall.



**Figure 2** TRIMprob Medical Device. The system is composed of the exploratory probe, the receiver (a spectrum analyzer), and a computer with dedicated software to record patient information and store TRIMprob data.

the passive oscillator (the tissue) in the radiofrequency range of the electromagnetic spectrum. Tissue suspected of harboring disease is irradiated by means of a hand-held probe placed 1 to 2 m from the patient, captured by using a special antenna and analyzed through a spectrum analyzer (Figure 1). The device has a high dynamic range, in the order of 30 decibel (dBm) or more, and can thus detect small lesions<sup>[8,15,16]</sup>. Originally the apparatus was developed for military purposes and is another example of technology being adapted for medical use.

Prior clinical experience with the TRIMprob has proven the method to be simple and reliable with high diagnostic yield when used for detection of prostate<sup>[9,15-17]</sup>, breast<sup>[18]</sup>, and bladder cancers<sup>[19]</sup>, thyroid carcinoma in patients with multinodular goiter<sup>[20]</sup>, gastric cancer<sup>[21]</sup>, and rectal cancer<sup>[22]</sup>. The aim of this study was to extend the use of the TRIMprob to the non-invasive detection of colon lesions. We therefore compared the TRIMprob method for detection of colonic cancer and polyps to the results of colonoscopy with histology.

## MATERIALS AND METHODS

### Study design

This was a prospective single-center, operator-blinded, pilot study. The clinicians, endoscopists and TRIMprob

operator remained blinded to the results of the alternate method until the study was completed.

### Inclusion criteria

Consecutive patients aged 18 years and older attending the general gastroenterology section at the University Hospital in Sassari, Italy (Clinica Medica), with an indication for colonoscopy for any reason, were invited to participate.

### Exclusion criteria

Patients with an implanted pacemaker and pregnant women.

### Ethics statement

Written informed consent was obtained from each participant. The study protocol was approved by the Local Ethics Committee, more specifically by the "Comitato di Bioetica dell'Azienda Sanitaria Locale (A.S.L.) n° 1 di Sassari" (supplemental material, Appendix 1). There was no device company or commercial sponsor for this study.

### TRIMprob examination

The system consists of a work-station plus a battery powered hand-held probe 30 cm long with a tunable, autonomous, non linear oscillator that emits low intensity electromagnetic waves (similar to that experienced during the use of a cordless telephone). The work station is composed by a personal computer assisted spectrum analyzer-receiver to process and display the interaction between the radiofrequency probe emission and the diseased tissue (Figure 2).

In this study, a hand-held computer (PSA2701T-2.7 GHz RF Spectrum Analyzer Thurlby Thandar Instruments, Ltd, Huntingdon, United Kingdom), was added to the system; wave variations were displayed on the personal computer screen using a logarithmic scale and expressed in arbitrary units of 0-255 and also in decibel (dBm) by the hand-held computer based spectrum analyzer to facilitate signal interpretation by the operator (Figure 1).

A single investigator (MOT), blind to any clinical information, performed the test using the TRIMprob system (Galileo Avionica, Turin, Italy) approved by the "Ministero della Salute" (corresponding to the Italian FDA) as a medical device, and certified by the European Union (supplemental material, Appendix 2-4). The operator is a physician radiologist with expertise in ultrasound and specifically trained on the TRIMprob system with a multi-organ experience of more than 10 thousand TRIMprob examinations.

The test was performed approximately 15 min before the scheduled colonoscopy. All patients had completed the polyethylene glycol (PEG)-containing bowel prep for the colonoscopy procedure before being screened by the TRIM system. During the procedure the subjects remained fully dressed. The probe was

**Table 1 Results of the preliminary study to establish criteria for the outcomes assessed in the blinded trial**

Spectral line	Normal mucosa ( <i>n</i> = 47)	Hyperplastic polyps ( <i>n</i> = 29)	Low-grade <sup>1</sup> adenoma ( <i>n</i> = 16)	High-grade <sup>2</sup> adenoma ( <i>n</i> = 4)	Adeno carcinomas ( <i>n</i> = 10)
465 MHz	0	0	25-30	30-35	60-70
930 MHz	0	> 40	> 40	> 40	35-45
1395 MHz	0	0	0-10	65-70	35-45

<sup>1</sup>Low-grade dysplasia; <sup>2</sup>High-grade dysplasia. Threshold values are represented in decibel by the hand-held computer (PSA2701T-2.7 GHz RF Spectrum Analyzer Thurlby Thandar Instruments, Ltd, Huntingdon, United Kingdom), spectrum analyzer, for each of the three spectral lines for normal and specific biological anomaly of colonic mucosa.

moved over the surface of the abdomen (Figure 1), from the upper to the lower right and left quadrants of the abdomen, including the pelvic area in order to perform a complete examination of the colon segments and rectum. In addition, the perineal area was screened in order to exclude malignancies from the uterus or prostate.

The colon was irradiated through the abdomen wall by the field resulting from the TRIM antenna. Nonlinear resonance interactions between the nonlinear oscillator and the tissue reduce the emitted wave energy at distinct frequencies on the basis of the pathological state of the tissue being examined and a reduction in signal amplitude indicates the presence of abnormal tissues or structures. Amplitude changes of the emitted signals at the established frequencies of 465, 930, and 1395 MHz were recorded in an electronic file as a value of the corresponding spectral line expressed in decibel, for each position. The entire patient examination took  $8 \pm 1.5$  min.

### Colonoscopy

Patients were provided written and verbal instructions about bowel preparation. The conventional four liter-dose PEG regimen combined with a strict liquid diet for a full day was used (supplemental material, Appendix 5).

Polyp size was assessed by comparing the polyp dimensions with the maximum jaw width of a fully open biopsy forceps (9 mm). Polyps equal to or smaller than one-half of the distance between the tips of the open biopsy forceps were termed diminutive polyps ( $\leq 5$  mm), polyps between 6-9 mm small polyps, and those greater in size were considered as clinically significant polyps. Attempts were made to remove and retrieve all polyps irrespective of size and these, as well as biopsies from suspected cancers and other mucosal abnormalities, were sent for histological evaluation. Diminutive and small polyps were also recorded and biopsied or cold snared.

Historically, in our endoscopic unit cecal intubation had ranged from 95% to 97%. The proportion of polyps of all types identified in 100 screening colonoscopies in our population, for which there is no regional screening program, is generally typically

greater than 60%.

### Statistical analysis

A preliminary study was done prior to starting the blinded study and included 100 patients and 10 stool samples which were examined *in vitro* to determine the best threshold values for each of the three spectral lines using Receiver Operator Characteristics curves. The best cut-off for each frequency was determined by maximizing the sum of sensitivity + specificity (supplemental material, Appendix 6). Results of the interactions between the electromagnetic field emitted by the probe and normal/pathological tissue are shown in Table 1.

Sessile serrated adenoma/polyps were not found in this cohort of patients independently evaluated. The presence of stool appears as a decrease of 25 dBm in the first spectral line.

**Analysis of the data from the blinded trial:** The results of TRIMprob analysis were compared to that of the reference gold standard in a  $2 \times 2$  contingency table, the absolute number of true positives (TPs), false positives (FPs), true negatives (TNs), and false negatives (FNs) was determined. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the TRIM procedure compared to the gold standard (colonoscopy with histologic confirmation) were calculated and 95% confidence intervals (CI) were estimated with the Wilson score method<sup>[23]</sup>. The Matthews correlation coefficient (MCC) was calculated<sup>[24]</sup>, as a balanced measure of agreement between the two methods under comparison. For each study participant the most advanced colon lesion was considered in order to assign the patient to a diagnostic category. All analyses were performed with the SPSS statistical package (v. 16.0, Chicago, United States) and *P*-values lower or equal than 0.05 were considered statistically significant. The statistical review of the study was performed by a biomedical statistician. This report was done following STARD guidelines<sup>[25]</sup>.

## RESULTS

A total of 305 consecutive patients fulfilling the inclusion

**Table 2** Sensitivity, specificity, positive and negative predictive value of the TRIMprob analysis compared to the gold standard (colonoscopy and histology) in detecting biological anomalies of colonic mucosa for the whole cohort, and for the non-inflammatory bowel disease cohort

Histology	Colonoscopy (No. of positive cases/ total No. of cases)	TRIMprob (No. of TP, FP, FN, TN)				Sensitivity, % (95%CI)	Specificity, % (95%CI)	Positive predictive value, % (95%CI)	Negative predictive value, % (95%CI)
All cases (IBD + Non IBD)	156/305	154	12	2	137	98.7 (95.4-99.6)	91.9 (86.4-95.3)	92.8 (87.8-95.8)	98.6 (94.9-99.6)
Non IBD cases	149/281	147	5	2	127	98.7 (95.2-99.6)	96.2 (91.4-98.4)	96.7 (92.5-98.6)	98.4 (94.5-99.6)
Hyperplastic <sup>1</sup>	73/281	71	4	2	204	97.3 (90.5-99.2)	98.1 (95.2-99.2)	94.7 (87.0-97.9)	99 (96.5-99.7)
Adenomas <sup>2</sup>	47/281	40	4	7	230	85.1 (72.3-92.6)	98.3 (95.7-99.3)	90.9 (78.8-96.4)	97 (94.0-98.6)
Advanced adenomas <sup>3</sup>	17/281	14	1	3	263	82.4 (58.9-93.8)	99.6 (97.9-99.9)	93.3 (70.2-98.8)	98.9 (96.7-99.6)
Cancer	12/281	12	0	0	269	100 (75.7-100.0)	100 (98.5-100.0)	100 (75.7-100.0)	100 (98.6-100.0)

<sup>1</sup>Hyperplastic polyps of every size; <sup>2</sup>Every size adenomas with tubular or villus morphology and low grade dysplasia; <sup>3</sup>Every size adenomas with tubular or villus morphology and high grade dysplasia. TP: True positives; FP: False positive; TN: True negative; FN: False negative; IBD: Inflammatory bowel disease.

criteria were enrolled over a period of 12 mo. Patients were scheduled for colonoscopy for a variety of reasons (supplemental material Appendix 7).

The TRIMprob was well accepted by all patients, none spontaneously complained about the procedure, and no adverse effects were observed. Cecal intubation was performed in all patients, however in 10 cecum visualization was suboptimal because of a poor bowel prep.

TRIM proved inaccurate for polyp detection in patients with inflammatory bowel disease (IBD) likely because mucosal inflammation produced false positive results for polyps. Because IBD patients are not candidates for routine screening colonoscopy of normal patients, the analysis was performed both on all patients examined and separately without the 24 IBD patients to provide a better approximation of the intended population (Table 2) (*i.e.*, as an adjunct to screening colonoscopy). Overall, the results were similar whether the IBD patients were included or excluded. Additional material about IBD patients is provided in the online supplemental material (Appendix 8).

### Comparison of colonoscopy and TRIMprob

In the 281 non-IBD patients TRIM was able to detected all adenocarcinomas (12: 3 were Tis; 4 were T1 N0 M0; 2 were T2 N0 M0; 2 Any T N M0; Any T Any N M1b respectively) (Figure 3), and 135 of 137 polyps (98.5%) (including 100% of the 64 adenomas) found at colonoscopy (supplemental material, Appendix 9). Among the 135 polyps detected, TRIMprob was able to correctly categorize 125 (Table 2). Ten TRIMprob diagnoses of the polyp histology were incorrect: 5 adenomas were thought to be hyperplastic polyps and 2 adenomas were recognized as adenomas but thought to be advanced adenomas. Among the advanced adenomas 2 were falsely characterized as

hyperplastic polyps and 1 as an adenoma.

### Sensitivity and specificity

Assuming colonoscopy/histology as the gold standard for detecting adenomas, the overall sensitivity, specificity, positive and negative predictive values of TRIMprob among the non-IBD patients were 98.7%, 96.2%, 96.7%, and 98.4%. For the entire group examined the results were: sensitivity 98.7%; specificity 91.9%, positive and negative predictive value 92.8% and 98.6% respectively (Table 2).

### Discordant cases

Among the 281 non-IBD subjects, there were 7 cases with discordant results (2.5%) between TRIMprob and the reference standard including 5 false positive results (1.8%) and 2 false negative (0.7%) results (Table 3).

### False positive TRIMprob

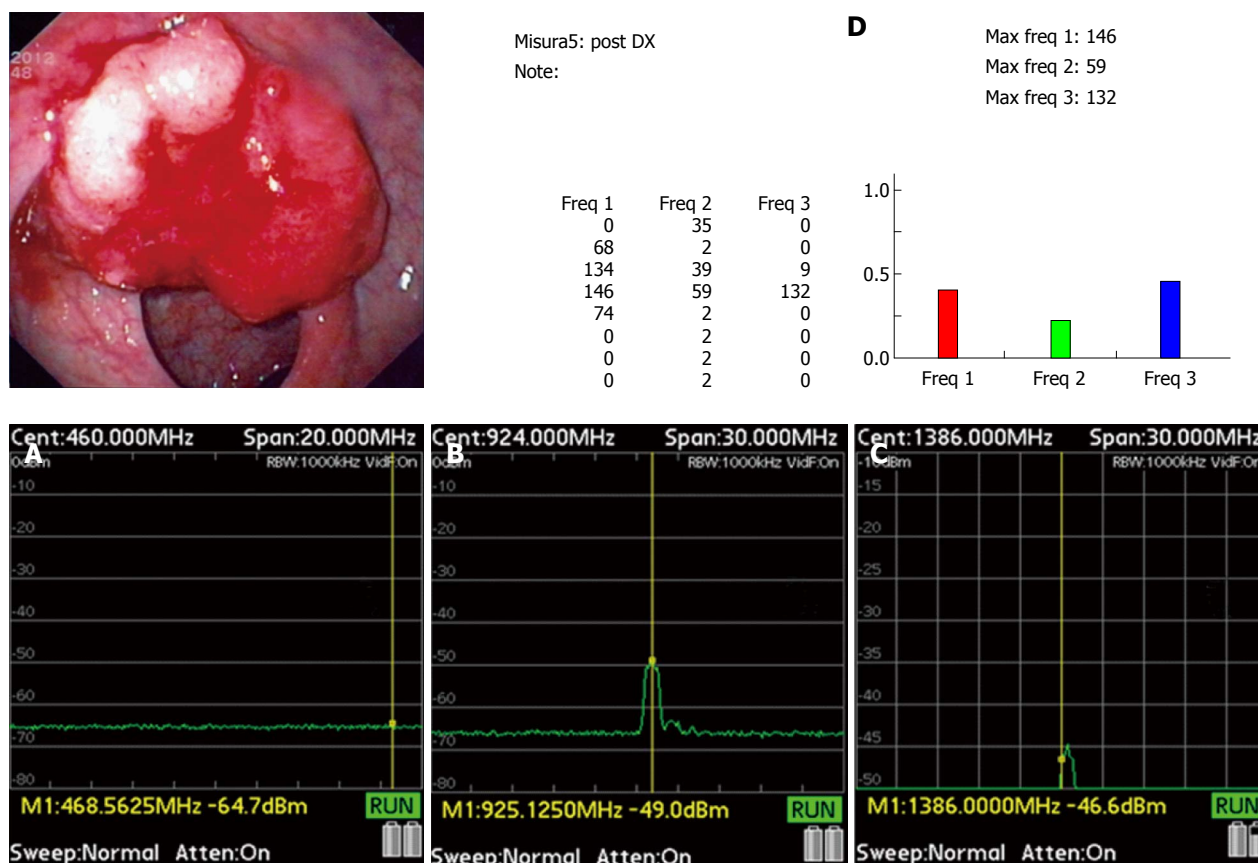
The 5 false positives that were not confirmed by the colonoscopy included one thought to be an advanced adenoma in the transverse and 4 thought to be hyperplastic polyps. There were no false positives for the presence of cancer. Importantly, second look endoscopy was not done to confirm that the false positive results were truly false positive results.

### False negative TRIMprob

Two patients had hyperplastic polyps that went undetected. Both were between 6 and 9 mm and located in the sigmoid colon. One possible speculation could be a difficult niche, or the small size of the polyp and subsequent studies will need to look specifically at this region of the colon.

**Patients with poor bowel prep:** In the 10 patients with a poor bowel prep there were no false positive or





**Figure 3** Colon cancer as observed by colonoscopy and by the TRIMprob. Spectral lines A, B and C correspond to the red, green and blue bars respectively in D. The interaction between the electromagnetic field emitted by the probe and cancerous tissue results in a significant decrease of 40 dBm at 465 MHz (first spectral line), a modest attenuation at 930 MHz (second spectral line); and at 1395 MHz (third spectral line).

**Table 3** Two hundred eighty-one non-inflammatory bowel disease subjects

281 non-IBD patients	Normal	Hyperplasia	LGD	HGD	Cancer	Total
Colonoscopy						
Normal	127	2	0	0	0	129
Hyperplasia	0	73	5	2	0	80
LGD	4	0	40	1	0	45
TRIM						
HGD	1	0	0	14	0	15
Cancer	0	0	0	0	12	12
Total	132	75	45	17	12	281

LGD: Low grade dysplasia; HGD: High grade dysplasia; TRIM: Tissue Resonance Interaction Method; IBD: Inflammatory bowel disease.

false negative TRIMprob results. All patients with poor bowel preps had the colonoscopy repeated without additional finding.

The TRIMprob test displayed good performance distinguishing the number of polyps (supplemental material, Appendix 10). The consistency between the TRIM assay and the colonoscopy/histology was high as reflected by a Matthews correlation coefficient (MCC) of 0.897.

## DISCUSSION

Adenoma detection and removal is one of the primary

targets for prevention of CRC<sup>[4]</sup>. Currently, colonoscopy is the best available screening tool because it can both detect and remove precancerous lesions<sup>[4]</sup>. However, colonoscopy is labor intensive, expensive, and its availability is considerably less than the size of the population at risk. Attempts have been made to enrich the screening population in terms of significant abnormalities by the use of fecal occult blood testing (FOBT), fecal DNA testing, or computed tomographic (CT) colonography<sup>[26]</sup>. However, FOBT has a relatively poor sensitivity for adenoma detection and CT colonography is associated with significant radiation<sup>[27-30]</sup>.

This pilot study evaluated a non-invasive detection method to identify premalignant and malignant colon lesions. Compared with colonoscopy/histology TRIMprob yielded a sensitivity as high as 98.7% with a high concordance (up to 90%) between the two methods. These results are similar to those reported for use of the TRIMprob method in prostate cancer (95.5%-86%)<sup>[9,15-17]</sup>, breast cancer (84%)<sup>[18]</sup>, for carcinomas detection in patients with multinodular goiter (100%)<sup>[20]</sup>, gastric cancer (100%)<sup>[21]</sup>, and for rectal malignant lesions (94%)<sup>[22]</sup>. More specifically in that pilot study, Vannelli *et al.*<sup>[22]</sup> reported a diagnostic accuracy of the TRIMprob of 89.5% when a cutoff of 50 arbitrary units was chosen for the 465-MHz frequency. The specificity was 85.1% and lower than what was observed in our study (97.5%) suggesting a lower proportion of false positive attributable to a more expert operator<sup>[22]</sup>.

For bladder cancer the level of agreement between TRIMprob and cystoscopy was also high (Cohen's  $K = 0.77$ ,  $P < 0.001$ )<sup>[19]</sup>.

In this preliminary study, false-negative results among the non-IBD patients consisted of 2 cases thought to have hyperplastic polyps (0.7%). There was 100% TRIM-endoscopy concordance for actual cancers. One other case had a false positive diagnosis of an advanced adenoma that was not confirmed at the blinded endoscopy. Endoscopy was however not repeated to ensure that it was a true false positive result. In ten cases TRIM was able to detect benign lesions but failed to correctly categorize the type of benign polyp histologically.

This study was designed to evaluate the accuracy of the TRIM approach for identifying and correctly categorizing colonic lesions in a mixed population scheduled for endoscopy for any reason with the goal to detect polyps or cancer and thus potentially reduce the incidence of negative colons. The study was not restricted to those meeting the criteria for screening and contained patients with indications for colonoscopy which is likely responsible for the higher prevalence of CRC detected.

The TRIMprob demonstrated to provide excellent results except in patients with ulcerative colitis or Crohn's disease where 7 of the 24 subjects had false positive results for polyps. IBD patients have marked mucosal inflammation and those with Crohn's disease have full bowel wall thickness inflammation. As this group of patients is markedly different from healthy subjects who would participate in colon cancer screening in retrospect, they should not have been included. However, the data with and without IBD patients is given. Future studies will be required to determine what is responsible for the TRIMprob findings in IBD patients and include comparisons of the histology and radiologic findings in the areas with TRIMprob abnormalities.

One limitation of the TRIMprob system is the requirement for trained operators but this requirement

is no different from any diagnostic technique including colonoscopy. Those who are already expert in interpreting ultrasound, computer tomography or magnetic resonance images should be able to quickly become proficient in this technology. Interobserver evaluations of trained TRIMprob operators for other indications have shown excellent correlations close to 100%<sup>[9,17]</sup>. These data suggest that developing a cadre of individuals with expertise in colon screening should not be a major problem.

The probe oscillations of biological tissues produce the phenomenon of "non linear resonance interaction" which is detected by the TRIM receiver. Because the required intensities of the electromagnetic waves are very low, there is thought to be no health hazard. The current price of the TRIMprob system is about € 60000. For the single patients we can hypothesize, at least in Europe, a cost no more expensive than an abdominal ultrasound (€ 100). An additional side benefit of the device is the possibility to detect other potential lesions located in the scanned areas, regardless of their origin.

Our results should encourage additional studies with different designs to confirm these results and explore other parameters. For example, to examine the need for the colon prep one might examine patients scheduled for screening endoscopy (*e.g.*, one might perform TRIM before colon prep, immediately following the colon prep, and then perform the screening endoscopy). Those with positive TRIM but negative colonoscopy could be identified immediately after the procedure such as by opening a sealed opaque envelope to allow immediate comparison prompting the endoscopist to reevaluate a certain segment of the colon where lesions were identified but not seen on endoscopy. Alternate designs would couple TRIM with iFOBT to ask whether the combination would be complementary for identification of those most likely to benefit from colonoscopy. Other areas of research include evaluation of methods to automate the interpretation of the TRIMprob results and thus reduce the need for highly trained operators.

Overall our results are consistent with the notion that the non-invasive TRIMprob method is a novel, highly effective method for identifying which patients would likely benefit most from colonoscopy. The TRIMprob examination has the potential to become a first line tool in the armamentarium for CRC prevention. TRIMprob colon screening virtually offers an inexpensive, noninvasive approach that could make colonoscopic screening both more efficient and cost effective. Future research includes development of software to analyze the images to reduce the need for highly trained operators as well testing whether a bowel prep is needed prior to TRIM screening.

## COMMENTS

### Background

Colorectal cancer is a common and lethal disease. Colonoscopy is currently

the preferred modality of screening, although associated with high costs and low patient compliance. Recently, a non invasive device was developed for detecting differences in electromagnetic properties of cancerous and normal tissues, using a non linear tuneable oscillator, the tissue resonance interaction method (TRIM) that proved to accurately identify those patients who would likely benefit from colonoscopy.

### Research frontiers

The diagnosis of cancer in humans is mainly based on morphological changes in cells and irregularities in tissues confirmed using cytological and histological methods. The TRIMprob focuses on differences in the biochemical metabolism between cancer and normal cells which produce electromagnetic fields inducing alignment in dipole movements. Most of the molecules in the body are electrical dipoles which electronically function like transducers in that they are able to transform acoustic waves into electrical waves and electrical waves into acoustic waves. The natural properties of biomolecular structures enable cell components and whole cells to oscillate and interact resonantly with other cells. The cells of the body and cellular components possess the ability to function as electrical resonators. A dipole movement is a function of polarization processes and the strength of the electric field. When cell membranes in the biological tissue are exposed to an electric field in the right frequency and amplitude windows a preferential alignment of dipoles occurs. TRIMprob utilizes frequency selective (resonant) absorption of electromagnetic waves in malignant tumors. The biochemical interactions are dislodged from electromagnetic fields in this way the TRIMprob represents a revolution in devising a new way of screening. This method has previously been proven to be highly accurate in detection of other cancers such as breast and prostate.

### Innovations and breakthroughs

Colorectal cancer is one of the most common malignancies. Current colon cancer prevention programs focus on polyp detection and removal and detection of early cancers using colonoscopy to reduce cancer incidence and mortality. However, colonoscopy is associated with high costs, poor patient acceptability, and the majority of examinations are negative making the method very inefficient. A method to non-invasively detect lesions would allow to target colonoscopy to those who would most likely benefit. Here, the authors show that using a non-linear tunable oscillator to detect differences in electromagnetic properties of biological abnormal and normal tissues (*i.e.*, TRIM) can achieve that goal.

### Applications

This operator-blinded pilot study shows that TRIM is a rapid, highly accurate, and noninvasive method to identify individuals most likely to have polyps or cancers and thus to benefit from colonoscopy. If confirmed by subsequent studies, TRIM is likely to revolutionize colon cancer prevention programs.

### Peer-review

This article reported the TRIMprob used to detect colon polyps and cancers. TRIM present highly sensitivity and specificity. TRIMprob scanning is a valuable tool in diagnosing carcinoma of prostate, breast, gastric, rectal and so on. In this study, the authors compared TRIMprob with colonoscopy. It's of clinical significance and convincing.

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## Prospective Study

# Resolution of non-alcoholic steatohepatitis by rosuvastatin monotherapy in patients with metabolic syndrome

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**Author contributions:** Kargiotis K designed the study and recruited patients; Athyros VG designed the study, followed-up patients and wrote the paper; Giouleme O performed biopsies; Katsiki N followed-up patients; Katsiki E did pathology; Anagnostis P recruited patients; Boutari C followed up patients; Doumas M wrote the paper; Karagiannis A designed the protocol; Mikhailidis DP interpreted the results wrote the paper; all authors approved the final version of the paper.

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## Abstract

**AIM:** To investigate the effect of rosuvastatin monotherapy on non-alcoholic steatohepatitis (NASH). At present there is no effective treatment for non-alcoholic fatty liver disease or its advanced form NASH.

**METHODS:** This prospective study included 20 biopsy proven patients with NASH, metabolic syndrome (MetS) and dyslipidaemia. Biochemical parameters of the blood of the patients and an ultrasonography of the liver were performed at baseline. Then patients received

lifestyle advice and were treated for a 12 mo period with rosuvastatin (10 mg/d) monotherapy. Patients were re-evaluated during the study at 3 mo intervals, during which biochemical parameters of the blood were measured including liver enzymes. A repeat biopsy and ultrasonography of the liver were performed at the end of the study in all 20 patients. Changes in liver enzymes, fasting plasma glucose, serum creatinine, serum uric acid (SUA), high sensitivity C reactive protein (hsCRP) and lipid profile were assessed every 3 mo. The primary endpoint was the resolution of NASH and the secondary endpoints were the changes in liver enzyme and lipid values.

**RESULTS:** The repeat liver biopsy and ultrasonography showed complete resolution of NASH in 19 patients, while the 20<sup>th</sup>, which had no improvement but no deterioration either, developed arterial hypertension and substantial rise in triglyceride levels during the study, probably due to changes in lifestyle including alcohol abuse. Serum alanine transaminase, aspartate transaminase, and  $\gamma$ -glutamyl transpeptidase were normalised by the 3<sup>rd</sup> treatment month (ANOVA  $P < 0.001$ ), while alkaline phosphatase activities by the 6<sup>th</sup> treatment month (ANOVA,  $P = 0.01$ ). Fasting plasma glucose and glycated haemoglobin were significantly reduced ( $P < 0.001$ ). Lipid values were normalised by the 3<sup>rd</sup> treatment month. No patient had MetS by the 9<sup>th</sup> treatment month. Body mass index and waist circumference remained unchanged during the study. Thus, changes in liver pathology and function should be attributed solely to rosuvastatin treatment. A limitation of the study is the absence of a control group.

**CONCLUSION:** These findings suggest that rosuvastatin monotherapy could ameliorate biopsy proven NASH and resolve MetS within 12 mo. These effects and the reduction of fasting plasma glucose and SUA levels may reduce the risk of vascular and liver morbidity and mortality in NASH patients. These findings need confirmation in larger studies.

**Key words:** Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Metabolic syndrome; Dyslipidaemia; Rosuvastatin; Fasting blood glucose; Serum uric acid

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**Core tip:** We treated 20 patients with metabolic syndrome (MetS) and biopsy proven non-alcoholic steatohepatitis (NASH) with rosuvastatin monotherapy for one year. Repeat liver biopsy and ultrasonography showed complete resolution of NASH in 19 patients, and normalization of liver enzymes, lipid profile and blood glucose; no patient had MetS at the end of the study. These findings suggest that rosuvastatin monotherapy could ameliorate biopsy proven NASH and resolve MetS within 12 mo. These effects and the reduction of fasting plasma glucose and serum uric

acid levels, if confirmed by larger studies, may reduce the risk of vascular and liver morbidity and mortality in NASH patients.

Kargiotis K, Athyros VG, Giouleme O, Katsiki N, Katsiki E, Anagnostis P, Boutari C, Doumas M, Karagiannis A, Mikhailidis DP. Resolution of non-alcoholic steatohepatitis by rosuvastatin monotherapy in patients with metabolic syndrome. *World J Gastroenterol* 2015; 21(25): 7860-7868 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7860.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7860>

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a term describing a histological spectrum of the most common liver disease (affects approximately 15%-30% of the general population in Western Countries) characterized by accumulation of fat ( $> 5\%$ ) in liver cells in the absence of excessive alcohol intake, chronic viral hepatitis or other liver disease<sup>[1]</sup>. The histological manifestations of NAFLD range from simple steatosis, steatohepatitis (NASH), liver fibrosis, cirrhosis, and may progress to hepatocellular carcinoma<sup>[2]</sup>. NASH is characterised by steatosis plus necro-inflammation, and fibrosis, which can be diagnosed by liver biopsy<sup>[2]</sup>. Recent data suggest that NAFLD is linked to increased cardiovascular disease (CVD) risk, independently of the risk related to components of the metabolic syndrome (MetS); NAFLD is the hepatic manifestation of MetS<sup>[3]</sup>. It has also been shown that patients with NASH are at higher CVD risk than those with simple steatosis, emphasizing the role of chronic liver inflammation in the pathogenesis of atherosclerotic CVD<sup>[3]</sup>.

Statin treatment is safe in patients with mild to moderate elevations of liver enzymes due to NAFLD/NASH<sup>[4]</sup>. It has been established that statin regimens substantially reduce the risk of death or atherosclerotic CVD events in a wide range of individuals<sup>[5]</sup>. We reported that atorvastatin treatment is safe, improves liver tests, and liver ultrasonography as well as reduces CVD events in patients with NAFLD<sup>[6,7]</sup>. We have also shown, in a pilot study ( $n = 6$ ) involving liver biopsy, that rosuvastatin can have a beneficial effect on NASH resolution<sup>[8]</sup>. In the present paper we report results of rosuvastatin monotherapy (10 mg/d) on liver enzymes, ultrasonography and biopsy of 20 patients with NASH and MetS within 12 mo of treatment. The patients were followed for an additional (mean) 18-mo period to ensure sustainability of results.

## MATERIALS AND METHODS

Methods were described in the pilot study paper ( $n = 6$ )<sup>[8]</sup>. Here we report the results of rosuvastatin (10 mg/d) monotherapy in 20 patients (this includes

the 6 patients included in the preliminary report). In short, this is a prospective, randomized, open-label study that involved patients with MetS, without overt type 2 diabetes mellitus (T2DM), not taking any previous hypolipidaemic therapy. The study protocol was approved by the ethical committee of Aristotle University and written informed consent was obtained from all patients before inclusion in the study.

Twenty MetS patients with increased serum liver enzyme activity and ultrasonographic image compatible with NAFLD were included. The presence of NASH was validated by liver biopsy. The age range was 18–70 years. Patients with overt CVD, congestive heart failure (CHF), stage 3 or higher of chronic kidney disease (CKD), rheumatic diseases, chronic viral hepatitis, congenital disorders of the liver, autoimmune hepatitis or excessive alcohol intake (21 and 14 u/wk for men and women, respectively) were excluded.

The study was designed to include 40 patients; 20 on rosuvastatin monotherapy and 20 on rosuvastatin-fenofibrate combination. All participants had MetS<sup>[9]</sup> and dyslipidaemia [total cholesterol > 200 mg/dL, high density lipoprotein cholesterol (HDL-C) < 40 for men and < 50 mg/dL for women, triglycerides (TGs) > 150 mg/dL or combinations]<sup>[10]</sup>. Participants received lifestyle advice with suggestions to adopt a hypocaloric and hypolipidaemic diet<sup>[10]</sup> and regular exercise (at least 1 h of walking every day, if possible, or equivalent physical activity). For safety reasons we started treatment with 5 mg/d of rosuvastatin and if there were no side effects we titrated this dose to 10 mg/d at the end of the 1<sup>st</sup> treatment month (safety and titration visit).

The primary endpoint was the degree of resolution of NASH in the repeat biopsy compared with the baseline biopsy. Secondary endpoints were safety of treatment and degree of normalization of liver enzymes, lipid profile and liver ultrasonography. At every visit safety parameters [alanine transaminase (ALT), aspartate transaminase (AST), and creatine kinase (CK)] were assessed as well as gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP). The lipid profile (total cholesterol, TGs, LDL-C, HDL-C), fasting plasma glucose, serum creatinine, blood urea nitrogen (BUN) and serum uric acid (SUA) were assessed by standard methods. Body mass index (BMI), waist circumference, blood pressure (BP) and smoking habits were recorded at each visit.

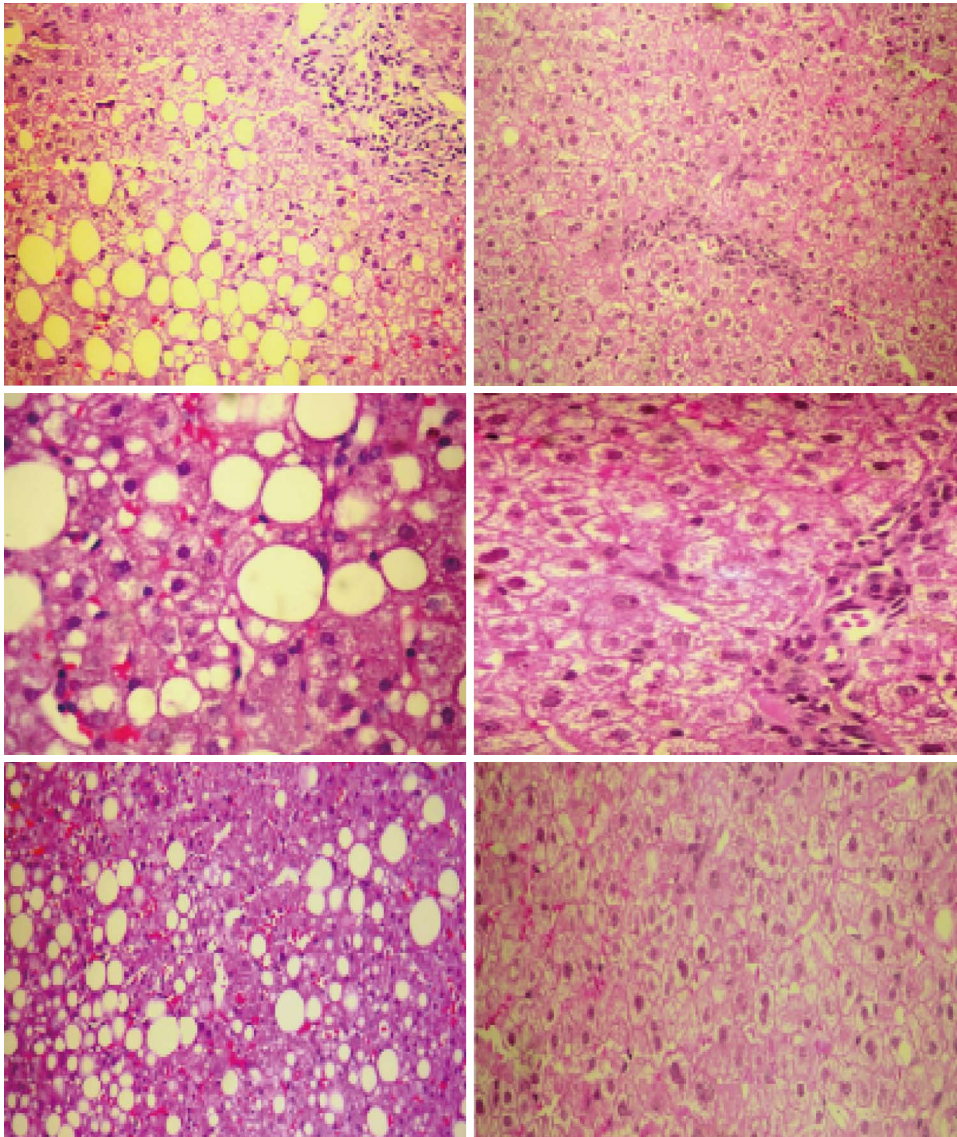
### Statistical analysis

All measured parameters had normal distribution and are reported as mean values and standard deviations. Paired *t*-tests and ANOVA for repeat measurements were used. A 2-tailed *P* < 0.05 was considered significant. All analyses were carried out using the SPSS 21.0 software (SPSS Inc., Chicago, IL).

