

# World Journal of *Gastroenterology*

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2014-2017

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## Micronutrient deficiencies in patients with chronic atrophic autoimmune gastritis: A review

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### Abstract

Chronic atrophic autoimmune gastritis (CAAG) is an organ-specific autoimmune disease characterized by an immune response, which is directed towards the parietal cells and intrinsic factor of the gastric body and fundus and leads to hypochlorhydria, hypergastrinemia and inadequate production of the intrinsic factor. As a result, the stomach's secretion of essential substances, such as hydrochloric acid and intrinsic factor, is reduced, leading to digestive impairments. The most common is vitamin B12 deficiency, which results in a megaloblastic anemia and iron malabsorption, leading to iron deficiency anemia. However, in the last years the deficiency of several other vitamins and micronutrients, such as vitamin C, vitamin D, folic acid and calcium, has been increasingly described in patients with CAAG. In addition the occurrence of multiple vitamin deficiencies may lead to severe hematological, neurological and skeletal manifestations in CAAG patients and highlights the importance of an integrated evaluation of these patients. Nevertheless, the nutritional deficiencies in CAAG are largely understudied. We have investigated the frequency and associated features of nutritional deficiencies in CAAG in order to focus on any deficit that may be clinically significant, but relatively easy to correct. This descriptive review updates and summarizes the literature on different nutrient deficiencies in CAAG in order to optimize the treatment and the follow-up of patients affected with CAAG.

**Key words:** Chronic atrophic autoimmune gastritis; Nutritional deficiency; Vitamin B12; Iron; Vitamin C; Vitamin D; Calcium; Malabsorption

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**Core tip:** Chronic atrophic autoimmune gastritis is an autoimmune disease characterized by progressive parietal cells destruction leading to hypochlorhydria and intrinsic factor deficiency. These alterations may result in vitamin B12 deficiency and iron malabsorption. A possible role of chronic atrophic autoimmune gastritis in the development of several nutritional deficiencies (*e.g.*, calcium, vitamin D, vitamin C) has been reported. However, the prevalence and clinical impact of these deficiencies has not been elucidated. The present paper aims at investigating the relevance, frequency and clinical presentation of nutritional deficiencies in chronic atrophic gastritis to enable clinicians to promptly identify and correct any possible nutritional impairment.

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## INTRODUCTION

Chronic atrophic autoimmune gastritis (CAAG) is an organ-specific autoimmune disease characterized by the presence of autoantibodies against gastric parietal cells and intrinsic factor<sup>[1,2]</sup>. CAAG occurs in approximately 2% of the general population, with a higher prevalence in elderly females<sup>[3]</sup>. Early alterations are characterized by the chronic inflammation in the submucosa that extends into the lamina propria of the mucosa, with a progressive destruction of both gastric and zymogene cells<sup>[4]</sup>. In the advanced stages of the disease, the gastric mucosa presents a dramatic reduction or absence of gastric glands. In particular, the parietal cells and zymogenic cells are absent from the gastric mucosa and are replaced by intestinal metaplasia<sup>[4,5]</sup>. These histological changes result in achlorhydria, hypergastrinemia and intrinsic factor deficiency<sup>[6]</sup>.

The reduction of intrinsic factor levels results in vitamin B12 malabsorption. Indeed, intrinsic factor has a key-role in binding vitamin B12 in the duodenum and transporting it to the terminal ileum for absorption<sup>[7,8]</sup>. More recently, iron deficiency and iron deficiency anemia have been reported in the setting of CAAG, particularly in younger patients<sup>[9]</sup>.

Moreover, the deficiency of several vitamins and micronutrients, such as vitamin C, vitamin D, folic acid and calcium, has been described in patients with CAAG<sup>[10,11]</sup> or long-standing achlorhydria due to proton pump inhibitors (PPIs) therapy or gastrectomy<sup>[12]</sup>. The pathogenic mechanism underlying these changes seems to be the increased destruction or decreased

absorption of nutrients in the gastric mucosa, possibly due to the elevated pH and bacterial overgrowth<sup>[11]</sup>.

This paper aims to critically review the current knowledge on CAAG and nutritional deficiency in order to focus on the existence of any deficit that may be clinically significant, and to optimize the treatment and the follow-up of patients affected with CAAG.

## MICRONUTRIENT DEFICIENCIES

### Vitamin B12

Pernicious anemia (PA) is the most common cause of megaloblastic anemia in Western countries, resulting from impaired cobalamin (*i.e.*, vitamin B12) absorption due to the lack of intrinsic factor production caused by the destruction of parietal cells. Cobalamin cannot be synthesized in the human body, but should be introduced with food, where it is bound to proteins. After hydrolysis by gastric pepsin and chlorhidric acid, cobalamin binds to the intrinsic factor, which is released by the gastric parietal cells and is essential for its absorption in the distal ileum<sup>[13,14]</sup>. As a cofactor for two enzymes, the adenosylcobalamin-dependent methylmalonyl-CoA mutase in mitochondria and the methylcobalamin-dependent methionine synthase in the cytoplasm, cobalamin is important in several biological processes, such as DNA synthesis and regulation, energy production and erythropoiesis<sup>[15]</sup>. The spectrum of diseases associated with vitamin B12 deficiency is very wide, varying from asymptomatic to life-threatening pancytopenia or myelopathy<sup>[16]</sup>. Vitamin B12 deficiency can cause macrocytic anemia, mild hyperhomocysteinemia that however increase the risk of atherothrombosis, neuropsychiatric manifestations, as paraesthesia, weakness, gait abnormalities, and cognitive or behavioral changes (Table 1).

Recent epidemiological studies support the evidence that CAAG and PA are found across all the continents<sup>[17,18]</sup> and are probably under-diagnosed, since most patients with microcytic or macrocytic anemia are treated with iron, folates, and cobalamin, without any investigation of other causes of anemia. A study, which analyzed blood B12 levels in 729 American people aged 60 years or older, observed that 1.9% of the survey population had unrecognized and untreated PA: the prevalence was 2.7% in women, 1.4% in men and similar in black and white women (4.3% vs 4.0%)<sup>[19]</sup>. In the literature the prevalence of vitamin B12 deficiency among elderly people can range between 5% and 40% depending on the cut-off value of vitamin B12 used: the most frequent serum cut-off to diagnose vitamin B12 deficiency is 150 pmol/L (corresponding to 203 pg/mL)<sup>[20-22]</sup>. Autoimmune gastritis (pernicious anemia) is the most common cause of severe vitamin B12 deficiency due to food-cobalamin malabsorption in the elderly, nevertheless use of medications, as proton pump inhibitors, histamine H<sub>2</sub> blockers, metformin or cholestyramine can interfere



**Table 1 Clinical and laboratory findings in vitamin B12 deficiency**

General symptoms	Weight loss observed in most patients
Gastrointestinal symptoms	Low-grade fever occurs in one third of newly diagnosed patients and promptly disappears with treatment Smooth tongue (50% of patients) with loss of papillae. Changes in taste and loss of appetite Patients may report either constipation or having several semi-solid bowel movements daily Anorexia, nausea, vomiting, heartburn, pyrosis, flatulence and a sense of fullness
Brain	Altered mental status. Cognitive defects ("megaloblastic madness"): depression, mania, irritability, paranoia, delusions, lability
Sensory organs	Optic atrophy, anosmia, loss of taste, glossitis
Bone marrow	Hypercellular bone marrow Increased erythroid precursors Open, immature nuclear chromatin Dyssynchrony between maturation of cytoplasm and nuclei Giant bands, metamyelocytes Karyorrhexis, dysplasia Abnormal results on flow cytometry and cytogenetic analysis
Spinal cord	Myelopathy Spongy degeneration Paresthesias Loss of proprioception: vibration, position, ataxic gait, limb weakness/spasticity (hyperreflexia) Positive Romberg sign Lhermitte's sign Segmental cutaneous sensory level
Autonomic nervous system	Postural hypotension Incontinence Impotence
Peripheral nervous system	Cutaneous sensory loss Hyporeflexia symmetric weakness Paresthesias
Genitourinary symptoms	Urinary retention and impaired micturition may occur because of spinal cord damage. This can predispose patients to urinary tract infections
Reproductive system	Infertility
Abnormalities in infants and children	Developmental delay or regression, permanent disability The patient does not smile Feeding difficulties Hypotonia, lethargy, coma Hyperirritability, convulsions, tremors, myoclonus Microcephaly Choreoathetoid movements, peripheral blood Macrocytic red cells, macro-ovalocytes Anisocytosis, fragmented forms Hypersegmented neutrophils Leukopenia, possible immature white cells Thrombocytopenia Pancytopenia Elevated lactate dehydrogenase level (extremes possible) Elevated indirect bilirubin and aspartate aminotransferase levels Decreased haptoglobin level Elevated levels of methylmalonic acid, homocysteine, or both

with or reduce vitamin B12 absorption. Although autoimmune gastritis is known to be a major cause of vitamin B12 deficiency, the exact prevalence of vitamin B12 deficiency in CAAG has not been fully elucidated, being reported in a percentage varying from 37% to 69% of the cases (Table 2)<sup>[23-27]</sup>, this probably being due to the high heterogeneity of the populations considered and the limited availability of prospective studies. Moreover, chronic *Helicobacter pylori* (*H. pylori*) infection is frequently associated with atrophic gastritis and a study reported that *H. pylori* was found in 56% of people with vitamin B12 deficiency<sup>[28]</sup>. Pernicious anemia accounts for 15% to 25% of vitamin B12 deficiency in elderly people<sup>[29]</sup>. In a study on 296 Chinese patients, PA was diagnosed in

61% of the patients having megaloblastic anemia with vitamin B12 or folate deficiency<sup>[30]</sup>.

The variability of vitamin B12 levels in CAAG seems to be influenced by a large number of genetic and environmental factors<sup>[31]</sup>. The genotypes HLA-DRB1\*03 and DRB1\*04, which are known to be associated with other autoimmune diseases (*e.g.*, diabetes mellitus type 1 and autoimmune thyroid disease), were significantly associated with PA<sup>[32]</sup>.

Recently, several studies about genome-wide association have shown that some single nucleotide polymorphisms (SNPs) are linked with the vitamin B12 serum/plasma levels. A meta-analysis of three genome-wide association scans on Caucasians has found genome-wide associations between plasma

**Table 2** Demographic and biochemical characteristics of chronic atrophic autoimmune gastritis patients with vitamin B12 deficiency

Ref.	Total No. of patients	Gender (M/F)	Age (yr), median	Gastrin (pg/mL), median	Prevalence Vit. B12 deficiency, n (%)	Vitamin B12 (pg/mL) median <sup>1</sup>	Prevalence of neurological complications
Marignani <i>et al</i> <sup>[23]</sup> , 1999	80	24/56	56	491	44 (55.0)	87.5	NA
Hershko <i>et al</i> <sup>[24]</sup> , 2006	160	53/107	50	846	111 (69.4)	82.0	17%
Annibale <i>et al</i> <sup>[25]</sup> , 2005	140	49/91	55	500	65 (46.5)	80.0	NA
Miceli <i>et al</i> <sup>[27]</sup> , 2012	99	72/27	59	726	37 (37.4)	NA	6%
Lahner <i>et al</i> <sup>[26]</sup> , 2015	83	42/41	59	NA	43 (51.8)	138.0	NA

<sup>1</sup>Median vitamin B12 levels in patients with macrocytic anemia at presentation. NA: Not assessed.

vitamin B12 and SNPs on the methylmalonyl-CoA mutase (MUT), the intrinsic factor-cobalamin receptor cubilin (CUBN), the transcobalamin I (TCN1) and the fucosyltransferase 2 (FUT2) genes<sup>[33]</sup>. A more recent study has showed that, among 14 SNPs associated with vitamin B12 levels, a genetic variant of transcobalamin II (TCN2), encoding for the transport protein transcobalamin 2, and a genetic variant of fucosyltransferase 6 (FUT6), encoding for the enzyme fucosyltransferase 6, were significantly more frequent in CAAG patients with PA compared to healthy controls<sup>[26]</sup>. This observation was in contrast with the association of this variant of FUT6 and higher vitamin B12 plasma levels detected in a genome-wide association study performed on the Chinese male population<sup>[34]</sup>. Furthermore, another study observed a link between *FUT* gene variants, especially *FUT2*, *FUT3* and *FUT6*, *H. pylori* status and intestinal-type gastric cancer risk<sup>[35,36]</sup>.

Even if in healthy older adults the recommended therapy is the oral replacement of crystalline vitamin B12, the patients with CAAG and secondary vitamin B12 malabsorption, will require its parenteral replacement with intramuscular cyanocobalamin at a dose of 1000 µg daily for one week, then weekly for 4 to 8 wk, and then monthly for life. In case of mild deficiency with mild atrophic gastritis high-dose oral cyanocobalamin at 500 to 1000 µg daily can be considered adequate<sup>[16]</sup>. Concomitant iron and folate replacement is needed to achieve a full hemoglobin response<sup>[16]</sup>.

Moreover, PA is frequently associated (up to 27% of cases) with iron deficiency anemia (IDA)<sup>[37]</sup>. In a study by Hershko *et al*<sup>[24]</sup> low serum vitamin B12 levels were found in 100% macrocytic, 92% normocytic and 46% microcytic patients with CAAG, whereas iron deficiency was found in all the patients with microcytic anemia, but also in 50% of the normocytic and 10% of the macrocytic patients. Thus, a considerable proportion of patients had combined iron and cobalamin deficiencies. The mean age was 41 ± 15 years in those CAAG patients presenting with IDA and 59 ± 16 years in those patients presenting with PA. Whilst autoimmune atrophic gastritis impairs both food iron and cobalamin absorption, age, sex, the co-presence of *H. pylori* infection, duration and severity

of disease may determine the clinical presentation of CAAG as microcytic IDA or macrocytic megaloblastic anemia. In fact, in female patients presenting with IDA menstrual blood loss may have been an important role in the development of iron deficiency, aggravated by the inability to compensate it by improving food iron absorption. Co-existing *H. pylori* gastritis, which is more frequent in young patients, may contribute to the development of IDA. Conversely, the depletion of cobalamin stores may take many years longer and manifest in elderly patients, implying a severe impairment of the intrinsic factor secretion.

Finally, vitamin B12 is important to bone development, in particular for the osteoblastic function, as demonstrated by *in-vitro* studies<sup>[20,38]</sup> and population studies showing lower bone mineral density and greater fracture risk in patients with vitamin B12 deficiency<sup>[39,40]</sup>. In a two-year-long randomized controlled trial a 80% reduction in the hip fracture risk was observed among stroke patients after vitamin B12 repletion<sup>[41]</sup>, probably due to hypergastrinemia, which has been shown to stimulate parathyroid activity in animal models and in humans, with the consequent hyperparathyroidism and increased bone turnover<sup>[42-44]</sup>. However, a study by Merriman *et al*<sup>[45]</sup> did not confirm these data, as no reduction of risk of hip fracture showed among patients with PA after B12 repletion, thus suggesting that the presence of mechanisms other than B12 deficiency mediated the fracture risk.

Therefore, PA does not only influence cobalamin plasma levels, but also iron absorption and bone development: therefore patients with CAAG should undergo the evaluation of martial pool, B12 stores and 25-OH vitamin D levels.

### Iron

Alimentary iron is available in two forms: heme and non-heme iron. Heme iron, present in meat hemoglobin and myoglobin, is in a ferrous form which is soluble at alkaline pH and is easily absorbed in the duodenum without any need for chelation. However, non-heme ferric form represents about 80% of dietary iron and is less absorbable. Ferric iron is insoluble, precipitates at pH > 3, and is not absorbed unless reduced to the ferrous or chelated form. Different observations have demonstrated the importance of

gastric secretion of both hydrochloric and ascorbic acid in the solubilization and reduction of non-heme food iron for a normal iron absorption<sup>[24,46,47]</sup>.

Moreover, a possible role of achlorhydria in the development of iron malabsorption has been suggested in different hypo/achlorhydria models<sup>[48]</sup>.

A possible association between CAAG and iron malabsorption was initially proposed in 1930<sup>[7,49]</sup>, and it was most commonly assumed that iron deficiency anemia was caused by achlorhydria-induced malabsorption of dietary iron. In 1963 Cook and colleagues observed a significant impairment of iron absorption in patients with PA, which was corrected by adding gastric juice<sup>[50]</sup>. On the other hand, later studies did not confirm those observations and reported that the achlorhydria-induced malabsorption of dietary iron was unlikely to be the primary etiology of iron deficiency<sup>[51,52]</sup>. In recent years, however, the co-existence of CAAG and iron deficiency anemia (IDA) has been increasingly reported. In 1966 Dagg<sup>[53]</sup> introduced the concept of IDA progressing to classic PA in CAAG patients, demonstrating a 19% prevalence of achlorhydria and anti-parietal cell antibodies (APCA) positivity in patients with IDA and predicting that 32% of such patients would develop PA. More recently<sup>[23]</sup>, based on the observation of high prevalence of *H. pylori* positivity in young CAAG patients, it has been proposed that *H. pylori* infection may represent an early stage of gastritis. In later stages the infectious process is gradually replaced by an autoimmune disease with the irreversible destruction of the gastric body mucosa. Indeed, with the advancing age of presentation there is a progressive increase in the mean corpuscular volume of erythrocytes and in the severity of hypergastrinemia and cobalamin deficiency in CAAG patients<sup>[23]</sup>. Histological active chronic inflammation has been reported to be 4 times more common in patients with IDA than in classic PA patients presenting with macrocytosis, probably due to their higher rate of active *H. pylori* infection<sup>[23]</sup>.

In 2002 Dickey<sup>[46]</sup> observed a significant proportion of IDA patients having a concomitant CAAG (8/41). None of such patients had concomitant vitamin B12 deficiency, suggesting that CAAG may present with IDA from the onset of the condition. In addition, 20%-40% of PA patients developed IDA after treatment with parenteral vitamin B12, those observations suggesting the presence of subclinical iron deficiency in patients with clinically manifest PA<sup>[46]</sup>.

Moreover, a prospective study by Hershko *et al.*<sup>[24]</sup> showed a high (27%) proportion of CAAG among patients with iron deficiency anemia (IDA) without any apparent gastrointestinal diseases. Differently from classic PA, most of these patients were women, 20 years younger and had coexistent *H. pylori* infection. The authors then postulated that iron deficiency would develop earlier than vitamin B12 deficiency in young women with menstrual blood loss<sup>[24]</sup>. Annibale

*et al.*<sup>[54-56]</sup> found atrophic body gastritis in the 27% of patients with refractory IDA without gastrointestinal symptoms, in accordance with previous studies<sup>[23]</sup>. The same authors in 1999 compared CAAG patients presenting with IDA with others presenting with PA, and found that in 45% of CAAG patients IDA was the first clinical manifestation of the disease<sup>[23]</sup>. Finally, some recent studies have reported the occurrence of IDA in pediatric patients affected by CAAG, highlighting that CAAG should be considered when investigating refractory iron deficiency anemia in children<sup>[9]</sup>.

In conclusion, iron malabsorption and the onset of iron deficiency anemia appear to be biologically plausible and supported by different models of achlorhydria. Therefore, the patients with PA should be evaluated for concomitant iron deficiency development<sup>[57]</sup>. On the other hand, CAAG should be taken in consideration when evaluating patients with unexplained IDA, after the proper exclusion of any bleeding lesions.

Since patients with iron deficient anemia and autoimmune atrophic gastritis may be refractory to oral iron treatment, *H. pylori* eradication in combination with continued oral iron therapy have been suggested<sup>[23,24,54-56]</sup>.

### Vitamin C

Ascorbic acid, which is important in the production of key proteins such as collagen, norepinephrine and serotonin, is not synthesized *ex-novo* by the human body, but can only be introduced through the diet and then absorbed in the stomach and along the entire length of the small intestine<sup>[58]</sup>.

Ludden *et al.*<sup>[11]</sup> noted that CAAG patients had a diminished absorption of ascorbic acid and suggested that was due to the destruction of ascorbic acid in the gastric mucosa because of elevated pH and bacterial overgrowth, as suggested by more recent studies showing destruction of ascorbic acid caused by hypochlorhydria induced by potent acid suppression<sup>[12,59]</sup>.

Alt *et al.*<sup>[60]</sup> evaluated the effect of pH on ascorbic acid stability *in vitro* and demonstrated the destruction of 65% of the ascorbic acid at pH 7.95 vs only 14% at pH 1.45.

Ascorbic acid is an important antioxidant that inhibits the generation of N-nitroso compounds (NOC) and scavenges nitrites in the gastric juice by converting them to nitric oxide<sup>[61,62]</sup>. The ability of ascorbic acid to scavenge nitrite depends on the ratio of vitamin C/nitrite and gastric pH, thus increased NOC levels are generated in case of a decreased ratio of vitamin C/nitrite and pH > 2-4<sup>[63]</sup>. Finally, since some population-based epidemiologic studies have showed negative correlations between the vitamin C intake and gastric cancer, ascorbic acid is thought to decrease the oxidative damage to the gastric mucosa by scavenging free radicals and NOCs and attenuating the *H. pylori*-related inflammation<sup>[10]</sup>. This antioxidant role may also reduce the inflammation present in the gastric

mucosa of CAAG patients, although further studies are necessary to confirm this hypothesis.

### Calcium

Calcium is absorbed as calcium ion ( $\text{Ca}^{2+}$ ) in the proximal small intestine in both active and passive way. The absorption process begins in the stomach with the dissolution of calcium salts (e.g., calcium carbonate) to calcium chloride ( $\text{CaCl}_2$ ) which easily dissociates to  $\text{Ca}^{2+}$  which are highly water-soluble<sup>[64]</sup>. The bio-availability of dietary calcium salts depends on several factors, including the gastric acid secretion, physiological function of the stomach and intestine, levels of vitamin D in the tissues and circulation and the chemical structure and quantity of the calcium compounds ingested<sup>[65-67]</sup>.

Gastric acid plays an important role as it increases the dissolution and ionization of poorly soluble calcium. It has been reported that conditions causing a decrease in gastric acid secretion, such as gastric surgery, use of PPIs and CAAG, lead to a reduction in the dissolution of calcium salts, which may not be properly absorbed<sup>[66]</sup>.

There have been a few reports since the 1960s emphasizing the risk of malabsorption of calcium in patients with achlorhydria and atrophic stomach mucosa<sup>[66,68]</sup>. In 1985 Recker<sup>[66]</sup> compared the absorption of calcium carbonate and calcium citrate, measured by a modified double-isotope procedure, in normal subjects and patients with achlorhydria. The study showed that the absorption of calcium carbonate in patients with achlorhydria was significantly lower than in the normal subjects, supporting the role of gastric acid in calcium homeostasis. Calcium malabsorption has been demonstrated in animal models with gastric acid suppression<sup>[69,70]</sup>, as well as in humans with achlorhydria under fasting conditions. However, the degree of calcium malabsorption in patients with CAAG remains controversial, as Eastell *et al.*<sup>[71]</sup> have found normal calcium absorption with meals in patients with pernicious anemia and achlorhydria.

Interestingly, a few studies have reported the possible association between pernicious anemia and/or CAAG, and osteopenia and osteoporosis<sup>[71,72]</sup>. The study by Eastell *et al.*<sup>[71]</sup> has reported a significant lower bone mineral density in post-menopausal women affected by pernicious anemia as compared to healthy controls. In the same study the authors showed that the decrease in bone mineral density was linearly related to a decline in serum levels of pepsinogen I, which is a serum biomarker of the structure and function of the gastric oxyntic mucosa. Its levels tend to decrease with the increase in the grade of atrophy of the oxyntic mucosa<sup>[71]</sup>. Another study, instead, has recently observed the reduction in bone mineral density and an increased frequency of osteopenia and osteoporosis in male, but not female patients with CAAG<sup>[73]</sup>. Moreover, the incidence of

fractures has been reported increased in patients with pernicious anemia/CAAG<sup>[45,72]</sup>. On the other hand, Kakehasi *et al.*<sup>[74]</sup> in a cross-sectional study did not find any significant difference in bone mineral densities between patients with autoimmune gastritis and *H. pylori* gastritis and the controls. However, it should be noted that the lack of significance in the reduction of bone mineral density might be due to the low number of patients with CAAG included in the study. In addition, a paper from the Finnish group has suggested that the administration of supplementary calcium in patients with osteoporosis may require the evaluation of gastric acid secretion, atrophic gastritis and the use of PPIs<sup>[64]</sup>.

Further evidence on a possible role of gastric acidity in calcium absorption results from gastric surgery and the use of anti-acid drugs: in these setting an increased risk for low bone mass or fractures has been reported.<sup>75</sup> Gastrectomy increases the risk of osteoporosis and fractures due to weight loss and changes of body composition as well as calcium malabsorption<sup>[75,76]</sup>. More recently, Krause *et al.*<sup>[77]</sup> demonstrated a statistically significant increase of osteomalacia, marrow fibrosis, and impaired calcium distribution within the mineralized matrix in gastrectomized patients as compared to controls. Long-term acid-suppressive therapy can also raise the risk of fractures<sup>[78-82]</sup>. A meta-analysis revealed that proton pump inhibitors can increase the risk of hip, spine, and any site fractures by 30%, 56% and 16%, respectively<sup>[83]</sup>.