## RESULTS

We report the results of 20 patients on rosuvastatin monotherapy (10 mg/d). Patients on rosuvastatin-fenofibrate combination had identical (serum enzyme, ultrasonography, and liver biopsy) results (only TGs were lower but in both groups were within normal range) with those on rosuvastatin monotherapy. The rosuvastatin-fenofibrate combination was discontinued, because patients did not have MetS any longer and it was considered futile to insist on an unnecessary remedy<sup>[11]</sup>. Patients on the rosuvastatin-fenofibrate combination continued with rosuvastatin monotherapy, but their results are not included in the present analysis, because for some period of time they were on combination therapy and this could have affected the results. We recruited 5 rosuvastatin-fenofibrate patients in the initial phase of this project, but there was no difference in results between those and the rosuvastatin monotherapy patients and thus these were put on rosuvastatin monotherapy. It was difficult to continue with the statin-fibrate combination (only 3 patients had a repeat biopsy), because most patients expressed the wish to discontinue the fibrate. This was due to the fear of their attending physicians (4 years ago physicians were reluctant to prescribe statin-fibrate combinations) due to liver or muscle side-effects of this combination. The results of these 5 patients are not included in the present study and the results of the 3 that made it to biopsy are not included either in the pilot or in the present study, because the number was too low to allow statistical analysis. In the rosuvastatin monotherapy group (*n* = 20) liver biopsy at baseline showed steatosis (fat content of the liver > 30%), hepatocyte ballooning degeneration, diffuse lobular mixed acute and chronic inflammation, and perivenular as well as perisinusoidal collagen disposition. Repeat liver biopsies in all 20 patients showed that all patients but one had a complete resolution of NASH (Figure 1 shows 3 pairs of biopsies at baseline and 12 mo later). At baseline, 2 pathologists, blinded to the decision of each other, graded the histology in the liver biopsies. Disease activity was assessed using a score that included NAFLD activity (scale: 0 to 3), lobular inflammation (scale: 0 to 3) and hepatocellular ballooning (scale: 0 to 2); higher scores indicate NASH severity<sup>[12]</sup>. The study only included patients with a histology score of 8, to make certain that NASH was present at baseline<sup>[12]</sup>. According to this classification<sup>[12,13]</sup>, the comparison of liver biopsies at one year after rosuvastatin treatment with those at baseline showed that in 19 patients there was a NAFLD activity score (based on the standardized grading system of measuring steatosis mentioned above<sup>[12]</sup>) reduction from 3 to 0 (*P* < 0.001), while in one patient this fell from 3 to 1; lobular inflammation





**Figure 1** Presentation of the baseline and repeat liver biopsies in 3 metabolic syndrome patients with non-alcoholic steatohepatitis on rosuvastatin (10 mg/d) monotherapy for 12 mo. On the left panel liver biopsies of patients with non-alcoholic steatohepatitis presenting steatosis (fat content of the liver > 30%), hepatocyte ballooning degeneration, diffuse lobular mixed acute and chronic inflammation, and perivenular, perisinusoidal collagen disposition. On the right panel liver biopsies of the same 3 patients after one year monotherapy with 10 mg/d of rosuvastatin presenting total normal liver tissue.

was reduced from 3 to 0 in 19 patients ( $P < 0.001$ ), while in the same patient mentioned above it changed from 3 to 2; hepatocellular ballooning changed from 2 to 0 ( $P < 0.001$ ), while in the same patient mentioned above it changed from 2 to 1. Even in the one patient (without full histological improvement) the total histological NASH score was 4 (*i.e.*, < 5 that indicates definite or possible NASH)<sup>[12,13]</sup>. There were no significant differences between the evaluations of the 2 pathologists in grading NASH activity in all 20 patients.

The patient, as also reported in the pilot study, which did not show either any improvement or deterioration of NASH in the second biopsy, was the one that had arterial hypertension and high TG levels, attributed to a change in life-style habits, including excess alcohol consumption. We advised him to adopt a healthier lifestyle but we were not successful. This

patient was not submitted to a third biopsy later, but he had normalization of the liver enzymes and the ultrasonographic image of the liver by the end of second year of the study while on rosuvastatin and adopting a healthier lifestyle for at least a year.

The changes in measured parameters during the 12 mo of the duration of the study, between the two biopsies, are reported in Table 1 and Figure 2.

There were no statin-related safety problems-side effects from the liver or the muscles. In contrast, liver enzymes were normalized by the 6<sup>th</sup> treatment month and remained normal thereafter. Even the one patient that had no improvement in the repeat liver biopsy had normal liver enzyme levels.

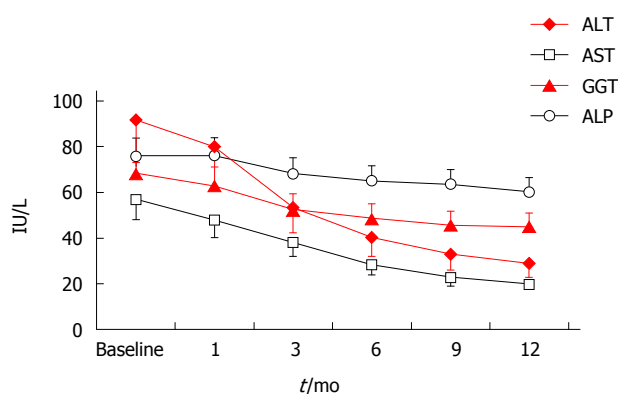
The lipid profile was completely normalized by the 3<sup>rd</sup> treatment month (Table 1), while all 20 patients did not have MetS any more from the 9<sup>th</sup> treatment month



**Table 1** Changes in measured parameters during the study

Parameter	Baseline	1 <sup>st</sup> mo	3 <sup>rd</sup> mo	6 <sup>th</sup> mo	9 <sup>th</sup> mo	12 <sup>th</sup> mo	P value (ANOVA)
Age (yr)	40.5 ± 5.6	-	-	-	-	-	-
Gender (male)	16	-	-	-	-	-	-
Cigarette smoking	13	13	13	12	12	11	NS
BMI (kg/m <sup>2</sup> )	31.5 ± 1.1	31.3 ± 1.0	31.4 ± 1.0	31.6 ± 1.1	31.6 ± 1.2	31.5 ± 1.2	NS
Waist circumference (cm)	110.5 ± 6.2	110.4 ± 6.0	109.9 ± 6.1	110.6 ± 6.3	110.7 ± 6.2	110.4 ± 6.2	NS
Total cholesterol (mg/dL)	251 ± 22	226 ± 17	192 ± 16	185 ± 12	181 ± 8	179 ± 9	< 0.001
Triglycerides (mg/dL)	187 ± 19	161 ± 20	143 ± 26	123 ± 11	121 ± 22	117 ± 18	< 0.001
HDL-cholesterol (mg/dL)	38 ± 5	40 ± 5	42 ± 7	42 ± 4	43 ± 3	44 ± 5	< 0.001
LDL-cholesterol (mg/dL)	180 ± 23	152 ± 15	121 ± 17	118 ± 14	114 ± 9	110 ± 11	< 0.001
Serum creatinine (mg/dL)	0.93 ± 0.2	0.92 ± 0.2	0.94 ± 0.2	0.92 ± 0.2	0.91 ± 0.2	0.90 ± 0.2	NS
hsCRP (mg/L)	4.2 ± 1.3	-	-	2.7 ± 0.8	-	1.6 ± 0.5	< 0.001
BUN (mg/dL)	34 ± 8	34 ± 8	35 ± 8	34 ± 7	33 ± 6	31 ± 6	NS
SUA (mg/dL)	5.5 ± 1.1	5.4 ± 1.0	5.2 ± 0.9	5.0 ± 0.7	4.9 ± 0.8	4.8 ± 0.9	0.016
Plasma glucose (mg/dL)	102 ± 8	101 ± 8	96 ± 6	93 ± 7	89 ± 5	87 ± 5	< 0.001
HbA <sub>1c</sub> (%)	5.3 ± 0.4	-	5.1 ± 0.4	5.0 ± 0.5	4.9 ± 0.3	4.8 ± 0.3	< 0.001
Metabolic Syndrome, <i>n</i>	20	20	18	9	0	0	< 0.001

Data are presented as mean ± SD. BMI: Body mass index; HDL: High density lipoprotein; LDL: Low density lipoprotein; NS: Not significant; BUN: Blood urea nitrogen; SUA: Serum uric acid; HbA<sub>1c</sub>: Glycosylated haemoglobin.



**Figure 2** Liver enzyme changes in 20 patients with non-alcoholic steatohepatitis during the 12 mo of rosuvastatin (10 mg/d) monotherapy. The reduction in serum alanine transaminase (ALT), aspartate transaminase (AST) and  $\gamma$ -glutamyl transpeptidase (GGT) levels became statistical significant by the 3<sup>rd</sup> month of treatment (ANOVA for the 12 mo period  $P < 0.001$ ) and for alkaline phosphatase (ALP) by the 6<sup>th</sup> mo of treatment (ANOVA for the 12 mo period  $P = 0.01$ ).

(Table 1). It should be noted that these happened in the absence of any change in waist circumference (and BMI), the only MetS component that persisted after 9<sup>th</sup> month of treatment. Mean SUA levels were significantly reduced (Table 1)

Fasting plasma glucose levels and HbA<sub>1c</sub> were significantly reduced by rosuvastatin in all patients, by the 6<sup>th</sup> treatment month, even in the patient with no

improvement in the repeat liver biopsy (Table 1).

## DISCUSSION

The main findings of this prospective study, which included 20 MetS patients with biopsy proven NASH, were that monotherapy with 10 mg/d of rosuvastatin was linked to resolution of NASH (as established by a second liver biopsy, measurement of serum liver enzymes and liver ultrasonography), complete regression of MetS, reduction in SUA levels, and a large reduction in plasma glucose levels. These happened within 12 mo of treatment, although some changes were evident within the first treatment months. The lipid profile was totally normalized by rosuvastatin early during follow-up. Follow-up of patients for a mean period of 18 mo after the second biopsy did not show any indication of NASH or MetS relapse.

Five years ago we have shown in a post hoc analysis of the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) Study ( $n = 1600$ ; 437 patients had moderately abnormal liver tests at baseline)<sup>[6]</sup> that atorvastatin therapy was safe in patients with coronary heart disease (CHD) and mild to moderate elevations of serum transaminases, probably due to NAFLD as indicated by liver ultrasonography after exclusion of other liver diseases. Not only atorvastatin did not increase liver enzymes but it normalised liver

tests and liver ultrasonography within the duration of the study<sup>[6]</sup>. Moreover, atorvastatin treatment induced substantial reductions in CVD events (68% vs usual care) during the 3-year follow-up period compared with the participants with CHD and normal liver enzymes (39% vs usual care);  $P = 0.007$ <sup>[6]</sup>. These findings were confirmed one year later by the Assessing The Treatment Effect in Metabolic Syndrome Without Perceptible diabeTes (ATTEMPT) study in patients with MetS but without overt CVD or T2DM ( $n = 1123$ ; 326 had modestly elevated liver enzymes and ultrasonographic evidence of NAFLD)<sup>[7]</sup>. In 2013 the post hoc analysis of the Incremental Decrease in End Points Through Aggressive Lipid Lowering [IDEAL] trial ( $n = 8863$ ) showed that high dose atorvastatin treatment (80 mg) in 1,081 (12.2%) patients, who had an ALT  $\geq$  ULN, normalised ALT values and substantially reduced 5-year CVD event rates compared with simvastatin [11.5% for simvastatin and 6.5% for atorvastatin, hazard ratio (HR) 0.556; 95% confidence interval (CI) 0.367-0.842;  $P = 0.0056$  vs 20-40 mg simvastatin treatment], confirming our findings<sup>[14]</sup>.

A previous study with rosuvastatin showed a suboptimal clinical, laboratory and biopsy proven benefit in 9 patients with NASH and dyslipidaemia<sup>[15]</sup>. This may be attributed to the very low dose of rosuvastatin administered (2.5 mg/d)<sup>[15]</sup>. A study with pitavastatin included 20 patients with biopsy-proven NASH with dyslipidaemia<sup>[16]</sup>. After treatment for 12 mo with pitavastatin 2 mg/d NASH-related metabolic parameters improved, including histology in some patients, however, 3 of 13 patients had progression of fibrosis during treatment<sup>[17]</sup>. Studies with simvastatin<sup>[17]</sup> or pravastatin<sup>[18]</sup> in NASH patients showed that NASH alters the expression of hepatic uptake transporters which may increase the risk of statin-induced adverse drug reactions (myopathy). In a pilot study that included 16 patients with biopsy proven NASH (14 completed the study and 10 underwent 1-year repeat liver biopsy), simvastatin monotherapy did not seem to be an effective treatment for NASH<sup>[19]</sup>. A recent study in rats showed that simvastatin delays the evolution of NASH-related fibrosis, improving its prognosis<sup>[20]</sup>. Thus, clinical, laboratory, ultrasonography and liver biopsy benefits related to statin treatment may be compound specific and dose related.

The mechanisms involved in the biochemical, ultrasonographic, and histological improvement with some statins at specific doses is not clear<sup>[21]</sup>. An animal study evaluated whether rosuvastatin changes the carbohydrate and lipid metabolism and the development of NAFLD in male C57Bl/6 mice (3-month-old) on a high-fat diet (60% lipids) compared with standard chow (10% lipids) for 15 wk<sup>[22]</sup>. Rosuvastatin improved glucose intolerance, insulin sensitivity and NAFLD in this animal model of diet-induced obesity in a dose-dependent manner and changed the fat distribution from visceral to subcutaneous<sup>[22]</sup>.

Therefore, rosuvastatin therapy may help patients with MetS because of beneficial pleiotropic effects<sup>[22]</sup>.

Data on the mechanisms of improvement of NASH patients who are at a higher risk for liver- and vascular-related morbidity and mortality than those with simple steatosis<sup>[23]</sup>, are scarce<sup>[23]</sup>. An open-label prospective study of atorvastatin (10 mg/d) for 24 mo included 31 patients with biopsy-proven NASH and hyperlipidaemia<sup>[24]</sup>. Follow-up liver biopsy was performed in 17 patients. BMI and plasma glucose levels did not change during the treatment, while 23 patients (74.2%) presented normal transaminase levels. During the study adiponectin levels increased significantly and the levels of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) decreased significantly<sup>[24]</sup>. Liver steatosis and NASH-related metabolic parameters improved with treatment, including fibrosis in some patients. However, 4 of 17 patients had progression of fibrosis over the 2-year period, with 3 of them progressing to stage 3<sup>[24]</sup>. These results suggest that atorvastatin may have acted *via* a reduction in markers of systemic inflammation (*e.g.*, TNF- $\alpha$ ), as well as increased adiponectin levels<sup>[24]</sup>. It has been suggested that oxidative stress that promotes the pathogenesis of atherosclerosis might be one key link between NASH and CVD<sup>[25]</sup>. Thus, atorvastatin may be effective in NASH treatment not only through reduction of inflammatory cytokine production, but also reactive oxygen species generation in the liver. These are also the two major pathophysiological mechanisms involved in the progression from NAFLD to NASH<sup>[25]</sup>. Similar effects were shown with 20 mg of rosuvastatin<sup>[26]</sup>. Moreover, it has been shown that atorvastatin treatment (10 mg/d for 12 mo) improved metabolic and histological parameters in 43 biopsy proven NASH patients with dyslipidaemia and that this effect was probably related to the reduction in serum levels of advanced glycosylated end products (AGEs) that might be useful as a marker for NASH presence<sup>[27]</sup>. The above, as well as our findings, suggest that the effect of statins on NASH is dose dependent<sup>[24-27]</sup>.

Another important finding of the present study was the complete resolution of MetS by the 9<sup>th</sup> treatment month. This was mainly due to the substantial reduction in TG levels, a significant increase in HDL-C levels and (paradoxically) to the reduction in fasting plasma glucose levels. Rosuvastatin treatment has been reported to have a negative effect on plasma glucose homeostasis<sup>[28]</sup> and has been linked to new onset diabetes (NOD)<sup>[29]</sup>. However, it has been shown that the risk of NOD in statin-treated patients was related to female gender (16/20 of our patients were males), old age (the mean age of our patients was 40.5 years), and to intensity of the statin and its dose (in our study rosuvastatin, a potent statin, was prescribed at 10 mg/d; a relatively low dose)<sup>[29]</sup>. These factors may explain the lack of NOD but not the great reduction of fasting plasma glucose levels. In a prospective randomized open-label study in non-diabetic patients with dyslipidaemia, rosuvastatin

(10 mg) exerted a favourable effect on glucose homeostasis, by improving insulin resistance index. This effect was not shown with atorvastatin (20 mg), although the effect of both statins on blood glucose and HbA<sub>1c</sub> levels was neutral<sup>[30,31]</sup>. There are data showing that NAFLD/NASH play a central role in the genesis of an insulin-resistant state in obese subjects, independent of the role of visceral fat, suggesting that the improvement of NASH might contribute to the reduction of insulin resistance<sup>[32,33]</sup>. It has been shown in NAFLD patients that exercise-induced reduction in fetuin-A (a blood protein synthesized in the liver that may be associated with the pathogenesis of NAFLD and T2DM) levels is closely linked to exercise-induced improvement in glucose tolerance, due to the reduction of skeletal muscle insulin resistance<sup>[34]</sup>. Thus, early resolution of NASH might have been involved in the reduction in fasting plasma glucose levels and HbA<sub>1c</sub> in our patients.

From the other MetS components waist circumference (as well as body weight and BMI) was not reduced and BP showed a small but non-significant reduction. Thus, any improvement of NASH cannot be attributed to these MetS components.

SUA is considered by some as a MetS component<sup>[35]</sup> possibly associated with CVD risk in MetS and NAFLD/NASH<sup>[36]</sup>. Thus, SUA levels reduction by rosuvastatin in the present study might have contributed to a further reduction of CVD risk, beyond the improved lipid profile and amelioration of NASH. We have shown in the GREACE study that all CVD patients were independently (after backwards regression analysis) benefited (had fewer vascular events) by SUA level reduction<sup>[37]</sup>. However, those with MetS benefited more from statin treatment than those without MetS<sup>[38]</sup>. Furthermore, an atorvastatin-based multifactorial intervention in MetS patients without established CVD or T2DM reduced SUA levels, especially in stage 3 CKD patients; this might have contributed to the reduction in CVD events in these patients<sup>[39]</sup>.

This study did not include a control group and each patient acted as his/her control. This was because, based on our and other previous findings and lipid guidelines it was unethical to deprive statin treatment in NASH patients with MetS.

In conclusion, Rosuvastatin (10 mg/d) monotherapy for 12 mo was associated with resolution of NASH, regression of MetS and reduction in plasma glucose and SUA levels, without any change in body weight, BMI, or waist circumference, in patients with MetS and dyslipidaemia. These results will hopefully reduce the risk of NOD and vascular and liver morbidity and mortality related to MetS and its liver manifestation, NAFLD/NASH. There is a need to confirm these results in larger prospective studies, given that NAFLD might have already affected almost 1 billion people (the most common chronic hepatic disorder in Western countries, with a prevalence of 20%-30%<sup>[40,41]</sup>, increasing to 57%-74% among obese patients<sup>[42]</sup>, and 5%-18% in

Asia with a strong trend to increase over time<sup>[40]</sup>). At present, three decades of research on pharmacological treatment have provided limited options<sup>[1]</sup>; especially for NASH there is very little evidence supporting the efficacy of most regimens<sup>[43]</sup>.

## COMMENTS

### Background

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide. It may evolve to non-alcoholic steatohepatitis (NASH), cirrhosis and in a few patients to hepatoma. NAFLD is considered the hepatic manifestation of metabolic syndrome (MetS). NAFLD, but mainly NASH, are related to increased cardiovascular disease (CVD) risk and more patients die from vascular than liver disease.

### Research frontiers

Currently there is no generally acceptable treatment for NASH.

### Innovations and breakthroughs

Up until recently statins were not prescribed to patients with NAFLD/NASH and thus high CVD risk patients were deprived from an effective treatment. Data from *post hoc* analyses showed a benefit from statin treatment in these patients in reducing both the liver and the CVD risk without major adverse events. However, this had to be proven by liver biopsy.

### Applications

The results of the study suggest that in patients with MetS and biopsy proven NASH rosuvastatin monotherapy resulted in complete resolution of both NASH and MetS after a year of treatment.

### Terminology

Thus, NASH and MetS two major CVD risk factors were effectively treated with a statin that may not have been allowed in these patients some years ago.

### Peer-review

A novel paper suggesting a possible treatment for a wide spread disease worldwide.

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## Prospective Study

# Long-term antiviral efficacy of entecavir and liver histology improvement in Chinese patients with hepatitis B virus-related cirrhosis

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## Abstract

**AIM:** To evaluate the clinical outcomes of 240-wk treatment with entecavir (0.5 mg) in Chinese nucleoside-naive patients with cirrhosis.

**METHODS:** A total of 204 nucleoside-naive patients with compensated ( $n = 96$ ) or decompensated ( $n = 108$ ) hepatitis B virus (HBV)-induced cirrhosis at the Department of Gastroenterology of the China-Japan Union Hospital (Jilin University, Changchun, China) who were treated with entecavir (0.5 mg) for 240 wk were enrolled in this study. Liver biopsy samples obtained from 38 patients prior to treatment (baseline) and at week 240 were evaluated by different independent histopathologists. Efficacy assessments included the proportions of patients who achieved an HBV DNA level  $< 500$  copies/mL, the association of interleukin-28B genetic variation with antiviral therapy, clinical outcomes, and histologic improvement. Changes in liver disease severity were analyzed, and liver histologic evaluation was performed in 38 patients with paired biopsies. Student  $t$  tests were used to compare the means of continuous variables between the groups, and the proportions of patients who achieved the endpoints were compared using the  $\chi^2$  test.

**RESULTS:** At week 240, 87.5% of the patients with compensated cirrhosis and 92.6% of the patients with decompensated cirrhosis achieved a HBV DNA level  $< 500$  copies/mL. Three patients had genotypic entecavir resistance within the 240-wk period. No significant association was observed between virologic response and interleukin-28 genotype (CT, 88.2% vs CC, 90.6%). The proportion of patients with Child-Pugh

class A disease was significantly increased at week 240 (68%) from the baseline (47%;  $P < 0.01$ ). The proportion of patients with Child-Pugh class B disease was significantly decreased at week 240 (25%) from the baseline (39%;  $P = 0.02$ ). In the patients with paired liver biopsies, the mean reduction in the Knodell necroinflammatory score from the baseline was  $3.58 \pm 1.03$  points ( $7.11 \pm 1.80$  vs  $3.53 \pm 1.35$ ,  $P < 0.01$ ). The mean reduction in Ishak fibrosis score from the baseline was  $1.26 \pm 0.64$  points ( $5.58 \pm 0.50$  vs  $4.32 \pm 0.81$ ,  $P < 0.01$ ).

**CONCLUSION:** Entecavir is an effective treatment option for patients with HBV-related compensated or decompensated cirrhosis that can result in sustained virologic suppression and histologic improvement.

**Key words:** Decompensated cirrhosis; Hepatic function; Histologic improvement; Knodell histologic activity index score; Nucleoside analog

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**Core tip:** Entecavir is a potent antiviral agent that is effective and safe for the treatment of chronic hepatitis B. However, data on its clinical benefits in patients with cirrhosis, especially in long-term treatment, are limited. The aims of this prospective study were to evaluate the antiviral efficacy and clinical outcomes of entecavir treatment for 240 wk in nucleoside-naïve Chinese patients with chronic hepatitis B, and compensated or decompensated cirrhosis.

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## INTRODUCTION

Chronic hepatitis B (CHB) remains a serious global public health problem, with an estimated 350-400 million people affected worldwide<sup>[1]</sup>. Such patients are at increased risk of developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC)<sup>[2]</sup>. In the absence of treatment, 15%-20% of patients develop cirrhosis within five years<sup>[3,4]</sup>. Patients who subsequently progress to decompensated cirrhosis have a poor prognosis, with a five-year survival rate of only 14%-28% compared with 84% for patients with compensated cirrhosis<sup>[5,6]</sup>. Elevated serum hepatitis B virus (HBV) DNA levels are an independent risk factor of progression to cirrhosis, hepatic decompensation, HCC, and death<sup>[7,8]</sup>. Conversely, sustained reductions in

viral load associated with antiviral therapy are strongly correlated with decreased risk of disease progression and improvements in liver histology and clinical signs or symptoms<sup>[9,10]</sup>.

Multiple clinical studies have demonstrated that nucleos(t)ide analogs are effective in suppressing viral replication and reducing disease progression in patients with HBV-related cirrhosis<sup>[11-13]</sup>. In a randomized clinical trial, long-term lamivudine treatment (median duration, 32.4 mo) significantly reduced overall disease progression (increase in Child-Pugh score, hepatic decompensation, or HCC) compared with placebo (7.8% vs 17.7%,  $P = 0.001$ ) in patients with hepatitis B e antigen-positive CHB and advanced fibrosis/compensated cirrhosis<sup>[12]</sup>. In contrast, data on clinical outcomes with nucleos(t)ide analogs in patients with decompensated cirrhosis are limited.

Entecavir is a potent antiviral agent that has been shown to be effective and safe for the treatment of CHB<sup>[14-17]</sup>. A subanalysis of phase III clinical data found that 57%-59% of patients with CHB and advanced liver fibrosis/cirrhosis experienced improvements in terms of Ishak fibrosis score at 48 wk of entecavir therapy<sup>[18]</sup>. More recently, the Shim *et al*<sup>[19]</sup> research group observed the clinical efficacy of one-year entecavir therapy in 55 patients with decompensated cirrhosis and found that 66% of the patients had improved Child-Turcotte-Pugh scores, which comprises individual scores for five parameters, namely total bilirubin level, serum albumin level, prothrombin time, ascites level, and hepatic encephalopathy. Patients with scores of 5 or 6, 7-9, or 10-15 were classified as having Child-Pugh class A, B, or C liver disease, respectively. Of the patients, 49% had increased Child-Turcotte-Pugh scores by  $\geq 2$ . Clinical trial data from patients with advanced fibrosis/cirrhosis found that after approximately six years of cumulative entecavir therapy, all ten patients showed improvement in liver histology and Ishak fibrosis score. In particular, four patients had Ishak fibrosis scores  $\leq 4$  after the entecavir therapy<sup>[20]</sup>.

Although the efficacy and safety of entecavir in nucleoside-naïve patients without cirrhosis have been demonstrated in multiple studies, limited data are available on the clinical benefits in patients with cirrhosis. The aims of this prospective study were to evaluate the antiviral efficacy and clinical outcomes of entecavir treatment for 240 wk in nucleoside-naïve Chinese patients with CHB and compensated or decompensated cirrhosis.

## MATERIALS AND METHODS

### Study design

This prospective study evaluated the efficacy of entecavir (Bristol-Myers Squibb, Wallingford, CT, United States) at 0.5 mg once daily for 240 wk in patients with cirrhosis. Nucleoside-naïve patients ( $n = 204$ ) with HBV-related cirrhosis who attended the Department of Gastroenterology at the China-Japan Union Hospital

(Jilin University, Changchun, China) were recruited and enrolled in the study beginning in June 2006. Diagnoses of compensated and decompensated cirrhosis were based on liver biopsy and/or clinical, radiologic, and laboratory criteria according to disease management guidelines<sup>[21]</sup>. Liver disease severity was graded according to Child-Pugh score. Patients with scores of 5 or 6, 7-9, or 10-15 were classified as Child-Pugh class A, B, or C, respectively. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Jilin University. Written informed consent was obtained from all participants.

### Study population

Eligible patients were adults aged  $\geq 16$  years with CHB infection (defined as hepatitis B surface antigen positive for  $\geq 6$  mo with persistent detectable hepatitis B surface antigen and/or serum HBV DNA) and compensated or decompensated cirrhosis. All of the patients were nucleoside-naïve prior to entecavir treatment and had serum HBV DNA levels  $\geq 500$  copies/mL as measured using a PCR assay (Da An Gene Co. Ltd, Guangzhou, China; lower limit of detection, 500 copies/mL). The exclusion criteria included patients with coinfection with HIV or hepatitis A, C, D, or E viruses, and active alcohol abuse or dependence. Women who were pregnant or breastfeeding were also excluded.

### Efficacy assessments

HBV DNA, interleukin (IL)-28B genotype, and serum biochemical profiles were analyzed at baseline, weeks 4 and 12 of treatment, and every 12 wk thereafter up to 240 wk of treatment. Efficacy was defined when patients achieved a HBV DNA level  $< 500$  copies/mL (via PCR) at week 240. The clinical endpoint assessed was disease progression in the total study population. Disease progression was defined as an increase in Child-Pugh score of  $\geq 2$ , hepatic decompensation, HCC, spontaneous bacterial peritonitis, bleeding gastroesophageal varices, or death related to liver disease.

Liver biopsy samples obtained from 38 patients prior to treatment (baseline) and at week 240 were evaluated by different independent histopathologists. The proportions of patients with improvements in Knodell histologic activity index (HAI), fibrosis, and necroinflammatory scores from the baseline were assessed at week 240. Histologic improvement was defined as a decrease in Knodell necroinflammatory score of  $\geq 2$  points from the baseline and no worsening of fibrosis score, or a decrease in Ishak fibrosis score of  $\geq 1$  point from the baseline. Histologic worsening was defined as an increase in Knodell necroinflammatory score of  $\geq 2$  points or an increase in Ishak fibrosis score of  $\geq 1$  point from the baseline.

### Resistance analysis

Patients with a virologic breakthrough ( $> 1\text{-log}_{10}$

increase in HBV DNA level higher than the nadir) were monitored for resistance mutations. Nucleotide sequence analysis of the HBV polymerase gene to detect genotypic entecavir resistance was performed for on-treatment samples by an independent laboratory (TaKaRa Biotechnology Co., Ltd., Dalian, China).

### Safety

The incidence of adverse events, treatment discontinuation, deaths, and on-treatment alanine aminotransferase flares (defined as a serum level  $> 2 \times$  baseline and  $> 10 \times$  upper limit of normal) were documented. Renal impairment (defined as an elevation in serum creatinine to  $> 3 \times$  upper limit of normal vs baseline) was also monitored. In all the patients with increased lactate serum concentrations, arterial blood gas analysis was performed immediately.

### Statistical analysis

Statistical analysis was performed using SPSS v13.0 (SPSS Inc., Chicago, IL, United States). Continuous variables are expressed as mean  $\pm$  SD and categorical data are expressed as proportions or percentages. The Student's *t* test was used to compare the means of the continuous variables between the groups. The proportions of patients who achieved the end points were compared using the  $\chi^2$  test. All of the tests were two-sided, and  $P < 0.05$  was considered as statistically significant.

## RESULTS

### Patient disposition

A total of 204 patients with HBV-related cirrhosis who were treated with entecavir for 240 wk at the China-Japan Union Hospital (Jilin University, Changchun, China) beginning in June 2006 were enrolled in this study. Of these patients, 96 had compensated cirrhosis (Child-Pugh class A) and 108 had decompensated cirrhosis (Child-Pugh classes B and C). Thirty-eight patients had paired liver biopsies at baseline and week 240 of treatment. The patients were predominantly male (67%-78%), and patients in the compensated cirrhosis group were significantly younger ( $P < 0.05$ ) (Table 1). The compensated cirrhosis group also had higher alanine aminotransferase levels and fewer patients with HBV genotype C than the decompensated cirrhosis group (both  $P < 0.05$ ).

### Virologic response

At 240 wk of treatment, 87.5% of the patients with compensated cirrhosis and 92.6% of the patients with decompensated cirrhosis had achieved serum HBV DNA levels  $< 500$  copies/mL. No significant differences in virologic response were observed between the two groups at week 240.

### IL-28 genotypes vs efficacy

The genotype distributions of rs12979860 C/T in all



**Table 1** Demographics and baseline characteristics of patients

Characteristic	Compensated cirrhosis group (n = 96)	Decompensated cirrhosis group (n = 108)	P value
Age (yr)	33.4 ± 10.6	42.4 ± 14.5	< 0.05
Male	64 (67)	84 (78)	0.085
HBsAg-positive	72 (75)	46 (43)	< 0.05
HBV DNA (log <sub>10</sub> copies/mL)	6.5 ± 1.3	5.6 ± 1.5	0.077
ALT (IU/L)	131.4 ± 125.7	72.5 ± 63.1	< 0.05
HBV genotype			
B	40 (42)	16 (15)	< 0.05
C	48 (50)	78 (72)	< 0.05
Other (A and D)	8 (8)	14 (13)	0.367

Data are presented as mean ± SD or n (%). ALT: Alanine aminotransferase; HBsAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

the patients were analyzed. For the genotypes, the proportion of the CT genotype in the patients was 16.7% and that of the CC genotype was 83.3%. No significant association was observed between virologic response and IL-28 genotype (CT, 88.2% vs CC, 90.6%).

### Histologic improvement

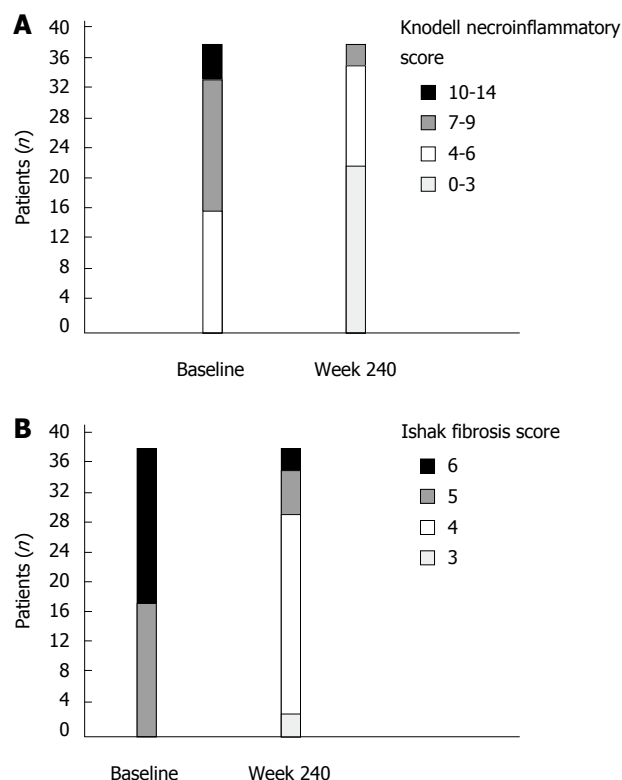
Histologic evaluation of liver biopsy samples from 38 patients with HBV-related cirrhosis indicated that 20 (52.6%) patients had a Knodell HAI score of 0-3 points at week 240 of entecavir treatment. The mean reduction in the Knodell necroinflammatory score from the baseline was  $3.58 \pm 1.03$  points ( $7.11 \pm 1.80$  vs  $3.53 \pm 1.35$ ,  $P < 0.01$ ; Figure 1A).

With respect to fibrosis, 89.5% of the patients achieved improvement ( $\geq 1$  point decrease from the baseline) in terms of Ishak fibrosis score. The mean reduction in Ishak fibrosis score from the baseline was  $1.26 \pm 0.64$  points ( $5.58 \pm 0.50$  vs  $4.32 \pm 0.81$ ,  $P < 0.01$ ; Figure 1B).

A total of 89.5% of the patients achieved improvement (decrease in Knodell necroinflammatory score of  $\geq 2$  points from the baseline and no worsening of fibrosis score, or a decrease in Ishak fibrosis score of  $\geq 1$  point from the baseline).

### Relationship between HBV DNA level and histologic improvement

The relationships between HBV DNA level and the Knodell HAI and Ishak fibrosis scores at baseline and after entecavir treatment were analyzed by performing linear regression using data from the histology subgroup of patients ( $n = 38$ ). As shown in Figure 2A, viral load at baseline was significantly correlated with Knodell HAI and Ishak fibrosis scores in the untreated patients ( $r = 0.880$  and  $r = 0.876$ , respectively;  $P = 0.01$ ). Similarly, decreases in HBV DNA level from the baseline were strongly correlated with decreases in Knodell HAI ( $r = 0.60$ ;  $P = 0.01$ ), but not Ishak fibrosis score ( $r = 0.17$ ) at week 240 of entecavir treatment (Figure 2B).