In conclusion, even if biologically plausible, the evidence regarding the role of CAAG and/or hypochlorhydria in calcium malabsorption remains controversial; however, the increasing evidence on a relationship between CAAG/hypochlorhydria and the risk of osteoporosis suggests that in this setting an important impairment of bone mineralization exists and can be secondary to long-term calcium malabsorption.

### Vitamin D

To date a few studies have investigated the association between CAAG and vitamin D deficiency<sup>[84,85]</sup>.

In 1992 Eastell *et al.*<sup>[71]</sup> did not observe the presence of vitamin D deficiency in a group of 21 patients with CAAG as compared to healthy subjects. However, the very low number of cases considered have possibly limited the results of this study<sup>[71]</sup>. In 2012 a study<sup>[85]</sup> observed, for the first time, significantly lower 25-OH vitamin D levels in patients with CAAG as compared to non-specific gastritis or the general population. In this study, the 25-OH vitamin D mean concentration in subjects with CAAG was  $9.8 \pm 5.6$  ng/mL (CI: 8.4-11.2) vs  $21.3 \pm 12.2$  ng/mL (CI: 19.7-22.9) in healthy control subjects. Based on these observations, the authors hypothesized that hypovitaminosis D might be a risk factor for the development of



**Table 3** Summary of the main types of deficit described in chronic atrophic autoimmune gastritis patients

Deficit	Mechanism of action	Effects	Reported prevalence
Vitamin B12	Lack of intrinsic factor reduced vitamin B12 absorption in terminal ileum	Pernicious anemia Neurological alteration Osteopenia/osteoporosis	37%-69% <sup>[24,27]</sup>
Iron deficiency	Gastric acid increases the dissolution and ionization of poorly soluble calcium salt	Microcytic anemia	41% <sup>[24]</sup>
Vitamin C	Destruction of ascorbic acid in the gastric mucosa for elevated pH and bacterial overgrowth	Reduced and oxidative effects	Not known
Calcium	Gastric acid increases the dissolution and ionization of poorly soluble calcium salt	Osteopenia/osteoporosis	Not known
Vitamin D	Not clarified	Secondary hyperparathyroidism Osteopenia/osteoporosis Increased incidence of autoimmune diseases	12.1% <sup>[84]</sup>

autoimmune diseases<sup>[85]</sup>. More recently, a paper from our group<sup>[84]</sup> has reported the increased prevalence of hyperparathyroidism secondary to vitamin D deficiency in patients affected by CAAG. This finding can suggest that the regulation of calcium and/or vitamin D metabolism may be impaired in patients with CAAG, potentially because of the malabsorption of vitamin D in the intestine.

Moreover, as stated in the earlier Vitamin B12 and Calcium sections, different studies have reported on the risk of calcium malabsorption<sup>[64,66,68]</sup> and an increased risk of osteoporosis and pathological fractures in CAAG patients<sup>[45,72,86]</sup>. Remarkably, the active transcellular absorption of  $\text{Ca}^{2+}$  in the duodenum and proximal small intestine depends on vitamin D and represents the most important physiological pathway for the absorption of the calcium. Thus, it seems possible that vitamin D deficiency in CAAG patients can also explain calcium malabsorption and alterations in bone mineralization.

Further prospective studies are needed to fully elucidate the association between CAAG and vitamin D deficiency.

## CONCLUSION

Although pernicious anemia is the most frequent deficiency observed in patients with CAAG, the deficiency of other vitamins and micronutrients, such as vitamin C, vitamin D and calcium, has been described in the current literature (Table 3)<sup>[11,60]</sup>. The pathogenic mechanisms seem to be the increased destruction or decreased absorption of nutrients in the gastric mucosa, because of elevated pH or bacterial overgrowth<sup>[11]</sup>.

Vitamin B12 deficiency, causing hematological and neurological consequences remains the most extensively investigated nutritional impairment in CAAG. However, even in this setting further studies appear to be necessary to clarify the exact prevalence of vitamin B12 deficiency in CAAG patients. Moreover, a large number of environmental and genetic factors (such as some single nucleotide polymorphisms on

particular genes) may influence the levels of vitamins in patients with CAAG<sup>[31,26]</sup>, and their impact remains to be fully elucidated. With regard to iron absorption, the current literature reports IDA occurrence in up to 27% of patients with PA, this suggesting that CAAG should be taken into consideration in patients with unexplained IDA, after the proper exclusion of potential bleeding lesions. On the other hand, it appears relevant to evaluate the iron status in CAAG patients at diagnosis and during follow-up<sup>[57]</sup>.

Finally, as some reports suggest, CAAG may lead to an increased risk of osteoporosis and pathological fractures secondary to the deficiency of vitamin B12 and/or calcium and vitamin D: the prompt recognition of such deficiencies is crucial to reduce the risk of bone fractures.

Overall, the increasing amount of evidence on the possible occurrence of multiple vitamin deficiencies in CAAG, leading to hematological, neurological and skeletal manifestations, highlights the importance of an integrated evaluation of these patients.

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## Intraepithelial lymphocytes, scores, mimickers and challenges in diagnosing gluten-sensitive enteropathy (celiac disease)

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### Abstract

The upper digestive tract is routinely scoped for several causes of malabsorption, and the number of duodenal biopsy specimens has increased notably in the last 10 years. Gluten-sensitive enteropathy (GSE) is an autoimmune disease, which shows an increasing prevalence worldwide and requires a joint clinico-pathological approach. The classical histopathology of GSE with partial or total villous blunting is well recognized, but the classification of GSE is not straightforward. Moreover, several mimickers of GSE with intraepithelial lymphocytosis have been identified in the last 20 years, with drug interactions and medical comorbidities adding to the conundrum. In this review, we report on the normal duodenal mucosa, the clinical presentation and laboratory diagnosis of GSE, the duodenal intraepithelial lymphocytes and immunophenotype of GSE-associated lymphocytes, the GSE mimickers, the differences "across oceans" among guidelines in diagnosing GSE, and the use of a synoptic report for reporting duodenal biopsies in both children and adults in the 21<sup>st</sup> century.

**Key words:** Gluten; Duodenum; Lymphocytes

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**Core tip:** Striking and unique microphotographs with comparison of classification of gluten-sensitive enteropathy across oceans and tables useful for the practice of gastroenterology.



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## INTRODUCTION

In the last decade, there have been a plethora of publications in refining several gastrointestinal diseases. It has emerged as a period of an unceasing interest, particularly for diseases of the upper gastrointestinal tract<sup>[1-14]</sup>. In the hands of physicians reading histopathology reports, the number of duodenal biopsies with normal or near normal villous architecture and increased intraepithelial lymphocytes (IELs) appears to be collectively growing. Increased IELs or intraepithelial lymphocytosis in an otherwise apparently normal villous architecture can be a puzzle for both pathologists and treating physicians and may raise several differential diagnoses<sup>[15]</sup>. Moreover, the histopathology report may not be useful as it is, if it is not complemented with clinical and laboratory information. The report may be unsatisfactory due to lack of knowledge, incomplete performance of special stains or inadequate application of technical or professional skills. One or more of these issues may contribute to miscommunication between pathologists and clinical colleagues.

At first glance, gluten-sensitive enteropathy (GSE) or celiac disease seems to be straightforward, but it is not, neither from the clinical nor the pathologic point of view. There are mysteries behind this disease. Both the pathology and the pathogenesis are not yet fully unveiled. A few years ago, the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) issued some guidelines on GSE, defining it as an "immune-mediated systemic disorder, elicited by gluten and related prolamins in genetically susceptible individuals"<sup>[16]</sup>. GSE was first described around 200 AD and is known in some countries as "sprue", recalling the 18<sup>th</sup> century-old Samuel Gee's work "*On the Coeliac Affection*"<sup>[17]</sup>.

To date, genetic studies have identified 43 predisposing loci that collectively explain some 50% of the genetic variance in GSE, but more than 90% of GSE-associated single nucleotide polymorphisms (SNPs) localize to the non-coding genome<sup>[18-20]</sup>. There is indeed a large epigenomic component that may play a contributive role, and a better understanding of the genomic-epigenomic relationship may be needed to translate genetic knowledge into future clinical practice<sup>[21,22]</sup>. In the meantime, pathology remains key in the diagnostic procedures and this review is composed of six parts, focusing on (1) the composition

of normal duodenal mucosa; (2) the clinical presentation and laboratory diagnosis of GSE; (3) the immunophenotype of GSE-associated lymphocytes; (4) the GSE mimickers; (5) the differences "across oceans" among guidelines for diagnosing GSE; and (6) the use of a synoptic report for reporting duodenal biopsies in both children and adults.

## NORMAL DUODENAL MUCOSA

The villous character of the small bowel is intrinsically linked to the aim of an organism to increase its absorptive surface area. In early embryogenesis, development of the duodenal epithelium takes place from simple endodermal tubules between the 9<sup>th</sup> and 10<sup>th</sup> wk of gestation, when the epithelium converts to simple columnar epithelium. The epithelium ends its differentiation just 4-5 d before birth<sup>[23]</sup>. The usual configuration of the duodenal mucosa contains slender structures protruding from the surface, with 3-5 times the height of the crypts. The patchiness of the lymphoid nodules or mucosa-associated lymphatic tissue (MALT) needs to be considered in assessing the duodenal histology and can constitute one of the first pitfalls in interpreting a small intestinal biopsy.

According to our more than 20 years' experience of reading duodenal biopsies of healthy individuals across ages, we can state that only very few lymphocytes can be usually seen among the epithelial cells. However, the IELs may vary during life and possibly in a circadian cycle. The IELs usually do not go over 5-10 per 100 epithelial cells in healthy individuals. The cut-off between pathological and normal has been decreased in the last three decades from 40 to either 20 or 25 lymphocytes per 100 epithelial cells<sup>[24]</sup>. Between 5-10 and the pathological threshold (20 or 25), there is a gap that has probably been inadequately investigated. The unveiled and/or underlying causes of the "near normal" cases (5-20 IELs/100 epithelial cells) may be intriguing. The presence of scattered normal lymphocytes in the surface epithelium of the duodenum is not well understood, although the prominent role of the duodenum in assessing the epitopes present in the food should be considered.

MALT of the gut is, indeed, crucial for the immunology and preservation of the microbiome<sup>[1,18,25]</sup>. Lymphocytes are recognized in the duodenal surface epithelium, because of some characteristics that allow them to be differentiated from the epithelial cells. Lymphocytes are characterized by their roundness, cell hyperchromasia, high nucleus-to-cytoplasm ratio, and quite constant intercellular distribution. However, the counting may be jeopardized by a number of factors, including the intrinsic and extrinsic conditions of biopsy grasping by the endoscopist, the laboratory processing of the tissue biopsy, and the individual evaluation of the pathologist<sup>[26-28]</sup>.

Processing of a duodenal biopsy may represent a challenge for some laboratories. In fact, tangentially cut villi of appropriate duodenal regions may look like blunted and, thus, these areas should be avoided when an assessment of the villous architecture is crucial. Although criteria of adequacy are variable among authors of excellent reviews<sup>[29,30]</sup>, in our opinion, it is desirable that biopsies containing at least 5 consecutive, intact villi that are well-oriented in the plane of section are sent for assessment to the pathologist. The conditions of "consecutiveness" and "intactness" are extremely important for the standardization of studies involving duodenal mucosal tissue. The correct orientation of the biopsy before paraffin embedding may be crucial and 3, but preferably 5 consecutive, intact and well-oriented villi are the minimum to adequately evaluate the villous architecture. In our opinion, if at least 5 villi are not consecutive, the diagnosis may be uncertain.

IELs are usually localized at the base of the surface epithelium in biopsies from healthy individuals. If the IELs are slightly increased in number, they tend to arrange themselves generally throughout the full thickness of the epithelium. Some ancillary studies have been proposed to give an accurate value of the lymphocytosis. Immunohistochemical typing of the cells has been suggested as an ancillary technique, but the normal upper limit has been suggested to be set higher than normal, at 29 IELs instead of 25 (or 24 instead of 20) per 100 epithelial cells<sup>[31]</sup>. The rationale for it is not fully clear, but values between 26 and 29 CD3-positive IELs may be empirically stated as borderline IEL. The term IEL should be reserved when IELs are equal to 30 or superior to this value. Duodenal portions may be different in the number of IELs present and this may also vary according to the ingested food.

Duodenal bulb biopsies may show more IELs than distal portions, and the other portions of the small bowel are also quite different from duodenum. In fact, villi of the distal bowel tend to be slightly taller, apart from areas overlying lymphoid aggregates, where they acquire broad based or flat shape. Underneath the surface epithelium, the lamina propria of the small bowel contains typically an infiltrate of scattered or mildly dense lymphocytes, plasma cells and eosinophils, which constitute the usual complement of the small bowel wall. The number of these three cell types in the lamina propria varies, but usually they are low in number. All three types can be easily recognized in normal biopsies and highlighted by immunohistochemistry or histochemistry using antibodies against CD3 (lymphocytes), CD138 (plasma cells) and Luna special stain (eosinophils), respectively. The presence of more than occasional plasma cells and more than 30 eosinophils per high power field (ocular  $\times 10$  and objective  $\times 40$ ) should be considered anomalous<sup>[28]</sup>.