**Figure 1** Improvements in liver histology at week 240 of entecavir treatment in patients ( $n = 38$ ) with paired liver biopsies (baseline and week 240). A: Distribution of Knodell necroinflammatory scores at baseline and at week 240; B: Distribution of Ishak fibrosis scores at baseline and at week 240.

### Clinical outcomes

The proportion of patients with disease progression in the decompensated cirrhosis group was 4.6% within the 240 wk. Three patients were found to have HCC at weeks 40, 60, and 72, and two patients had bleeding gastroesophageal varices at weeks 36 and 48. None of the patients had worsened compensated cirrhosis (Figure 3).

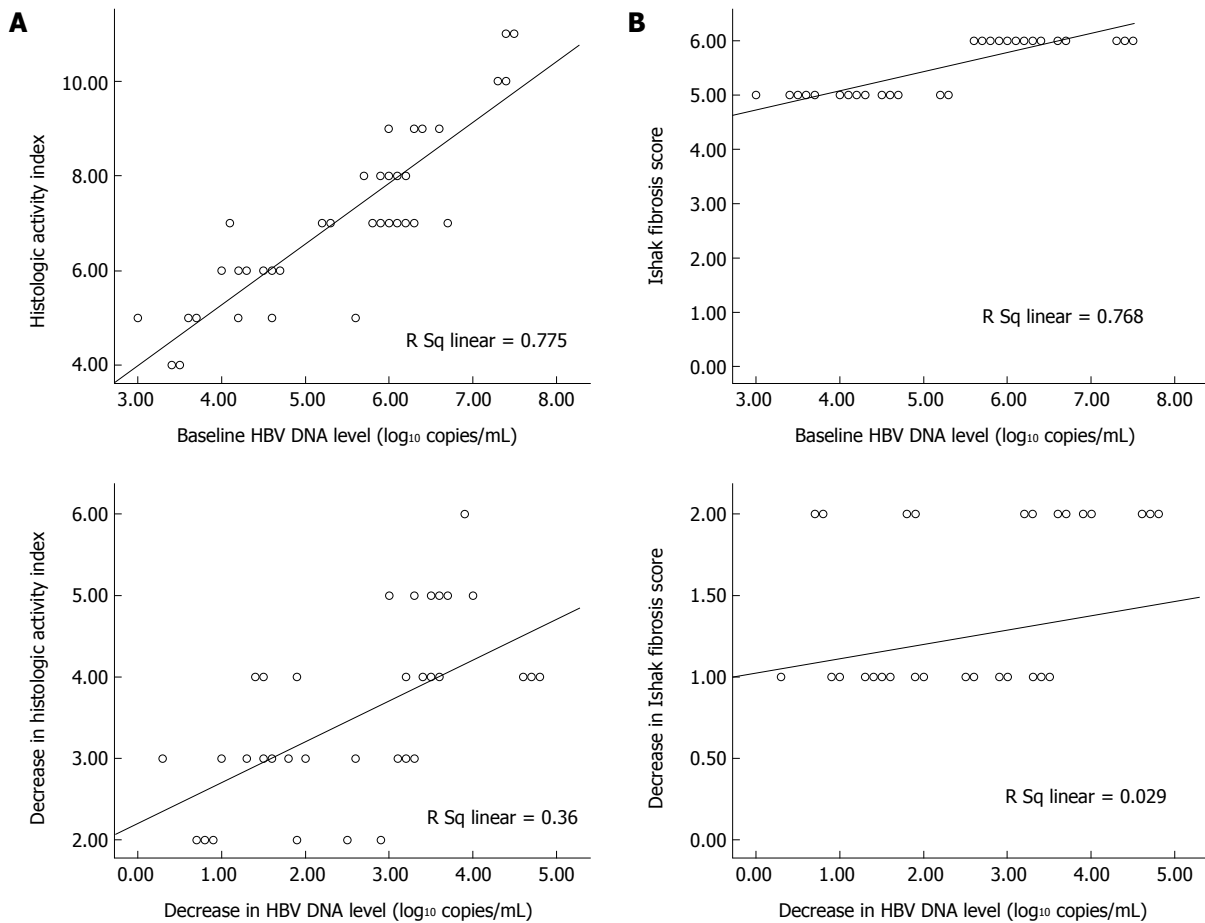
Liver disease severity (Child-Pugh class) at baseline, week 96, and week 240 in the total study population is shown in Figure 4. The proportion of patients with Child-Pugh class A disease significantly increased from the baseline at week 240 (47% vs 68%,  $P < 0.01$ ). The proportion of patients with Child-Pugh class B disease significantly decreased from the baseline at week 240 (39% vs 25%,  $P = 0.02$ ), with corresponding decreases occurring in the proportions of patients with Child-Pugh class C disease.

### Resistance

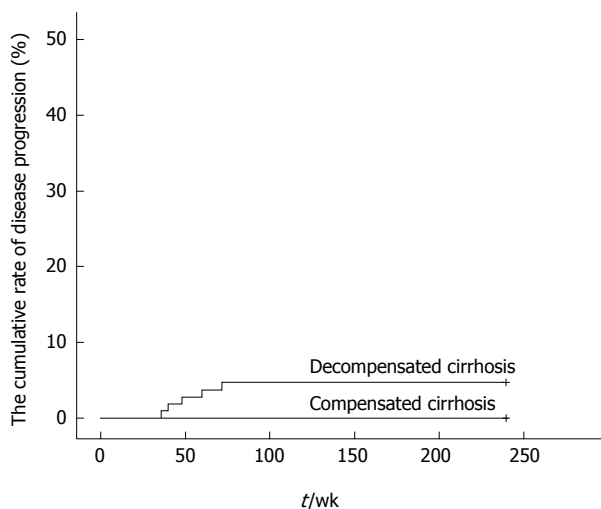
Three patients (1.5%) experienced a virologic breakthrough during 240 wk of entecavir treatment. They had the same mutations, and resistance mutation occurred in rtM204I/V, rtL180M, and rtT184, respectively.

### Safety

None of the patients discontinued treatment with



**Figure 2 Relationship between hepatitis B virus DNA level and histologic improvement.** A: Relationships between hepatitis B virus (HBV) DNA level and Knodell histologic activity index and Ishak fibrosis score at baseline; B: Relationships between changes from baseline in HBV DNA level and Knodell histologic activity index and Ishak fibrosis score at week 240 of entecavir treatment.



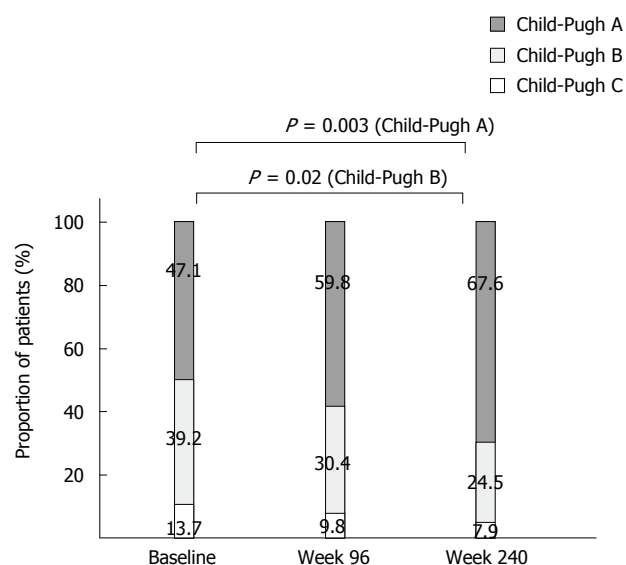
**Figure 3 Proportion of patients with disease progression in compensated and decompensated cirrhosis groups.**

entecavir, experienced renal function impairment, or developed lactic acidosis throughout the 240-wk period of treatment with entecavir.

## DISCUSSION

Data on the efficacy and safety of entecavir in patients with CHB-related cirrhosis are limited. This prospective study demonstrates that entecavir is an effective treatment option for Chinese nucleoside-naïve patients with CHB and compensated or decompensated cirrhosis. Most of the patients achieved virologic suppression (HBV DNA level < 500 copies/mL) by week 240 of therapy. Furthermore, histologic improvement was observed in most (89.5%) of the patients with paired biopsies at baseline and week 240. The most important finding is that the entecavir treatment was associated with significant improvements in hepatic functional reserve in the patients with decompensated cirrhosis.

Sustained suppression of HBV replication is recommended as a primary aim of therapy for CHB<sup>[22-24]</sup>. In this study, 88% and 93% of patients with compensated and decompensated cirrhosis, respectively, achieved an HBV DNA level < 500 copies/mL by week 240. These results confirm previous findings that demonstrated the efficacy of entecavir in nucleoside-naïve patients



**Figure 4** Improvements in Child-Pugh grade at weeks 96 and 240 of entecavir treatment in patients with compensated and decompensated cirrhosis.

and extended these findings to patients with CHB and compensated or decompensated cirrhosis.

The long-term goal of treatment for CHB is to arrest or reverse liver disease progression<sup>[22,23]</sup>. After week 240 of entecavir therapy, all of the 38 patients showed improvement in liver histology and Ishak fibrosis score. The mean change in Knodell necroinflammatory and Ishak fibrosis scores from the baseline were -1.3 and -3.5, respectively. These findings are consistent with previous studies that demonstrated histologic improvement with nucleoside analog treatment in patients with bridging fibrosis/cirrhosis<sup>[12,18,20,25,26]</sup>. Clinical trial data from ten patients with advanced fibrosis/cirrhosis (Ishak fibrosis scores, 4-6) found that after approximately six years of cumulative entecavir therapy (range: 267-297 wk), all of the patients showed improvement in liver histology and Ishak fibrosis score; mean changes in Ishak fibrosis and Knodell necroinflammatory scores from the baseline were 2.2 and 7.6, respectively. A reduction in Ishak fibrosis score to 4 or lower was observed for all four patients who had cirrhosis at baseline<sup>[20]</sup>.

In a previous study of one-year entecavir treatment in Korean patients with decompensated cirrhosis, genotypic resistance to entecavir was not evaluated<sup>[7]</sup>. In contrast, comprehensive resistance monitoring of all the patients in the present study found a virologic breakthrough in three patients. These results are consistent with the low cumulative probability of genotypic entecavir resistance (0.5% at two years to 1.2% over six years) observed in clinical trials with nucleoside-naïve patients without cirrhosis<sup>[27,28]</sup>. Considering that current CHB guidelines recommend long-term treatment for patients with cirrhosis<sup>[22-24]</sup>, the low rate of genotypic entecavir resistance in this study provides further evidence to support the

use of entecavir in patients with CHB and either decompensated or compensated cirrhosis.

Recently, genome-wide association studies have shown that several single-nucleotide polymorphisms in the IL-28B gene (*IL28B*) on chromosome 19q13, which encodes type III interferon (IFN; also named IFN-λ3), are strongly associated with not only spontaneous and treatment-induced clearance of hepatitis V virus (HCV) infection, but also the course of HCV-related disease<sup>[29]</sup>. Moreover, our recent study also showed that *IL28B* polymorphism rs12979860 is associated with response to treatment in Chinese hepatitis C patients<sup>[30]</sup>. Considering that HBV and HCV are both hepatotropic viruses that can establish chronic infections that persist for the lifetime of the host and are sensitive to the antiviral activity of IFN-λ in cell culture models of virus replication, it might be possible that genetic variants of *IL28B* play a similar functional role during chronic HBV infection<sup>[31]</sup>. Several previous studies in different ethnic groups have suggested that *IL28B* genetic variation is associated with HBV-related disease and IFN-based treatment outcomes<sup>[32-35]</sup>. However, the present study shows that virologic response and IL-28 genotype are not significantly associated.

In conclusion, this study demonstrates that entecavir is safe and provides potent virologic suppression and improvement in overall liver disease severity in nucleoside-naïve patients with HBV-related decompensated or compensated cirrhosis. Sustained virologic suppression, biochemical response, and improvements in liver histology were achieved by most of the patients throughout the 240 wk of treatment. These findings, together with a high genetic barrier to resistance, provide evidence that support the use of entecavir as a first-line treatment for patients with CHB and advanced liver disease.

## COMMENTS

### Background

Entecavir is a potent antiviral agent that has been shown to be effective and safe for the treatment of chronic hepatitis B (CHB). However, data on its clinical benefits in patients with cirrhosis, especially in long-term treatment, are limited.

### Innovations and breakthroughs

Prospectively evaluate the antiviral efficacy and clinical outcomes of entecavir treatment for 240 wk in nucleoside-naïve Chinese patients with CHB, and compensated or decompensated cirrhosis.

### Applications

This study demonstrates that entecavir is an effective treatment option for Chinese nucleoside-naïve patients with CHB, and compensated or decompensated cirrhosis, can result in sustained virological suppression and histological improvement.

### Peer-review

The paper is informative and very interesting, which evaluate the efficacy of entecavir in the treatment of hepatitis B virus-cirrhosis. Although it does not offer any very novel insights, it does provide worthwhile "real world" data in this group of patients.

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## Utility of fluorescent cholangiography during laparoscopic cholecystectomy: A systematic review

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### Abstract

**AIM:** To verify the utility of fluorescent cholangiography for more rigorous identification of the extrahepatic biliary system.

**METHODS:** MEDLINE and PubMed searches were performed using the key words "fluorescent cholangiography", "fluorescent angiography", "intraoperative fluorescent imaging", and "laparoscopic cholecystectomy" in order to identify relevant articles published in English, French, German, and Italian during the years of 2009 to 2014. Reference lists from the articles were reviewed to identify additional pertinent articles. For studies published in languages other than those mentioned above, all available information was collected from their English abstracts. Retrieved manuscripts (case reports, reviews, and abstracts) concerning the application of fluorescent cholangiography were reviewed by the authors, and the data were extracted using a standardized collection tool. Data were subsequently analyzed with descriptive statistics. In contrast to classic meta-analyses, statistical analysis was performed where the outcome was calculated as the percentages of an event (without comparison) in pseudo-cohorts of observed patients.

**RESULTS:** A total of 16 studies were found that involved fluorescent cholangiography during standard laparoscopic cholecystectomies ( $n = 11$ ), single-incision robotic cholecystectomies ( $n = 3$ ), multiport robotic cholecystectomy ( $n = 1$ ), and single-incision laparoscopic cholecystectomy ( $n = 1$ ). Overall, these preliminary studies indicated that this novel technique was highly sensitive for the detection of important biliary anatomy and could facilitate the prevention of bile duct injuries. The structures effectively identified before dissection of Calot's triangle included the cystic duct (CD), the common hepatic duct (CHD), the common bile duct (CBD), and the CD-CHD junction. A review of the literature revealed that the frequencies

of detection of the extrahepatic biliary system ranged from 71.4% to 100% for the CD, 33.3% to 100% for the CHD, 50% to 100% for the CBD, and 25% to 100% for the CD-CHD junction. However, the frequency of visualization of the CD and the CBD were reduced in patients with a body mass index  $> 35 \text{ kg/m}^2$  relative to those with a body mass index  $< 35 \text{ kg/m}^2$  (91.0% and 64.0% *vs* 92.3% and 71.8%, respectively).

**CONCLUSION:** Fluorescent cholangiography is a safe procedure enabling real-time visualization of bile duct anatomy and may become standard practice to prevent bile duct injury during laparoscopic cholecystectomy.

**Key words:** Extrahepatic biliary system; Laparoscopic cholecystectomy; Bile duct injury; Biliary anomalies; Fluorescent cholangiography

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**Core tip:** Fluorescent cholangiography (FC) is a safe and effective novel procedure that enables real-time visualization of the biliary system. Intraoperative FC has been successfully performed during mini-invasive cholecystectomies in various studies, including standard laparoscopic cholecystectomies, single incision cholecystectomies, and robotic cholecystectomies. The primary aim of this review is to verify the utility of this technique for more rigorous identification of the extrahepatic biliary system in order to prevent bile duct injuries intraoperatively. The second aim is to illuminate potential benefits and limitations in the application of FC.

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## INTRODUCTION

Laparoscopic cholecystectomy (LC) is one of the most commonly performed surgical procedures worldwide. Annually, more than 750000 procedures are performed in the United States<sup>[1]</sup> and approximately 60000 in Japan<sup>[2]</sup>. Bile duct injury (BDI) is a rare but very serious complication of LC, with an incidence of 0.3%-0.7%<sup>[3-7]</sup> and a significant impact on quality of life and overall survival<sup>[8]</sup>.

The high frequency of BDI with laparoscopic cholecystectomy was first considered to be a consequence of the initial learning curve of the surgeon, but it later became clear that the primary cause of BDI was misinterpretation of biliary anatomy (71%-97% of all cases)<sup>[9]</sup>. Intraoperative cholangiography (IOC) has been advised by many authors as the technique

reduces the risk of BDI<sup>[1,4,6,10]</sup>. However, the procedure also has inherent limitations and is therefore reserved for select cases<sup>[11,12]</sup>. Moreover, worldwide consensus regarding the implementation of IOC is still lacking<sup>[13]</sup>.

Hepatobiliary surgery has become increasingly safe as a result of considerable progress in equipment, technology, perioperative management, and surgical technique. Fluorescent cholangiography (FC) is a novel approach, which offers real-time intraoperative imaging of the biliary anatomy. The first intraoperative use of FC in humans was described by Ishizawa *et al*<sup>[14]</sup> in 2010. The method involves the administration of indocyanine green (ICG) by either intrabiliary injection or intravenous injection 30 min before surgery. ICG binds to proteins present in bile and is excreted exclusively by the liver when administered intravenously. The excitation of protein-bound ICG by near-infrared light causes it to fluoresce, thereby delineating components of the biliary system for the surgeon. Fluorescence and imaging is achieved through a system consisting of a small control unit, a charge-coupled device camera, a xenon light source, and a 10 mm laparoscope containing specially coated lenses that transmit near-infrared light.

Intraoperative FC has been successfully performed during mini-invasive cholecystectomies in various studies, including standard LCs, single-incision cholecystectomies (SILCs), and robotic cholecystectomies (RCs)<sup>[15-34]</sup>. The primary aim of this review was to verify the utility of this technique for the intraoperative visualization of the extrahepatic biliary system in order to reduce the incidence of BDIs. The second aim was to illuminate the potential benefits and relative limitations of intraoperative FC.

## MATERIALS AND METHODS

### Literature search

MEDLINE and PubMed searches were performed using the key words "fluorescent cholangiography", "fluorescent angiography", "intraoperative fluorescent imaging", and "laparoscopic cholecystectomy" in order to identify relevant articles published in English, French, German, and Italian from 2009 to 2014. Reference lists from the articles were reviewed to identify additional relevant articles. For studies published in languages other than those mentioned above, all available information was taken from their English abstracts. All studies that contained material applicable to the topic were considered. Retrieved manuscripts (case reports, reviews, and abstracts) were reviewed by the authors, and the data were extracted using a standardized collection tool. Data were analyzed using descriptive statistics.

### Statistical analysis

In contrast to classic meta-analyses, the outcome is defined here as the percentages of an event (without comparison) in pseudo-cohorts of observed

**Table 1** Detection rates of biliary and vascular structures using fluorescent cholangiography *n* (%)

Ref.	Technique	<i>n</i>	CD	CHD	CD-CHD junction	CBD	CA
Larsen <i>et al</i> <sup>[17]</sup>	LC	35	35 (100)	35 (100)	35 (100)	35 (100)	29 (83.0)
Daskalaki <i>et al</i> <sup>[19]</sup>	RC	184	180 (97.8)	173 (94.0)	154 (83.6)	177 (96.1)	-
Dip <i>et al</i> <sup>[16]</sup>	LC	45	44 (97.7)	27 (60.0)	-	36 (80.0)	-
Osayi <i>et al</i> <sup>[18]</sup>	LC	82	78 (95.1)	57 (69.5)	63 (76.8)	63 (76.8)	-
Dip <i>et al</i> <sup>[20]</sup>	LC	43	42 (97.6)	25 (58.1)	-	34 (79.1)	-
Verbeek <i>et al</i> <sup>[21]</sup>	LC	14	14 (100)	-	-	-	-
Buchs <i>et al</i> <sup>[22]</sup>	SIRC	23	23 (100)	-	-	-	-
Spinoglio <i>et al</i> <sup>[23]</sup>	SIRC	45	42 (93.0)	40 (80.0)	40 (80.0)	41 (91.0)	-
Schols <i>et al</i> <sup>[24]</sup>	LC	15	15 (100)	-	-	15 (100)	-
Buchs <i>et al</i> <sup>[27]</sup>	SIRC	12	11 (91.7)	4 (33.3)	3 (25.0)	6 (50.0)	-
Kaneko <i>et al</i> <sup>[25]</sup>	LC	28	26 (92.9)	27 (96.4)	-	-	25 (89)
Ishizawa <i>et al</i> <sup>[28]</sup>	SILC	7	5 (71.4)	7 (100)	7 (100)	-	4 (57.1)
Aoki <i>et al</i> <sup>[30]</sup>	LC	14	10 (71.4)	-	-	10 (71.4)	-
Ishizawa <i>et al</i> <sup>[32]</sup>	LC	52	52 (100)	50 (96.2)	50 (96.2)	-	-
Ishizawa <i>et al</i> <sup>[32]</sup>	LC	1	1 (100)	1 (100)	1 (100)	-	-
Weighted average, % (95%CI)			96.2 (94.7-97.7)	78.1 (74.8-81.4)	72.0 (69.0-75.0)	86.0 (83.3-88.8)	69.4 (61.8-77.1)

CA: Cystic artery; CBD: Common bile duct; CD: Cystic duct; CHD: Common hepatic duct; LC: Standard laparoscopic cholecystectomy; RC: Robotic cholecystectomy; SILC: Single-incision laparoscopic cholecystectomy; SIRC: Single-incision robotic cholecystectomy.

patients. Overall proportions can be estimated from the weighted mean of percentages measured in each study. The weight in this case is derived from the number of subjects included in the study out of the total number of subjects in all studies, which is inverse of the variance in the classic meta-analyses. The confidence interval is calculated through the use of the normal distribution to approximate the binomial probabilities given that the condition "product of the probability and sample size ( $np$ ) is more than 5" is fulfilled.

## RESULTS

### Identification of biliary system with FC

At the time of this review, a total of 16 studies were found which involved FC during standard LCs ( $n = 11$ ), single-incision RCs (SIRCs;  $n = 3$ ), multiport RCs ( $n = 1$ ), and SILC ( $n = 1$ ).

The detection rates of major extrahepatic biliary structures with FC during laparoscopic or robotic cholecystectomy before dissection of Calot's triangle are summarized in Table 1. Overall, the potential of this novel technique for the detection of important biliary anatomy was revealed in these preliminary studies. The structures successfully identified before dissection of Calot's triangle included the cystic duct (CD), the common hepatic duct (CHD), the common bile duct (CBD), and the CD-CHD junction. A review of the literature revealed that the rates of detection of extrahepatic biliary system with this strategy ranged from 71.4 to 100% for CD, 33.3% to 100% for CHD, 50.0% to 100% for CBD, and 25.5% to 100% for the CD-CHD junction, with weighted averages of 96.2%, 78.1%, 72.0%, and 86.0%, respectively (Table 1).

Daskalaki *et al*<sup>[19]</sup> published the largest series to date of RCs performed with ICG fluorescence for the visualization of the biliary tree anatomy. Visualization

of at least one biliary structure was possible in 99% of cases, whereas all four main structures were detected (CD, CHD, CBD, and CD-CHD junction) in 83% of cases. No major complications, including biliary injury or conversion to open or laparoscopic approach, occurred in this series.

Using near-infrared FC (NIRF-C), Osayi *et al*<sup>[18]</sup> reported rates of visualization of the CD, CBD, and CHD after complete dissection of Calot's triangle of 95.1%, 76.8%, and 69.5%, respectively, compared to 72.0%, 75.6%, and 74.3% for IOC. In general, biliary structures were successfully identified with NIRF-C without biliary injuries or other major complications in 80% of cases.

These data indicated that FC provided a reliable roadmap of the bile duct anatomy, enabling surgeons to avoid BDIs while dissecting Calot's triangle.

### Identification of biliary structures with FC before and after Calot's dissection

A review of the literature revealed that a preliminary dissection of Calot's triangle led to an overall increase in the identification of all biliary structures. Ishizawa *et al*<sup>[14]</sup> in 2010 reported results on the first large cohort of patients ( $n = 52$ ) who had undergone LC with ICG FC. Rates of visualization for the CD and the CHD were found to be 100% and 96% before dissection and 100% after dissection for both structures. Buchs *et al*<sup>[22]</sup> concurrently published preliminary results on a series of 12 SIRC cases performed with intraoperative ICG FC. The rates of visualization for the CD, CHD, CBD, and the CD-CHD junction before Calot's dissection were 91.7%, 33.3%, 50.0%, and 25.0%, and 100.0%, 66.0%, 83.3%, and 58.0% after Calot's dissection, respectively. More recently, Spinoglio *et al*<sup>[23]</sup> reported more encouraging data based on results from a cohort of patients ( $n = 45$ ) who had undergone SIRC performed with routine ICG FC to evaluate the



**Table 2** Identification of biliary structures before and after Calot's dissection

Vascular structure	Ishizawa <i>et al</i> <sup>[14]</sup>		Osayi <i>et al</i> <sup>[18]</sup>		Spinoglio <i>et al</i> <sup>[23]</sup>		Buchs <i>et al</i> <sup>[22]</sup>		Weighted average, % (95%CI)
	<i>n</i> /total	%	<i>n</i> /total	%	<i>n</i> /total	%	<i>n</i> /total	%	
Before dissection of Calot's triangle									
CD	52/52	100.0	46/82	56.1	42/45	93.3	11/12	91.7	79.1 (74.0-84.1)
CHD	50/52	96.1	29/82	35.4	40/45	88.8	4/12	33.3	64.4 (59.0-69.8)
CBD	-	-	31/82	37.8	41/45	91.1	6/12	50.0	56.1 (48.9-63.3)
CD-CHD junction	50/52	96.1	20/82	24.4	40/45	88.8	3/12	25.0	59.1 (54.1-64.1)
After dissection of Calot's triangle									
CD	52/52	100.0	78/82	95.1	44/45	97.7	12/12	100.0	97.4 (94.9-99.8)
CHD	52/52	100.0	57/82	69.5	44/45	97.7	8/12	66.6	84.3 (79.6-89.0)
CBD	-	-	63/82	76.8	44/45	97.7	10/12	83.3	84.1 (78.3-90.0)
CD-CHD junction	52/52	100.0	63/82	76.8	44/45	97.7	7/12	58.3	86.9 (82.5-91.3)

CD: Cystic duct; CHD: Common hepatic duct; CBD: Common biliary duct.

extrahepatic biliary anatomy. The visualization rates of the CD, CHD, and CBD before the dissection of Calot's triangle were 93%, 88%, and 91%, respectively. After dissection of Calot's triangle, all of the rates increased to 97%. Statistical analysis confirmed that the increases in visualization rates from all studies were statistically significant (Table 2).

#### Identification of biliary structures with FC in obese patients

Although FC during LC appears to be a safe and effective procedure enabling real-time visualization of the biliary duct anatomy, limited results have been reported for when patients present with more challenging clinical conditions, such as obesity or acute cholecystitis. One of the potential limiting factors of the procedure is that near-infrared light has a penetration capability of only 5-10 mm. Therefore, the identification of the Calot's triangle structures can be challenging, especially in cases where there is an abundance of fatty tissue or severe inflammation of the gallbladder and surrounding tissues. In a cohort of obese patients<sup>[19]</sup>, the CD, CHD, and CBD were successfully identified with ICG fluorescence in 97%, 94%, and 95% of the cases, respectively, and the CD-CHD junction in 82% of the cases. However, some differences between patients were reported based on a body mass index (BMI) > 30 kg/m<sup>2</sup> or < 30 kg/m<sup>2</sup><sup>[19]</sup>.

NIRF-C was also used to evaluate the extrahepatic biliary structures, before and after complete dissection of Calot's triangle, in patients ( $n = 82$ ) who had undergone elective LC<sup>[18]</sup>. A number of obese patients were also included (39/82; 47.6%)<sup>[18]</sup>, and the results were compared to the routine use of IOC. A modestly improved rate for the identification of biliary structures was observed in patients with BMI < 30 kg/m<sup>2</sup> (43/82; 52.4%) relative to those with BMI > 30 kg/m<sup>2</sup>. Only a statistical difference for the visualization of the CD-CHD junction emerged (24.4% vs 76.8%,  $P = 0.04$ ). However, the rates of visualization of the CD and the CBD were decreased in patients with a BMI >

35 kg/m<sup>2</sup> (22/82; 26.8%) relative to patients with a BMI < 35 kg/m<sup>2</sup> (91.0% and 64.0% vs 92.3% and 71.8%, respectively). Finally, in the case with the highest BMI (63 kg/m<sup>2</sup>), the only structure visualized was the CD. In all patients, the CD was visualized at a significantly higher rate with NIRF-C than IOC (95.1% vs 72.0%,  $P < 0.001$ ), while there was no difference in visualization of the CD in the subgroup of patients ( $n = 62$ ) who had undergone both NIRF-C and IOC (98.4% vs 95.2%).

Overall, few results regarding the use of FC in obese patients have been reported. According to the data available, no statistically significant difference exists between patients with BMI < 30 kg/m<sup>2</sup> compared to patients with BMI > 30 kg/m<sup>2</sup>, regarding improved visualization of the biliary structures. The visualization frequency of the biliary structures in obese relative to non-obese patients, ranges from 92.3% to 100% vs 90.0% to 98.7% for the CD, 61.5% to 94.0% vs 40.0% to 93.9% for the CHD, and 50.0% to 95.0% vs 50.0% to 97.5% for the CBD, respectively (Table 3). There was an apparent difference only with regard to the visualization of the CD-CHD junction (61.0% to 82.3% vs 76.7% to 85.3%, respectively).

#### Identification of biliary structures with FC in patients with cholecystitis

Data were analyzed in the patients presenting with a second complicating clinical factor, cholecystitis, excluding patients with acute and gangrenous cholecystitis undergoing emergency surgery. Even in this subset of challenging cases, the successful identification of the CD, CHD, CBD and the CD-CHD junction was reported to be 91.6%, 79.1%, 79.1%, and 75.0%, respectively<sup>[19]</sup>. Similar results for visualization rates in such patients have been reported in a second study: 94.5%, 57.0%, and 72.0% for the CD, CHD, and CBD respectively<sup>[20]</sup> (Table 4). The number of patients examined, however, was too small for conclusive determination of the utility of FC in patients with cholecystitis. For these patients, Dip *et al.*<sup>[20]</sup> has advocated for the combined

**Table 3 Identification of biliary structures using fluorescent cholangiography in obese patients**

Vascular structure	Daskalaki <i>et al</i> <sup>[19]</sup>		Osayi <i>et al</i> <sup>[18]</sup>		Buchs <i>et al</i> <sup>[22]</sup>		Weighted average, % (95%CI)
	n/total	%	n/total	%	n/total	%	
BMI > 30 kg/m <sup>2</sup>							
CD	99/102	97.0	36/39	92.3	2/2	100	95.8 (92.5-99.0)
CHD	96/102	94.1	24/39	61.5	0/2	0	83.9 (80.0-87.8)
CBD	97/102	95.0	28/39	71.8	1/2	50	88.0 (83.1-93.0)
CD-CHD junction	84/102	82.3	26/39	66.6	0/2	0	76.9 (70.2-83.5)
BMI < 30 kg/m <sup>2</sup>							
CD	81/82	98.8	42/43	97.7	9/10	90	97.1 (94.6-99.5)
CHD	77/82	93.9	33/43	76.7	4/10	40	84.4 (78.8-90.0)
CBD	80/82	97.5	35/43	81.4	5/10	50	88.9 (84.0-93.7)
CD-CHD junction	70/82	85.3	37/43	86.0	3/10	30	81.4 (75.3-87.5)

BMI: Body mass index; CBD: Common biliary duct; CD: Cystic duct; CHD: Common hepatic duct.

**Table 4 Identification of biliary structures using fluorescent cholangiography in patients with acute cholecystitis**

Vascular structure	Daskalaki <i>et al</i> <sup>[19]</sup>		Dip <i>et al</i> <sup>[20]</sup>	
	n/ total	%	n/total	%
CD	22/24	91.6	-	94.5
CHD	19/24	79.1	-	57.0
CBD	19/24	79.1	-	72.0
CD-CHD junction	18/24	75.0	-	-

CBD: Common biliary duct; CD: Cystic duct; CHD: Common hepatic duct.

use of FC to identify the CD, followed by IOC to verify the CD-CHD junction. Preoperative magnetic resonance cholangiopancreatography (MRCP) offers an alternative to this strategy. In addition to excluding the concomitant lithiasis of the CBD, MRCP imaging allows for accurate visualization of the intra- and extrahepatic biliary tracts and can reveal a greater number of primary or secondary anatomical variations due to acute inflammation.

#### Detection of biliary stones

One of the important applications of standard fluoroscopic IOC is for the detection of biliary stones. To date, there is no evidence that FC can effectively identify CBD stones. However, the ability of this technique to detect stones elsewhere in the biliary tree has not been thoroughly investigated. Currently, results do not support the replacement of standard IOC with FC in cases where biliary stones are suspected preoperatively. Biliary stones in the cystic duct observed in preoperative cholangiography were correctly diagnosed in four patients with fluoroscopic IOC<sup>[14]</sup>. The fluorescent images were helpful for determining the optimal point for dividing the cystic duct without leaving stones in the remaining cystic duct. In contrast, FC failed to detect CBD stones diagnosed before surgery in one patient. According to Daskalaki *et al*<sup>[19]</sup>, FC could help to reveal a dilation or gallstones in the CD, but the method cannot exclude

the presence of CBD stones.

#### Detection of the biliary anomalies and leaks

The ability to detect biliary anomalies with FC has been investigated. Accessory bile ducts were diagnosed before surgery by drip infusion cholangiography and/or MRCP in 8/52 (15%) patients<sup>[14]</sup>; a right lateral, right paramedian, or a left paramedian sector branch were all draining directly into the CHD. In two patients, the accessory bile duct was detected with FC before dissection of Calot's triangle. In the remaining six patients, the accessory bile duct was observed only after dissection of Calot's triangle. Fluorescent imaging also revealed communicating accessory bile ducts between the left and right lobes of the liver in two patients. Anatomical variations were also identified with FC in an additional five patients (2.7%)<sup>[19]</sup>. In two cases, the CD was joined directly to the right hepatic duct, while in a third, the CBD was completely posterior to the hepatic artery. A fourth patient had an extended CD that was observed running parallel to the right hepatic duct before joining the CBD, and the last patient presented with an aberrant canaliculus from liver segment VI to the CHD.

The ability to detect intraoperative bile leaks with FC has been investigated to a limited extent. Bile leakage caused by cannulation of the CD in humans during IOC was easily visualized with fluorescent imaging<sup>[35]</sup>. However, detection of previously unknown bile leaks has not been reported.

#### Fluorescent angiography concomitant with cholangiography

Coupling of the fluorescent angiography with cholangiography has been described to enable identification of the cystic artery<sup>[14,17,25,34]</sup>. A second intraoperative bolus injection of ICG was required, however. In the largest study, the cystic artery began to fluoresce 20 to 30 seconds after the bolus, and it was identified in 25/28 (89.3%) patients<sup>[25]</sup>. In two additional studies, the cystic artery was successfully localized in 57%<sup>[14]</sup>

and 83%<sup>[17]</sup> of cases.

## DISCUSSION

FC has several potential advantages over conventional radiographic IOC. First, FC saves time, and second, FC prevents BDIs typically associated with a conventional IOC approach. Third, the technique is more convenient, as it requires only a preoperative intravenous ICG injection, and fluorescent images of the biliary tract are obtained in real time at any point during surgery without the assistance of radiation technicians. Fourth, fluorescent imaging enables surgeons to evaluate the extrahepatic biliary system easily and within a short timeframe. Lastly, the procedure is safe. There is no exposure to radiation, and the risk of adverse reactions to the ICG injection is very small (about 0.003% at doses exceeding 0.5 mg/kg)<sup>[35]</sup>. In short, ten characteristics have been highlighted for the use of FC in LC over IOC: feasibility, cost (cheaper), operating time (faster), specificity, instructional applications, safety, lack of learning curve, lack of X-ray exposure, simplicity, and real-time surgery<sup>[20]</sup>.

FC, however, also has some inherent deficiencies, namely, the limited tissue penetration of near-infrared light. Limited penetration of light results in the inability to visualize deep intrahepatic ducts or extrahepatic ducts obscured by surrounding organs and tissue. Practically, the technique is severely limited in patients with specific clinical conditions, such as obesity and cholecystitis, due to obstruction of near-infrared light.

In conclusion, ICG FC is a safe and effective procedure that enables real-time visualization of the biliary system. For these reasons, this novel procedure may become standard practice in order to prevent BDI during LC. Furthermore, the technique may replace RC as it allows for a more accurate and less invasive identification of the extrahepatic biliary anatomy tract, which reduces operative time, medical costs, and major postoperative complications<sup>[14,15]</sup>. Further research should aim to assess the impact of this technique on adverse events and long-term patient outcomes.

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## COMMENTS

### Background

Intraoperative cholangiography has been advised by many authors, as it reduces the risk of bile duct injury. However the technique has inherent limitations and is therefore generally reserved for select cases. Moreover, worldwide consensus regarding the implementation of intraoperative cholangiography is still lacking. Fluorescent cholangiography (FC) is a safe

and effective novel procedure that enables real-time visualization of the biliary system. The primary aim of this review is to verify the utility of this technique for more rigorous identification of the extrahepatic biliary system in order to reduce bile duct injuries intraoperatively. The second aim is to illuminate the limitations of the procedure as well as the potential benefits.

### Research frontiers

The first intraoperative use of FC in humans was described by Ishizawa *et al* in 2010. The method involves the administration of indocyanine green (ICG) by either intrabiliary injection or intravenous injection 30 min before surgery. ICG binds to proteins present in bile and is excreted exclusively by the liver when administered intravenously. The excitation of protein-bound ICG by near-infrared light causes it to fluoresce, thereby delineating components of the biliary system for the surgeon. A fluorescent imaging system consisting of a small control unit, a charge-coupled device camera, a xenon light source, and a 10 mm laparoscope containing specially coated lenses that transmit near-infrared light has been devised specifically for this surgical application.

### Innovations and breakthroughs

Intraoperative FC has been successfully performed during mini-invasive cholecystectomies in various studies, including standard laparoscopic cholecystectomies, single incision cholecystectomies, and robotic cholecystectomies. Retrieved manuscripts (case reports, reviews, and abstracts) concerning the utility of FC were reviewed by the authors, and the data were extracted using a standardized collection tool.

### Applications

This review suggests that FC is a valid method to detect extrahepatic biliary anatomy during laparoscopic cholecystectomy, and that the technique may become standard practice in order to prevent bile duct injuries.

### Terminology

FC is a novel method that involves the administration of ICG by either intrabiliary injection or intravenous injection 30 min before surgery, which is excited by a near-infrared light source, thus permitting visualization of the extrahepatic biliary system intraoperatively.

### Peer-review

In this systematic review, the authors have presented a thorough and critical analysis of the utility of FC for more rigorous identification of biliary anatomy in order to prevent bile duct injury during laparoscopic cholecystectomies.

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## Diagnostic performance of magnifying narrow-band imaging for early gastric cancer: A meta-analysis

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### Abstract

**AIM:** To investigate the performance of magnifying endoscopy with narrow-band imaging (ME-NBI) in the diagnosis of early gastric cancer (EGC).

**METHODS:** Systematic literature searches were conducted until February 2014 in PubMed, EMBASE, Web of Science, Ovid, Scopus and the Cochrane Library databases by two independent reviewers. Meta-analysis was performed to calculate the pooled sensitivity, specificity and diagnostic odds ratio and to construct a summary receiver operating characteristic (ROC) curve. Subgroup analyses were performed based on the morphology type of lesions, diagnostic standard, the size of lesions, type of assessment, country and sample size to explore possible sources of heterogeneity. A Deeks' asymmetry test was used to evaluate the publication bias.

**RESULTS:** Fourteen studies enrolling 2171 patients were included. The pooled sensitivity, specificity and diagnostic odds ratio for ME-NBI diagnosis of EGC were 0.86 (95%CI: 0.83-0.89), 0.96 (95%CI: 0.95-0.97) and 102.75 (95%CI: 48.14-219.32), respectively, with the area under ROC curve being 0.9623. Among the 14 studies, six also evaluated the diagnostic value of conventional white-light imaging, with a sensitivity

of 0.57 (95%CI: 0.50-0.64) and a specificity of 0.79 (95%CI: 0.76-0.81). When using "VS" (vessel plus surface) ME-NBI diagnostic systems in gastric lesions of depressed macroscopic type, the pooled sensitivity and specificity were 0.64 (95%CI: 0.52-0.75) and 0.96 (95%CI: 0.95-0.98). For the lesions with a diameter less than 10 mm, the sensitivity and specificity were 0.74 (95%CI: 0.65-0.82) and 0.98 (95%CI: 0.97-0.98).

**CONCLUSION:** ME-NBI is a promising endoscopic tool in the diagnosis of early gastric cancer and might be helpful in further target biopsy.

**Key words:** Narrow-band imaging; Early gastric cancer; Magnifying endoscopy; Meta-analysis; Conventional white-light imaging

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**Core tip:** This is the first meta-analysis to systematically evaluate the diagnostic performance of magnifying endoscopy with narrow-band imaging (ME-NBI) for early gastric cancer (EGC) and the pooled results showed that ME-NBI was an effective endoscopic tool in EGC diagnosis, which has a better performance than conventional white-light imaging. Moreover, the morphology type of lesions, diagnostic standard and the size of lesions might influence the diagnostic value of ME-NBI.

Hu YY, Lian QW, Lin ZH, Zhong J, Xue M, Wang LJ. Diagnostic performance of magnifying narrow-band imaging for early gastric cancer: A meta-analysis. *World J Gastroenterol* 2015; 21(25): 7884-7894 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7884.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7884>

## INTRODUCTION

Gastric cancer remains the second leading cause of cancer-associated death worldwide<sup>[1]</sup>. Early detection and therapy for gastric cancer can improve 5 year survival rates to 96%, compared to the high mortality of advanced gastric cancer<sup>[2]</sup>. Therefore, it is a top priority to make a diagnosis at an early stage in the management of gastric cancer.

Conventional white-light imaging (C-WLI) has been applied as the standard endoscopic examination for the identification of suspicious lesions but it is difficult to make an accurate diagnosis of early neoplastic lesions in most cases<sup>[3]</sup>. Several studies have indicated that the sensitivity of C-WLI for diagnosing early gastric cancer varied from 33% to 75% and specificity from 57.0% to 93.8%<sup>[3-8]</sup>. The ultimate goal of endoscopists is to make a reliable diagnosis under microscopic view, with a decreased number of biopsies<sup>[3]</sup>. To this end, C-WLI would not be entitled and it is urgent to

find a novel endoscopic imaging technology with high diagnostic accuracy.

Magnifying endoscopy with narrow-band imaging (ME-NBI) is an advanced endoscopic imaging technology launched recently, in which spectral bandwidth filters in a red-green-blue (R/G/B) sequential illumination system are used to improve the accuracy of diagnosis<sup>[9]</sup>. It has been developed to enhance the visualization of the superficial mucosal structure and vascular architecture<sup>[10]</sup>. So far, ME-NBI has been applied in the diagnoses of various diseases, such as Barrett's esophagus<sup>[11,12]</sup>, esophageal carcinoma<sup>[13]</sup>, *Helicobacter pylori*-associated chronic gastritis<sup>[14]</sup>, intestinal metaplasia<sup>[15]</sup>, colonic polyps<sup>[10]</sup> and so forth. Moreover, it has also been applied to evaluate the histological type of early gastric cancer (EGC)<sup>[16]</sup> and to measure the horizontal extent and invasion depth of the tumor before endoscopic submucosal dissection<sup>[17,18]</sup>.

A randomized and controlled trial reported that ME-NBI was more accurate than C-WLI endoscopy in identifying small, depressed gastric mucosal cancers<sup>[3]</sup>. However, the accuracy of ME-NBI for the diagnosis of EGC was variable, with the sensitivity ranging from 60% to 100% and the specificity ranging from 84% to 100%<sup>[3-8,19-26]</sup>. The aim of this meta-analysis was to systematically assess the diagnostic performance of ME-NBI in EGC.

## MATERIALS AND METHODS

### Search strategy

We systematically searched in PubMed, EMBASE, Web of Science, Ovid, Scopus and the Cochrane Library databases up to February 2014 to identify relevant articles. The search terms were as follows: ("narrow band" OR "narrow band imaging" OR "NBI" OR "electronic chromoendoscopy" OR "digital chromoendoscopy" OR "optical chromoendoscopy") AND ("gastric cancer" OR "gastric carcinoma" OR "gastric neoplasm" OR "stomach cancer" OR "stomach carcinoma" OR "stomach neoplasm"). To avoid missing studies, we also read through the reference lists of relevant articles and reviews. The retrieved studies were carefully examined to exclude duplicate data. After scanning titles and abstracts of articles selected from the initial search, we reviewed the full text of potential eligible studies. This meta-analysis was designed, conducted and reported according to the PRISMA statement.

### Selection criteria

Articles were included if they met all the following inclusion criteria: (1) ME-NBI was used for the diagnosis of EGC; (2) numbers of true-positive (TP), false-positive (FP), true-negative (TN) and false-negative (FN) cases were reported or could be calculated; (3) histopathology was applied as a reference standard; and (4) published

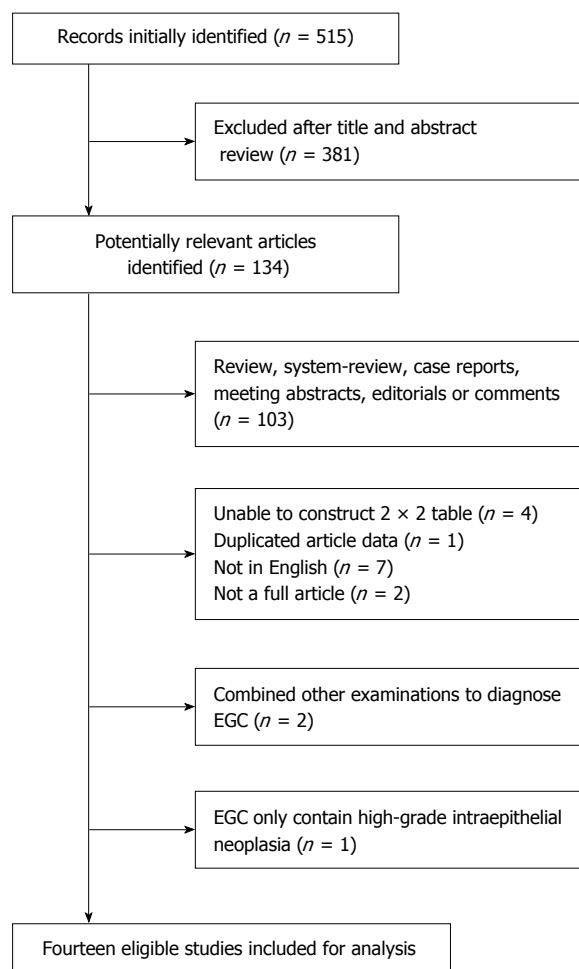


Figure 1 Literature search flow diagram. EGC: Early gastric cancer.

as full articles in English. Articles that met any of the following exclusion criteria were excluded: (1) combined examinations for EGC diagnosis, such as ME-NBI combined with trimodal imaging endoscopy or AFI; (2) previous known gastric cancer lesions; (3) only high-grade intraepithelial neoplasia; (4) hereditary diffuse gastric cancer or gastric remnant carcinoma; and (5) review articles, case reports, editorials, comments, letters to the editor, meeting abstracts.

#### Data extraction and quality assessment

Data were extracted independently by 2 reviewers and the following information was obtained from each study: the first author, year of publication, age and gender, morphology type of lesions, diagnostic standard, lesion size, type of assessment, endoscopic system and number of endoscopists. Numbers of TP, FP, TN and FN were also extracted. Discrepancies were resolved by a third investigator. The quality of the included studies was estimated using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS). A total of 14 items were assessed, with each item assessed as "yes", "no" or "unclear".

#### Statistical analysis

Meta-analysis was carried out to evaluate the accuracy of ME-NBI in differentiating malignant from benign early gastric lesions. The pooled sensitivity, specificity, positive LR, negative LR and diagnostic OR (with corresponding 95%CI) were estimated by a fixed-effect model (Mantel-Haenszel method) when significant heterogeneity was absent or a random effect model (DerSimonian-Laird method) when there was significant heterogeneity. Heterogeneity among the included studies was tested by the Cochrane Q test. Inconsistency ( $I^2$ ) was used to express the percentage variability attributable to heterogeneity.  $I^2$  greater than 50% was considered significant for heterogeneity. A summary receiver operating characteristic (SROC) curve was constructed. The area under the curve (AUC) was an overall summary measure index of the diagnosis and a perfect test would have an AUC close to 1. To explore possible sources of heterogeneity among the studies, subgroup analyses were performed with the following covariates such as the morphology type of lesions (depressed vs not depressed), diagnostic standard (an irregular microvascular (MV) pattern or/and an irregular microsurface (MS) pattern with a demarcation line vs others), lesion size (the diameter more than 10 mm vs less than 10 mm), type of assessment (real-time vs post-procedure), country (China vs Japan) and sample size (< 100 patients vs  $\geq$  100 patients). Spearman coefficient was assessed to assess threshold effect. A strongly positive correlation between the log of sensitivity and the log of 1-specificity indicates the presence of threshold effect.

Deeks' asymmetry was employed to evaluate the publication bias by constructing a funnel plot of diagnostic log odds ratio vs  $1/\sqrt{\text{effective sample size}}$ . The pooled sensitivity, specificity, positive LR, negative LR, diagnostic OR, SROC curve, Spearman coefficient and subgroup analysis were performed using Meta-Disc version 1.4 (Ramony Cajal Hospital, Madrid, Spain). Meta-regression and publication bias were analyzed using STATA version 12.0 (Stata Corporation, College Station, Tex).  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Included studies

After searching PubMed, EMBASE, Web of Science, Ovid, Scopus and the Cochrane Library databases, 515 articles were identified. On the basis of titles and abstracts, 343 articles were excluded, leaving 172 studies for further selection. The selection process and reasons for exclusion are summarized in Figure 1. Fourteen studies comprising 2171 participants were eligible for final analysis.

Table 1 Characteristics of the selected studies

Ref.	No. of patients	Sex, male/female	Age, mean	Country	Diagnostic standard	Morphology type of lesions (depressed/not depressed)	Lesion size (mm)	Type of assessment	Endoscopic system (Lucera/Exera)	Endoscopists number
Liu <i>et al</i> <sup>[19]</sup>	90	49/41	57.5	China	Type <sup>1</sup> A-E	47/160	NA	Real-time	NA	2
Yao <i>et al</i> <sup>[20]</sup>	310	183/127	66	Japan	Irregular MV and/or irregular MS with a demarcation line	231/134	NA	Real-time	Lucera	20
Kanesaka <i>et al</i> <sup>[21]</sup>	49	35/16	NA	Japan	Presence of dense-type crypt openings	0/51	NA	Post-procedure	Lucera	4
Tao <i>et al</i> <sup>[4]</sup>	508	316/192	63	China	Irregular MV and/or irregular MS with a demarcation line	192/451	7	Post-procedure	NA	NA
Horuchi <i>et al</i> <sup>[22]</sup>	51	31/20	65	Japan	Micrification of fine mucosal structure	0/64	NA	Post-procedure	Lucera	NA
Maki <i>et al</i> <sup>[5]</sup>	93	73/20	NA	Japan	Irregular MV and/or irregular MS with a demarcation line	0/93	NA	Post-procedure	Lucera	NA
Miwa <i>et al</i> <sup>[6]</sup>	135	77/58	70.1	Japan	Irregular MV and/or irregular MS with a demarcation line	26/109	NA	Post-procedure	Lucera	NA
Tsuji <i>et al</i> <sup>[24]</sup>	137	101/36	NA	Japan	Irregular MV and/or irregular MS with a demarcation line	19/118	NA	Post-procedure	Lucera	4
Li <i>et al</i> <sup>[23]</sup>	146	88/58	59.3	China	Type <sup>2</sup> A-C	52/112	21.9	Real-time	Lucera	2
Ezoe <i>et al</i> <sup>[5]</sup>	353	278/75	69	Japan	Irregular MV with a demarcation line	353/0	5.6	Real-time	Lucera	31
Nonaka <i>et al</i> <sup>[25]</sup>	93	71/22	70	Japan	Type <sup>3</sup> I-V	0/93	15.1	Real-time	NA	4
Ezoe <i>et al</i> <sup>[7]</sup>	53	NA	NA	Japan	Irregular MV with a demarcation line	57/0	≤ 10	Real-time	Lucera	5
Kato <i>et al</i> <sup>[8]</sup>	111	98/13	66.3	Japan	Disappearance of fine mucosal structure, microvascular dilation, heterogeneity	NA	7.0	Real-time	Lucera	NA
Yao <i>et al</i> <sup>[26]</sup>	42	NA	NA	Japan	Either WOS with a regular distribution or a regular MV	0/46	NA	Real-time	Lucera	1

<sup>1</sup>Type A-E: Type A, short rod-like pits with regular microvasculature inside pits; Type B, elongated open branch-like pits with regular microvasculature; Type C, dilated pits and increased branching microvasculature; Type D, villus-like appearance or light blue crest sign; Type E, the appearance of new tumor vessels; <sup>2</sup>Type A-C: Type A, clear regular surface patterns and microvascular architecture; Type B, obscure irregular surface patterns or microvascular architecture; Type C, no surface pattern and sparse microvessels or with avascular areas; <sup>3</sup>Type I-V: Type I, clear MS, unclear MV; type II, clear MS, clear MV; type III, clear MS, abnormal MV; type IV, slightly obscured MS, abnormal MV; type V, markedly obscured MS, abnormal MV. NA: Not available; MV: The microvascular pattern; MS: The microsurface pattern; WOS: White opaque substance.

### Study characteristics and quality assessment

The characteristics of the 14 studies are presented in Table 1. Among them, 6 studies<sup>[3-8]</sup> compared the diagnostic values between ME-NBI and C-WLI. Eleven studies were performed in Japan<sup>[3,5-8,20-22,24-26]</sup> and three in China<sup>[4,19,23]</sup>. Seven studies used "an irregular microvascular (MV) pattern and/or an irregular microsurface (MS) pattern with a demarcation line" as a diagnostic standard<sup>[3-7,20,24]</sup> and others used less defined criteria of fine mucosal structure, white opaque substance<sup>[26]</sup> or crypt openings<sup>[21]</sup>. Real-time assessment was conducted in eight studies<sup>[3,7,8,19,20,23,25,26]</sup> and post-procedure assessment in the remaining ones<sup>[4-6,21,22,24]</sup>. The macroscopic appearance of lesions was limited to depressed type in three studies<sup>[3,7,20]</sup>, with "VS" (vessel plus surface) diagnostic systems applied, and the rest of the studies recruited non-depressed lesions<sup>[4-6,8,19,21-26]</sup>. The overall quality of the selected studies was excellent according to the QUADAS questionnaires and the details are listed in Table 2.

### Diagnostic performance of ME-NBI

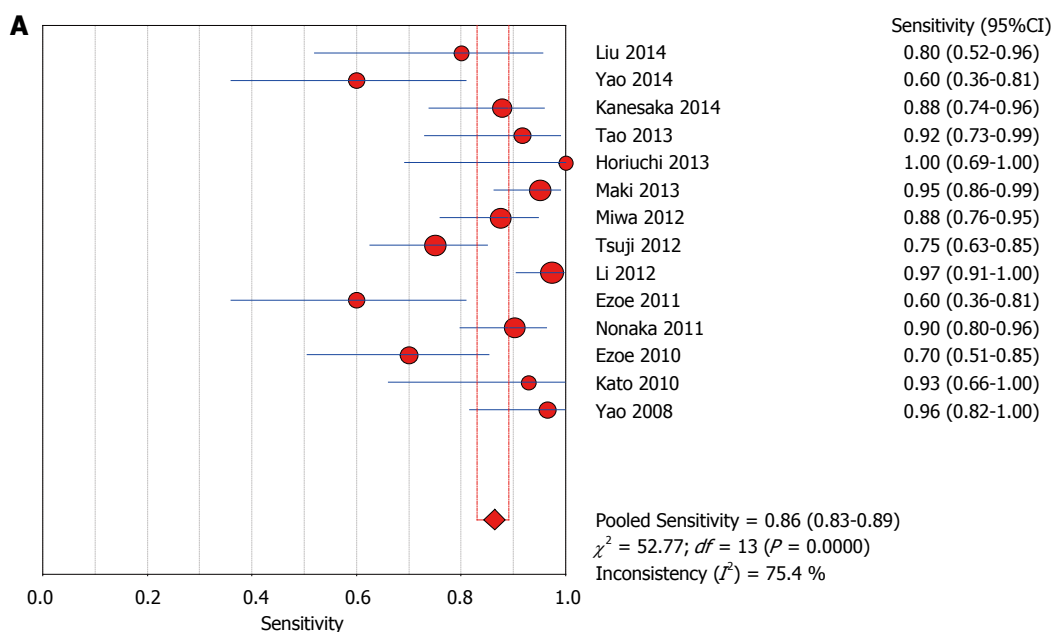
Calculated by a random effect model, the pooled sensitivity and specificity of ME-NBI for diagnosing early gastric cancer were 0.86 (95%CI: 0.83-0.89) and 0.96 (95%CI: 0.95-0.97), respectively (Figure 2). Significant heterogeneities were found in sensitivity ( $I^2 = 75.4\%$ ) and specificity ( $I^2 = 86.9\%$ ). The AUC was 0.9623 ( $E = 0.0127$ ).



**Table 2** Quality of articles using the quality assessment of diagnostic accuracy studies tool

Ref.	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Item 7	Item 8	Item 9	Item 10	Item 11	Item 12	Item 13	Item 14	Scores
Liu <i>et al</i> <sup>[19]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14
Yao <i>et al</i> <sup>[20]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14
Kanesaka <i>et al</i> <sup>[21]</sup>	N	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	Y	12
Tao <i>et al</i> <sup>[4]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	13
Horiuchi <i>et al</i> <sup>[22]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	U	Y	Y	Y	12
Maki <i>et al</i> <sup>[5]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	U	Y	Y	Y	12
Miwa <i>et al</i> <sup>[6]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	U	Y	Y	Y	12
Tsuji <i>et al</i> <sup>[24]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14
Li <i>et al</i> <sup>[23]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14
Ezoe <i>et al</i> <sup>[3]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	N	12
Nonaka <i>et al</i> <sup>[25]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14
Ezoe <i>et al</i> <sup>[7]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	13
Kato <i>et al</i> <sup>[8]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	13
Yao <i>et al</i> <sup>[26]</sup>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	13

Item 1: Was the spectrum of patients representative of the patients who will receive the test in practice? Item 2: Were selection criteria clearly described? Item 3: Is the reference standard likely to correctly classify the target condition? Item 4: Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests? Item 5: Did the whole sample or a random selection of the sample receive verification by using a reference standard of diagnosis? Item 6: Did patients receive the same reference standard regardless of the index test result? Item 7: Was the reference standard independent of the index test? Item 8: Was the execution of the index test described in sufficient detail to permit replication of the test? Item 9: Was the execution of the reference standard described in sufficient detail to permit its replication? Item 10: Were the index test results interpreted without knowledge of the results of the reference standard? Item 11: Were the reference standard results interpreted without knowledge of the results of the index test? Item 12: Were the same clinical data available when test results were interpreted as would be available when the test is used in practice? Item 13: Were uninterpretable/intermediate test results reported? Item 14: Were withdrawals from the study explained? Y: Yes; N: No; U: Unclear.



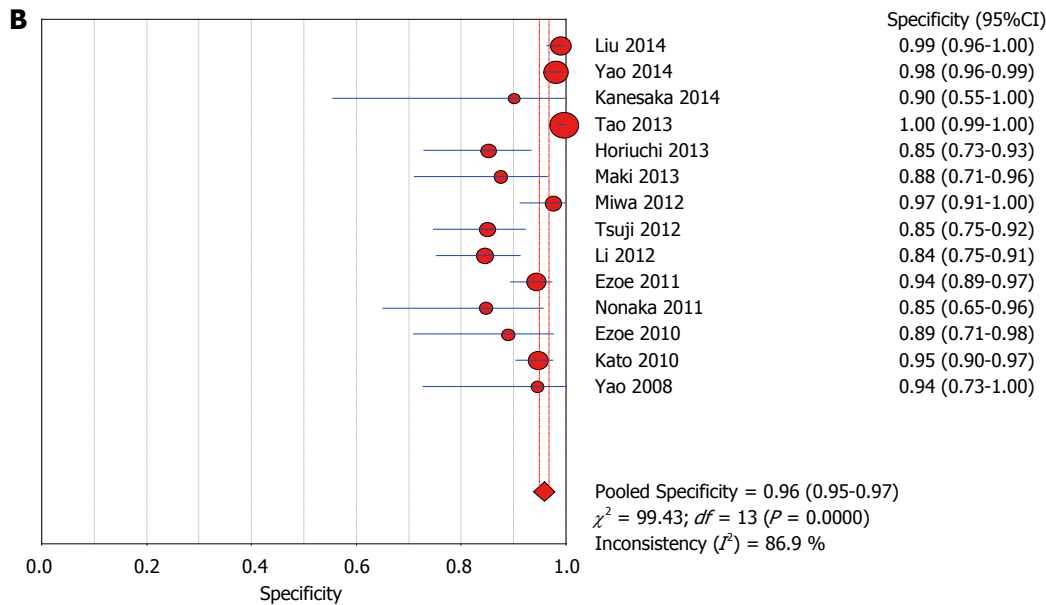


Figure 2 Forest plot showing pooled sensitivity and specificity of magnifying endoscopy with narrow-band imaging for early gastric cancer.

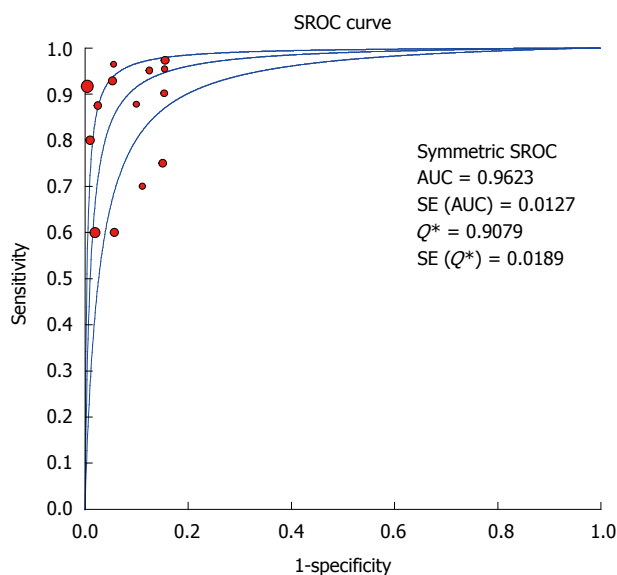


Figure 3 Summary receiver operating characteristic curve showing the diagnostic performance of magnifying endoscopy with narrow-band imaging for early gastric cancer.

(Figure 3), indicating an excellent performance of ME-NBI in the diagnosis of EGC. For C-WLI, the sensitivity, specificity and AUC for diagnosing EGC were 0.57 (95%CI: 0.50-0.64), 0.79 (95%CI: 0.76-0.81) and 0.6634, respectively, indicating a limited diagnostic performance of C-WLI compared with ME-NBI. Moreover, for the depressed-type lesions, C-WLI has a low sensitivity of 0.30 (95%CI: 0.18-0.45) and specificity of 0.68 (95%CI: 0.10-0.24). The overall positive LR, negative LR and diagnostic OR for diagnosing EGC by ME-NBI were 13.49 (95%CI: 8.14-22.37), 0.16 (95%CI: 0.10-0.24) and 102.75 (95%CI: 48.14-219.32), respectively (Figures 4 and 5).

Significant heterogeneities were found in positive LR ( $I^2 = 79.0\%$ ), negative LR ( $I^2 = 73.6\%$ ) and diagnostic OR ( $I^2 = 69.3\%$ ).

The Spearman correlation coefficient was 0.367 ( $P = 0.196$ ), suggesting no evidence of significant threshold effect. Meta-regression and subgroup analyses were performed in order to explore the potential sources of heterogeneity. The results indicated that the morphology type of lesions, diagnostic standard, lesion size and sample size might be the possible sources of heterogeneity (Table 3). Studies in which "VS classification system" was regarded as diagnostic standard revealed reduced benefits over those using other diagnostic standards in terms of sensitivity (81% vs 93%,  $P = 0.01$ ) but not in the specificity (97% vs 93%,  $P = 0.36$ ). When the "VS" was applied in the depressed lesions, the sensitivity and specificity were 0.64 (95%CI: 0.52-0.75) and 0.96 (95%CI: 0.95-0.98), while in the non-depressed lesions, the sensitivity and specificity were as high as 0.86 (95%CI: 0.81-0.91) and 0.98 (95%CI: 0.96-0.98). The diagnostic sensitivity of ME-NBI was strikingly lower in depressed lesions than that in non-depressed ones (64% vs 90%,  $P < 0.001$ ). Nevertheless, the difference of the specificity between them was not significant (96% vs 96%,  $P = 0.75$ ). ME-NBI had a significantly higher sensitivity when assessing lesions with a diameter more than 10 mm than those less than 10 mm (90% vs 74%,  $P = 0.04$ ), while there was an opposite result in terms of specificity (88% vs 98%,  $P = 0.02$ ). For the studies with a sample size less than 100 patients, the specificity of ME-NBI for EGC was higher than that of other studies (97% vs 87%,  $P = 0.04$ ), while no marked difference of sensitivity between them was observed (84% vs 90%,  $P = 0.22$ ). Meta-regression

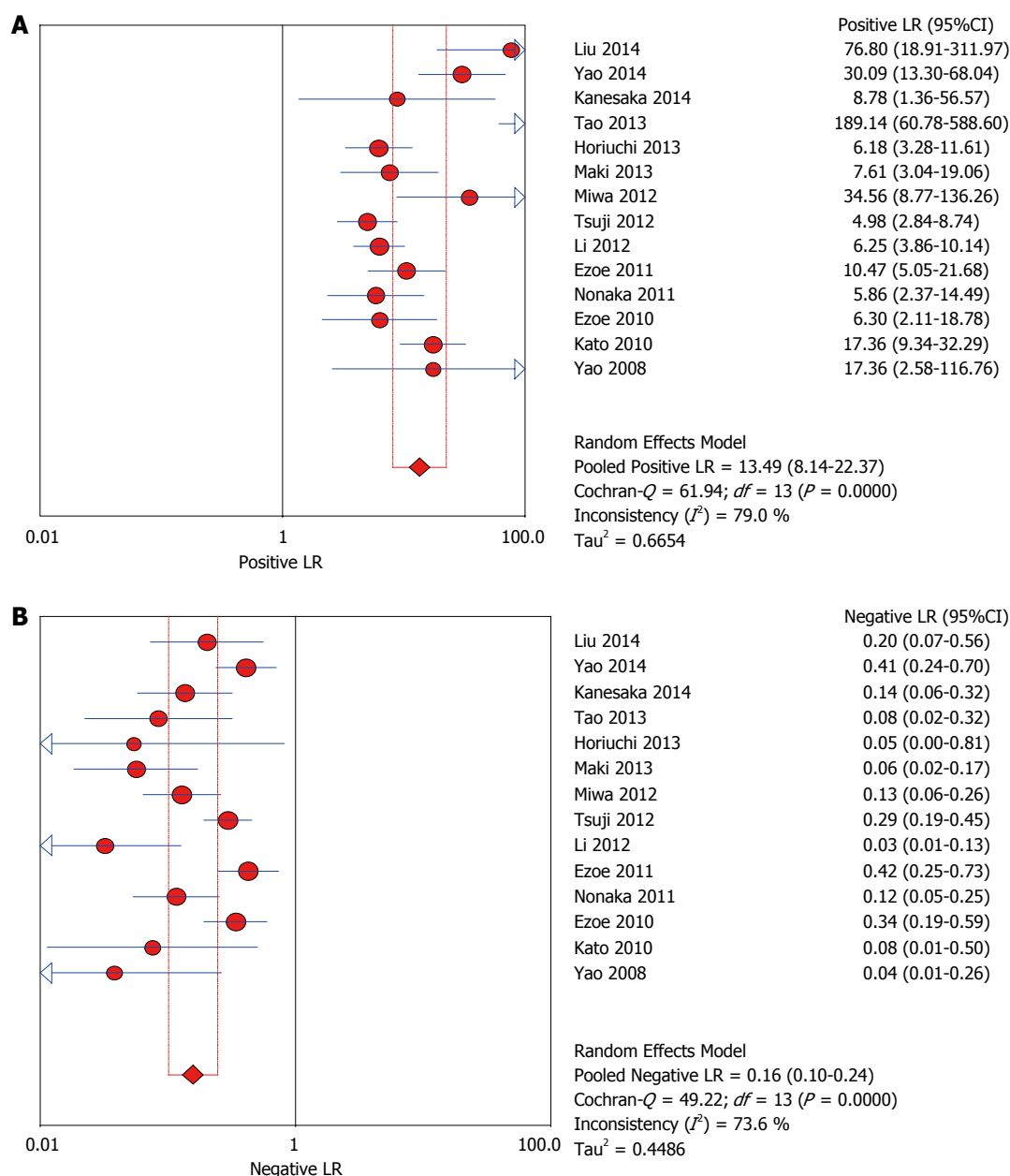


Figure 4 Forest plot showing the positive likelihood ratio and negative likelihood ratio of magnifying endoscopy with narrow-band imaging for early gastric cancer.

and subgroup analysis based on type of assessment and country did not show remarkable significance considering the values of sensitivity and specificity (Table 3).

Deeks' funnel plot did not display significant asymmetry ( $P = 0.967$ ), indicating that no striking publication bias was present in this study (Figure 6).

## DISCUSSION

Nowadays, ME-NBI has been applied in the diagnostic workup of gastrointestinal tumors, especially in the differentiation of colonic lesions<sup>[10]</sup>, while its diagnostic accuracy for EGC is unclear. In this meta-analysis, we demonstrated that ME-NBI is a highly specific

diagnostic tool for EGC, with a high sensitivity (86%), specificity (96%) and diagnostic odds ratio (102.75), which were higher than those of C-WLI (57%, 79% and 3.46), indicating that ME-NBI had a better diagnostic performance for EGC.

This meta-analysis also demonstrated that the diagnostic performance of ME-NBI was influenced by the type and size of gastric lesions, especially the depressed type and in lesions less than 10 mm. Since the depressed mucosa type is the predominant morphology among gastric cancers, early detection and diagnosis of depressed type cancer are an effective way to decrease the mortality of gastric cancer<sup>[27-29]</sup>. However, we observed that ME-NBI had a relatively lower sensitivity of 64% for lesions of a

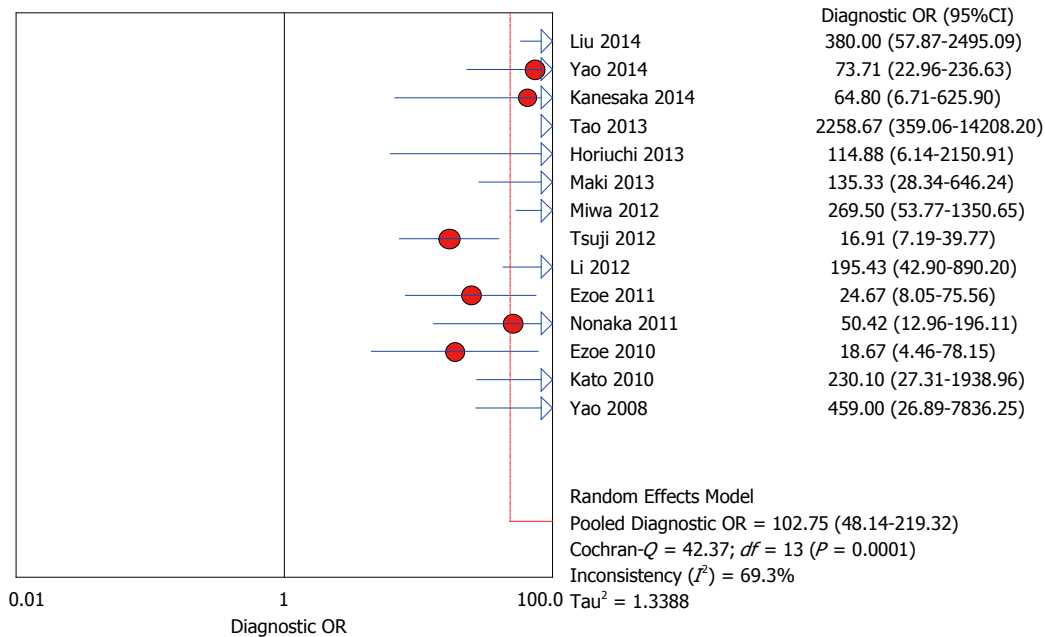


Figure 5 Forest plot showing diagnostic odds ratios of magnifying endoscopy with narrow-band imaging for early gastric cancer.