## CLINICAL PRESENTATION AND LABORATORY DIAGNOSIS OF GSE

GSE is a dysregulation of the genome-epigenome, with abnormal reaction of the body to gluten-containing food<sup>[2,32-40]</sup>. In Figure 1, the plant taxonomy and celiac toxicity are depicted. Gluten is a protein that gives dough its elasticity, allowing it to rise without collapsing while trapping the CO<sub>2</sub>. It is important to remember that gluten is within wheat, rye and barley, but also in wheat derivatives: bulgar, couscous, mataza, seitan, semolina, triticale, spelt, kamut, einkorn, emmer and anything with "wheat" in the name (except buckwheat) as well.

The presentation of GSE in childhood is quite protean, including abdominal distension, diarrhoea, anorexia, weight loss, dermatitis herpetiformis and irritability. Conversely, the presentation of GSE in adulthood includes usually abdominal distension, steatorrhea, oedema and lethargy. Infrequent ways of presentation have been described in the literature<sup>[18,19]</sup>. In case of a suspicion of GSE, both children and adults are first screened using serologic studies for autoantibodies including IgA anti-tissue transglutaminase (aTTG)<sup>[35,41]</sup>. IgA anti-endomysial autoantibodies (EMA) have been previously used. IgA aTTG antibodies is the preferred test worldwide, showing a sensitivity of 94% and specificity of 97%<sup>[42,43]</sup>. Both tests are useful in IgA competent patients. The institution of a gluten-free diet (GFD) starts the decline of the titres of aTTG.

False-negative aTTG results may be seen in patients harbouring IgA deficiency, which is detected in 1/10 of the GSE patients and testing of IgG isotype of aTTG is mandatory. To date, deamidated gliadin peptide (DGP) seems to have better sensitivity in detecting early-stage GSE as compared to the TTG and EMA, and has been proposed to be the first line of investigation in IgA deficient patients (see below for differences among gastroenterological societies)<sup>[25,41,44,45]</sup>. False-positive results may occur in the setting of patients suffering from inflammatory bowel disease (IBD), primary biliary cirrhosis, cardiovascular disease, autoimmune enteropathy and other immune-mediated disorders<sup>[25,29,41,44,46]</sup>. Clinical correlation is paramount.

In the absence of supportive histologic or laboratory findings, a strong clinical suspicion should be followed by evaluation of high-susceptibility alleles of the human leukocyte antigens (HLA), HLA-DQ2 or DQ8, or repeating testing before starting gluten withdrawal<sup>[47]</sup>. If endoscopy is performed, the gross findings remain quite nonspecific and very subjective according to the experience of the endoscopist<sup>[48,49]</sup>. It is essential to not rely exclusively on the endoscopy, because up to 43% of paediatric patients harbouring GSE may show normal-appearing mucosa<sup>[50]</sup>. Thus, endoscopy should be almost exclusively associated with biopsy.

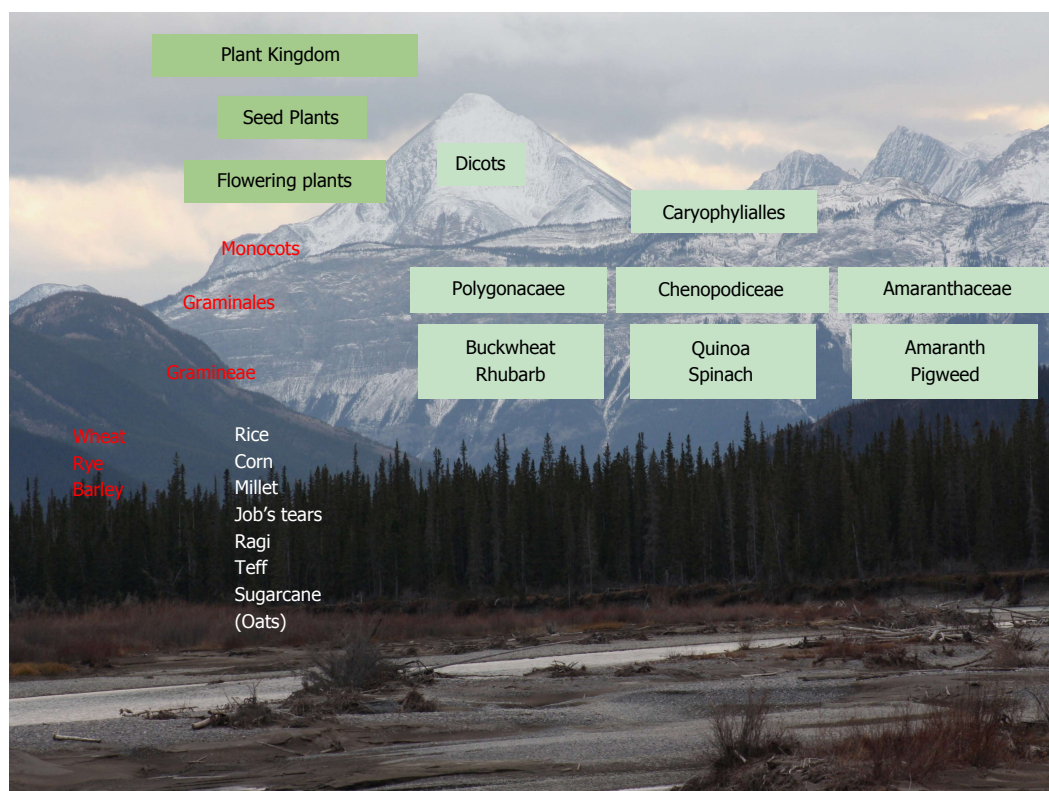


Figure 1 Plant taxonomy and celiac toxicity (red).

The histologic examination of duodenal biopsies should be performed by a pathologist with specific skills in reading gastrointestinal biopsies. The routine collection of oesophageal and gastric biopsy specimens during upper endoscopy should be considered mandatory, particularly to clarify if gastritis or eosinophilic esophagitis are present.

In 2017, the gold standard for the diagnosis of GSE remains the tissue biopsy obtained at endoscopy. The histology of GSE relies on villous atrophy, IEL with or without enterocyte damage, increased inflammatory cells in the lamina propria and crypt hyperplasia<sup>[50,51]</sup>. If we consider the normal upper limit of 20 IELs or 25 IELs per 100 epithelial cells in sections stained with haematoxylin and eosin (HE), the normal ratio of IELs to epithelial cells is 1:5 or 1:4, respectively<sup>[2,31,36,52,53]</sup>. The presence of neutrophils is a common finding in the histopathology of GSE<sup>[54]</sup>. Neutrophilic infiltration of the lamina propria may occur in up to 1/3 of patients with GSE. Conversely, neutrophilic infiltration of the surface epithelium is seen more rarely.

Neutrophilic crypt abscesses are usually not seen in GSE, but are frequently seen in GSE mimickers, such as patients with infection, peptic duodenitis or autoimmune enteropathy (see below). The sensitivity of crypt hyperplasia may harbour a higher inter-individual variability and may not be obvious in some patients. Enterocyte damage, as defined by increased nuclear size and decreased cytoplasmic volume, and increased inflammation of the lamina propria, including

increased numbers of lymphocytes and plasma cells in the lamina propria, are neither specific nor sensitive for GSE. These features are usually present in biopsies of different aetiology with marked abnormality of the mucosal architecture<sup>[54]</sup>.

Eosinophils are not uncommon in the gastrointestinal tract<sup>[28,55]</sup>. Eosinophilic infiltration of the lamina propria may constitute a separate subgroup of GSE and it has been suggested to report clearly their presence only if sheets of eosinophils are seen. There is an association with eosinophils in the small bowel and oesophageal mucosa that has been increasingly recognized<sup>[28,56-58]</sup> and may require individualized assessment and treatment<sup>[30,59,60]</sup>.

The presence of all histologic components makes the diagnosis of GSE certain indeed, but GSE may show only some of these features and some classifications have been proposed. The Oberhuber *et al.*<sup>[61]</sup> modification of the original classification proposed by Marsh and Crowe remains a cornerstone for both pathologists and clinicians. Marsh classification identifies type I as an infiltrative lesion, characterized by IEL and a normal villous architecture of the duodenal mucosa, type II as an hyperplastic lesion, characterized by IEL and crypt hyperplasia and a normal villous architecture, type III as a destructive lesion, characterized by IEL, crypt hyperplasia and villous atrophy, and type IV as a hypoplastic lesion, characterized by a normal IEL count, normal crypt length and villous atrophy<sup>[62,63]</sup>.



**Table 1 Revised Updated Marsh-Oberhuber classification of gluten-sensitive enteropathy (5 states of submucosal injury 0-4)**

Type 0
Pre-infiltrative: normal V:C ratio and crypts with < 20-25 IELs per 100 enterocytes (1:5 or 1:4)
Type 1
Infiltrative type: normal V:C ratio and crypts, but ↑ IELs (≥ 20-25 IELs/100 enterocytes)
Type 2
Infiltrative-hyperplastic type: normal V:C ratio, but crypt hyperplasia with ↑ IELs
Type 3
Destructive (flat mucosa) type of GSE lesion according to the degree of villous atrophy
Type 3a: mild villous atrophy with V: C < 3:1, and ↑ IELs
Type 3b: marked villous atrophy with V: C < 1:1, and ↑ IELs
Type 3c: total villous atrophy with completely flat mucosa and ↑ IELs
Type 4
Atrophic type (hypoplastic); flat mucosa with only a few crypts and near-normal IEL count

V:C: Villous to crypt ratio (normal, V:C > 3:1); GSE: Gluten-sensitive enteropathy; IEL: Intraepithelial lymphocytes. The upper limit of IEL may be considered 20 or 25 according to the country, institution, and physician's preference, although mostly 25 seems to be the most accepted current threshold.

**Table 2 Corazza-Villanacci classification**

Grade A
Nonatrophic, with normal V:C ratio and ↑ IELs (> 25 IELs/100 enterocytes)
Grade B1
"Atrophic", V:C < 3:1, but villi still detectable and ↑ IELs (> 25 IELs/100 enterocytes)
Grade B2
Atrophic and flat, villi not detectable and ↑ IELs (> 25 IELs per 100 enterocytes)

V:C: Villous to crypt ratio (normal, V:C > 3:1); GSE: Gluten-sensitive enteropathy; IEL: Intraepithelial lymphocytes.

Oberhuber *et al*<sup>[61]</sup> modified the Marsh classification by dividing the type III lesions into three subtypes, including A (*alike* = near normal) or mild villous atrophy, B (*broad villi*) or marked villous atrophy, and C (*complete*) or completely flat mucosa<sup>[61,62]</sup> (Table 1). Corazza and Villanacci proposed to keep the type I infiltrative lesion with a setting of an upper limit of 25 IELs per 100 enterocytes<sup>[64]</sup> (Table 2). The type II hyperplastic lesion is rarely seen, while Oberhuber types IIIA and IIIB are grouped into a single category or grade B1. Corazza-Villanacci's argument is pointing to the extreme variability between the same pathologist and different pathologists carrying a kappa divergence that is not minimal. Oberhuber stage IIIC is maintained in the revised classification as grade B2. Marsh-Oberhuber's type IV hypoplastic lesion may now be considered obsolete.

Since the histological changes of GSE may be patchy in nature, a satisfactory number of biopsies

need to be taken. It has been suggested that at least 4 distal duodenal biopsies and at least 2 biopsies of the duodenal bulb should be performed<sup>[61,65]</sup>. In consideration of the patchiness, mainly in the paediatric age, many institutions advocate for 6-8 distal duodenal biopsies and 2 biopsies of the duodenal bulb (CS, personal communication).

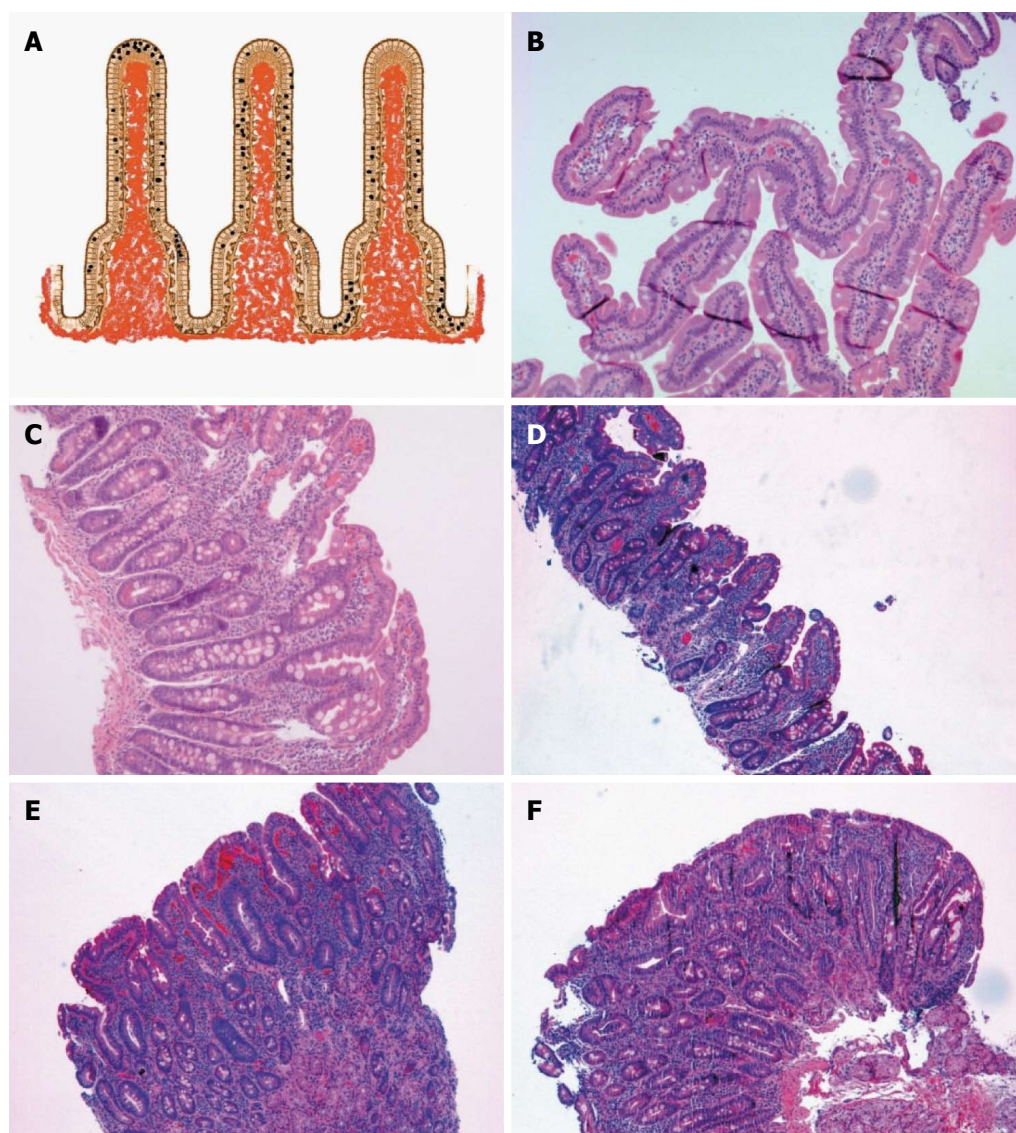
## IEL-IMMUNOPHENOTYPE

T cell receptors (TCRs) and surface co-receptors are used to characterize the immunological phenotype of the IELs. Normal duodenal biopsies should show in about 90% of healthy individuals a population of IELs which are CD3- and CD8-positive and mostly bearing TCRαβ. Conversely, CD4-positive T lymphocytes are few. In 1/10 of healthy individuals, a distinct population of TCRγδ-expressing lymphocytes has been recognized<sup>[39,66,67]</sup>. It is indeed an integrin of the β7 family, precisely CD103, which is responsible for the adhesion of the T lymphocytes to epithelial cells<sup>[68-70]</sup>. If frozen tissue is available, immunohistochemical typing for TCRγδ of T lymphocytes can be performed. In GSE, TCRγδ may reach up to 30%<sup>[71]</sup>.

IEL is constituted mainly by CD8-positive CD3-positive lymphocytes representing the most sensitive immunohistochemical features of GSE. From 40 IELs to 25 or 20 IELs per 100 epithelial cells has been a long journey and immunohistochemistry may help, but may also lead to over-diagnosis of GSE<sup>[72]</sup>. This may be the case in infiltrative-type lesions in an individual patient with suspected GSE, where the duodenal biopsy fails to show an abnormal architecture and the IEL count is difficult to perceptively be assessed adequately. Such situation, although uncommon, may be a sign of latent GSE, despite other causes possibly being at the origin of this finding. To the best of our knowledge, its clinical relevance remains to be adequately assessed by long-term follow-up studies.

An increased IEL count in an otherwise normal small bowel biopsy specimen is obviously not specific for GSE and may be associated with numerous conditions such as non-steroidal anti-inflammatory drugs (NSAIDs) use, microorganisms, bacterial overgrowth, immunological disorders, and lymphocytic or collagenous colitis among others. In examining the biopsies of patients with GSE, the number of CD8-positive CD3-positive T-lymphocytes is in crescendo towards the villous tips, while normal villi or non-GSE lymphocytes show a crescendo towards the base of the villi (crescendo vs decrescendo pattern) (Figure 2)<sup>[9,13,59]</sup>. Immunohistochemical investigation for TCRγδ in IEL is as sensitive and specific as the villous tip IEL count and may result in distinguishing other intestinal disorders from GSE in an effective way; but, to date, TCRγδ immunohistochemistry in early and latent GSE remains still controversial<sup>[24,73]</sup>. Moreover, the initial attempts to perform an assay using formalin-fixed and





**Figure 2** Intraepithelial lymphocytes and Marsh classification. A: Schema of the intraepithelial distribution of the intraepithelial lymphocytes (top, side and bottom, see text); B: Marsh 0, normal villous architecture with en-face cut (HE,  $\times 100$ ); C: Marsh I (HE,  $\times 100$ ); D: Marsh IIIA (HE,  $\times 100$ ); E: Marsh IIIB (HE,  $\times 100$ ); F: Marsh IIIC (HE,  $\times 100$ ). Marsh-Oberhuber classification is often shortened as Marsh.

paraffin-embedded tissue blocks have been in vain<sup>[24]</sup>.

Refractory gluten-sensitive enteropathy (RGSE) is a term used to define a pathological condition affecting the small bowel, histologically resembling GSE but not responding to a strict GFD of at least 6 mo<sup>[74]</sup>. In RGSE, most IELs have an abnormal immunophenotype, characterized by intracytoplasmic CD3 $\epsilon$  and CD103 and loss of expression of CD3, CD4 or CD8 as well as TCR on the cell surface in 52%-98% of cases associated with a restricted rearrangement of the TCR $\gamma$  gene<sup>[74,75]</sup>. In about 3/4 of patients with refractory sprue, clonal TCR $\gamma$  gene rearrangement is seen and the CD3 T cell lymphocytes of the lamina propria are constituted by a mixture of both CD4 and CD8 T lymphocytes<sup>[67]</sup>.

Type I RGSE is characterized by a normal T cell phenotype (CD3<sup>+</sup>/CD8<sup>+</sup>), while type II RGSE shows, by molecular investigations, loss of CD8 expres-

sion and clonality. Type II RGSE may progress to enteropathy-associated T cell lymphoma. In addition to the absolute number of IELs, the distribution of CD8-positive CD3-positive T lymphocytes along the villous has been observed to vary in GSE as well as in RGSE.

## GSE-MIMICKERS - "COMMON, LESS COMMON AND HIGHLY UNCOMMON"

GSE mimickers are defined as diseases that may mimic GSE leaving the patients to a wrong clinical management. The Latin poet Virgil (70-19 BC) wrote in his book of the Georgics of the 1<sup>st</sup> century BC a quite famous sentence, "*Felix, qui potuit rerum cognoscere causas*" (literally translated as: Privileged who was able to know the causes of things) that may be appropriate in this context. IELs alone may not

be diagnostic of GSE, because there are many GSE mimickers. In determinate situations, the location of IELs may help. Top or apical IEL may be suggestive of GSE and particularly of latent GSE or GSE at early stage with preserved villous architecture.

IELs are more likely to decrease along the villous tip in non-GSE, laterally located and patchy distributed IEL may be seen in IBD, while low down cryptically located IEL may suggest graft vs host disease (GvHD) or allograft rejection in an appropriate clinical setting. Indeed, the initial manifestation of an IBD has been recorded in the duodenum, before changes occur in the terminal ileum or large bowel. Focal acute inflammation is defined by the presence of a cluster of more than one ( $> 1$ ) neutrophilic granulocyte in the lamina propria or epithelium and more than one ( $> 1$ ) focus in a tissue biopsy<sup>[76-78]</sup>. Some other authors suggest that neutrophilic granulocytes may be normal components of the lamina propria, provided no invasion of the crypt or surface epithelium is detected<sup>[29]</sup>, but we do not agree because of the specific nature of this inflammatory cell.

Focal acute duodenitis is not a sensitive feature in Crohn's disease, but has high specificity (92%) and high predictive value (93%-95%)<sup>[78]</sup>. Precursors of aphthoid ulcers may be considered foci of acute inflammation detected in the surface epithelium and deep stroma of the duodenum. The duodenum is also affected by acute inflammation with or without stomach involvement, but the incidence of granulomas is quite variable depending on the age of the patients and duration of the disease. The interobserver variability of interpreting duodenal biopsies may show different kappa factor depending from the institution<sup>[60,79]</sup>.

IEL distribution seems to be highly sensitive, but it may require additional training in the interpretation of the histology of the upper gastrointestinal tract. The diagnosis of GSE may remain problematic, because no single test shows 100% sensitivity and 100% specificity in every patient<sup>[12]</sup>. GSE mimickers may be indeed behind the scene, and there is undoubtedly no other field in gastroenterology better pictured by the Virgilian sentence (Figures 2-6).

The three most common GSE mimickers are gastric *Helicobacter pylori* (*H. pylori*) infection, medications, especially NSAIDs or proton-pump inhibitors (PPIs), and IBD<sup>[80]</sup>. *H. pylori* infection is associated with chronic active gastritis, ulcer disease, chronic active duodenitis and bulbitis, while non-specific duodenitis or peptic duodenitis are conditions associated with acid injury. *H. pylori* infection is typically associated with duodenal gastric metaplasia, characterized by foci of gastric-type mucus-secreting cells interspersed between duodenal enterocytes, which may be easily recognized by the periodic acid Schiff (PAS)-positivity of the cells containing neutral mucin and the lack of the brush border<sup>[81,82]</sup>. An increased IEL count is observed in the duodenum of patients with *H. pylori* gastritis<sup>[9,15,83,84]</sup>.

There is still some debate about the specificity of the findings and more longitudinal studies may be needed, but in any case, correlation with serology and gastric biopsies is still recommended.

The use of NSAIDs has been associated in a few cases of duodenal IEL<sup>[85,86]</sup>. Brunner gland hyperplasia, originally thought of as a feature of peptic duodenitis, is now considered not relevant, because it may be encountered in the normal duodenum as well<sup>[29]</sup>. Other medications associated with villous architectural changes include colchicine, mycophenolate mofetil, ipilimumab and several chemotherapy agents or radio-/chemotherapy protocols among others.