**Table 3 Subgroup analysis on diagnostic performance of magnifying endoscopy with narrow-band imaging for differentiating early gastric cancer from non-cancer**

	Number of studies (lesions examined)	Sensitivity (95%CI)	Specificity (95%CI)	AUC	$I^2$	
					Sensitivity	Specificity
Overall	14 (2433)	0.86 (0.83-0.89)	0.96 (0.95-0.97)	0.9623	75.4%	86.9%
Type of lesion						
Depressed	3 (605)	0.64 (0.52-0.75)	0.96 (0.95-0.98)	0.7877	0.0%	74.1%
Not depressed	10 (1828)	0.90 (0.86-0.92)	0.96 (0.95-0.97)	0.9694	64.9%	90.1%
Diagnosis standard						
Diagnosis (IMVP/IMSP + DL)	7 (1613)	0.81 (0.76-0.85)	0.97 (0.96-0.98)	0.9407	78.0%	88.8%
Diagnosis (others)	7 (820)	0.93 (0.89-0.96)	0.93 (0.91-0.95)	0.9719	36.6%	80.5%
Lesion size						
Diameter (> 10 mm)	8 (777)	0.90 (0.86-0.93)	0.88 (0.85-0.91)	0.9585	71.2%	44.7%
Diameter ( $\leq$ 10 mm)	5 (1449)	0.74 (0.65-0.82)	0.98 (0.97-0.98)	0.9458	67.1%	86.6%
Sample size						
$\geq$ 100 patients	8 (2035)	0.84 (0.79-0.88)	0.97 (0.96-0.97)	0.9663	79.1%	90.7%
< 100 patients	6 (398)	0.90 (0.85-0.93)	0.87 (0.81-0.92)	0.9422	67.3%	0.0%
Type of assessment						
Real-time	8 (1310)	0.85 (0.81-0.90)	0.95 (0.94-0.96)	0.9617	81.6%	81.2%
Post-procedure	6 (1123)	0.87 (0.82-0.91)	0.97 (0.95-0.98)	0.9608	65.5%	91.6%
Country						
China	3 (1014)	0.94 (0.88-0.97)	0.98 (0.97-0.99)	0.9864	62.5%	95.7%
Japan	11 (1419)	0.84 (0.80-0.88)	0.94 (0.92-0.95)	0.9526	74.6%	71.2%

$I^2 > 50\%$  was considered significant for heterogeneity. AUC: Area under the curve.

depressed type than those of a non-depressed type (90%), while the specificity was similar as 96% for both of them. These results indicated that ME-NBI was more reliable in identifying non-depressed EGC lesions. Interestingly, the size of most depressed lesions was less than 10 mm in enrolled studies and thus the diagnostic performance for depressed type lesions is representative of lesions with a diameter less than 10 mm. As for the depressed type lesions with a diameter over 10 mm, we failed to find relevant studies and further research might be required in

the future. In addition, our results showed that the diagnostic sensitivity was as low as 74% in gastric lesions with a diameter less than 10 mm, but the sensitivity for lesions with a diameter over 10 mm was 90%. Although the sensitivity was low in the lesions with a diameter less than 10 mm, the specificity for these lesions was as high as 98%, while for the lesions with a diameter over 10 mm, the specificity was only 88%. Accordingly, depressed lesions and lesions with a diameter less than 10 mm might limit the application of ME-NBI in the diagnosis of EGC. When identifying



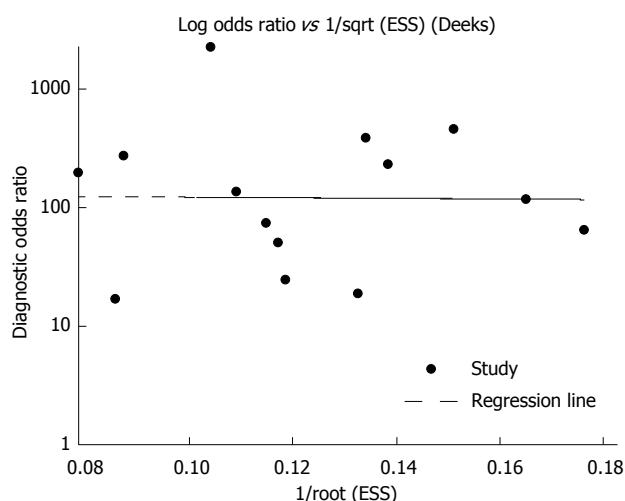


Figure 6 Deeks' funnel plot for publication bias. ESS: Effective sample size.

depressed gastric lesions with a diameter less than 10 mm, it would be better to combine ME-NBI with other examinations to improve the diagnosis of EGC.

Although ME-NBI was far from enough to identify depressed type lesions, it was a better option than C-WLI. Ezoe *et al.*<sup>[7]</sup> reported that ME-NBI was more accurate than C-WLI in identifying small, depressed gastric mucosal cancers, with a higher sensitivity (70% vs 33%) and specificity (89% vs 67%). Kaise *et al.*<sup>[30]</sup> discovered that in the differential diagnosis of superficial depressed gastric lesions, ME-NBI showed a superior specificity (85%) than C-WLI (65%), but the sensitivities for both of them were comparably moderate. In our meta-analysis, two studies<sup>[3,7]</sup> reported the role of C-WLI in the diagnosis of depressed type EGC, with a sensitivity of 30% and a specificity of 68%, both of which were lower than those of ME-NBI (sensitivity: 64%; specificity: 96%). Our study also demonstrated a higher diagnostic performance of ME-NBI than C-WLI in depressed EGC diagnosis.

Several diagnostic criteria have been developed to guide endoscopists in optical diagnosis EGC by ME-NBI. Yao *et al.*<sup>[9]</sup> firstly proposed a simple classification system called the "VS (vessel plus surface) classification system", in which an irregular microsurface pattern and/or an irregular microvascular pattern with a demarcation line are significant markers of EGC. This diagnostic reference has been used in several studies with variable diagnostic performance. This author also demonstrated that in cases of gastric neoplasia of 0-IIa type, a white opaque substance (WOS) obscured the subepithelial capillaries of the lesion and 83% of EGC showed an irregular distribution<sup>[26]</sup>. In another study, Kato *et al.*<sup>[8]</sup> used a triad-based diagnosis of disappearance of fine mucosal structure, microvascular dilation and heterogeneity to identify superficial gastric lesions. In our study, when the "VS (vessel plus surface) classification system" was applied as the diagnostic criteria, the sensitivity was 81%, which

was lower than that using other diagnostic criteria. For the lesions of depressed type, the sensitivity and specificity were 64% and 96% with the "VS classification system" diagnostic criteria and there were no other diagnostic criteria applied in these lesions in our enrolled studies. In contrast, when the diagnostic criteria of "VS" was used in non-depressed lesions, it had a higher sensitivity of 86% and the specificity was 98%. This indicates that the "VS classification system" was limited in depressed EGC. However, it has been reported that when only microvascular irregularity was used as the diagnostic standard for depressed gastric cancers, the mean sensitivity was as high as 86.7%<sup>[31]</sup>. These variable results suggest that further research is still required to evaluate the "VS classification system" in the differential diagnosis of depressed gastric lesions. There were other diagnostic standards in depressed gastric cancer but the results were not entirely optimistic. It was reported that when the triad of FMS disappearance, microvascular dilation and heterogeneity was used as the diagnostic standard for superficial depressed gastric cancer, the sensitivity was only 69.1%<sup>[30]</sup>. Thus, it is urgent to improve the sensitivity of ME-NBI, especially for depressed gastric lesions.

The variability of observers in the diagnostic performance of ME-NBI has increasingly caught our attention. Mochizuki *et al.*<sup>[32]</sup> reported that among the experts, the interobserver  $\kappa$  value was 0.85, with 88.0% consensus of diagnoses in the differential diagnosis of gastric adenoma and carcinoma, while with the two inexperienced endoscopists, the interobserver  $\kappa$  value was 0.44, with 68.0% consensus of diagnoses, implying that the diagnostic performance of ME-NBI might be improved through specific training. Kaise *et al.*<sup>[30]</sup> evaluated the interobserver concordance among 11 endoscopists, concerning the triad of FMS disappearance, microvascular dilation and heterogeneity, and the  $\kappa$  values from 0.34 to 0.54 showed low-to-moderate reliability. Yoo *et al.*<sup>[33]</sup> discovered that the  $\kappa$  value for interobserver agreement of experts and trainees were similar as 0.49 and 0.40 when using the "VS classification system" for the gastric mucosal surface. In view of these inconsistent results, further studies are still required to find how to improve diagnostic performance of ME-NBI in a multiple-observer setting.

This meta-analysis has several limitations. First, we could not make a clear distinction between expert and non-expert. In some studies, an endoscopist was regarded as an expert after a specific training but in others, only those who have done a specific number of ME-NBI had this honor. Second, the cost-effectiveness of ME-NBI was not reported as well as the comparison with that of histopathology. Recently, Takeuchi *et al.*<sup>[34]</sup> proposed that "a new resect and discard strategy" with ME-NBI in colorectal cancer screening might reduce the costs of histopathology. Third, the heterogeneity of this study was relatively high. We showed that the morphology type of lesions,

diagnostic standard, lesion size and sample size were the possible sources. In addition, the inequality of expertise, the absence of a validated training, different disease spectrum and pathological type might reduce the generalizability of the overall performance and increase the heterogeneity of this study. Fourth, all the selected articles were conducted in China and Japan so the overall performance of ME-NBI for early gastric cancer may not represent other populations. Finally, only articles written in English were selected.

In conclusion, ME-NBI is a reliable technique for EGC diagnosis and has a better diagnostic performance than C-WLI. Further research should be focused on establishing a standard classification system and specific training of ME-NBI to reduce various biases and improve its diagnostic accuracy.

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## COMMENTS

### Background

Early detection and therapy of gastric cancer can improve 5 year survival rates of gastric cancer and therefore the diagnosis of early gastric cancer is significant in clinical work. Magnifying endoscopy with narrow-band imaging (ME-NBI) is a recently advanced endoscopic imaging technology which can enhance visualization of the superficial mucosal structure and vascular architecture. However, the diagnostic performance of ME-NBI in early gastric cancer (EGC) remains unclear.

### Research frontiers

Although ME-NBI has been applied in EGC diagnosis, the accuracy of ME-NBI for the diagnosis of EGC was variable. Therefore, the authors performed a meta-analysis to analyze the diagnostic performance of ME-NBI in EGC.

### Innovations and breakthroughs

In this study, the authors found that ME-NBI was a promising technique for EGC diagnosis, with a better diagnostic performance than conventional white-light imaging (C-WLI). To the best of our knowledge, this is the first meta-analysis to systematically calculate the diagnostic performance of ME-NBI in EGC diagnosis, which will provide valuable information for clinical work.

### Applications

ME-NBI may be a clinically useful tool to diagnose EGC and it can improve the diagnostic performance of EGC, compared with C-WLI.

### Terminology

Generally speaking, EGC is defined as gastric cancer which invades, limited to the mucosa or submucosa layer, regardless of lymphatic metastasis.

### Peer-review

The article is a complete, systematic literature review investigating the utility of ME-NBI in the diagnosis of EGC. The results indicate that ME-NBI is a reliable technique for EGC diagnosis and has a better diagnostic performance than C-WLI in EGC diagnosis, which could be used for effective clinical work.

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## Thrombocytopenia for prediction of hepatocellular carcinoma recurrence: Systematic review and meta-analysis

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**Author contributions:** Pang Q and Qu K contributed equally to this work; Pang Q, Dong YF and Liu C designed the study; Pang Q and Zhang JY performed the literature search; Pang Q, Qu K, Song SD extracted the data; Lin T, Xu XS and Tai MH contributed to quality evaluation for each study and drew the forest plots; Pang Q, Qu K and Zhang JY wrote the paper; and Bi JB, Liu SS, Lin T, Xu XS, Wan Y and Liu HC revised the paper.

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### Abstract

**AIM:** To investigate the association between thrombocytopenia and relapse after treatment for hepatocellular carcinoma (HCC).

**METHODS:** We searched the PubMed, EMBASE, and Web of Science databases to obtain eligible studies. The hazard ratios (HRs) values and 95% confidence intervals (CIs) were pooled by random effects model. Subsequently, we estimated the heterogeneity, performed a sensitivity analysis, determined the publication bias, and performed subgroup and meta-regression analyses. Study quality was assessed by using the Oxford Center for Evidence Based Medicine tool.

**RESULTS:** We identified 18 eligible studies by retrieval (published during 2000-2014). Out of the 4163 patients with HCC who were recruited, 2746 (66.0%) experienced recurrence. In general, our meta-analysis suggested that low platelet count (PLT) before therapy significantly increased the probability of postoperative recurrence (HR = 1.53, 95%CI: 1.29-1.81). PLT was also valuable in the prediction of intrahepatic distant recurrence (HR = 1.49, 95%CI: 1.25-1.77). Subgroup



and meta-regression analyses identified various therapeutic modalities as the source of a high degree of heterogeneity. The pooled HR values showed no obvious change when a single study was removed, but otherwise, an opposite-effects model was used. In addition, no significant publication bias was detected.

**CONCLUSION:** Thrombocytopenia before treatment might be an inexpensive and useful predictor of postoperative recurrence in patients with HCC.

**Key words:** Hepatocellular carcinoma; Blood platelets; Thrombocytopenia; Recurrence; Prognosis

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**Core tip:** The probability of high postoperative recurrence is the greatest problem that affects the potential curative treatment of patients with hepatocellular carcinoma (HCC). No factors have been widely considered as useful predictors for postoperative recurrence in HCC. We analyzed 18 relevant studies to determine the significance of platelet count. We included 4163 patients with HCC and found that thrombocytopenia before therapy significantly increased the probability of postoperative recurrence as well as intrahepatic distant recurrence. We emphasize that thrombocytopenia is an inexpensive, helpful predictor of postoperative recurrence in patients with HCC.

Pang Q, Qu K, Bi JB, Liu SS, Zhang JY, Song SD, Lin T, Xu XS, Wan Y, Tai MH, Liu HC, Dong YF, Liu C. Thrombocytopenia for prediction of hepatocellular carcinoma recurrence: Systematic review and meta-analysis. *World J Gastroenterol* 2015; 21(25): 7895-7906 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7895.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7895>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy with an increasing incidence worldwide. Chronic infections with several types of hepatitis viruses, especially hepatitis B virus (HBV) and hepatitis C virus (HCV), are well-recognized risk factors for HCC<sup>[1]</sup>. Between these two, HBV infection is a major etiological factor, and thus, the majority of patients with HCC reside in developing countries where HBV is prevalent<sup>[2]</sup>.

With the advancement in techniques, substantial progress has been made in the diagnosis and treatment of HCC over the past few decades. Current therapies primarily consist of liver resection, transplantation, radiofrequency ablation (RFA), and transcatheter arterial embolization (TACE). However, despite these treatments, the overall 5-year survival rate is 5%-6%, and therefore, the prognosis of HCC

is unsatisfactory<sup>[3]</sup>. Many factors are related to the prognosis of HCC<sup>[4,5]</sup>. Among these factors, high probability of postoperative recurrence is the greatest issue that affects the potential curative treatments for HCC<sup>[5]</sup>.

Platelet function in thrombosis, the inflammatory response, and liver regeneration *via* the release of several inflammatory mediators such as serotonin, platelet derived growth factor (PDGF), and transforming growth factor (TGF)- $\beta$ <sup>[6,7]</sup>. Abnormalities in platelets, either quantitative or functional, would result in a series of pathological changes and would subsequently lead to some disorders. Platelet count (PLT) and/or several platelet-based noninvasive models have been validated as valuable indices for the detection of liver cirrhosis<sup>[8-10]</sup>; a disorder that is strongly associated with the incidence and outcome of HCC. Moreover, thrombocytopenia has been proposed as a useful tool to identify carcinogenesis in basic liver diseases<sup>[11,12]</sup>, as well as to predict postoperative morbidity and mortality of patients with HCC<sup>[13]</sup>. Furthermore, our previous study and other reports have suggested that thrombocytopenia is related to poor survival of patients with liver cancer<sup>[14-16]</sup>.

However, whether or not preoperative thrombocytopenia might increase the risk of relapse in patients with HCC is unclear and controversial. Some studies found that thrombocytopenia was significantly associated with HCC recurrence, whereas others failed to show a significant association between thrombocytopenia and HCC recurrence. However, we previously suggested that a higher preoperative PLT might lead to a higher postoperative recurrence rate<sup>[16]</sup>. Here, we further summarize all of the relative articles in order to clarify this issue.

## MATERIALS AND METHODS

### Search strategy and selection criteria

A systematic search in the PubMed, EMBASE, and ISI Web of Science databases for studies published until 31 August 2014 was performed by two independent investigators (Pang Q and Zhang JY). Our core search consisted of the terms (PLT or platelet or platelets or thrombopenia or thrombocytopenia or thrombocytosis) and (recurrence or relapse) and (hepatocellular carcinoma or HCC or liver cancer or hepatic carcinoma or hepatic cancer). In addition, we manually retrieved the reference lists of relevant reviews and included those studies as well.

We included studies that met the following predetermined criteria: (1) published as an original article; (2) HCC was determined by pathology/imaging; (3) studied the relationship between PLT and HCC recurrence and reported the hazard ratios (HRs) and 95% confidence intervals (CIs), or provided sufficient data to calculate them; (4) PLT was expressed as a binary variable (with a lower or a higher category as a reference); and (5) recruited no fewer than 20 patients.

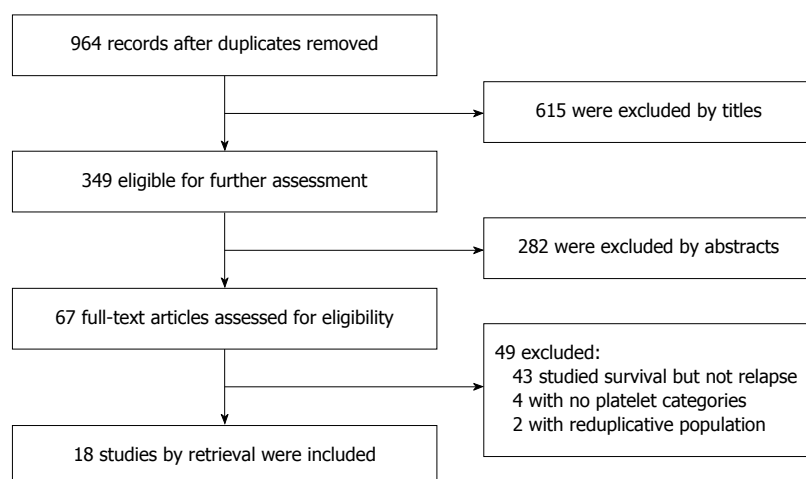


Figure 1 Flow chart of the search strategy and study selection.

The exclusion criteria were: (1) studies of secondary liver cancer or other liver diseases; (2) HCC was assessed by serum markers; (3) studies of the effect of PLT on survival but not on recurrence; (4) studies that only provided the *P* value or other conditions that could not be used to calculate HR and 95%CI values; (5) PLT was expressed as a continuous variable; and (6) conference abstracts or letters. If two or more publications recruited identical populations, we only included the one with the largest number of cases or the one with the most adjusted HR values.

### Data abstraction

Based on the above selection criteria, two researchers (Pang Q and Qu K) independently evaluated the retrieved studies. The  $\kappa$  statistic was calculated to assess the variability between the observers, and all discrepancies were resolved by discussion. For each included study, we abstracted the following data with a standardized data-collection protocol: first author, publication year, country where the study population lived, gender distribution, method of treatment, total cases, recurrent cases, duration of follow-up, cut-off value of PLT, and adjusted (prior use) or crude HR value and 95%CI. The quality of each study was appraised using the Oxford Center for Evidence-based Medicine appraisal approach<sup>[17]</sup>. We followed the MOOSE (Meta-analysis of Observational Studies in Epidemiology) guidelines<sup>[18]</sup> for the reporting of this meta-analysis. All data were verified by one author (Pang Q).

### Statistical analysis

If a study considered a lower PLT as a reference, we converted its HR value to an estimated value with a higher category as a reference (which reflected the influence of a low PLT on recurrence). We assessed the heterogeneity among the studies with the *Q* value and the  $I^2$  statistic value (25%, 50%, and 75% correspond to the cut-off points for low, moderate, and high degrees of heterogeneity, respectively). Heterogeneity

was considered to be statistically significant if *P* was  $< 0.10$  for *Q* statistic, or  $I^2$  was  $> 50\%$ ; otherwise, no significant heterogeneity was observed. We calculated the pooled HR value for recurrence (or distant recurrence) by random-effects model. If high heterogeneity was found, we explored the potential source of heterogeneity by performing a subgroup analysis and meta-regression. Subsequently, we performed an influence analysis to evaluate whether any single study could markedly affect the result. Moreover, we compared the HR values that were pooled by a fixed-effect model and that were summed by a random-effects model. Eventually, publication bias was examined by funnel plots with Begg's and Egger's tests. The statistical methods of this study were reviewed by Dr. Kai Qu from the Department of Epidemiology, MD Anderson Cancer Center, University of Texas, and Prof. Ya-Feng Dong from the University of Kansas School of Medicine, United States.

We used STATA 12.0 software to analyze the data. A bilateral *P* value  $< 0.05$  was indicated a significant difference.

## RESULTS

The flow chart that details our literature search and selection process is shown in Figure 1. Of the total 964 citations, we finally included 18 studies. There was good agreement between the two observers on which studies to include ( $\kappa$ : 0.940). No additional studies were found within the references of reviews and included studies. All 18 studies were published in English.

### Characteristics of the included studies

The baseline values of the included studies in this meta-analysis are summarized in Table 1. Our meta-analysis consisted of 4163 patients, of which 2784 (66.9%) were men. During the mean or median follow-up period, which ranged from 12 to 151.2

**Table 1** Baseline characteristics of the studies included in the meta-analysis

Ref.	Year	Country	Cutoff (10 <sup>9</sup> /L)	Therapy	Adjusted HR	Recurrence type	Men	Women	Recurrence	Follow-up time (mo)	LoE
Saito <i>et al</i> <sup>[32]</sup>	2014	Japan	100	PH + RFA	No	Overall	38	13	39	39.9-46.6	4
Kaibori <i>et al</i> <sup>[19]</sup>	2013	Japan	150	PH	Yes	Overall	60	14	14	151.2	2a
Shiina <i>et al</i> <sup>[24]</sup>	2012	Japan	100	RFA	Yes	Distant	751	419	740	38.2	2b
Nishikawa <i>et al</i> <sup>[25]</sup>	2012	Japan	100	RFA	Yes	Distant	114	68	86	36.4	2b
Kao <i>et al</i> <sup>[26]</sup>	2012	Taiwan	100	RFA	Yes	Overall	162	96	163	28.5	2b
Miyatake <i>et al</i> <sup>[33]</sup>	2012	Japan	100	PH + PTA	Yes	Overall	260	135	277	42-46	2b
Ikeda <i>et al</i> <sup>[20]</sup>	2011	Japan	150	RFA	No	Overall	68	39	55	12	4
Amano <i>et al</i> <sup>[21]</sup>	2011	Japan	100	PH	No	Overall	127	24	107	49.2	4
Ishikawa <i>et al</i> <sup>[31]</sup>	2011	Japan	100	TACE	Yes	Distant	47	31	46	36.6	1b
Hagihara <i>et al</i> <sup>[34]</sup>	2011	Japan	100	PH + PTA	No	Overall	124	58	81	44.4	4
Nonaka <i>et al</i> <sup>[22]</sup>	2010	Japan	100	PH	No	Overall	42	22	32	12	4
Kang <i>et al</i> <sup>[23]</sup>	2009	Korea	100	PH	No	Overall	125	42	78	38	4
Fuke <i>et al</i> <sup>[28]</sup>	2008	Japan	100	RFA	Yes	Distant	80	37	90	29.1	2b
Nouso <i>et al</i> <sup>[27]</sup>	2008	Japan	100	RFA	No	Overall + distant	415	206	589	34.7	4
Jeong <i>et al</i> <sup>[35]</sup>	2007	Japan	100	PH + medical	Yes	Overall	65	19	50	32	2b
Tateishi <i>et al</i> <sup>[29]</sup>	2006	Japan	100	RFA	No	Overall	276	140	277	NR	4
Yamanaka <i>et al</i> <sup>[30]</sup>	2005	Japan	100	RFA	Yes	Distant	17	9	14	18	2b
Ikeda <i>et al</i> <sup>[36]</sup>	2000	Japan	100	PH + RFA	No	Overall	13	7	8	25	4

LoE: Level of evidence; NR: Not reported; PH: Partial hepatectomy.

mo, 2746 (66.0%) patients experienced recurrence. Sixteen studies adopted a cut-off value of  $100 \times 10^9/\text{L}$  for PLT while the remaining two<sup>[19,20]</sup> considered  $150 \times 10^9/\text{L}$  as the cut-off point. There was one individual inception cohort study with > 80% follow-up and this was assessed as level 1b using the Oxford Center for Evidence-based Medicine tool. Others were retrospective cohort studies and were identified as either level 2a/2b or 4. All studies were performed in Asia, and the effect of PLT on intrahepatic distant recurrence was estimated in six of them.

#### **Pooled HR values for all of the studies**

After calculation of the summed effects, we demonstrated that low PLT before treatment significantly increased the risk of HCC recurrence (HR = 1.53, 95%CI: 1.29-1.81). The forest plot is shown in Figure 2. We observed a moderate degree of heterogeneity between studies ( $I^2 = 50.8\%$ ,  $P = 0.007$ ).

#### **Impact of thrombocytopenia on recurrence**

Subsequently, we summed the 17 studies, which used a cutoff value of  $\times 100 \times 10^9/\text{L}$  for PLT and consisted of 4035 patients, in order to investigate the influence of thrombocytopenia on recurrence after treatment. Thrombocytopenia was found to be a useful tool for the prediction of recurrence in HCC (HR = 1.42, 95%CI: 1.27-1.60) (Table 2).

#### **Pooled HR value for patients who underwent liver resection, RFA**

Different treatment modalities, which are given according to the tumor characteristics and stage, may influence the survival and risk of recurrence. Among these therapies, surgical resection is the primary curative treatment modality for HCC. Subsequently, we

explored the impact of PLT on patients who received partial hepatectomy. Overall, 231 of the 470 (49.1%) patients who underwent resection experienced recurrence. By pooling the four eligible studies<sup>[19,21-23]</sup>, we demonstrated a significant association between the preoperative PLT level and postoperative recurrence (HR = 4.46, 95%CI: 1.57-12.65), with a high degree of heterogeneity (Figure 3A).

Eight studies<sup>[20,24-30]</sup> recruited patients with HCC who underwent RFA. Of these included patients, 2014 of the 2897 (69.5%, about 1.42 times the number of patients who underwent resection) experienced recurrence during follow-up. Figure 3B indicates that PLT is still a useful indicator for the prediction of recurrence in patients who received RFA. There was no significant heterogeneity and the pooled HR value was 1.43 (95%CI: 1.24-1.65).

#### **Effect of PLT on distant recurrence**

Recurrence is divided into local and intrahepatic distant recurrence. Only one study<sup>[27]</sup> in our meta-analysis estimated the association between thrombocytopenia and local recurrence. In contrast, six studies<sup>[24,25,27,28,30,31]</sup>, which consisted of 2193 patients (1541 had a relapse), reported the effect of PLT on distant recurrence. A cutoff point of  $100 \times 10^9/\text{L}$  for PLT was used in all six studies. This meta-analysis showed that thrombocytopenia was also a significant indicator for the prediction of distant recurrence (HR = 1.49, 95%CI: 1.25-1.77) (Figure 3C). Little heterogeneity ( $I^2 = 15.8\%$ ,  $P = 0.312$ ) was found among these studies.

#### **Effect of PLT on recurrence of HCV-related HCC**

Etiology is another major factor that is associated with the prognosis of HCC. Most of our included studies calculated the HR value without a distinction among

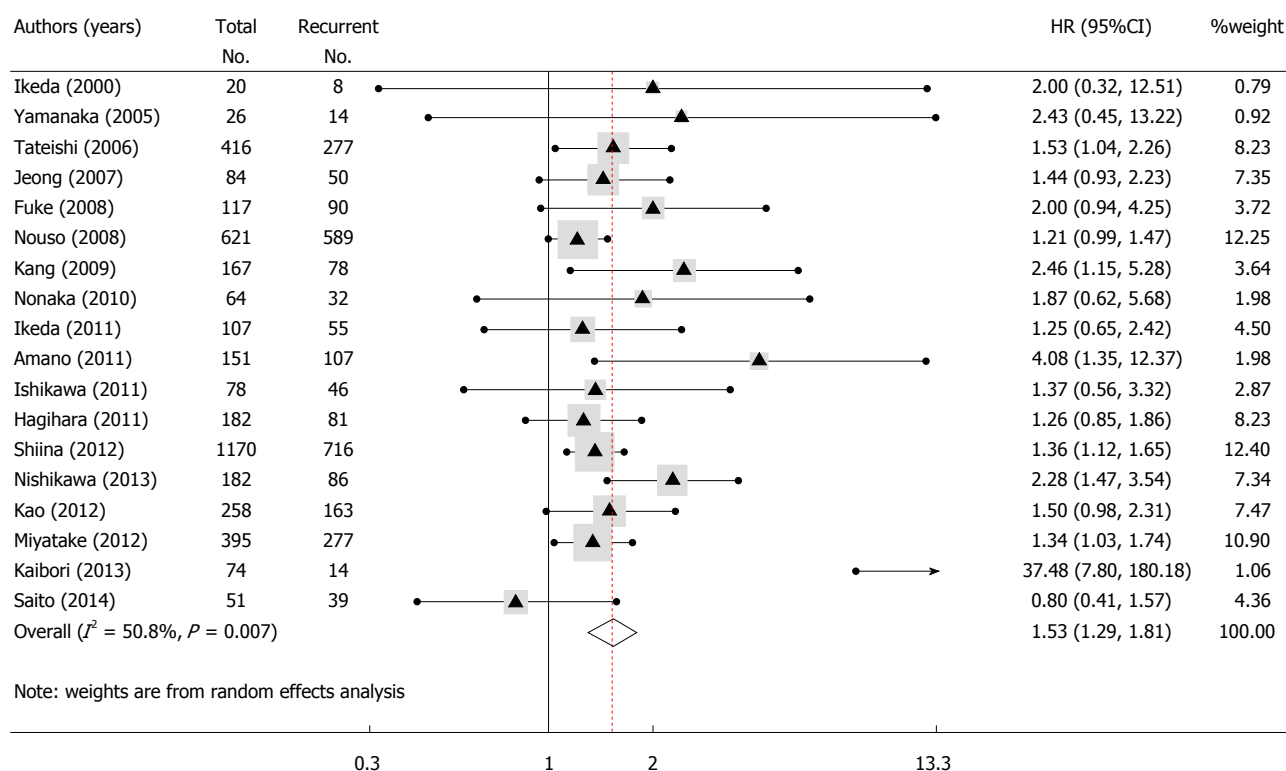


Figure 2 Effect of platelet count on hepatocellular carcinoma recurrence.

**Table 2** Effects of platelet count in different studies using the two effects model

	Random-effects model	Fixed-effect model
	HR (95%CI)	HR (95%CI)
All studies	1.53 (1.29-1.81)	1.41 (1.28-1.55)
Thrombocytopenia	1.42 (1.27-1.60)	1.39 (1.26-1.54)
Liver resection	4.46 (1.57-12.65)	3.48 (2.07-5.83)
RFA	1.43 (1.24-1.65)	1.39 (1.24-1.56)
Distant recurrence	1.49 (1.25-1.77)	1.45 (1.26-1.67)
HCV-HCC	1.33 (1.12-1.60)	1.33 (1.12-1.60)

RFA: Radiofrequency ablation; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

the various etiologies. Seven studies<sup>[28,30,32-36]</sup> only recruited patients with HCV-related HCC. Similarly, thrombocytopenia also had significant potential in the prediction of relapse in these patients (HR = 1.33, 95%CI: 1.12-1.60), and no significant between-study heterogeneity was observed ( $I^2 = 0\%$ ,  $P = 0.651$ ) (Figure 3D).

### Exploration of heterogeneity

To explore the source of heterogeneity, we performed subgroup and meta-regression analyses. We analyzed the covariates that might be responsible for the potential heterogeneity. We had at least two studies in each subgroup and included treatment method; follow-up time ( $\geq 3$  or  $< 3$  years); whether adjustment for confounding factors was performed; type of recurrence (overall or distant recurrence); and the number of recruited patients. The results of our exploration are

shown in Table 3. We suggested that the treatment method might affect the pooled effect size ( $P < 0.05$  in subgroup and meta-regression analysis). All of the subgroups showed a significant association between PLT and recurrence except the one subgroup whose studies recruited patients who received palliative therapies<sup>[31,35]</sup> (TACE or medical therapy, HR = 1.43, 95%CI: 0.96-2.11). For all five covariates, no significant between-group differences (all  $P > 0.05$ ) were found when a multivariable meta-regression was performed.

### Sensitivity analysis and determination of publication bias

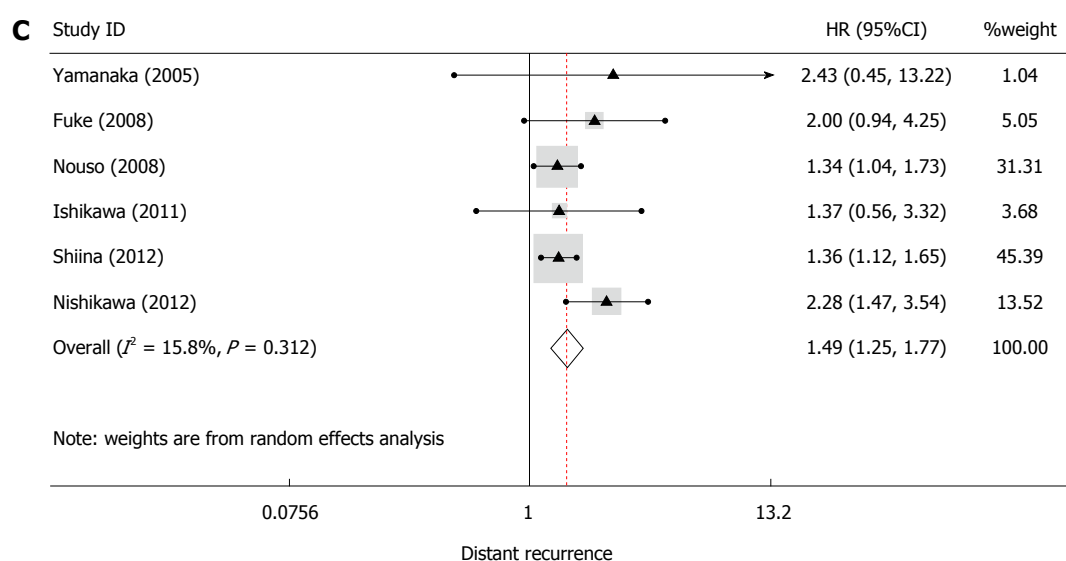
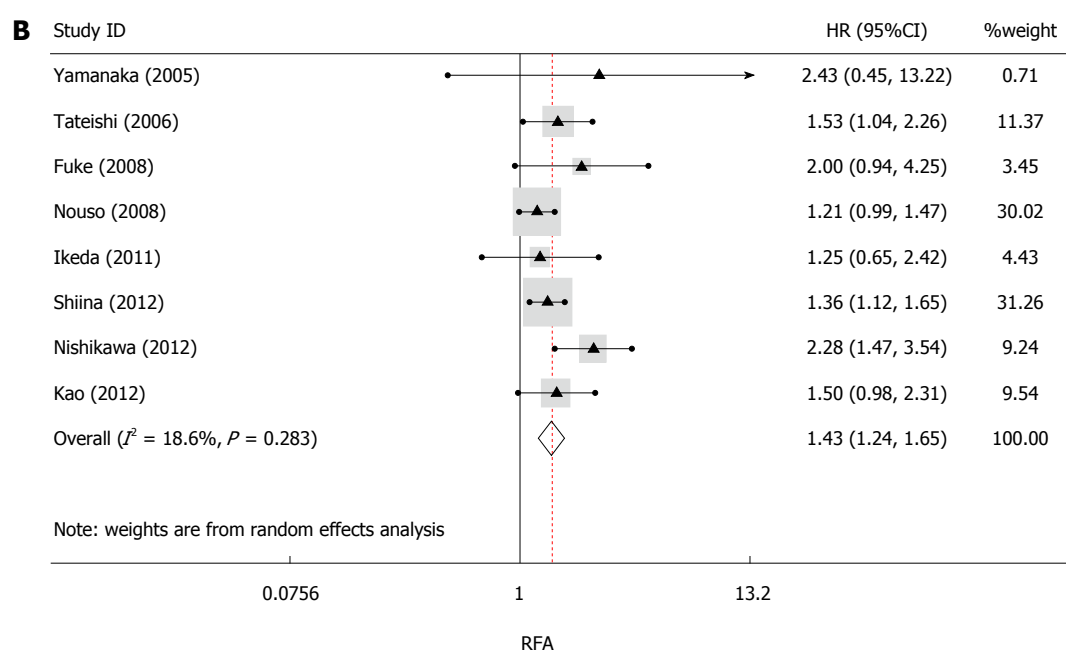
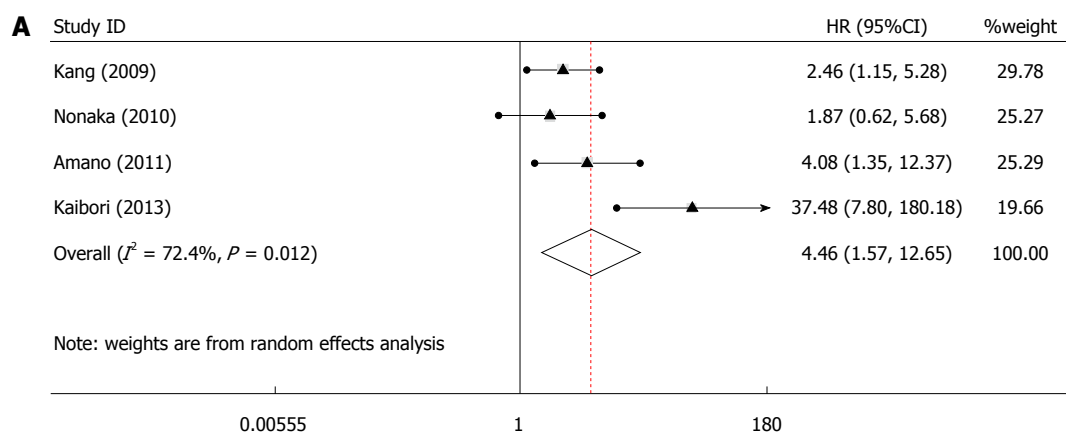
Furthermore, a sensitivity analysis was conducted to validate the certainty of our findings. When the opposite-effects model was used, the results did not significantly change (Table 2). Then, we performed an influence analysis and found that no single study affected the summary estimation (Figure 4).

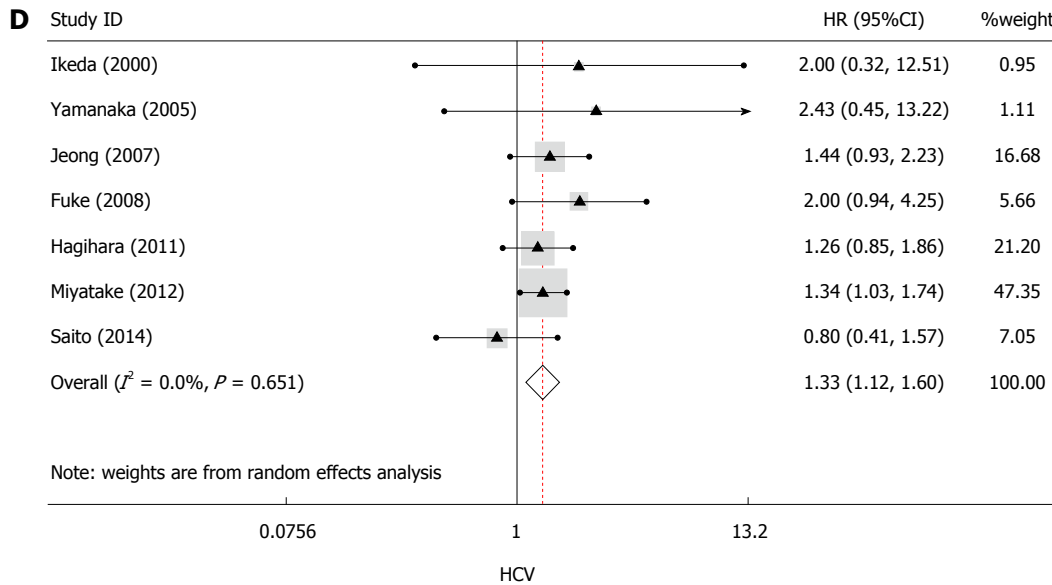
Eventually, we constructed a funnel plot to detect the existence of publication bias, and the figure reflects the basic symmetry (Figure 5). Indeed, no significant evidence of publication bias was found ( $P$  values were 0.11 and 0.36 in Begg's test and Egger's test, respectively).

## DISCUSSION

A high risk of recurrence is considered one of the greatest concerns with regard to the treatment of patients with HCC<sup>[5]</sup>. The postoperative recurrence rate







**Figure 3** Effect of platelet count on hepatocellular carcinoma recurrence in patients who underwent liver resection (A) and RFA (B), on distant recurrence (C), and on recurrence in patients with hepatitis C virus-related hepatocellular carcinoma (D).

**Table 3** Subgroup and meta-regression analyses

Covariates	Subgroup	No.	HR (95% CI)	Heterogeneity			Meta-regression	
				$P^a$	$I^2$	$P^b$	Crude $P$	Adjusted $P$
Treatment	Resection	4	4.46 (1.57-12.65)	0.012	72.4	0.005	0.048	0.212
	RFA	8	1.43 (1.24-1.65)	0.283	18.6			
	Resection+RFA	4	1.26 (1.03-1.55)	0.532	0			
	Others	2	1.43 (0.96-2.11)	0.916	0			
Follow-up (mo)	$\geq 36$	9	1.71 (1.25-2.35)	0.000	73.4	0.454	0.596	0.989
	$< 36$	9	1.35 (1.17-2.56)	0.859	0			
Confounder	Adjusted	9	1.70 (1.30-2.22)	0.004	64.1	0.198	0.345	0.863
	Unadjusted	9	1.37 (1.12-1.68)	0.225	24.6			
Recurrence type	Overall	13	1.51 (1.21-1.87)	0.005	57.6	0.351	0.720	0.999
	Distant	5	1.64 (1.25-2.14)	0.247	26.1			
Recruited No.	$\geq 150$	9	1.49 (1.26-1.69)	0.103	39.7	0.600	0.443	0.944
	$< 150$	9	1.78 (1.10-2.88)	0.007	61.8			

<sup>a</sup> $P$  value for  $Q$  statistic of between-study heterogeneity in each subgroup; <sup>b</sup> $P$  value for  $Q$  statistic of heterogeneity between the subgroups.

was reported to range from 50% to 100%<sup>[19]</sup>. To obtain a satisfactory prognosis in cases of HCC, it is crucial to determine the predisposing factors for recurrence and improve these factors before treatment. Platelets are associated with the prognosis of various solid tumors, including HCC<sup>[37]</sup>. However, the significance of platelets in the risk of recurrence of HCC remains unknown. This is the first time that an estimation by a quantitative summary of all relative studies has been performed. Our meta-analysis showed that patients with a low PLT before treatment had a significantly increased risk of postoperative relapse. In general, thrombocytopenia could increase the risk of recurrence by 53%. Specifically, thrombocytopenia was demonstrated to be a useful tool for the prediction of recurrence, no matter whether hepatic resection or RFA was performed for HCC.

Recurrence comprises both local and intrahepatic distant recurrence. The factors that influence the two

types of recurrence are different<sup>[27,28]</sup>. It is essential to identify the risk factors separately for the two types in order to prevent recurrence. However, few studies have differentiated local from intrahepatic distant recurrence. Only one study exclusively reported the local recurrence, and six studies exclusively estimated the distant recurrence in our meta-analysis. By pooling the latter studies, we found that thrombocytopenia was also significantly associated with distant recurrence.

Our results are important in that they provide crucial guidance for the estimation of the prognosis of patients with HCC. No significant difference was found between our use of the random-effects and fixed-effect models. The pooled HR values were not markedly affected by a single study, which indicated the robustness of our results. By subgroup and meta-regression analyses, we demonstrated that the various treatment modalities might be a source of heterogeneity.

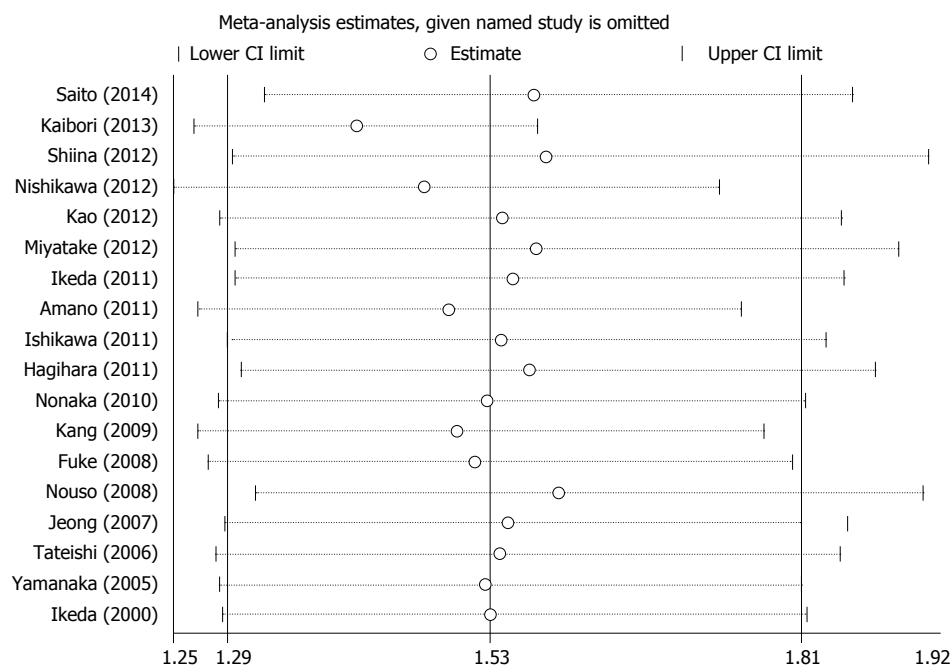


Figure 4 Influence analysis of our included studies.

This study did have some limitations. First, gender, age, Child-Pugh grade, and several other parameters that are important prognostic factors for HCC were not adjusted in this meta-analysis and several included studies were level 4. Whether or not the significance of PLT is independent of these confounders remains unknown. However, after pooling the adjusted HR values (Table 3), we suggest that platelets might be independently associated with postoperative relapse. Second, pathological examination is universally viewed as the gold standard for the diagnosis of HCC. However, to obtain more data, we did not exclude the studies that assessed HCC by imaging. Third, thrombocytopenia is strongly associated with the progression of liver cirrhosis and is considered an independent risk factor for multicentric HCC<sup>[38]</sup>. In other words, thrombocytopenia is only a surrogate marker or a confounding factor of multicentric carcinogenesis associated with liver cirrhosis. It is therefore necessary to evaluate the relationship between thrombocytopenia and recurrence of HCC in the cases with a corresponding amount of liver cirrhosis. However, because all the retrieved studies failed to stratify the patients with HCC according to the presence or degree of liver cirrhosis, we could not explain whether thrombocytopenia is independent of liver cirrhosis. Nevertheless, based on our previous report, in patients with HCC without cirrhosis, but not in those with cirrhosis, PLT was significantly associated with recurrence<sup>[16]</sup>. In another study, a lower PLT was also associated with a significantly worse prognosis in patients with HCC and cirrhosis<sup>[41]</sup>.

Due to portal hypertension, a decrease in thrombopoietin (TPO) production in the liver, and the capture of platelets by the liver<sup>[6]</sup>, PLT tends to decrease in

various liver diseases. Numerous studies have shown that thrombocytopenia is a major risk factor for the degree of cirrhosis, development of cirrhosis, as well as for carcinogenesis in patients with chronic hepatitis<sup>[8,39,40]</sup>. However, the exact mechanism of the effects of PLT in the prognosis of HCC is still unclear. Several clinical studies have demonstrated that thrombocytopenia could increase postoperative complications and morbidity, and could lead to the deterioration of liver function<sup>[41-43]</sup>. With the analysis of 202 patients with HCV-related HCC who underwent hepatectomy, Kubo *et al.*<sup>[38]</sup> emphasized that PLT was the only independent predictor for multicentric HCC; in addition, PLT was significantly associated with the severity of active hepatitis and hepatic fibrosis (both  $P < 0.05$ ). A previous study also indicated that PLT was a useful predictor of portal vein invasion<sup>[44]</sup>. Additionally, a decreased PLT level was found to be significantly associated with an elevated  $\alpha$ -fetoprotein level<sup>[12,45,46]</sup>. Furthermore, patients with a lower preoperative PLT showed a significantly higher probability of cirrhosis, a higher level of bilirubin, a greater amount of bleeding, and a high level of indocyanine green retention at 15 min<sup>[23]</sup>. The above-mentioned factors were all related to recurrence in HCC<sup>[27,29]</sup>, and thus, these findings may help hepatologists explore the exact mechanism. By both *in vitro* and *in vivo* studies, Nozaki *et al.*<sup>[47]</sup> proved that TPO could promote liver regeneration and improve cirrhosis *via* an increase in platelets. PDGF, which is mainly stored in platelets, is associated with tumor progression and prognosis in HCC<sup>[48]</sup>. These findings support the relationships between PLT and HCC recurrence. In addition to its influence on recurrence, our previous research and several other studies also showed that thrombocytopenia could result in poor

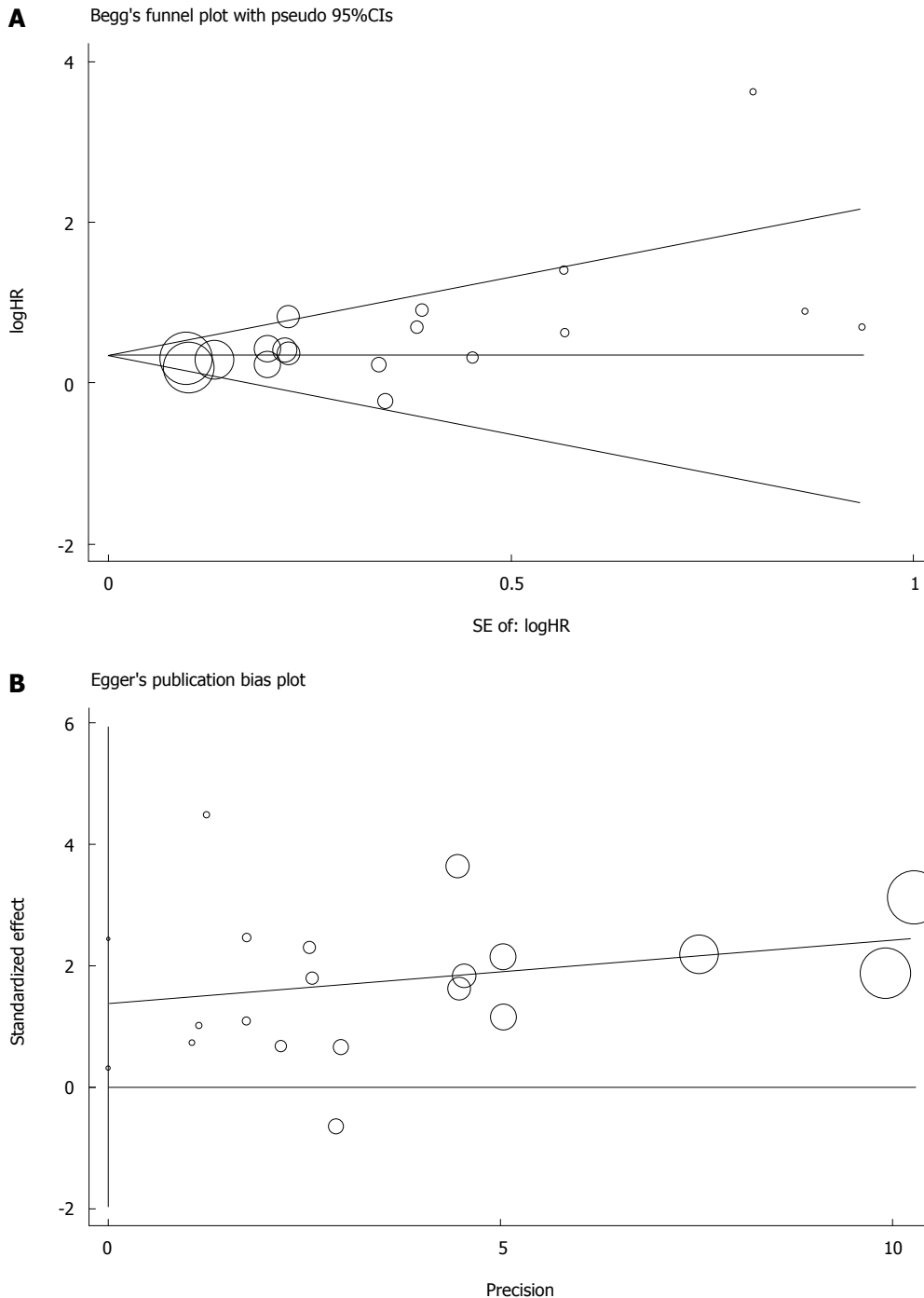


Figure 5 Begg's (A) and Egger's (B) plots of our included studies.

survival in patients with HCC<sup>[4,15,34]</sup>. However, data on the relationship between PLT and tumor stage or other crucial prognostic factors for HCC are lacking.

Additionally, thrombocytosis was found to be associated with the incidence of HCC as well<sup>[49]</sup> and could lead to an increase in the risk of death from HCC<sup>[37]</sup> and other cancers<sup>[50]</sup>. A positive correlation has been suggested between serum PLT and tumor size<sup>[49,51]</sup>. In *in vitro* studies, platelets are a stimulating factor for the growth and invasion of several HCC cell lines<sup>[52]</sup>, which further indicates the adverse effects of excessive platelets.

Therefore, it is believed that a huge gap in the cut-off values of PLT (such as  $400 \times 10^9/L$  vs  $100 \times 10^9/L$ ) may show opposite effects in the prognosis of HCC. Although thrombocytopenia and thrombocytosis are not contraindications for resection in patients with HCC<sup>[4]</sup>, it is still recommended to normalize the serum platelet level by prophylactic platelet transfusions or by taking agents before treatment. On the contrary, although sorafenib, interferon, or other chemotherapeutic/anticancer drugs might be effective in the delay and/or management of postoperative recurrence, they could lead to many complications



such as thrombocytopenia<sup>[32,33,53,54]</sup>. Therefore, for patients who are taking these drugs, a regular assessment of the platelet level in order to maintain the level within a normal range, is pivotal to obtain a favorable outcome.

In conclusion, our meta-analysis suggested that thrombocytopenia was a valuable, inexpensive predictor for recurrence in patients with HCC. Further experimental and clinical studies are needed to validate the results and to clarify the exact mechanism.

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## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is a common malignancy with an increasing incidence worldwide. No matter which treatments are given, the prognosis of HCC is poor. Many factors are related to the prognosis of HCC. Among these factors, a high postoperative recurrence rate is the greatest problem that influences potential curative treatments for HCC. It is crucial to seek several key risk factors that influence postoperative recurrence in HCC.

### Research frontiers

Thrombocytopenia is significantly associated with poor survival in HCC. However, whether or not a preoperative low platelet count could increase the risk of recurrence in patients with HCC remains uncertain. The authors endeavored to validate this issue in a meta-analysis.

### Innovations and breakthroughs

Several HCC prognostic models, which mainly focus on the tumor characteristics, have been proposed to assess postoperative survival. However, these models played a limited role in the prediction of recurrence. In contrast, the authors found that thrombocytopenia, a simple and inexpensive index, was significantly associated with a high probability of recurrence, including distant recurrence, in patients with HCC, no matter what treatments were given.

### Applications

Through a validation of this novel prognostic predictive parameter, this study may represent a future strategy for cancer prediction in the follow-up of patients with HCC.

### Terminology

Platelets are involved in thrombosis, inflammatory response, and liver regeneration via the release of several inflammatory mediators such as serotonin. The platelet count is a crucial reflection of platelet function, with a normal range of  $100 \times 10^9$  to  $300 \times 10^9/L$ . Thrombocytopenia occurs when the platelet count is  $< 100 \times 10^9/L$ .

### Peer-review

This is a prognostic meta-analysis and the manuscript is very well written.

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## Endovascular treatment of post-laparoscopic pancreatectomy splenic arteriovenous fistula with splenic vein aneurysm

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### Abstract

Splenic arteriovenous fistulas (SAVFs) with splenic vein aneurysms are extremely rare entities. There have been no prior reports of SAVFs developing after laparoscopic pancreatectomy. Here, we report the first case. A 40-year-old man underwent a laparoscopic, spleen-preserving, distal pancreatectomy for an endocrine neoplasm of the pancreatic tail. Three months after surgery, a computed tomography (CT) scan demonstrated an SAVF with a dilated splenic vein. The SAVF, together with the splenic vein aneurysm, was successfully treated using percutaneous transarterial coil embolization of the splenic artery, including the SAVF and drainage vein. After the endovascular treatment, the patient's recovery was uneventful. He was discharged on postoperative day 6 and continues to be well 3 mo after discharge. An abdominal CT scan performed at his 3-mo follow-up demonstrated complete thrombosis of the splenic vein aneurysm, which had decreased to a 40 mm diameter. This is the first reported case of SAVF following a laparoscopic pancreatectomy and demonstrates the usefulness of endovascular treatment for this type of complication.

**Key words:** Splenic arteriovenous fistula; Splenic vein aneurysm; Laparoscopic pancreatectomy; Percutaneous transarterial embolization; Endovascular treatment

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**Core tip:** This is the first reported case of a splenic arteriovenous fistula occurring after laparoscopic pancreatectomy and demonstrates the usefulness of endovascular treatment for these fistulas, especially when are associated with splenic venous aneurysms.

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## INTRODUCTION

Splenic arteriovenous fistulas (SAVFs) with splenic vein aneurysms are extremely rare<sup>[1,2]</sup> and may be either congenital or acquired. The majority of SAVFs occur after the rupture of a splenic artery aneurysm<sup>[2]</sup>. Other less common causes include splenectomies, gunshot injuries, congenital development, mycotic infections, and splenoportography<sup>[2]</sup>. However, to our knowledge, there have been no reports of SAVFs occurring after laparoscopic pancreatectomy. We report the first case of SAVF, which was successfully treated using percutaneous, transarterial embolization of the splenic artery, including the SAVF and drainage vein.

## CASE REPORT

A 40-year-old man underwent a laparoscopic, spleen-preserving, distal pancreatectomy for an endocrine neoplasm of the pancreatic tail. The surgery was performed uneventfully, and there were no complications such as bleeding, pancreatic leakage or infections during the hospitalization. The patient was discharged on postoperative day 7. Three months after surgery, a computed tomography (CT) scan demonstrated an SAVF with a dilated splenic vein measuring 56 mm × 64 mm (Figure 1). The patient's medical history included diabetes mellitus but no history of splenic artery aneurysm, splenectomy, or trauma. He was asymptomatic, and his blood test results were normal. Because of the rapid enlargement of the splenic vein aneurysm, after consultation with a surgeon, we decided to perform endovascular treatment of the SAVF.

Under local anesthesia, the Seldinger technique was used to place a 5-F sheath (Medikit, Tokyo, Japan) into the right femoral artery. Selective celiac angiography and selective splenic arteriography were performed using a 4-F shepherd-type catheter (Medikit). The angiograms revealed a fistula between the proximal splenic artery and the aneurysm-like, dilated splenic vein (Figure 2A). Contrast medium from the splenic vein aneurysm flowed into the portal vein through



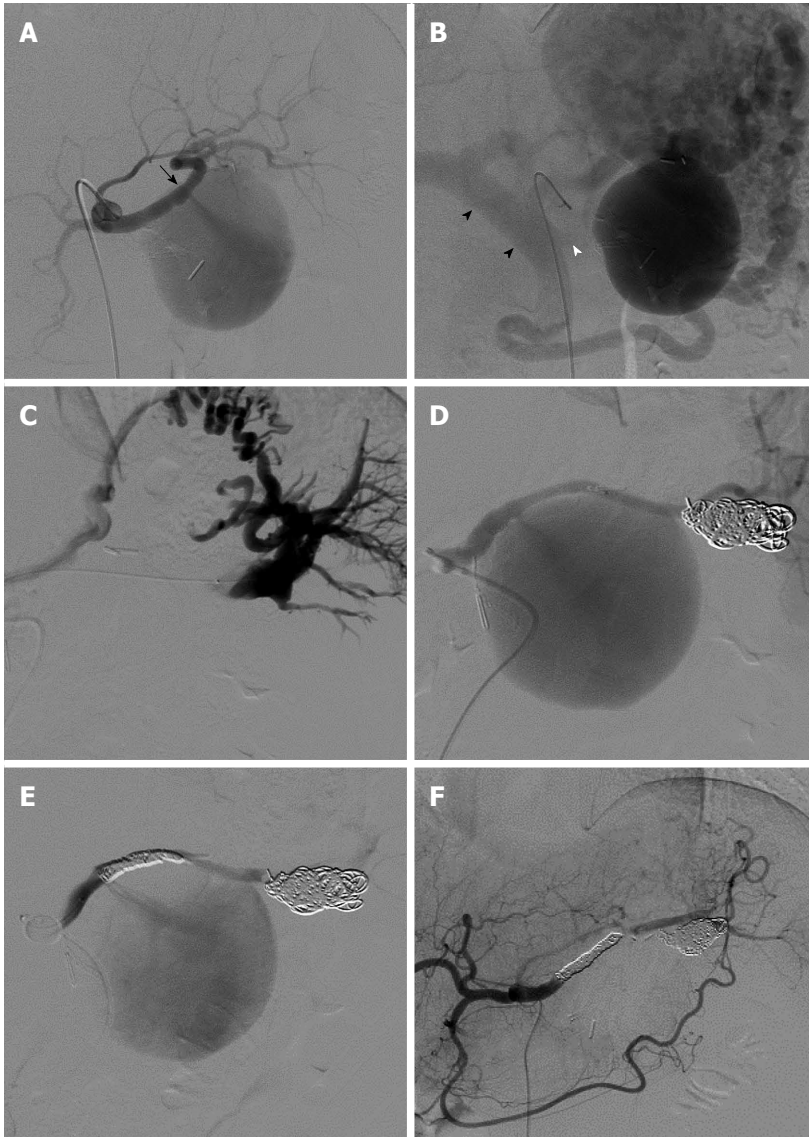
**Figure 1** Abdominal computed tomography images demonstrating a splenic vein aneurysm approximately 56 mm × 64 mm in size.

the collateral vessels that had developed; the splenic vein main trunk was occluded on the portal vein side (Figure 2B). The flow to the fistula was controlled with a 5-F balloon catheter (Terumo, Tokyo, Japan) placed in the splenic artery. A micro-catheter was advanced to the drainage vein through the aneurysm, and the drainage vein was embolized using 7 detachable coils (Boston Scientific, Tokyo, Japan) (Figure 2C and D). Embolization of the splenic artery, including the SAVF, was then performed successfully using 8 detachable coils (Figure 2E and F).

After the endovascular treatment, the patient had a slight fever on postoperative day 4, and he received antibiotic medication for 3 d. He was discharged on postoperative day 6 and continues to be well 3 mo after discharge. An abdominal CT scan performed at his 3-mo follow-up demonstrated complete thrombosis of the splenic vein aneurysm, which had decreased to a diameter of 40 mm. There was a small splenic infarction on the CT scan 4 d after endovascular treatment; however, the infarction had almost disappeared by 3 mo after endovascular treatment.

## DISCUSSION

SAVFs are rare, and the most occur after the rupture of a splenic artery aneurysm into the corresponding vein. However, their origins can also be congenital, traumatic (iatrogenic or accidental) or through infection<sup>[2-4]</sup>. Here, we described the first case of an SAVF with a splenic vein aneurysmal dilatation that occurred after a laparoscopic pancreatectomy. Laparoscopic partial pancreatectomies for pancreatic endocrine tumors were first reported in 1996 by Gagner *et al.*<sup>[5]</sup> and are now considered to be indicated for that condition<sup>[6]</sup>. One of the most common complications after a pancreatectomy is pancreatic leakage, which may result in massive hemorrhage. In the present case, a pancreatic leak was considered to be one of the possible causes of the SAVF. SAVFs can be asymptomatic or may result in a variety of portal vein hypertension symptoms, *i.e.*, upper abdominal pain, ascites (35%), esophageal or gastric varices



**Figure 2 Angiographic imaging.** A: Celiac artery angiogram demonstrating a fistula (black arrow) between the proximal splenic artery and the splenic venous aneurysm; B: The contrast medium from the splenic vein aneurysm continued to the portal vein (black arrowhead) through the collateral vessels; the splenic vein main trunk on the portal vein side was occluded (white arrowhead); C: A micro-catheter is shown advancing to the drainage vein through the aneurysm; D: The drainage vein after embolization with 7 coils; E: The splenic artery, including the splenic arteriovenous fistula, after coil embolization; F: The splenic vein aneurysm disappeared after embolization of the splenic artery, including the splenic arteriovenous fistula and drainage vein.

(52%), or splenomegaly (55%)<sup>[2,7,8]</sup>. Diarrhea, due to abrupt elevation of mesenteric venous pressure, and congestive heart failure have also been described<sup>[4,9]</sup>.

SAVFs can be diagnosed using contrast-enhanced CT and confirmed using celiac or splenic arteriography. An elongated, tortuous splenic artery; early filling of the splenic vein during the arterial phase; dense opacification of the splenoportal venous system; and an aneurysm-like splenic vein are the characteristic findings of SAVFs<sup>[2]</sup>. Rapid development of an aneurysmal dilatation in the splenic vein, as in our case, is uncommon and may suggest occlusion of the main trunk of the vein on the portal vein side. In the present case, the occlusion may have been caused during the laparoscopic pancreatectomy. The shunt flow from the SAVF could not flow directly to the portal vein due to the splenic vein occlusion; therefore, the

pressure in the drainage vein was elevated causing it to enlarge. SAVFs should be promptly treated in high-risk patients with symptomatic or expanding aneurysms.

Traditionally, surgery is used to treat SAVFs<sup>[8,10]</sup>, but the surgical approach often presents technical challenges because of the distal lesion site, adhesion formation, and the presence of numerous portal collaterals<sup>[1]</sup>. Non-surgical, endovascular treatment of SAVFs has recently been described, particularly for patients with a high risk of surgical complications and for patients with lesions that are difficult to treat surgically<sup>[1-3,9,10]</sup>. Compared to surgery, endovascular treatment is a less invasive, relatively low-risk, and rapid procedure, regardless of the SAVF location. These advantages (over surgery) strongly support its use for the safe and efficacious treatment of patients

with SAVFs.

Aneurysmal exclusion using a stent graft is an option for patients who do not have a tortuous splenic artery. Embolization using an Amplatzer™ vascular plug (AVP; St. Jude Medical, St. Paul, MN, United States) is another option. The AVP can be used safely in high-flow, short-segment arteriovenous fistulas where it is difficult to release coils accurately. The device can be retrieved and repositioned if the initial location is unsatisfactory. In this case, however, we initially controlled the blood flow using a balloon catheter to avoid hemorrhage due to rupture of the fragile splenic artery.

Generally, the embolization of the fistula or splenic artery, including the SAVF, is sufficient for their endovascular treatment<sup>[1-3]</sup>. In this case, however, we embolized the splenic artery, including the SAVF and the drainage vein because the main trunk of the splenic vein was occluded on the portal vein side. Without embolization of the drainage vein, the occlusion might have elevated pressure in the drainage vein and led to an enlargement of the venous aneurysm due to retrograde blood flow. As a result of these steps, we obtained successful control of the SAVF and splenic vein aneurysmal dilatation using selective embolization of the splenic artery, including the SAVF and drainage vein.

In conclusion, this is the first report of an SAVF occurring after a laparoscopic pancreatectomy and demonstrates the usefulness of endovascular treatment of SAVFs with splenic vein aneurysms.

## COMMENTS

### Case characteristics

A 40-year-old man undergoing a laparoscopic, spleen-preserving, distal pancreatectomy presented with no symptoms.

### Clinical diagnosis

The patient demonstrated a splenic arteriovenous fistula (SAVF) with a dilated splenic vein.

### Differential diagnosis

Splenic artery pseudoaneurysm, pseudocyst of the pancreas.

### Laboratory diagnosis

HGB 15.1 g/dL; AMY 44 IU/L. All blood test results were within normal limits.

### Imaging diagnosis

Computed tomography (CT) demonstrated an SAVF with a dilated splenic vein measuring 56 mm × 64 mm.

### Treatment

The SAVF, together with a splenic vein aneurysm, was successfully treated using percutaneous, transarterial, coil embolization of the splenic artery, including the SAVF and drainage vein.

### Related reports

SAVFs with splenic vein aneurysms are extremely rare entities. There are no

prior reports of SAVFs developing after laparoscopic pancreatectomy.

### Term explanation

SAVFs with splenic vein aneurysms are extremely rare. SAVFs can be asymptomatic or may result in a variety of portal vein hypertension symptoms, and the rapid enlargement of a splenic vein aneurysm can cause the rupture.

### Experiences and lessons

This is the first report of an SAVF occurring after laparoscopic pancreatectomy and demonstrates the usefulness of endovascular treatment of SAVFs with splenic vein aneurysms.

### Peer-review

This is a well-written and adequately documented report of a postoperative arteriovenous fistula, following laparoscopic distal pancreatic resection. The authors complete their report with a comprehensive review of relevant literature evidence. The subject of this case report is relevant to multiple disciplines, including surgeons, gastroenterologists, and interventional radiologists. The differential diagnosis, which might define the therapeutic approach, is of utmost importance.

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## ***Helicobacter cinaedi* bacteremia with cellulitis after ABO-incompatible living-donor liver transplantation: Case report**

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**Author contributions:** Mishima K and Obara H wrote this paper; Sugita K performed analysis of blood culture and gene sequence; all other members equally contributed to medical treatment.

**Ethics approval:** The study was reviewed and approved by the Keio University School of Medicine Institutional Review Board.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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### Abstract

*Helicobacter cinaedi* (*H. cinaedi*), a Gram-negative spiral-shaped bacterium, is an enterohepatic non-*Helicobacter pylori* *Helicobacter* species. We report the first case of *H. cinaedi* bacteremia with cellulitis after liver transplantation. A 48-year-old male, who had been a dog breeder for 15 years, underwent ABO-incompatible living-donor liver transplantation for hepatitis C virus-induced decompensated cirrhosis using an anti-hepatitis B core antibody-positive graft. The patient was preoperatively administered rituximab and underwent plasma exchange twice to overcome blood type incompatibility. After discharge, he had been doing well with immunosuppression therapy comprising cyclosporine, mycophenolate mofetil, and steroid according to the ABO-incompatible protocol of our institution. However, 7 mo after transplantation, he was admitted to our hospital with a diagnosis of recurrent cellulitis on the left lower extremity, and *H. cinaedi* was detected by both blood culture and polymerase chain reaction analysis. Antibiotics improved his symptoms, and he was discharged at day 30 after admission. Clinicians should be more aware of *H. cinaedi* in immunocompromised patients, such as ABO-incompatible transplant recipients.



**Key words:** *Helicobacter cinaedi*; Bacteremia; Cellulitis; Liver transplantation; Hepatitis C; Living-donor; ABO-incompatible; HBc-Ab-positive donor

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**Core tip:** This is the first case report of *Helicobacter cinaedi* infection in a liver transplant recipient. Clinicians should be aware of this microorganism when treating immunocompromised patients, such as ABO-incompatible liver transplant recipients with symptoms of cellulitis.

Mishima K, Obara H, Sugita K, Shinoda M, Kitago M, Abe Y, Hibi T, Yagi H, Matsubara K, Mori T, Takano Y, Fujiwara H, Itano O, Hasegawa N, Iwata S, Kitagawa Y. *Helicobacter cinaedi* bacteremia with cellulitis after ABO-incompatible living-donor liver transplantation: Case report. *World J Gastroenterol* 2015; 21(25): 7911-7915 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7911.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7911>

## INTRODUCTION

*Helicobacter* is a genus of gram-negative bacteria possessing a characteristic spiral shape. The most well-known species of the genus is *Helicobacter pylori*, as some strains are associated with peptic ulcers, chronic gastritis, and gastric cancers. Nevertheless, several reports published during the last few decades have contributed to a better understanding of both human and animal infection with non-*Helicobacter pylori* *Helicobacter* species<sup>[1]</sup>. One such enterohepatic species is *Helicobacter cinaedi* (*H. cinaedi*), which colonizes the gastrointestinal tract mucosa of mammals, including humans<sup>[2]</sup>. Cellulitis due to *H. cinaedi* is occasionally reported in neutropenic patients with hematologic malignancies and less frequently in patients with immunocompromised conditions, such as diabetes mellitus and malnutrition. Here, we report the first case of *H. cinaedi* bacteremia with cellulitis after liver transplantation.