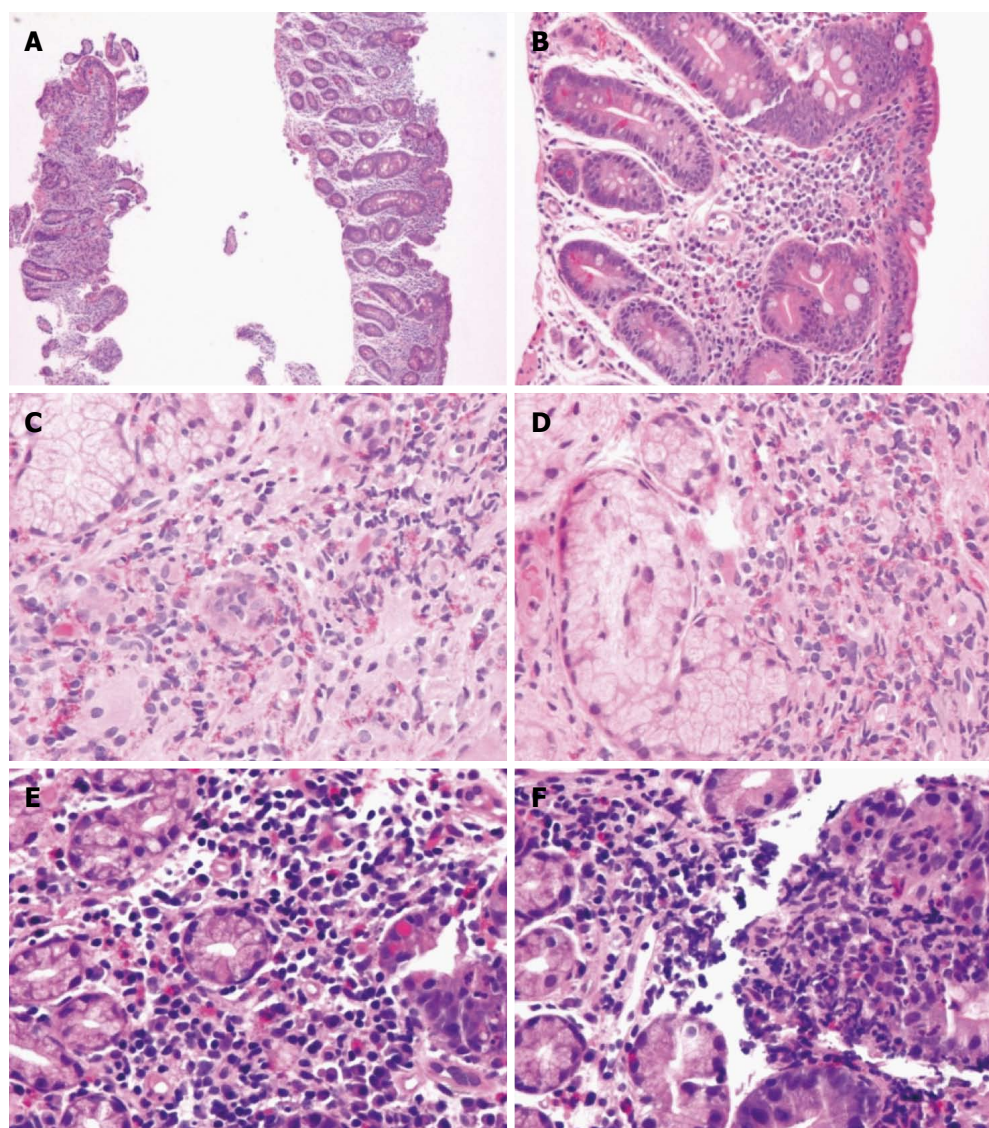
Both Crohn's disease and ulcerative colitis may have IELs in the duodenum<sup>[76,87-90]</sup>. Duodenal granulomas are a very helpful finding in confirming the diagnosis of Crohn's disease, but they are seen in less than half of the patients. In patients with classical presentation of Crohn's disease, villous shortening accompanied by neutrophil-rich inflammation, oedema in the lamina propria and crypt abscesses may also be encountered. Crucial is the correlation with biopsy findings arising from other sites, because isolated duodenal Crohn's disease is extremely rare<sup>[91]</sup>.

In up to 1/4 of patients with ulcerative colitis, variable villous blunting, inflammatory expansion of the lamina propria by plasma cells and active (neutrophilic) inflammation are also seen<sup>[90,92,93]</sup>. Food allergy, infections, small intestine bacterial overgrowth, tropical sprue and various immunological or autoimmune disorders have been described, but are less common. Uncommon events with IEL and villous blunting may include sickle cell anaemia-duodenitis with a potential ischemic background due to pileup of abnormally shaped erythrocytes<sup>[94,95]</sup> and non-*H. pylori* infection, such as with *Yersinia enterocolitis* and *Salmonella* spp.<sup>[96,97]</sup>.

Food allergy enteropathy (FAE) and cow's milk protein-sensitive enteropathy (CMSE) may be encountered in a duodenal biopsy and both conditions may show a wide variety of mucosal lesions in any part of the upper and lower gut. Although villous atrophy is not seen in food allergy, crypt hyperplasia and an increased number of inflammatory cells, particularly eosinophilic granulocytes, are detected more often in the lamina propria and rarely also in the surface epithelium<sup>[32,33,98-103]</sup>. In CMSE, the villous architecture is usually normal, but cytotoxic IEL count is increased, particularly in the descending part of the duodenum in contrast to GSE, which conversely shows the most severe changes in the proximal parts of the duodenum<sup>[104-109]</sup>.

Infections that are commonly seen in the duodenum include giardiasis, cryptosporidiosis, microsporidiosis, cyclosporiasis, isosporiasis, Whipple's disease, *Mycobacterium avium intracellulare*, visceral leishmaniasis, cryptococcosis and cytomegalovirus. The clinical history with the most recent travel history



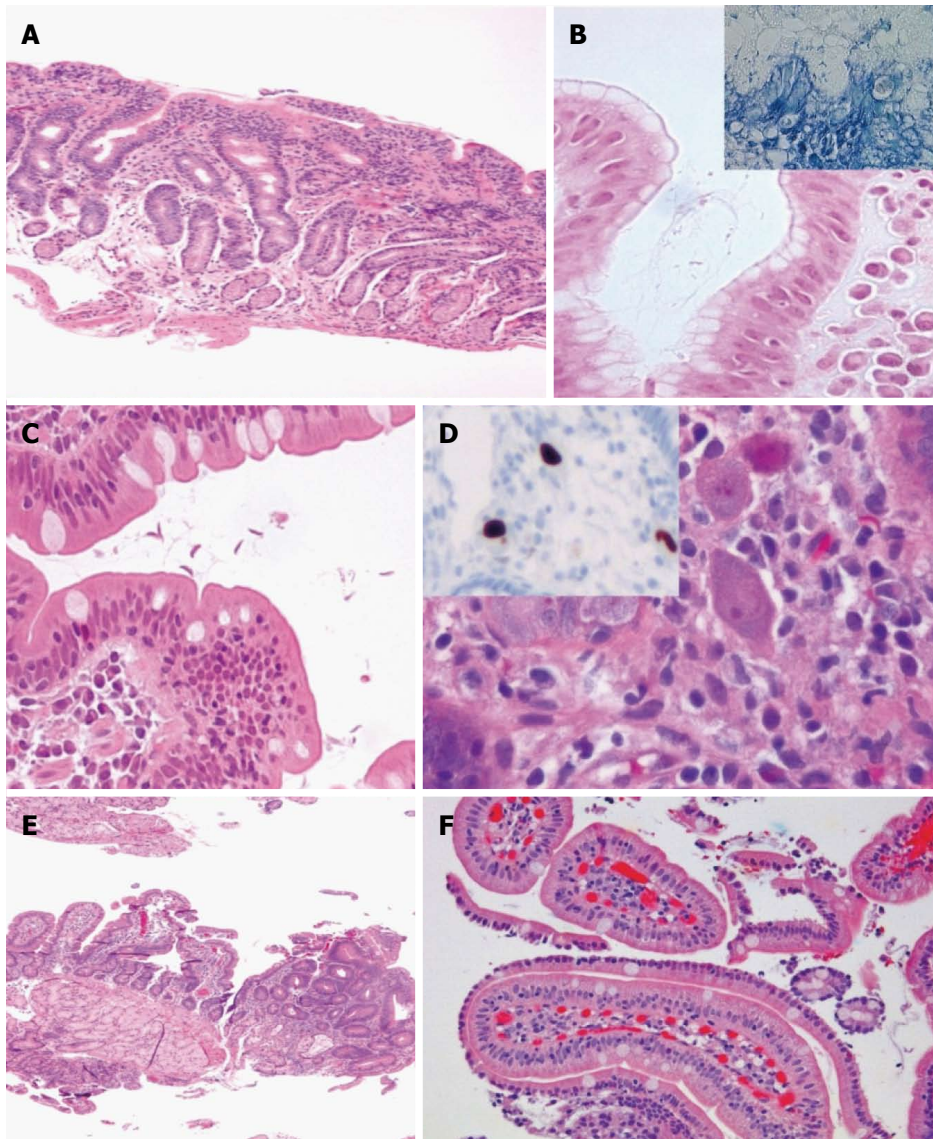


**Figure 3 Gluten-sensitive enteropathy and GSE mimickers.** A, B: Variable destructive patterns of Marsh III C of GSE (A: HE,  $\times 100$ ; B: HE,  $\times 200$ ); C, D: Eosinophilic duodenitis (HE,  $\times 400$ ); E, F: Peptic duodenitis (HE,  $\times 400$ ). GSE: Gluten-sensitive enteropathy.

of both parents and children, the geographical settings and the age of the patient may aim to restrict the diagnosis that needs to be confirmed by the laboratory and microbiologic analysis. Morphologically, *Giardia lamblia* is a pear-shaped microorganism if cut lengthwise and a binucleate, ventral disc if cut frontally, with 4 pairs of flagella. *Cryptosporidia* are basophilic merozoites (2-5  $\mu\text{m}$ ) of varying size. *Microsporidia* are supranuclear parasitophorous vacuoles indenting the nucleus. *Cyclospora* are round and fusiform merozoites (up to 6  $\mu\text{m}$  in length) with supra-nuclear parasitophorous vacuoles. *Isosporas* are subnuclear parasitophorous vacuoles (20-30  $\mu\text{m}$ ) containing banana-shaped merozoites and sexual forms. *Tropheryma whipplei* are PAS-positive bacilli. *Intracellular M. avia* are acid fast and diastase-resistant (D-)PAS-positive curved bacilli. *L. donovani* are 1-2  $\mu\text{m}$  basophilic amastigotes in an identifiable parasitophorous vacuole. Cryptococci are

microorganisms with narrow-neck budding, while the characteristic aspect of cytomegalovirus is the "owl's eye", which displays a dense nuclear inclusion and granular cytoplasmic inclusions. In Table 3, the differential diagnosis of these microorganisms, with the histological changes observed in the duodenum, are summarized.

Autoimmune enteropathy (AIE) is another GSE-mimicker because it is characterized by villous atrophy unresponsive to a GFD<sup>[25,110]</sup>. The histological evidence of enteropathy, a lack of any triggering food protein, anti-enterocyte antibodies as well as persistent diarrhoea after prolonged fasting and presence of organ-specific serum antibodies are essential for the diagnosis of this entity. The histology of AIE is characterized by variable degrees of architectural changes, including normal to total villous atrophy and a CD8-predominant immunophenotype of IELs. IEL count may be normal or increased and is mainly characterized by CD8-



**Figure 4 Gluten-sensitive enteropathy mimickers.** A: Lymphocytic gastritis with involvement of the duodenum (HE, × 100); B: *H. pylori* gastritis (inset, Giemsa staining) (HE and Giemsa × 630); C: Giardiasis (HE, × 400); D: Cytomegalovirus (CMV) infection (HE, × 6300) and inset showing anti-CMV antibody reacting against viral proteins using an avidin-biotin complex immunoperoxidase immunohistochemical detection (× 100); E: Focal adenomatous change in duodenum (HE, × 50); F: Sickle cell disease-related duodenitis (HE, × 200).

positive lymphocytes. Importantly, the number of lymphocytes harbouring  $\gamma\delta$  immunophenotype is normal in both surface epithelium and lamina propria help in distinguishing AIE from GSE. AIE may produce subtotal villous blunting and IEL simulating the appearance of GSE, but the absence of goblet cells and Paneth cells in AIE biopsies accompanied by a prominent crypt apoptosis are helpful clues<sup>[29]</sup>.

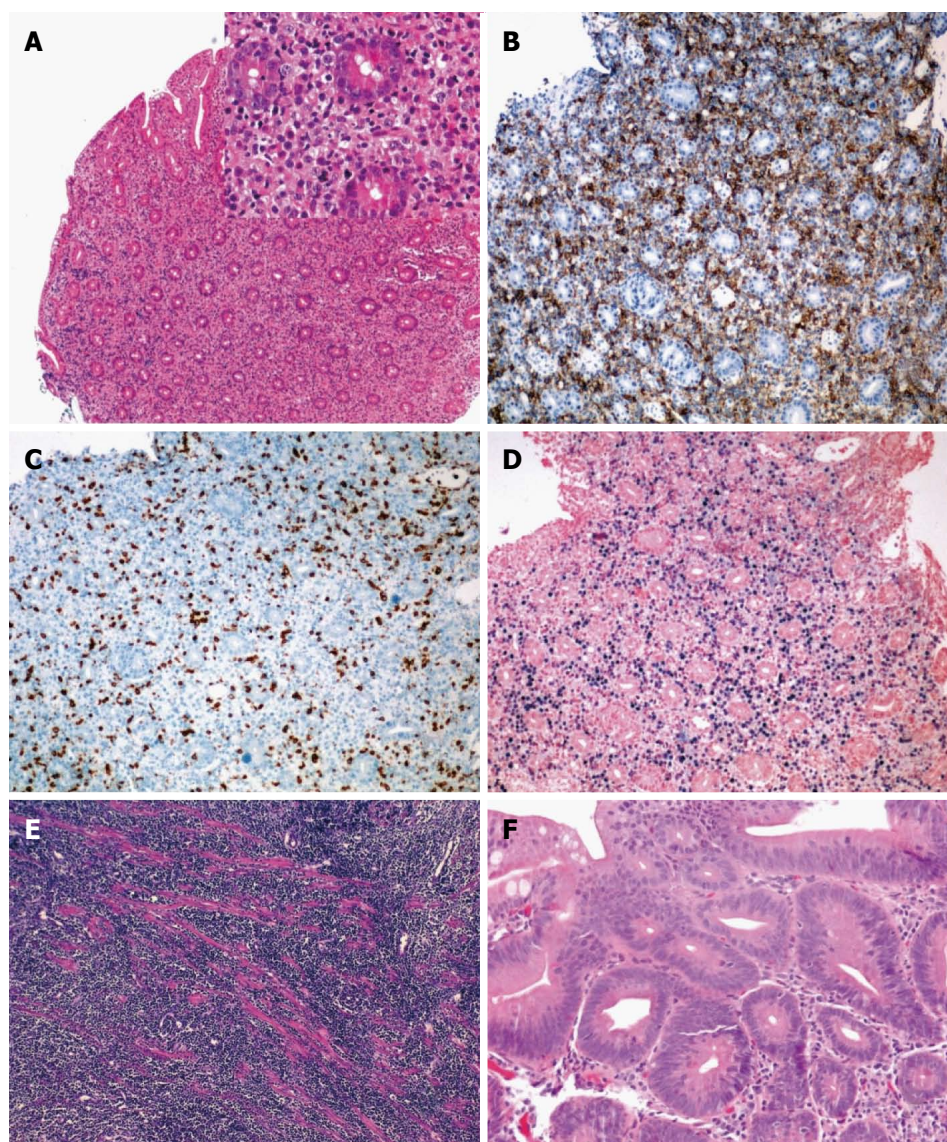
Common variable immunodeficiency (CVID) may also manifest with gastrointestinal symptoms, being the second most common primary immunodeficiency, and its diagnosis relies on recurrent infections, decreased IgG levels at least 2-standard deviations below normal with at least decreased levels of one other immunoglobulin subclass, exclusion of other causes of immunodeficiency, and a failure to mount a response to vaccination. In 2/3 patients with CVID

undergoing endoscopy, the duodenal biopsy shows IEL with or without villous architectural changes and 2 CVID characteristic clues are the paucity or absence of plasma cells with prominent crypt apoptosis in CVID<sup>[29,111-113]</sup>.

GvHD and allograft bowel rejection (AGBR) may be ruled out on clinical settings. GvHD may, however, come to the gastroenterologist or pathologist who are not provided with the history of bone marrow transplantation for instance. GvHD may have, although uncommonly, an increased IEL count in proximal small bowel biopsies. A decrescendo from base to apical villi and the finding of epithelial cell apoptosis in the deep crypts, with or without some degree of architectural disturbance, together with the clinical setting may help to address this diagnosis<sup>[114]</sup>.

Collagenous sprue (COS) is an GSE mimicker, originally described by Weinstein in 1970<sup>[115]</sup>, and





**Figure 5 Gluten-sensitive enteropathy mimickers.** A-D: Post-transplant lymphoproliferative disorder, polymorphic type (A: HE,  $\times 50$  and inset HE,  $\times 200$ ) showing mononuclear epithelial and stromal infiltration with blasts and high CD20 (B,  $\times 100$ ) on CD3 (C,  $\times 100$ ) lymphocytes and high Epstein-Barr virus replication (*in situ* hybridization or Epstein-Barr encoding region,  $\times 100$ ); E: Burkitt lymphoma of the duodenum (HE,  $\times 100$ ); F: Tubular adenoma of the duodenum of a patient with familial adenomatous polyposis (HE,  $\times 200$ ).

shares several aspects of GSE, including villous architectural abnormalities, IEL and crypt hyperplasia; but, an irregularly thickened layer of type 1 collagen just subjacent to the surface epithelium is extremely useful for distinguishing COS from GSE. A monotypic, truncated immunoglobulin  $\alpha$  heavy chain lacking an associated light chain secreted by plasma cells infiltrating the bowel wall characterizes a condition called immunoproliferative small intestine disease (IPSID), which is a MALT lymphoma<sup>[116-118]</sup>. The early stages of IPSID may be quite challenging, because the duodenal mucosa may appear normal or near-normal, but thickening, erythema and nodularity of the mucosal folds may be observed in the duodenum and upper jejunum at later stages<sup>[117]</sup>. IPSID is mostly reported in individuals from the Middle East, North and

South Africa and the Far East, and the epidemiological background may be quite helpful.

Several immune-related disorders, including Hashimoto thyroiditis, Graves' disease, rheumatoid arthritis, psoriasis and systemic lupus erythematosus may cause IEL. These diseases need to be carefully ruled out on both clinical and laboratory grounds. Other etiologic factors that have been associated with IEL are quite more uncommon and infrequently associated to IEL, but they also need clinical and laboratory correlation. These disorders involve the nervous system mainly, the two major diseases of which include autism and multiple sclerosis<sup>[119,120]</sup>.

The enteropathy-type intestinal T cell lymphoma (EITL) may be considered a complication of long-standing GSE<sup>[121-123]</sup>. EITL is frequently multifocal with

**Table 3** Most common duodenal infections potentially mimicking gluten-sensitive enteropathy

	Agent	Villi	CH	LPI	IELs	PMNs	Sup.-D.	Target	Site	Stain
Giardiasis	<i>G. lamblia</i>	0-3A	Nil	0/+ / ++	< 20/↑	Rarely	Nil	None	IL	GS
Cryptosporidia	<i>C. parvum</i>	1-3A	+	+ / ++	< 20	Focal	Focally	Enterocytes	IE	WS
Microsporidia	<i>E. bienewisi</i>	1-3A	+	+ / ++	< 20	Nil	Focally	Enterocytes/	IEv/	WS
	<i>E. intestinalis</i>							macrophages	IEc	
Cyclospora	<i>C. coytanensis</i>	1-3A	+	+ / ++	< 20/↑	Nil	Focally	Enterocytes	IEv	NA
Isospora	<i>I. belli</i>	1-3A	+	+ / ++	< 20	Nil	Focally	Enterocytes	IEv	NA
Whipple D	<i>T. whipplei</i>	1-3A	Nil	++ <sup>1</sup>	↑	Nil	Nil	Macrophages	LP	PAS
MAI	<i>M. avium intracellulare</i>	1-3A	Nil	++ <sup>1</sup>	< 20	Nil	Nil	Macrophages	LP	ZN
										PAS
										AR
Leishmaniasis	<i>L. donovani</i>	1-3A	Nil	+ / ++	< 20	Nil	Nil	Macrophages	LP	NA
Cryptococcosis	<i>C. neoformans</i>	1-3A	Nil	0/+	< 20	Nil	Nil	None	LP	DPAS
										MS
CMV	<i>Cytomegalovirus</i>	Ulcers	<sup>2</sup>	+ / ++	< 20/↑	+/-	Focally	Epithelium/ endothelium	LP	IHC

<sup>1</sup>Pale macrophages; <sup>2</sup>Crypt damage; III A partial atrophy, III B subtotal atrophy, III C total atrophy. 0-3A: 0, no inflammation, 1, IELs, 2 mild hyperplasia/mild inflammation. IL: Intraluminal; IE: Intraepithelial (surface and crypt epithelium); IEv: Intraepithelial at the villous tips; IEC: Intraepithelial at the crypts; LP: Lamina propria; CH: Crypt hyperplasia; AR: Auramine-rhodamine stain; DPAS: Diastase-periodic acid Schiff stain; GS: Giemsa stain; IHC: Immunohistochemistry with antibodies against the cytomegalovirus antigens; MS: Methenamine-silver stain; WS: Warthin-Starry stain; ZN: Ziehl-Nielsen stain.

Name: ..... Case #:  N/Y: 0/1

Location: .....

Distal duodenum biopsies #:  Villous/crypt ratio #:  :1

Bulb biopsies #:

Lamina Propria inflammation #:  Lymphocytes  Plasma cells  Neutrophils

IELs #/100 epithelial cells (top):  Enterocyte damage:

IELs #/100 epithelial cells (side):  Brush border thickness (μm):

IELs #/100 epithelial cells (down):  Erosion:

Crypt hyperplasia  Micro-organisms:  .....

Benign (B)/dysplasia (D)/malignancy (M)  .....

**Figure 6** Synoptic report for gluten-sensitive enteropathy and gluten-sensitive enteropathy mimickers.

ulcerative lesions and a tendency to perforate either at presentation or during chemotherapy. Histologically, there is a pleomorphic medium-to-large cell population constituted by the expression of CD3 and lack of CD4 and CD8 expressions as well as a small and monomorphic cell population characterized by the expression of CD3, CD8 and CD56 and lack of CD4 expression. CD30 is always present in the tumour cells and may be seen in the adjacent villi of the lymphoma lesions, and is considered an ominous marker for prognosis. Among the neoplastic GSE mimickers, the tubular adenomas, post-transplant lymphoproliferative disorders (PTLDs) and lymphomas should be listed.

## GSE - CHALLENGES ACROSS OCEANS

In 2012<sup>[16]</sup>, the guidelines for GSE diagnosis were issued by the ESPGHAN suggesting that biopsies can be avoided in patients who have positive HLA-DQ test results. It has been suggested that HLA-DQ test may extend beyond these cases<sup>[41]</sup>. The main inhibitions in efficiently using molecular biology techniques are represented by cost and lack of automation, but RT-PCR, digital PCR and next-generation sequencing may today open interesting possibilities in tailoring the diagnostic algorithm for GSE in a more efficient way. The combined use of aTTG and anti-DGP assay is

**Table 4 North American - European divergences across oceans in gluten-sensitive enteropathy**

	Target	Screening	PS Tests <sup>1</sup>	HLA-DQ	EMA	AGA
ESPGHAN	Paediatric	Anti-tTG-IgA and IgA <sup>2</sup>	Anti-tTG-IgG/anti-DPG-IgG <sup>3</sup>	Yes, if ↑EMA/anti-tTG	Yes, in confirming PS tests	No
ACG	Paediatric/adult	Anti-tTG-IgA	Anti-tTG-IgG/anti-DPG-IgG <sup>3</sup>	Yes, if biopsy/serology disagreement	NS	No
WGO	NS	Anti-tTG-IgA/anti-DPG IgG	NS	Yes, if biopsy/serology disagreement	Yes, in confirming PS tests	No

<sup>1</sup>Postscreening tests; <sup>2</sup>Total serum IgA; <sup>3</sup>Anti-DPG: Anti-deamidated gliadin peptide. ESPGHA: European Society of Pediatric Gastroenterology, Hepatology and Nutrition; ACG: American College of Gastroenterology; WGO: World Gastroenterology Organization; NS: Not specified.

now recommended in young children, while HLA-DQ typing is useful in support of histology in seronegative patients, and to exclude patients at high risk for GSE. In patients with low risk for development of GSE, the presence of IgA aTTG-positive blood inclines towards endoscopy and duodenal biopsy. ESPGHAN emphasizes that patients with selective IgA deficiency should be tested for anti-DGP IgG and/or aTTG IgG and, if positive, a biopsy needs to be performed.

The guidelines of the American College of Gastroenterology and World Gastroenterology Organization are similar, but differences have been identified recently<sup>[41]</sup>. These two guidelines distinguish between patients at low and high risk of GSE and in screening the general population, with a GSE prevalence of 1%, the IgA aTTG and DGP assays are now recommended, either simultaneously, or in sequence. Thus, in the high-risk population, only one test is considered sufficient, because in these patients it is supposed that additional tests do not increase the reliability of screening results. Conversely, in low-risk patients, a positive serological test is a strong indication for duodenal biopsy that remains the gold standard in North America. For patients at risk of GSE, biopsy is always recommended, irrespective of serological results; if the results of both tests are positive, the diagnosis of GSE is confirmed. Conversely, if the serology is positive and histology is negative, it has been suggested that the biopsy is repeated at least after 1 year. If the histology is positive and serology is negative, HLA-DQ typing is counselled and other possible causes of duodenitis should be carefully evaluated. GSE is ruled out only when both serology and histology are negative. In Table 4, the main differences between the GSE guidelines across oceans are presented<sup>[41]</sup>.

## DUODENAL MUCOSA - SYNOPTIC REPORT

An integrated assessment of the histopathology elementary lesions and clinical and serological findings make consistent and reliable the diagnosis of GSE. The elementary lesions consist of (1) *increased IELs* or IEL with a value between 20 and 24 IEL/100 enterocy-

tes as borderline and  $\geq 25$  IEL/100 enterocytes representing a pathological lymphocytic infiltration of the surface epithelium; (2) *decreased height of the enterocytes* with flattening of enterocytes, intracytoplasmic vacuolation as well as reduction or absence of brush-border; (3) *crypt hyperplasia* as indicated by extension of the regenerative epithelial crypts associated with changes in the presence of more than 1 mitosis per crypt; and (4) *villous blunting* identified as decrease in villous height, alteration of normal crypt/villous ratio (3:1) until total disappearance of villi with proper orientation of the biopsies<sup>[124]</sup>.