In Japan, although the number of deceased donor liver transplantation (DDLT) has gradually increased, living-donor liver transplantation (LDLT) is still the most frequent treatment option because of organ shortages. Therefore, the use of liver grafts from hepatitis B core antibody (HBc-Ab)-positive<sup>[3,4]</sup> or ABO-incompatible (ABO-I) living donors are permitted in Japan. The present case is also a rare case of ABO-I LDLT from an HBc-Ab-positive donor to a recipient with hepatitis C virus (HCV).

## CASE REPORT

After receiving a detailed explanation, the patient

**Table 1** Preoperative blood type, viral marker status of the recipient and donor

Blood type	Recipient B Rh (+)	Donor AB Rh (+)
Viral marker status		
HBs-Ag (C.O.I)	< 1.0	< 1.0
HBs-Ab (mIU/mL)	186.9	< 10.0
HBc-Ab (% Inh)	92.6	95.8
HBe-Ag (S/CO)	< 0.50	< 0.50
HBe-Ab (% Inh)	73	48
HBV-RNA (log copy/mL)	–	–
HCV-Ab	+	–
HCV-RNA (log IU/mL)	5.8	–
HCV genotype	2a	–

HBs-Ag: Hepatitis B surface antigen; HBs-Ab: Hepatitis B surface antibody; HBc-Ag: Hepatitis B core antigen; HBc-Ab: Hepatitis B core antibody; HBe-Ag: Hepatitis B envelope antigen; HBe-Ab: Hepatitis B envelope antibody; HCV-Ab: Hepatitis C virus antibody.

provided informed consent to publish his case details.

A 48-year-old male, who had been a dog breeder for 15 years, underwent ABO-I LDLT for HCV-induced decompensated liver cirrhosis with an HBc-Ab-positive liver graft. His Model for End-stage Liver Disease (MELD) score was 9 at the time of LDLT. His notable medical history included was hypertension and diabetes mellitus.

The donor was the patient's 46-year-old younger brother who had no notable medical history except for resolved HBV infection. The viral marker statuses of the recipient and donor are summarized in Table 1; the results suggest that the recipient also had a history of resolved HBV infection. To overcome blood type incompatibility, 500 mg/body of rituximab (an anti-CD20 antibody) was administered to the recipient four weeks before LDLT and preoperative plasma exchange was performed twice according to our institution's protocol<sup>[5]</sup>. LDLT was performed routinely using a left lobe graft. Intraoperative liver wedge biopsy of the donor revealed no evidence of steatosis. Immediately after total hepatectomy was performed, 10000 IU of hepatitis B immunoglobulin (HBIG) was systemically infused into the recipient as anti-HBV prophylaxis. The liver graft was revascularized in the order of anastomosis of the hepatic vein to the inferior vena cava and reconstruction of the portal vein and hepatic artery. Immediately before reperfusion of the liver *via* the portal vein, 650 mg of methylprednisolone was infused intravenously. Splenectomy was performed following hepatic artery reconstruction, and a portal vein catheter was placed *via* a middle colic vein for local graft infusion therapy according to the immunosuppression protocol for ABO-I<sup>[5,6]</sup>.

In addition to routine postoperative treatment, the CD19- and CD20-positive B-cell counts, as well as isoagglutinin titers of anti-A and anti-B, were monitored frequently. At postoperative day 25, tacrolimus was stopped and cyclosporine was started

**Table 2** Case reports of *Helicobacter cinaedi* bacteremia and their associated symptoms *n* (%)

Ref.	Bacteremia, <i>n</i>	Fever	Cellulitis	Diarrhea
Kawakami <i>et al</i> <sup>[21]</sup>	46	43 (93)	8 (17.4)	4 (8.7)
Araoka <i>et al</i> <sup>[22]</sup>	63	ND	24 (38.0)	7 (10.4)
Mandai <i>et al</i> <sup>[23]</sup>	1	Yes	Yes	No
Kikuchi <i>et al</i> <sup>[24]</sup>	1	Yes	Yes	No
Kim <i>et al</i> <sup>[25]</sup>	1	Yes	Yes	No
Ishizawa <i>et al</i> <sup>[26]</sup>	1	No	Yes	No
Holst <i>et al</i> <sup>[19]</sup>	1	Yes	Yes	No
Matsumoto <i>et al</i> <sup>[16]</sup>	6	6 (100)	ND	0 (0)
Nishine <i>et al</i> <sup>[27]</sup>	1	Yes	No	No
Kitamura <i>et al</i> <sup>[17]</sup>	11	ND	11 (100)	ND
Uçkay <i>et al</i> <sup>[28]</sup>	1	Yes	No	No
Van Genderen <i>et al</i> <sup>[29]</sup>	1	Yes	Yes	No
Simons <sup>[30]</sup>	1	No	No	No
Murakami <i>et al</i> <sup>[8]</sup>	1	Yes	Yes	No
Lasry <i>et al</i> <sup>[31]</sup>	1	Yes	No	No
Hung <i>et al</i> <sup>[32]</sup>	1	Yes	No	Yes
Sullivan <i>et al</i> <sup>[33]</sup>	1	Yes	Yes	No
Tee <i>et al</i> <sup>[34]</sup>	3	ND	1 (33)	0
Mammen <i>et al</i> <sup>[35]</sup>	1	Yes	No	Yes
Burman <i>et al</i> <sup>[11]</sup>	7	5 (71.4)	4 (57.1)	1 (14.3)

ND: Not documented.

due to the possibility of thrombotic microangiopathy. Immunosuppression therapy at discharge (*i.e.*, postoperative day 63) comprised cyclosporine (130 mg/d), MMF (2000 mg/d), and PSL (15 mg/d). The doses of these drugs were gradually reduced during follow-up. He was followed almost every two weeks.

Unfortunately, four months after ABO-I LDLT, routine laboratory investigations and liver biopsy specimens showed early HCV relapse. The HCV-RNA level at that time had increased to 7.2 log IU/mL. As the patient had a history of progression of diabetic retinopathy due to interferon therapy and liver function tests at that time were almost normal, he did not start interferon therapy; he is planned to take sofosbuvir, which will be approved shortly in Japan.

Seven months after transplantation, he was hospitalized with complaints of high fever and swelling in the left lower extremity, which is compatible with cellulitis, without any signs of trauma. On admission, hemoglobin level was 11.9 g/dL, white blood cell count was 13000/μL with 80.5% neutrophils, and platelet count was 364000/μL. C-reactive protein level was elevated to 6.50 mg/dL. Blood culture was not analyzed at that time. Thereafter, cefazolin was empirically administered for seven days. His symptoms were relieved immediately, and he was discharged at day 10 after admission.

However, one week later, he was readmitted with a diagnosis of recurrent cellulitis on the left lower extremity. Blood culture was analyzed at this time, and cefazolin was empirically administered again. Although left lower leg swelling improved immediately, subfever was prolonged and gram-negative spiral bacteria were confirmed by both aerobic and anaerobic vials of two sets of blood cultures at day 5 after admission.

Considering the possibility of *Campylobacter* infection according to the results of gram-negative spiral bacteria, cefazolin was replaced with ciprofloxacin. The results of the API Campy kit (Sysmex bioMerieux Co., Ltd., Kobe, Japan) indicated that the causative microorganism was *H. cinaedi* with a 68.5% probability; 16S rRNA gene sequencing was performed for further identification. According to a search of the Basic Local Alignment Search Tool (BLAST) database (<http://www.ncbi.nlm.nih.gov/blast/>), the sequence of this isolate exhibited 99% similarity with that of *H. cinaedi*. As the swelling of the left lower extremity and high fever occurred simultaneously, we diagnosed *H. cinaedi* bacteremia with recurrent cellulitis. According to the result of antibiotic susceptibility testing (disk diffusion test), the microorganism was susceptible to tetracycline, third generation cefem, and carbapenem, and, on the contrary, resistant to first generation cefem and new quinolone antibiotics. Therefore, ciprofloxacin was switched to minocycline at day 20 after admission because of reports of increasing quinolone-resistant *H. cinaedi*. Thereafter, his subfever resolved, and he was discharged at day 30 after the latest admission. He has been on minocycline for more than 3 mo and is currently being followed up at our institution, without recurrence.

## DISCUSSION

We reported a case of bacteremia with cellulitis caused by *H. cinaedi* after LDLT for HCV-induced decompensated liver cirrhosis, using an HBc-Ab-positive liver graft. To our knowledge, this is the first case report of *H. cinaedi* infection in a liver transplant recipient; meanwhile, there is only one case report of ABO-I LDLT from an HBc-Ab-positive donor to an HCV recipient<sup>[7]</sup>. In the field of solid organ transplantation, only one case of *H. cinaedi* infection after renal transplant has been reported<sup>[8]</sup>. *H. cinaedi* was originally isolated as a *Campylobacter*-like organism from rectal swabs obtained from homosexual men infected with HIV in 1984<sup>[9]</sup>. Regarding the isolation of *H. cinaedi*, it takes  $4.1 \pm 1.60$  d to identify this species after blood culture. Therefore, at least 5 d of incubation is required to avoid overlooking the microorganism.

Some cases of *H. cinaedi* infection have been reported during the last few decades. In these reports, this microorganism is described as causing diverse symptoms, including erysipelas, cellulitis, arthritis, and neonatal meningitis, as well as gastroenteritis and proctitis<sup>[10-14]</sup>. A review of the literature on cases of bacteremia by *H. cinaedi* documenting the incidence of each symptom is shown in Table 2. Interestingly, cellulitis was observed in 56/150 cases (37.3%), whereas diarrhea was only reported in 14/150 cases (9.3%); thus, cellulitis is the predominant symptom caused by this microorganism compared with other gram-negative enteric bacilli, such as *Campylobacter* spp.

Regarding *H. cinaedi* pathogenesis, the secondary involvement of the skin and subcutaneous tissues in bacteremia is thought to be caused by its toxic factors<sup>[15]</sup>. In addition, immunodeficiency may allow continuous bacterial translocation resulting in high recurrence. In our case, recurrent cellulitis accompanied by bacteremia led to the diagnosis of *H. cinaedi* infection.

Regarding our patient's background, it has been reported that *H. cinaedi* bacteremia is rare but can occur in immunocompromised hosts by Matsumoto, Goto, who evaluated the prevalence of *H. cinaedi* as a bacteremia-causing pathogen by analyzing blood culture samples<sup>[16]</sup>. *H. cinaedi* infection is observed occasionally in patients with alcoholism, diabetes, and malignancy and less commonly in patients with no recognized host defense defect<sup>[17-19]</sup>. As this microorganism is presumably transmitted from animals to human *via* the fecal-oral route, our patient's work as dog breeder for 15 years may be associated with the infection route of *H. cinaedi*. In addition, splenectomy and the immunosuppression protocol for ABO-I comprising rituximab (anti-CD20 antibody), tacrolimus / cyclosporine, MMF, and PSL might have been associated with pathogenesis by strongly affecting the patient's immunity.

Of the drugs mentioned above, rituximab is a key drug for suppressing humoral immunity in ABO-I LDLT. In Japan, where LDLT has been developed more than DDLT because of a lack of brain-dead donors, donors are mostly limited to close family members. Therefore, ABO-I LDLT use in Japan is more common than in other countries. In ABO-I LDLT, B-cells and alloantibodies become pathogenic in terms of antibody-mediated rejection in addition to cell-mediated rejection, which is also observed in ABO-compatible LDLT. Rituximab is a monoclonal antibody usually used to treat B-cell non-Hodgkin lymphoma. In ABO-I LDLT, the effectiveness of rituximab is mostly explained by its depletion of specific antidonor antibodies and elimination of circulating and presumably tissue CD20<sup>+</sup> B-cells<sup>[20]</sup>. As the effect of rituximab persists for several months, serious fungal, bacterial, and new or reactivated viral infections can occur after treatment. The long-term effectiveness of rituximab may explain why cellulitis occurred in our patient, who was taking only cyclosporine when the pathogenesis of cellulitis occurred.

There are currently no clear guidelines in the literature concerning the choice or duration of antibiotic therapy for *H. cinaedi* infection. A large review of 23 cases of bacteremia reported that penicillins, tetracycline, and aminoglycosides are more effective than are cephalosporins, erythromycin, or ciprofloxacin<sup>[10]</sup>. Quinolones alone may not completely eradicate *H. cinaedi*, which explains the frequent reports of recurrent disease after quinolone monotherapy. In our case, recurrent cellulitis was observed in spite of the use of cefazolin for one week; therefore, oral minocycline was

continued for more than three months.

In conclusion, this is the first case report of *H. cinaedi* infection in a liver transplant recipient. Clinicians should be aware of this microorganism when treating immunocompromised patients such as ABO-I transplant recipients with symptoms of cellulitis.

## COMMENTS

### Case characteristics

A 48-year-old male who was diagnosed with recurrent cellulitis on the left lower extremity after liver transplantation.

### Clinical diagnosis

*Helicobacter cinaedi* (*H. cinaedi*) bacteremia with cellulitis.

### Differential diagnosis

*Campylobacter* species infection.

### Laboratory diagnosis

Spiral bacteria were confirmed by both aerobic and anaerobic vials of blood cultures, and 16S rRNA gene sequencing of this isolate exhibited 99% similarity with that of *H. cinaedi*.

### Imaging diagnosis

There are no imaging methods to confirm the diagnosis of *Helicobacter cinaedi* infection.

### Pathological diagnosis

There are no pathological methods to confirm the diagnosis of *Helicobacter cinaedi* infection.

### Treatment

At first, cefazolin was empirically administered for one week. Finally, oral minocycline was continued for more than three months after the diagnosis of *Helicobacter cinaedi* bacteremia with cellulitis was obtained.

### Related reports

To date, this is the first case report of *Helicobacter cinaedi* bacteremia with cellulitis after liver transplantation.

### Term explanation

*Helicobacter cinaedi* is one of the non-*Helicobacter pylori* *Helicobacter* species that colonizes the gastrointestinal tract mucosa of mammals, including humans.

### Experience and lessons

Review of literature showed that cellulitis is the predominant symptom caused by *Helicobacter cinaedi*. Clinicians should be aware of this microorganism when treating immunocompromised patients such as ABO-I liver living-donor transplant recipients with symptoms of cellulitis.

### Peer-review

This is the first case report of *Helicobacter cinaedi* infection in a liver transplant recipient. Due to the limited volume of patients, the underlying cause of bacteremia and cellulitis is unknown.

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## Intraductal papillary mucinous neoplasm of the ileal heterotopic pancreas in a patient with hereditary non-polyposis colorectal cancer: A case report

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**Author contributions:** Lee SH analyzed the data and wrote the paper; Lee SH, Kim WY and Han HS made the pathologic diagnosis and reviewed the manuscript; and Hwang DY performed surgery, clinical care of the patient and collected the patient's clinical data.

**Ethics approval:** The study was reviewed and approved by the Konkuk University Medical Center Institutional Review Board.

**Informed consent statement:** A study participant provided informed written consent prior to study enrollment.

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### Abstract

We report a case of intraductal papillary mucinous neoplasm (IPMN) originating from the ileal heterotopic pancreas in a patient with hereditary non-polyposis colorectal cancer (HNPCC). A 49-year-old woman had a past history of total colectomy and total hysterectomy with bilateral salpingo-oophorectomy due to colonic adenocarcinoma and endometrial adenocarcinoma 11 years ago. Her parents died from colonic adenocarcinoma and her sister died from colonic adenocarcinoma and endometrial adenocarcinoma. The clinician found an ileal mass with necrotic change and the mass increased in size from 1.7 cm to 2.2 cm during the past 2 years on computed tomography. It was surgically resected. Microscopically, the ileal mass showed heterotopic pancreas with IPMN high grade dysplasia. Immunohistochemical staining revealed positive reactivity for MLH1/PMS2 and negative reactivity for MSH2/MSH6. This is the first report of IPMN originating from the ileal heterotopic pancreas in a patient with HNPCC in the English literature.

**Key words:** Colorectal neoplasms; Hereditary nonpolyposis; Pancreatic neoplasms; Choristoma

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**Core tip:** Intraductal papillary mucinous neoplasm (IPMN) is a precursor lesion of pancreatic cancer. Although pancreatic cancer is one of the malignancies associated with hereditary non-polyposis colorectal cancer (HNPCC), only one case has been reported IPMN in a HNPCC patient. We report a first case of IPMN arising in heterotopic pancreas in a patient of HNPCC.

Lee SH, Kim WY, Hwang DY, Han HS. Intraductal papillary mucinous neoplasm of the ileal heterotopic pancreas in a patient with hereditary non-polyposis colorectal cancer: A case report. *World J Gastroenterol* 2015; 21(25): 7916-7920 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7916.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7916>

## INTRODUCTION

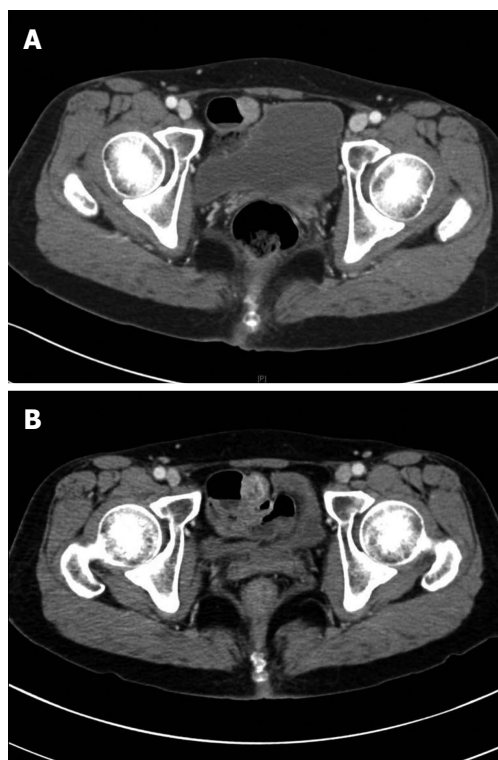
Hereditary non-polyposis colorectal cancer (HNPCC) is a most common form of hereditary colorectal cancer syndrome that accounts for approximately 3% of all colorectal cancers<sup>[1]</sup>. It is an autosomal dominant syndrome caused by germline mutations of mismatch repair genes including MLH1, MSH2, MSH6, and PMS2. It shows characteristic microsatellite instability (MSI).

HNPCC associated cancers include cancer of colon, rectum, stomach, ovary, ureter, renal pelvis, brain, small bowel, hepatobiliary tract, and skin<sup>[2]</sup>. Clinically these tumors are diagnosed at an early age, are multiple, and have a family history. Colonic cancers are usually located in the proximal colon.

Heterotopic pancreas is defined as pancreatic tissue located outside of the normal anatomic border of the pancreatic gland<sup>[3]</sup>. About 90% of heterotopic pancreas is localized in the stomach, duodenum, or upper part of jejunum, but ileal location is rare at approximately 1%<sup>[4]</sup>. It is usually asymptomatic, however it can produce size or location dependent symptoms and can show most pathologic changes of pancreas including benign or malignant tumors. Intraductal papillary mucinous neoplasm (IPMN) is one of the tumors arising in pancreas. It is characterized by cystic dilatation of pancreatic duct with intraductal papillary growth structures that are covered with mucin producing columnar cells. In this report, we describe a case of intraductal papillary mucinous neoplasm of the ileal heterotopic pancreas in a patient with hereditary non-polyposis colorectal cancer which, to our knowledge, is the first report in English literature.

## CASE REPORT

We present the case of a 49-year-old woman with IPMN of heterotopic pancreas of the HNPCC family. The patient had a past history of total colectomy, transabdominal hysterectomy, and bilateral salpingo-oophorectomy due to colonic adenocarcinoma on



**Figure 1** Abdominopelvic computed tomography in 2011 and B: 2014. A: Contrast enhanced computed tomography (CT) scan shows 1.7 cm sized enhancing mass arising in small bowel; B: Follow up CT shows a slightly increased size to 2.2 cm and new appearance of low density areas in the mass suggests necrotic change.

anal verge 75 cm, and endometrial adenocarcinoma 11 years ago. Lobectomy of thyroid due to thyroid cancer was also performed 3 years ago. Family history included death of her father due to colonic carcinoma and death of her mother due to right colonic carcinoma. Her sister had also suffered fatal right colonic carcinoma and endometrial carcinoma. Another sister died and the patient was unable to recall the cause of death.

The patient had no specific symptom and the clinician could not find any specific sign on physical examination. However clinician found a submucosal mass on the terminal ileum on follow up computed tomography (CT). It had increased in size from 1.7 cm to 2.2 cm and was suspected of necrotic change on abdominopelvic CT compared with 2 years ago CT (Figure 1). Surgical resection was performed under the presumption of gastrointestinal stromal tumor.

The cut surface of specimen showed an ill circumscribed grayish yellow, fibrotic and focal cystic mass in the submucosal layer (Figure 2). Microscopically, the mass was composed of pancreatic ducts and acini. The pancreatic ducts were cystically dilated. They showed intraductal papillary proliferative lesion with abundant mucin and some of the cells had marked nuclear atypia. We diagnosed an IPMN with high grade dysplasia arising in heterotopic pancreas of the terminal ileum (Figure 3). Immunohistochemically, the tumor cells were positive

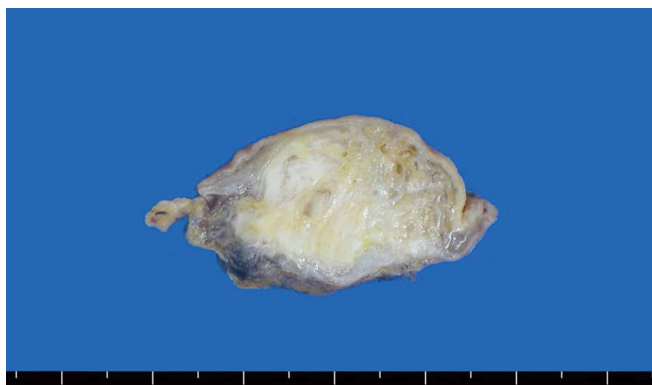
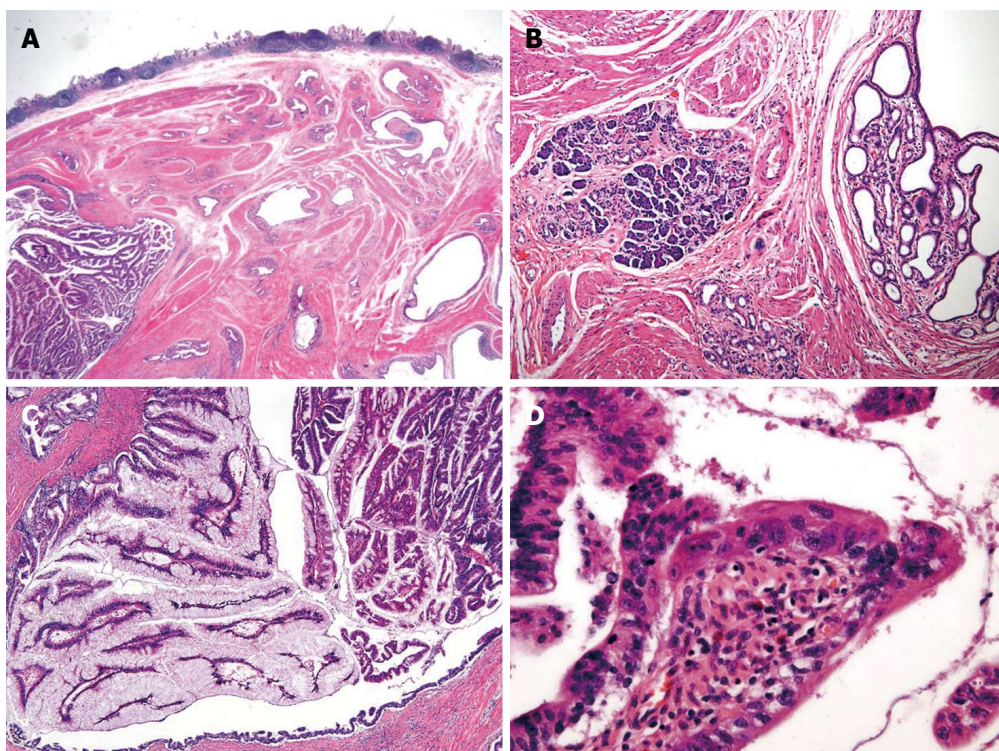


Figure 2 Cut surface shows a fibrotic and cystic lesion in the submucosa.



**Figure 3 Microscopic findings.** A: Microscopic examination shows dilated ducts [hematoxylin-eosin (HE) stain, original magnification  $\times 40$ ] with B: pancreas acini (HE stain, original magnification  $\times 100$ ); C: The ducts are filled with papillary neoplasm. The epithelium is covered with tall columnar cells that have abundant intracytoplasmic mucin (HE stain, original magnification  $\times 100$ ); D: Some of the epithelial cells have enlarged nuclei with nucleoli and irregular nuclear membrane consistent with high grade nuclear atypia (HE stain, original magnification  $\times 400$ ).

for MUC2, MUC5AC and negative for MUC1, MUC6, CDX2, indicative of intestinal type IPMN. Interestingly the tumor cells were positive for MLH1, PMS2 and negative for MSH2, MSH6 indicating defects in DNA mismatch repair (Figure 4).

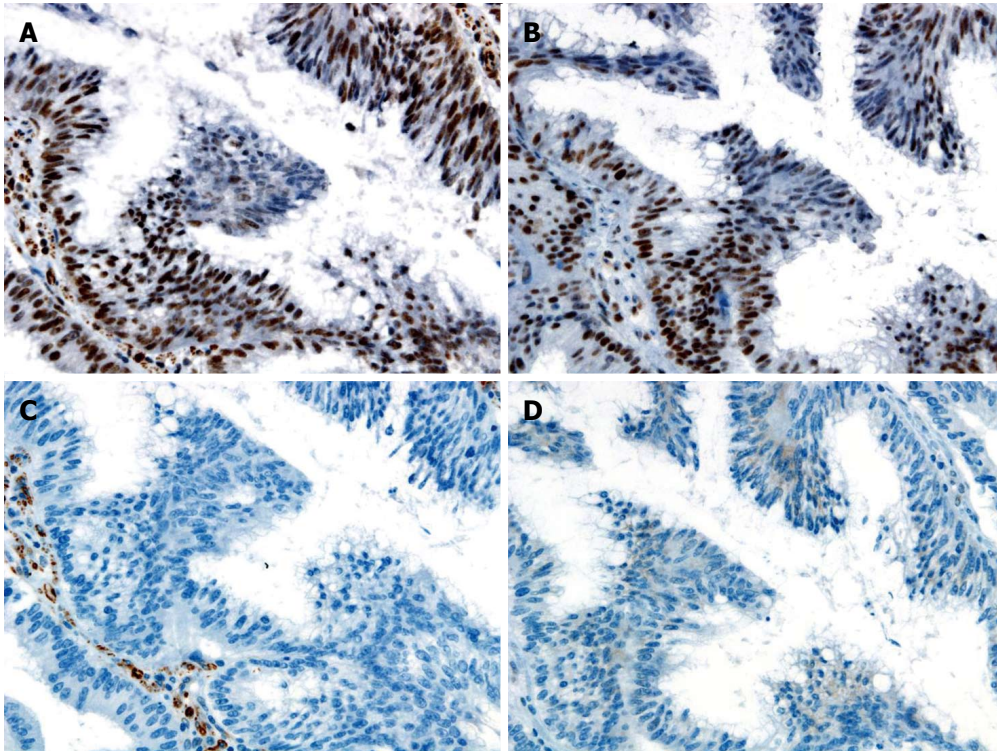
## DISCUSSION

We reported the first case of IPMN arising in heterotopic pancreas of a HNPCC patient. We could not confirm the germline mutation of mismatch repair genes and MSI status. However, clinically she was a HNPCC patient according to Amsterdam criteria II<sup>[2]</sup>. Also, immunohistochemical staining for MSH2 and MSH6

were negative. Loss of expression of the MLH1 or MSH2 genes is associated approximately 100% with an MSI-high phenotype<sup>[5]</sup>. The Revised Bethesda Guidelines recommend molecular evaluation in patients with loss of expression of one of the MMR genes<sup>[5]</sup>. Based on clinical and family history with immunohistochemical study, we diagnosed the patient with HNPCC.

IPMN is one of the premalignant lesions of the pancreas arising in elderly. Only one report of IPMN in a patient with HNPCC has been previously reported<sup>[6]</sup>. Briefly, the patient was a 61-year-old woman with multiple cancer history including unknown pathologic type brain tumor, endometrioid ovarian cancer, endometrial cancer, infiltrating duct carcinoma of





**Figure 4** Immunohistochemical staining. A: Immunohistochemical stain is positive for MLH1; B: PMS2; C: Negative for MSH2; D: MSH6. Magnification  $\times 400$  (A-D).

breast, malignant melanoma, multiple basal cell carcinomas, squamous cell carcinoma of skin, and adenocarcinoma contained within an adenoma at the splenic flexure, intramucosal adenocarcinoma of colon, and invasive adenocarcinoma of rectum. She had a germline mutation of the *MSH2* gene, however, family history was unavailable<sup>[6]</sup>.

In previous study, immunohistochemical staining for MLH1 and MSH2 showed 100% positivity in IPMN, suggestive that *MMR* genes do not play a significant role in development of IPMN<sup>[7]</sup>. Sparr *et al.*<sup>[6]</sup> suggested that IPMN may be part of extracolonic tumors in HNPCC because their study case showed loss of MSH2 expression, high level *MSI*, *MSH2* gene mutation and in general, IPMN is associated with extrapancreatic neoplasms, specifically colorectal adenomas and adenocarcinomas, as well as gastric carcinomas. Our case showed loss of expression of MSH2 and MSH6 protein by immunohistochemical staining. This finding suggested that *MMR* genes play a role in development of IPMN, especially in patients with HNPCC, in agreement with Sparr *et al.*<sup>[6]</sup>.

On the other hand, IPMN can very rarely originate from heterotopic pancreas. To the best of our knowledge, only 9 cases of IPMN or adenocarcinoma with IPMN arising in heterotopic pancreas were reported in English literature<sup>[3,8-15]</sup>. The mean age of these cases was 65.4 years (range: 44-80 years). They were located in stomach (4 cases), jejunum (2 cases), esophagus (1 case), duodenum (1 case), and Meckel's diverticulum (1 case). Most cases were located in common sites of heterotopic pancreas such as stomach, duodenum,

and jejunum<sup>[4]</sup>. Clinically our case of IPMN occurred at a relatively young age that is characteristic of tumors arising in HNPCC patient.

In conclusion, we reported the second case of IPMN in a patient with HNPCC and the first case of IPMN arising in heterotopic pancreas in a patient with HNPCC. Pancreatic cancer is one of the extracolorectal tumors associated with HNPCC. IPMN is a precursor lesion of pancreatic cancer but the relationship between IPMN and HNPCC is unknown. It is important to identify HNPCC for follow up and screening tests for the family. Further studies are needed to identify the relationship between IPMN and HNPCC.

## COMMENTS

### Case characteristics

The 49-year-old female patient was asymptomatic. Ileal mass was found on routine follow up computed tomography (CT).

### Clinical diagnosis

On physical examination there was no palpable mass.

### Imaging diagnosis

Follow up CT showed a submucosal tumor with increased in size from 1.7 cm to 2.2 cm during the past 2 years and newly appeared foci of suspicious for necrosis.

### Pathologic diagnosis

Histological examination demonstrated heterotopic pancreas with intraductal papillary proliferative tumor. Immunohistochemically, it was negative for MSH2 and MSH6.

### Treatment

The patient underwent local excision of small intestine.

### Related reports

This is a first report of intraductal papillary mucinous neoplasm (IPMN) arising in heterotopic pancreas with a patient with hereditary non-polyposis colorectal



cancer (HNPCC).

### Experience and lessons

This case report presents a first case of IPMN arising in heterotopic pancreas with a patient with HNPCC. We add a new case supporting the inclusion of IPMN in the list of extracolonic HNPCC associated tumors.

### Peer-review

This is a case report of an interesting case of IPMN originating from the ileal heterotopic pancreas in a patient with HNPCC. The manuscript is well written and structured in its composition. It contains four figures which appear to be complete and easily accessible.

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## Monitoring disease progression and treatment efficacy with circulating tumor cells in esophageal squamous cell carcinoma: A case report

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### Abstract

This study investigated whether changes in circulating tumor cell (CTC) numbers reflect tumor progression and treatment efficacy in esophageal squamous cell carcinoma (ESCC). A 47-year-old male patient with ESCC is presented in this case study. The patient was evaluated for a series of serum tumor markers and subjected to radiological examinations before and after surgery and during follow-up over the course of five years. In addition, the CTCs in 7.5 mL of peripheral blood were enriched by magnetic-activated cell sorting negative selection and identified by immunofluorescence staining. Serum tumor markers remained within normal ranges and were discordant with imaging scans during the follow-up. Initially, one

CTC was detected in the peripheral blood sample, and 14 were observed seven days after the operation. After 12 wk, subcutaneous metastases and bone metastases occurred, and the number of CTCs increased to 84. After 48 wk, lung metastases were noted, and the CTC level was 21. At 104 wk, the number of CTCs was 14, and disease recurrence was detected by positron emission tomography-computed tomography. The CTC counts were in accord with the imaging studies at several time points. The additional information provided by CTC enumeration could thus facilitate monitoring of disease status and treatment efficacy and provide support for treatment decisions.

**Key words:** Individualized treatment; Adjuvant therapy; Esophageal squamous cell carcinoma; Circulating tumor cells

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**Core tip:** We report a follow-up of a 47-year-old male patient with esophageal squamous cell carcinoma in this study. In addition to the conventional examination, a novel workflow was performed to detect circulating tumor cells (CTCs). We evaluated the relationship between CTC characteristics and other tests. The serum tumor markers were normal and thus did not appear to reflect changes in the disease, whereas the number of CTCs fluctuated with the disease progression and treatment and coincided with imaging studies performed during the follow-up. This case highlights CTCs as a useful diagnostic tool with potential applications during treatment.

Qiao YY, Lin KX, Zhang Z, Zhang DJ, Shi CH, Xiong M, Qu XH, Zhao XH. Monitoring disease progression and treatment efficacy with circulating tumor cells in esophageal squamous cell carcinoma: A case report. *World J Gastroenterol* 2015; 21(25): 7921-7928 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i25/7921.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i25.7921>

## INTRODUCTION

Esophageal carcinoma is a common malignancy, ranking sixth among global cancer-related deaths and thus representing a serious threat to human health. More than 90% of malignant esophageal tumors are esophageal squamous cell carcinomas (ESCCs), and the incidence of the disease has increased in recent decades. Due to the lack of early symptoms and specific diagnostic methods, ESCC is commonly diagnosed at an advanced stage and therefore has an extremely poor prognosis, with only 20%-30% survival at five years<sup>[1,2]</sup>.

Tumor metastasis and recurrence are the major causes of death. Tumor lesions are mainly revealed by radiological examination and serum tumor markers. However, tumor lesions that are small in diameter are difficult to detect by imaging scans. Highly specific tumor markers are currently unavailable, especially for ESCC. Histopathology is the "gold standard" of tumor diagnosis. However, due to the limitations of specimen collection, real-time monitoring of tumor progression cannot be realized. Thus, it is critical to introduce new tumor-detection methods into clinical practice.

Studies have shown that circulating tumor cells (CTCs) are closely related to tumor metastasis and can be useful as a "window" to monitor disease prognosis both initially and after therapy<sup>[3]</sup>. The number and phenotype of CTCs can reflect the disease progression in real time and guide the treatment. Furthermore, CTCs can be easily obtained for series detection because the collection method is noninvasive and causes no trauma to patients.

However, owing to the rarity of CTCs in peripheral blood, their detection requires a combination of high specificity and sensitivity. Several CTC detection methods have been developed, including magnetic-activated cell sorting (MACS), polymerase chain reaction, and microfluidic chips<sup>[4-6]</sup>. In recent years, the immune magnetic bead enrichment method has been shown to efficiently separate cells of epithelial origin from blood samples. Two strategies for immunological isolation have emerged: positive enrichment and negative enrichment<sup>[7]</sup>.

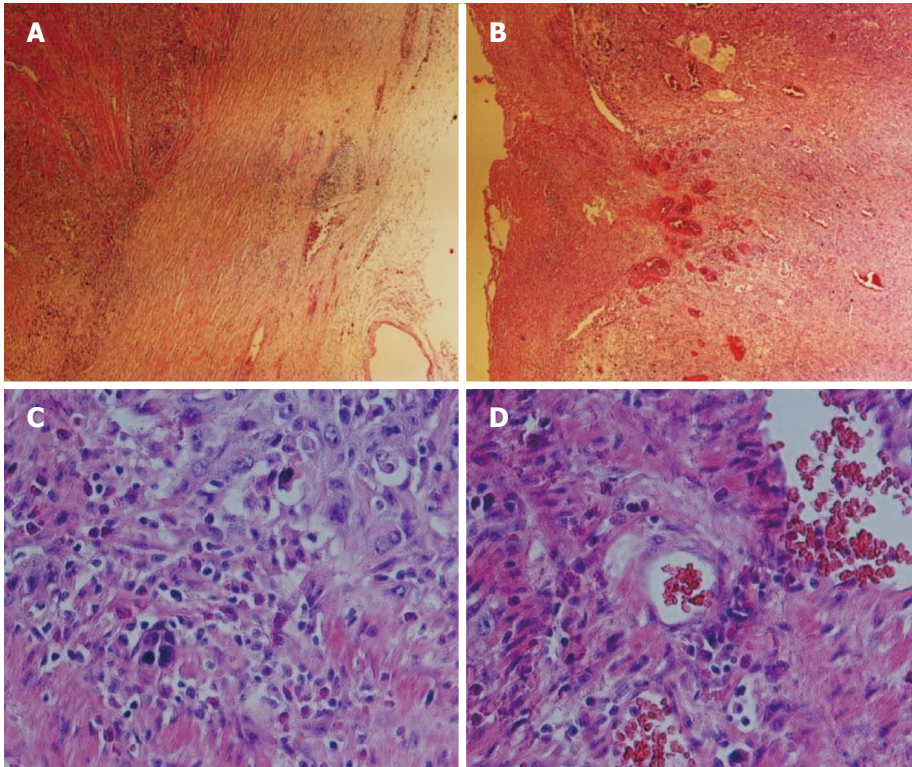
The negative selection strategy involves capturing cells of interest by depleting unwanted cells. During the metastatic cascade, tumor cells undergo epithelial-mesenchymal transition (EMT) and lose epithelial markers; therefore, positive enrichment may overlook CTCs. In the present study, we explored negative enrichment methods for obtaining CTCs. Negative enrichment has been confirmed as a promising approach for isolating CTCs<sup>[8,9]</sup>.

Here, we report a series study of CTCs from an ESCC patient and analyze the relationship between the CTC enumeration and other examination results. CTC counts appear to provide a solid basis for disease monitoring and individualized treatment.

## CASE REPORT

A 47-year-old male patient was referred for dysphagia in February 2009, and an esophageal barium meal and computerized tomography (CT) scan determined the presence of esophageal carcinoma. The diagnosis by gastroscopy and biopsy was poorly differentiated squamous cell carcinoma. The tumor lump was completely removed by surgical resection with mediastinal lymph node dissection. The final





**Figure 1** Histopathologic characterization of the esophageal squamous cell carcinoma. Hematoxylin and eosin staining of the patient's pathological tissue slide. A and B: Histologic sections revealed a papillary architecture (magnification  $\times 40$ ); C and D: Higher-magnification views of the slides. The tissue presents structural disorder involving abnormal organization, heterotypic cell number, deep nuclear staining, loss of normal epithelial polar structure, and increased mitotic activity. Obvious tumor nests are shown in (D) (magnification  $\times 200$ ).

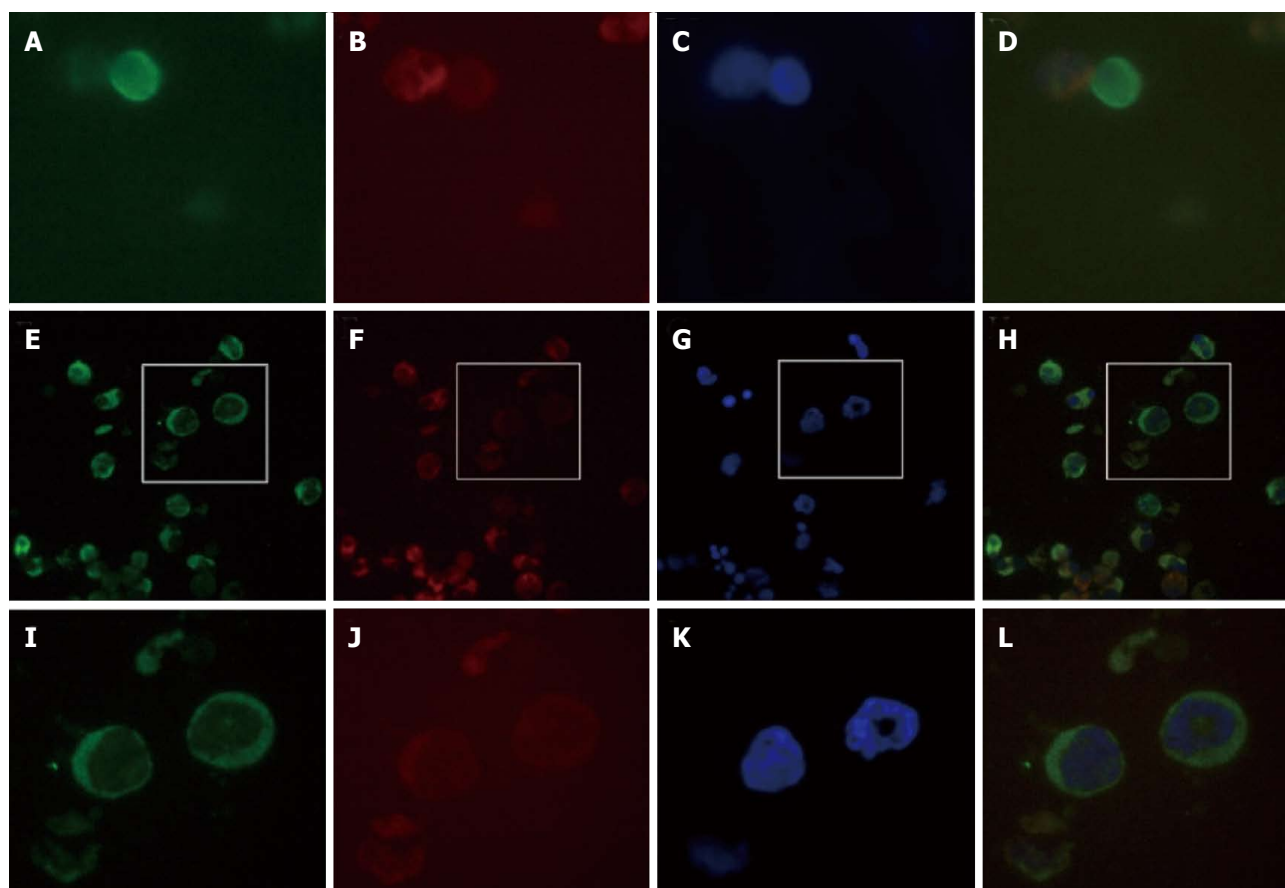
pathological result revealed a squamous cell carcinoma localized in the lower esophagus (Figure 1), 5.5 cm  $\times$  5.5 cm  $\times$  1.2 cm in size, with a stage classified as T3N2M; the carcinoma tissue invaded the entire esophageal wall. The patient presented lymph node metastases involving the paraesophageal lymph node (1/4), subcarinal lymph node (2/13), cardia lymph node (1/2), left gastric lymph node (2/5), and inferior pulmonary vein lymph node (1/1). He was treated with chemotherapy with an NP program (cisplatin + vinorelbine), followed by radiation therapy using DT4860cGy/27f/5w. After three months, when a metastasis was found in the bone, the patient received body  $\gamma$ -knife treatment using a boost dose in the mediastinal lymph nodes and abdominal lymph nodes, followed by completion of the second, third, and fourth cycles of chemotherapy, with the same treatment options as before (NP program). At 48 wk postoperation, a metastasis in the lower lobe of the right lung was identified, and a localized  $\gamma$ -knife at 4.8 Gy was employed for 10 cycles of treatment. The chemotherapy plan was then switched to the GF program (gemcitabine + fluorouracil + leucovorin) for three cycles (the fifth, sixth, and seventh cycles). Subsequently, the patient underwent traditional Chinese drug therapy and exhibited a stable condition. The follow-up process was conducted with informed consent by the patient, and the patient cooperated

actively.

We performed CTC detection by MACS negative selection<sup>[6]</sup>. After the patient signed an informed consent form, 7.5 mL of peripheral blood was collected at multiple treatment points. The blood samples were treated with erythrocyte lysate buffer to remove red blood cells, and the rest of the cells were mixed with the appropriate amount of magnetic beads and incubated for 15 min. The cell suspensions were applied to LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany), and the nucleated cells were collected under a strong magnetic field. The slide was incubated with fluorescent anti-CK8/18/19-FITC (1:100) and anti-CD45-PE (1:1000) (Miltenyi Biotec, Bergisch Gladbach, Germany) and mounted with 7  $\mu$ L 4',6'-diamidino-2-phenylindole (DAPI); the cells were observed and counted under a microscope (Table 1 and Figure 2).

The Protein Chip System for Multi-tumor Marker Detection (S20010007, Huzhou Shu Kang Biological Technology Co., Ltd.) was used in this study. This protein biochip measures 12 tumor markers in the serum, including cancer antigen CA125, CA15-3, CA19-9, CA242,  $\alpha$ -fetoprotein (AFP), carcinoembryonic antigen (CEA), human growth hormone (HGH), prostate specific antigen (PSA), free-prostate specific antigen (f-PSA),  $\beta$ -human chorionic gonadotropin ( $\beta$ -HCG), neuron-specific enolase (NSE), and ferritin (FER). The serum tumor markers were evaluated with





**Figure 2** Immunofluorescence analysis of peripheral blood circulating tumor cells collected three months postoperation. The circulating tumor cells (CTCs) were nucleated and elliptical or elongated, larger than 10  $\mu\text{m}$ , expressed cytokeratin (CK) (CK8/18/19-fluorescein isothiocyanate staining; the epithelial-derived cells are stained in green), lacked CD45 (CD45-phycoerythrin-stained leukocytes are in red), and were positive for 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (blue-stained nuclei). Some CTCs exhibited morphologically apoptotic features. A single CTC (green) and leukocytes (red) can be observed in fields A-D. A: A CTC stained with anti-CK8/18-FITC (green); B: A leukocyte stained with anti-CD45-PE (red); C: DAPI-stained nuclei; D: Merged image of A-C; E-H: Clusters of tumor cells, CTCs are green (E), leukocytes are red (F), and nuclei are stained with DAPI (G), the merged image of E-G (H); I-K: Enlargements of the boxes above, in which nuclear apoptotic features can be observed in the CTCs.

several replicates during the follow-up. The levels of these markers fluctuated slightly within normal ranges.

Under a fiber microscope, tissue abnormalities indicative of structural disorder and loss of normal polarization in the epithelial structure were observed. The cells were irregularly arranged, showing dense nuclear staining, a higher nucleus/cytoplasm ratio, and increased mitotic activity. The immunohistochemistry results revealed cytokeratin (CK) AE1/AE3 (+++), CK17 (++), p63 (++), p53 (++), epidermal growth factor receptor (EGFR) (+), CK7/20 (-) and CEA (-) in the tumor cells, with a Ki67 index of approximately 70%. At every patient visit, CT/positron emission tomography-CT (PET-CT), MRI, or a bone scan was performed at the physician's discretion.

Examination results (pre- and postoperative, as well as five-year follow-up) were collected. The follow-up occurred after every treatment or recurrence for a period of five years. The changes in CTC numbers were nearly consistent with the imaging results, thereby possibly reflecting the disease progression and treatment efficacy, as shown in Table 1.

## DISCUSSION

Esophageal carcinoma has a poor prognosis and high malignancy, with 20%-30% five-year survival rate<sup>[2]</sup>. Effective diagnosis and treatment can prolong the survival of patients with malignant tumors<sup>[10]</sup>. To date, p53, squamous cell carcinoma (SCC), p5-Ab, CEA, and cytokeratin 19 fragments (CYFRA21-1) are used for esophageal carcinoma, but their sensitivity is as low as 11%-40%<sup>[11]</sup>. The patient in this study successively underwent CEA, AFP and other serum tumor marker quantitative tests initially, after therapy and treatments or metastases points during five years of follow-up. The test results were continuously in normal range, suggesting that CEA and AFP levels did not reflect changes in his disease status. However, the SCC and CYFRA21-1 test was not performed at our hospital, and we unfortunately did not obtain these data.

Imaging scans have great significance for diagnosis of esophageal carcinoma. Our study showed that CTC changes were consistent with imaging results. CTCs may provide additional tests for metastatic cancer to

**Table 1 Relationship between the number of circulating tumor cells in the peripheral blood and disease progression**

Code	Time	Number of CTCs/7.5 mL	Results of the imaging study	Serum tumor marker levels	Disease progression	Treatment
Pre	Preoperative 2 d	1	CT revealed wall thickening of the mid-lower esophagus, with a high likelihood of esophageal carcinoma	Normal levels of CA125, CA15-3, CA19-9, CA242, AFP, CEA, HGH, PSA, f-PSA, $\beta$ -HCG, NSE and FER	Preoperative diagnosis of poorly differentiated squamous cell carcinoma	Surgical treatment
Post 1	Postoperative 1 wk	14		CEA 2.4 ng/mL; AFP 1.7 ng/mL	Tumor resection, lymph node dissection	Postoperative chemotherapy started
Post 2	Postoperative 12 wk	84	Bone imaging revealed an abnormal increase in salt metabolism in the 10th left front rib	Normal levels of CA125, CA15-3, CA19-9, CA242, AFP, CEA, HGH, PSA, f-PSA, $\beta$ -HCG, NSE and FER	Subcutaneous metastasis and bone metastasis	Treatment by a systemic $\gamma$ -knife with a boost dose and chemotherapy
Post 3	Postoperative 48 wk	21	CT revealed occupying nodules in the lower right lobe, suggesting metastatic cancer	CEA 2.0 ng/mL; AFP 1.5 ng/mL	Right lung metastasis	Treatment with a localized $\gamma$ -knife and chemotherapy
Post 4	Postoperative 55 wk	15		CEA 2.2 ng/mL; AFP 1.2 ng/mL	Stable condition	Systemic chemotherapy
Post 5	Postoperative 104 wk	41	PET-CT revealed a high density of radionuclide in the esophageal residue, suggesting disease recurrence	Normal levels of CA125, CA15-3, CA19-9, CA242, AFP, CEA, HGH, PSA, f-PSA, $\beta$ -HCG, NSE and FER	Recurrence	Systemic chemotherapy
Post 6	Postoperative 117 wk	14		CEA 2.1 ng/mL	Disease in progression	Chemotherapy with Chinese medicine
Post 7	Postoperative 178 wk	14		CEA 2.4 ng/mL	Disease in progression	Chemotherapy with Chinese medicine
Post 8	Postoperative 186 wk	5		CEA 0.5 ng/mL	Condition improved	Chinese medicine
Post 9	Postoperative 196 wk	3			Stable condition	Adjuvant treatment with traditional Chinese medicine
Post 10	Postoperative 207 wk	0			Stable condition	Adjuvant treatment with traditional Chinese medicine
Post 11	Postoperative 222 wk	1			Stable condition	Adjuvant treatment with traditional Chinese medicine
Post 12	Postoperative 238 wk	1 (suspicious)		Normal levels of CA125, CA15-3, CA19-9, CA242, AFP, CEA, HGH, PSA, f-PSA, $\beta$ -HCG, NSE and FER	Stable condition	Adjuvant treatment with traditional Chinese medicine
Post 13	Postoperative 262 wk	0			Stable condition	Adjuvant treatment with traditional Chinese medicine
Post 14	Postoperative 300 wk	0			Stable condition	Adjuvant treatment with traditional Chinese medicine

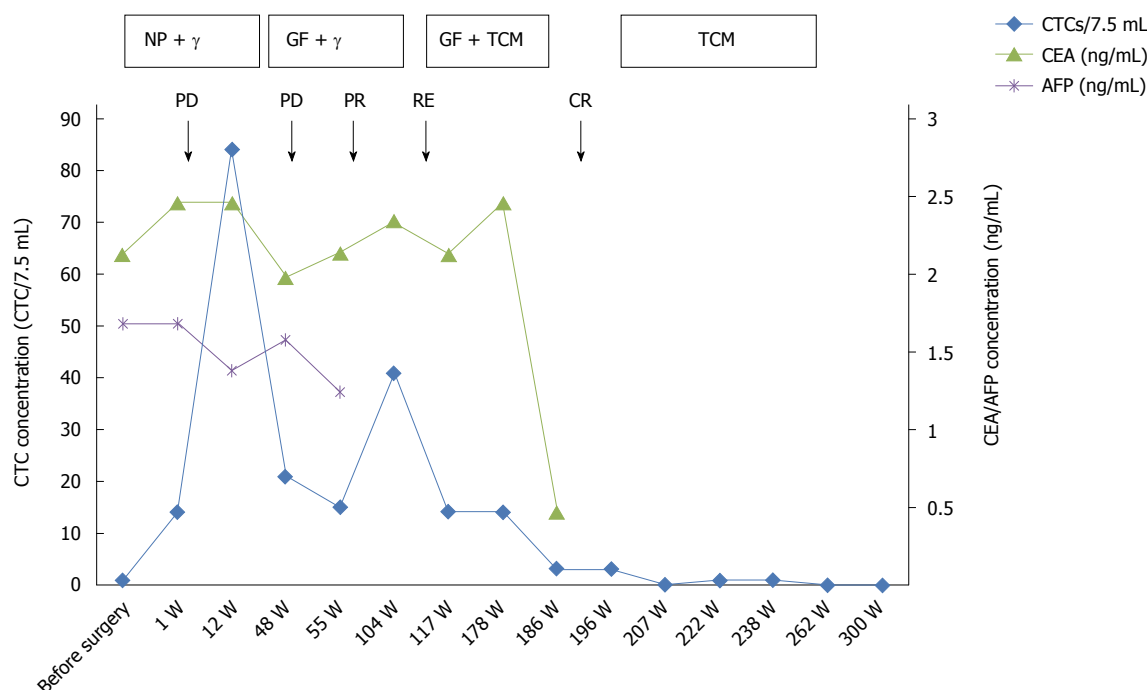
CTCs: Circulating tumor cells; AFP:  $\alpha$ -fetoprotein; CEA: Carcinoembryonic antigen; NSE: Neuron-specific enolase; FER: Ferritin; HGH: Human growth hormone;  $\beta$ -HCG:  $\beta$ -human chorionic gonadotropin; f-PSA: Free-prostate specific antigen; CT: Computed tomography.

supplement the current methods<sup>[12]</sup>. CTC are used to predict survival in several metastatic cancers and the studies investigated that increased number of CTCs suggested a high risk of disease progression and a poor prognosis<sup>[13-18]</sup>.

However, few studies have yet reported CTCs in ESCC patients. In a study of certain digestive tumors, the presence of two or more CTCs was significantly correlated with peritoneal dissemination of gastric or colorectal cancer and pleural dissemination of esophageal cancer<sup>[19]</sup>. We used negative selection method to obtain CTCs and analyzed by immunocytochemistry. In Figure 2, CTCs were nucleated and elliptical or elongated with a singular or clustered appearance and larger than 10  $\mu$ m, expressed cytokeratin, lacked

CD45, and were positive for DAPI staining. Some CTCs exhibited morphologically apoptotic features due to chemotherapy or radiotherapy.

Our group's previous studies found CTCs (using a cutoff value of 5) to be a significant prognostic factor. The results were confirmed in this case study (Table 1). Initially, one CTC was detected in the peripheral blood of the patient. The Multi-tumor Marker Detection was normal. Seven days after surgery, 14 CTCs presented. Two possible reasons were considered: first, the patient had lymph node metastases, and because some of the lymph nodes were around the gastric artery, they could not be excised completely; second, the operation procedure may promote tumor micrometastases. One study reported that CTCs



**Figure 3** Integrated schema of therapy, biomarkers, and assessments performed. The various assessments applied during the treatment in this case study are displayed. Boxes representing the duration of treatment are presented at the top of the graph. The names of the therapeutic regimens are shown in the boxes. Changes in circulating tumor cell (CTC) numbers occurred during the different treatments. The carcinoembryonic antigen (CEA) and  $\alpha$ -fetoprotein (AFP) markers maintained low or normal values. NP: Cisplatin + vinorelbine; GF: Gemcitabine + leucovorin calcium + fluorouracil; TCM: Traditional Chinese medicine; PD: Progressive disease; PR: Partial response; CR: Complete response; RE: Recurrence.

number in breast cancer patients was increased by 85% during the third to fourth postoperative days and may even rise to 1000 times the original level in some cases<sup>[20]</sup>. CEA and AFP were normal at this time point, 2.4 ng/mL and 1.7 ng/mL, respectively. The immunohistochemistry revealed cytokeratin (CK) AE1/AE3 (+++), CK17 (++), p63 (++), p53 (++), EGFR(+), CK7/20 (-), and CEA (-) in the tumor cells, with a Ki67 index of approximately 70%. These indicated that the tumor cells were cytokeratin-positive and actively proliferating and confirmed that we could obtain CTCs based on cytokeratin markers. After chemotherapy, three months (12 wk) postoperation, bone and subcutaneous metastases were found through imaging, and CTCs rose to 84 per 7.5 mL of peripheral blood. CEA and AFP remained within normal ranges. After an initial response, three additional rounds of chemotherapy were performed. After one year (48 wk), a lung metastasis was found by CT scan, and the number of CTCs was 21 per 7.5 mL of peripheral blood. CEA and AFP were still normal. Concurrently, the patient attempted to use traditional Chinese medicine (TCM) in addition to the chemotherapy. One part of the medicine was *Astragalus*. Many studies have shown that *Astragalus membranaceus* polysaccharide can promote antitumor activity via improving the immune responses of the host organism<sup>[21]</sup>.

After 104 wk, a PET-CT examination revealed a relapse, and CTCs was increased to 41 per 7.5 mL,

concordant with the PET-CT results. After integrative anti-tumor therapy with TCM, the patient was stable. CTCs after 186 wk decreased to three from 14 at 117 wk, and the patient had no abnormalities by radiographic examination. The patient is currently in a stable condition. The CTC counts and the treatment and test results are summarized in Figure 3. The CTCs were increased postoperatively, and the CTC concentration was maintained at a high level after radiotherapy and chemotherapy. Bone and lung metastasis relapses occurred during this period. With the integrative therapy, the CTC number dropped, and the patient attained a stable status. During the follow-up, CTC enumeration could effectively complement imaging studies, especially for patients with normal serum markers. CTC also indirectly reflects the reactivity of the metastatic cancer cells to a chemotherapeutic drug<sup>[22]</sup>. Most importantly, the CTC test is noninvasive and allows successive detection of CTCs to monitor tumor metastasis or recurrence. As a real-time dynamic screening method, CTC technology will be greatly improved and widely applied in cancer diagnosis and treatment.

## COMMENTS

### Case characteristics

A 47-year-old male patient was referred for dysphagia in February 2009. An esophageal barium meal and computerized tomography (CT) scan determined

the presence of esophageal carcinoma.

### Clinical diagnosis

The patient was diagnosed with poorly differentiated squamous cell carcinoma by imaging and biopsy. The authors conducted a 5 year follow-up of patients, to evaluate the therapeutic effect and disease progression.

### Differential diagnosis

Serum tumor markers, imaging and circulating tumor cells (CTCs) detection were used to monitor disease tumor progression and treatment efficacy of the patient.

### Laboratory diagnosis

Serum tumor markers of the patient maintained among normal range. The number of circulating tumor cells in patients fluctuated with the disease and treatment response and coincident with the imaging diagnosis.

### Imaging diagnosis

For this cases, CT scan and esophageal barium meal showed squamous cell carcinoma in the in the lower esophagus. Twelve weeks after operation, the patient performed Bone imaging.

### Pathological diagnosis

The immunohistochemistry results revealed cytokeratin (CK) AE1/AE3 (+++), CK17 (++), p63 (++), p53 (++), epidermal growth factor receptor (+), CK7/20 (-), and carcinoembryonic antigen (-) in the tumor cells, with a Ki67 index of approximately 70%.

### Treatment

The patient received tumor resection and lymph node dissection. Further treatments including radiotherapy, chemotherapy and traditional Chinese medicine were performed.

### Related reports

Very few cases of circulating tumor cells in esophageal squamous cell carcinoma (ESCC) patients have been reported in the literature.

### Experiences and lessons

CTCs detections of the ESCC patient were reported in this case and analyzed the consistence with the disease progression. With more researches, CTCs are expected to provide support to monitor the disease status and treatment efficacy.

### Peer-review

This manuscript is overall interesting, but some revision is needed.

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## Refractory diarrhea: A paraneoplastic syndrome of neuroblastoma

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**Author contributions:** Han W analyzed the data and wrote the paper; Wang HM designed the research and performed the operations.

**Ethics approval:** The study was reviewed and approved by the Beijing Children's Hospital Institutional Review Board.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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### Abstract

Neuroblastoma (NB) is the most common extracranial solid tumor in children. Diarrheal NB is quite rare and is not easy to diagnose in the early stage. Six cases of diarrheal NB in our hospital treated from 1996 to 2006 were retrospectively analyzed, including characteristics such as electrolyte imbalance, pathologic features, vasoactive intestinal peptide (VIP) immunohistochemical staining results, treatment, and prognosis. All patients were boys with 3-8 loose or watery stools each day and routine fecal tests were normal. Abdominal tumors were identified by B-ultrasound. Drugs were ineffective. Three patients underwent surgery, and the remaining three patients received surgery and chemotherapy. Diarrhea stopped after treatment in five patients. Two patients died due to intractable hypokalemia. The tumor was located in the adrenal gland in four patients, in the upper retroperitoneum in one patient, and in the presacral area in one patient. Pathologic findings were NB and ganglioneuroblastoma. Five patients were at clinical stage I - II, and one was at stage III. Four patients survived (followed-up for 6 mo to 4 years). Immunohistochemical staining for VIP was positive. Refractory diarrhea is a paraneoplastic syndrome of NB and is rare. Patients aged 1-3 years who present with chronic intractable diarrhea should be followed closely. Intractable diarrhea, hypokalemia, and dysplasia are the initial clinical manifestations. Increased VIP is characteristic of this disease. Potassium supplementation plays a vital role in the treatment procedure, especially preoperatively. The prognosis of diarrheal NB is good following appropriate treatment.

**Key words:** Diarrhea; Hypokalemia; Neuroblastoma; Paraneoplastic syndrome; Vasoactive intestinal peptide

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**Core tip:** Neuroblastoma (NB) is the most common extracranial solid tumor in children. Diarrheal NB is quite rare and is not easy to diagnose in the early stage. Six cases of diarrheal NB were retrospectively analyzed, including characteristics such as electrolyte imbalance, pathologic features, vasoactive intestinal peptide immunohistochemical staining results, treatment, and prognosis.

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## INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor in children, and the primary tumor can be located in any part of the sympathetic chain or the adrenal medulla<sup>[1]</sup>. The tumor is most commonly found in the abdomen (75%), followed by the mediastinum (20%) and the neck (5%)<sup>[2]</sup>. Retroperitoneal NB is initially enigmatic, leading to progression and a poor prognosis. Patients with diarrhea as the main symptom, namely diarrheal NB, are quite rare<sup>[3]</sup>. Consequently, diarrheal NB is not easy to diagnose in the early stage of the disease. In this article, data from six patients with diarrheal NB treated in our hospital from 1996 to 2006 were retrospectively analyzed, including characteristics such as electrolyte imbalance, pathologic features, vasoactive intestinal peptide (VIP) immunohistochemical staining results, treatment, and prognosis.

## CASE REPORT

According to the diagnostic criteria for NB, we developed the following criteria for inclusion of patients in the study: an increase in urinary catecholamine metabolites, consistent with a primary or metastatic tumor determined by NB or pathologic features from a tissue biopsy, and NB cells were detected in bone marrow. Patient information was collected, including sex, age, clinical manifestations, laboratory tests, imaging studies, treatment, pathologic findings, and follow-up results. VIP was measured by immunohistochemical staining. From the year 1996 to 2006, a total of six cases of diarrheal NB were admitted to our hospital. All patients were boys. The minimum age at diagnosis was 1 year and the maximum age was 2 years 6 mo. Diarrhea occurred from the age of 9 mo to 2 years. The time from when diarrhea occurred to diagnosis ranged from 4 mo to 1 year. Loose or watery stools occurred 3-8 times each day, and routine fecal tests were normal. Drugs were ineffective. Diarrhea was the first symptom and was

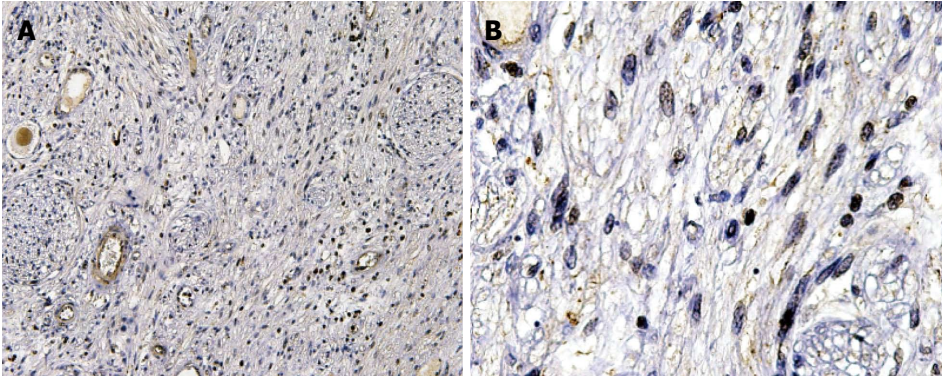
consistent in five cases, and abdominal tumors were identified by B-ultrasound.

Two patients underwent preoperative chemotherapy, in one case diarrhea stopped after 1 mo of chemotherapy, in the other patient, diarrhea was reduced and stopped after surgery. Three cases underwent surgery without preoperative chemotherapy, diarrhea stopped in two patients after surgery, and one patient died during surgery. One patient visited a doctor due to abdominal pain and a tumor was identified; diarrhea began after preoperative chemotherapy and the symptoms were obvious even after tumor resection. Water and electrolyte imbalance and malnutrition were difficult to correct and the patient died. The main characteristics were chronic dehydration, intractable hypokalemia, chronic malnutrition, and growth retardation. Potassium was as low as 1.3 mmol/L, and potassium supplementation by routine daily peripheral venous infusion was generally ineffective, and a high concentration of potassium infused intravenously was necessary. ECG monitoring was necessary in these patients to prevent cardiac arrest. Blood vessel leakage and skin necrosis occurred in one patient following the infusion of high concentrations of potassium via a peripheral vein. Although patients may adapt to chronic hypokalemia, correction of hypokalemia before surgery is necessary. One of the six cases, whose preoperative serum potassium was 2.8 mmol/L, was not cured, and cardiac arrest occurred during the operation; serum potassium at that time was 1.8 mmol/L. It is noteworthy that even if the preoperative serum potassium had been corrected (4.84 mmol/L), hypokalemia (1.8 mmol/L) would still have occurred during surgery, and serum potassium should be monitored frequently during surgery.

Of the six patients included in this study, the tumors were located in the adrenal gland in four cases, in the upper retroperitoneum in one case, and in the presacral area in one case. Pathologic findings were NB and ganglioneuroblastoma. Five patients were clinical stage I - II, and one case was stage III. Four patients survived (followed-up for 6 mo to 4 years). It is generally believed that diarrhea is related to VIP secretion by the tumor<sup>[4]</sup>. The immunohistochemical staining results are shown in Figure 1.

## DISCUSSION

NB is the most common extracranial solid tumor in children, and the primary tumor can be located in any part of the sympathetic chain or the adrenal medulla<sup>[5]</sup>. The tumor most commonly occurs in the abdomen (75%), followed by the mediastinum (20%) and the neck (5%). Retroperitoneal NB is initially occult, leading to progression and a poor prognosis<sup>[6]</sup>. Due to a lack of B-ultrasound newborn screening, early identification of NB is mainly dependent on initial symptoms<sup>[7]</sup>. Fever<sup>[8]</sup>, limb pain<sup>[9]</sup>, abdominal mass, abdominal pain<sup>[10]</sup>, and lower limb weakness



**Figure 1** Immunohistochemical staining showing vasoactive intestinal peptide positivity. A: The vasoactive intestinal peptide mainly expressed in cytoplasm [hematoxylin-eosin (HE)  $\times$  4]; B: The cytoplasm was dyed brown (HE  $\times$  100).

are the most common clinical manifestations in the majority of patients. Patients with diarrhea as the main symptom, suggestive of diarrheal NB, are quite rare. Consequently, it is not easy to diagnose in the early stage of the disease.

Intractable diarrhea is due to the tumor secreting large amounts of VIP, and these children usually present with secretory diarrhea, hypokalemia, and achlorhydria<sup>[11]</sup>. VIP acts on the intestinal epithelial cells via the blood circulation, causing excessive secretion of intestinal fluid and promoting pancreatic juice and bile secretion, and further exacerbating the loss of water and electrolytes. Secretory diarrhea is the most obvious symptom of the disease, and our patients suffered from serious watery diarrhea that lasted a long time, and was not relieved even after 72 h of fasting, which led to the diagnosis. In 1952, Hawfield and Daisly reported a case of adrenal neuroblastoma with chronic diarrhea where the diarrhea stopped after removal of the tumor<sup>[12]</sup>. Other investigators also reported ganglioneuroma and ganglion NB with watery diarrhea, hypokalemia, and achlorhydria syndrome in 1973<sup>[13]</sup>. They detected high levels of VIP in the patients' serum or tumor. Using peroxidase-anti-peroxidase staining assays, immunohistochemical methods, VIP serum level, and tumor tissue staining, they also found that the ganglion-like tumor cells were positive, but undifferentiated NB cells were negative.

VIP was first isolated in 1970 from pig small intestines<sup>[14]</sup>. It has been confirmed that the central nervous system and the sympathetic ganglion neurons are also rich in VIP. This peptide is composed of 28 amino acids and its main function is to dilate blood vessels, to inhibit histamine, which can stimulate gastric acid secretion resulting in achlorhydria, and stimulate the secretion of intestinal fluid resulting in excessive watery diarrhea. Normal amounts of VIP can be inactivated by the liver, and its physiologic function is insignificant. When ganglion cells proliferate, such as in tumors, and secrete excessive VIP, watery diarrhea syndrome occurs.

Some researchers have proposed the concept of "VIP secreting tumors", and these tumors include pheochromocytoma, mast cell tumors, non-B cell

hyperplasia, and medullary thyroid carcinoma. Refractory watery diarrhea in children is mainly due to high VIP levels in plasma. Such cases were first reported by Ghishan *et al.*<sup>[15]</sup> in 1979. VIP secreting tumor-induced diarrhea is characterized by watery diarrhea, hypokalemia, and alkalosis (WDHA syndrome). This syndrome is most common in children aged one year to three years, and should be included in the differential diagnosis of pediatric chronic diarrhea.

In summary, diarrheal NB is a rare disease that is difficult to identify in the early stage. Patients aged 1-3 years who present with chronic intractable diarrhea should be followed closely. Intractable diarrhea, hypokalemia, and dysplasia are the initial clinical manifestations. An increased level of VIP is characteristic. Attention should be paid to potassium supplementation, which plays a vital role in the treatment procedure, especially preoperatively. In order to prevent sudden cardiac death, monitoring of electrolytes and ECG changes during surgery is important. The prognosis of diarrheal NB is good following appropriate treatment.

## ACKNOWLEDGMENTS

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## COMMENTS

### Case characteristics

Intractable diarrhea, hypokalemia, and dysplasia are the initial clinical manifestations of diarrheal neuroblastoma.

### Clinical diagnosis

All patients were boys who had 3-8 loose or watery stools each day, abdominal tumors, and normal routine fecal tests.

### Differential diagnosis

The differential diagnosis of patients aged 1-3 years who present with chronic intractable diarrhea should include diarrheal neuroblastoma.

### Laboratory diagnosis

Routine fecal tests were normal.

### Imaging diagnosis

Abdominal tumors were identified by B-ultrasound in all cases.

### Pathological diagnosis

Pathologic findings were neuroblastoma or ganglioneuroblastoma with positive immunohistochemical staining for vasoactive intestinal peptide.



## Treatment

Tumor resection and potassium supplementation play a vital role in the treatment procedure.

## Related reports

Such cases were first reported by Ghishan *et al* in 1979.

## Experiences and lessons

Patients aged 1-3 years who present with chronic intractable diarrhea should be followed closely and potassium supplementation plays a vital role in the treatment procedure, especially preoperatively.

## Peer-review

This manuscript is very interesting. Diarrheal neuroblastoma is quite rare.

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