A synoptic report is commonly used for cancer pathologies, using checklists that allow a better management of patients with oncological disease<sup>[125,126]</sup>. Free text reports often demonstrate significantly impaired data collection when recording several parameters, and the number of words used is also significantly reduced using pre-formatted structured reports as compared to free text reports. In public healthcare, the introduction of a structured reporting dictation template improves data collection remarkably and reduces the subsequent administrative burden when dealing with phone calls and/or reviewing the number of cases reviewed at multidisciplinary team meetings, and external quality assurance programs provide a support for it<sup>[127]</sup>.

In our opinion, a biopsy report should include the number and site of the biopsy specimens, the pathology or normality of the tissue specimens, the villous-crypt ratio, the villous architecture (normal or blunted, partial/total), the IEL counts at the top, side and bottom, the morphology of the surface enterocytes (normal, flattened or damaged) with or without preservation or loss of the brush border, crypt hyperplasia, gastric metaplasia (e.g., chronic duodenitis), presence of microorganisms (e.g., *Giardia lamblia*, cryptosporidia, microsporidia, *Isospora belli*, cyclospora, *Mycobacterium avium intracellulare*, cytomegalovirus, *Cryptococcus neoformans*)<sup>[24]</sup>. A number of additional features have been suggested to be present in the histopathologic report<sup>[71,128]</sup>, including the search results for potential benign, dysplastic or neoplastic lesions (e.g., adenoma or carcinoma, carcinoid, lymphoma). Figure 6 displays a synoptic



report that may be considered useful for both clinics and research.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Partial and patchy villous blunting may be found in CMSE, in postenteritis enteropathy and in GSE. Thus, multiple biopsies should be taken to minimise the risk of misdiagnosis. The bulb mucosa may be the only duodenal area affected and total or moderate villous atrophy may affect the duodenal bulb exclusively with a normal distal duodenum. Therefore, careful appreciation with regard to whether specimens are taken from the bulb or the descending part of the duodenum is essential<sup>[129]</sup>. GSE is a common cause of an increased IEL count in the duodenum accounting, probably, for up half of the cases, but GSE mimickers should be taken into account. New molecular biology-supported methodologies may tailor and individualize the diagnostic algorithm in the future.

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## Basic Study

# Mutational analysis of hepatitis E virus ORF1 "Y-domain": Effects on RNA replication and virion infectivity

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## Abstract

### AIM

To investigate the role of non-structural open reading frame 1 "Y-domain" sequences in the hepatitis E virus (HEV) life cycle.

### METHODS

Sequences of human HEV Y-domain (amino acid sequences 216-442) and closely-related viruses were analyzed *in silico*. Site-directed mutagenesis of the Y-domain (HEV SAR55) was carried out and studied in the replicon-baculovirus-hepatoma cell model. *In vitro* transcribed mRNA (*pSK-GFP*) constructs were transfected into S10-3 cells and viral RNA replicating GFP-positive cells were scored by flow cytometry. Mutant virions' infectivity was assayed on naive HepG2/C3A cells.

### RESULTS

*In silico* analysis identified a potential palmitoylation-site (C<sub>336</sub>C<sub>337</sub>) and an  $\alpha$ -helix segment (L<sub>410</sub>Y<sub>411</sub>S<sub>412</sub>W<sub>413</sub>L<sub>414</sub>F<sub>415</sub>E<sub>416</sub>) in the HEV Y-domain. Molecular characterization of C<sub>336</sub>A, C<sub>337</sub>A and W<sub>413</sub>A mutants of the three universally conserved residues showed non-viability. Further, of the 10 consecutive saturation mutants covering the entire Y-domain nucleotide sequences (nts 650-1339), three constructs (nts 788-994) severely affected virus replication. This revealed the indispensability of the internal sequences but not of the up- or downstream sequences at the transcriptional level. Interestingly, the three mutated residues corresponded to the

downstream codons that tolerated saturation mutation, indicating their post-translational functional/structural essentiality. In addition, RNA secondary structure prediction revealed formation of stable hairpins (nts 788-994) where saturation mutation drastically inhibited virion infectivity.

### CONCLUSION

This is the first demonstration of the critical role of Y-domain sequences in HEV life cycle, which may involve gene regulation and/or membrane binding in intracellular replication complexes.

**Key words:** Hepatitis E virus; Open reading frame 1; Y-domain; Palmitoylation;  $\alpha$ -helix

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**Core tip:** The function of hepatitis E virus (HEV) Y-domain remains elusive. *In silico* analysis of closely-related virus sequences mapped a potential palmitoylation-site (CC) and  $\alpha$ -helix segment (LYSWLFE) in the Y-domain. Mutant replicons of the universally conserved residues C<sub>336</sub>, C<sub>337</sub> and W<sub>413</sub> showed non-viability. Saturation mutations in the Y-domain (nucleotide sequences 788-994) severely affected RNA replication, revealing their post-transcriptional indispensability. Notably, the three residues corresponded to the non-conserved codons, indicating their post-translational essentiality. RNA secondary structure prediction showed hairpin formations by the critical bases (788-994), where mutations drastically affected virion infectivity. This is the first demonstration of the critical role of Y-domain sequences in HEV life cycle that warrants further molecular/biochemical studies.

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## INTRODUCTION

The eukaryotic positive single-strand (+ss) RNA viruses share evolutionarily-conserved functional and putative domains, and even amino acid (aa) sequences in their nonstructural/replicase polyproteins<sup>[1]</sup>. In infected cells, one of the polyprotein proteolytic products, the methyltransferase (MTase), catalyzes 5' capping of viral mRNA and interacts with cytoplasmic membranes, essential for establishing replication complexes<sup>[2]</sup>. In addition to the MTase-domain, studies have also shown sequence and structural conservation of the downstream Y-domain in viral polyproteins<sup>[1,3,4]</sup>. Recently, sequence analysis of human, animal and plant viruses of the alphavirus-like superfamily has

suggested the Y-domain as an extension (“iceberg” region) of the MTase C-terminal (“core” region), and identified its homolog in nodaviruses<sup>[5]</sup>. Further, universally conserved cysteine residues have been identified in the core region of animal viruses, such as Semliki Forest virus (SFV) and Sindbis virus (SINV), and closely-related plant viruses, including bromo mosaic virus (BMV), bamboo mosaic virus (BaMV), alfalfa mosaic virus (AMV), tomato mosaic virus (ToMV), tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV), critical for RNA capping and replication<sup>[6-13]</sup>.

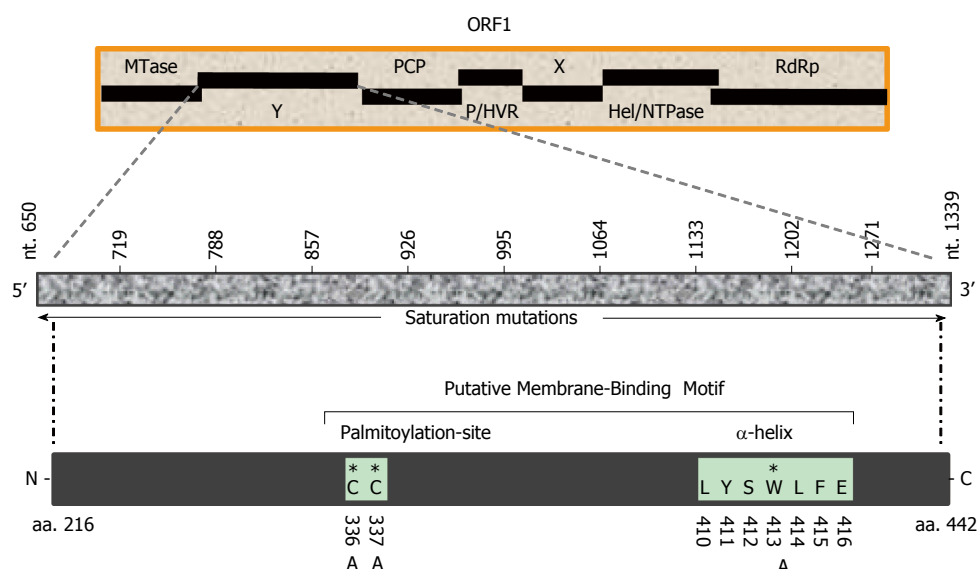
Moreover, mutational analysis of SFV-nonstructural protein 1 (nsP1) has shown indispensability of both the core and Y-domain residues for RNA capping activity<sup>[14,15]</sup>. In SINV-nsP1, deletions of core residues (aa 442-492) not only abolished MTase activity but also virus infectivity<sup>[16]</sup>. Notably, the capping was completely retained by truncated SINV-nsP1 (aa 1-448)<sup>[17]</sup> and BaMV-replicase (aa 1-442)<sup>[7]</sup>, which ended only 30-40 residues down in the Y-domain. The MTase-domain (N-terminal) alone, therefore, seems insufficient for viral 5' mRNA capping that is actually complemented by the combined sequences of core and Y. Thus, it is the “core-Y” region that undergoes post-translational palmitoylation, required for membrane binding through an amphipathic  $\alpha$ -helix to form replication complexes on cytoplasmic membranes<sup>[5,18-21]</sup>.

The hepatitis E virus (HEV), the only Hepevirus of the alphavirus-like superfamily, is the etiological agent of acute and chronic hepatitis E in humans<sup>[22,23]</sup>. The HEV +ssRNA genome (about 7.2 kb) contains three partially overlapping open reading frames (ORFs): ORF1, ORF2 and ORF3, flanked by 5' and 3' short untranslated-regions<sup>[24,25]</sup>. Of these, the largest gene, ORF1 (5109 bases), encodes the nonstructural polyprotein (1703 residues) essential for viral RNA replication in infected cells<sup>[26-28]</sup>. Homologous to the alphavirus polyprotein structural organization, the MTase-domain is followed by the Y-domain in HEV ORF1 (Figure 1). While the 5' mRNA capping activity of the ORF1 MTase-domain (N-terminal) is well characterized and implicated in RNA replication<sup>[29,30]</sup>, the function of the Y-domain remains completely unexplored. Therefore, the present study was postulated to investigate a potential role of Y-domain sequences in HEV life cycle, using the replicon-baculovirus-hepatoma cell model.

## MATERIALS AND METHODS

### *In silico* analysis

The Y-domain sequences of human HEV strains (GenBank;  $n = 206$ ), belonging to the four genotypes (HEV1, HEV2, HEV3 and HEV4) as well those of closely-related +ssRNA viruses were analyzed using the online bioinformatics tools *Multalin* (<http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl>) and *ClustalW* 1.8 (<http://embnet.vital-it.ch/software/ClustalW.html>).



**Figure 1** Schematic representation of hepatitis E virus nonstructural polypeptide (ORF1) domain organization, showing the undefined Y-domain. Saturation mutations covering the entire Y-domain (nts 650-1339; 10 constructs of 68 bases each) as well as specific amino acid (C<sub>336</sub>, C<sub>337</sub> and W<sub>413</sub>) mutations within the predicted membrane-binding motif are shown. MTase: Methyltransferase; Y: Undefined; PCP: Papin-like cysteine protease; P/HVR: Proline-rich/hypervariable region; X: Macro; Hel/NTPase: Helicase/nucleotide triphosphatase; RdRp: RNA-dependent RNA polymerase.

Prediction of peptide secondary structure/amphipathic helix was done by *PSIPRED* (<http://bioinf.cs.ucl.ac.uk/psipred>) and *PROFsec* (<http://bioinf.cs.ucl.ac.uk/psipred>). The program *RNAstructure* (<http://rna.urmc.rochester.edu/RNAstructureWeb/index.html>) was used to predict RNA secondary structures.

### Construction of Y-domain mutant replicons

Mutations were introduced in HEV1-SAR55 (accession no. AF444002) full-length genomic replicon (*pSK-GFP*; a kind gift from Dr Suzanne Emerson, National Institutes of Health, Bethesda, MD, United States) by site-directed mutagenesis as described elsewhere<sup>[27]</sup>. Ten consecutive saturation mutants that covered the entire Y-domain nucleotide sequences (nts 650-1339), including *pSK-GFP-Ydom1* (nts 650-718), *pSK-GFP-Ydom2* (nts 719-787), *pSK-GFP-Ydom3* (nts 788-856), *pSK-GFP-Ydom4* (nts 857-925), *pSK-GFP-Ydom5* (nts 926-994), *pSK-GFP-Ydom6* (nts 995-1063), *pSK-GFP-Ydom7* (nts 1064-1132), *pSK-GFP-Ydom8* (nts 1133-1201), *pSK-GFP-Ydom9* (nts 1202-1270) and *pSK-GFP-Ydom10* (nts 1271-1339), were constructed by changing every possible nucleotide without altering the aa sequences. In addition, three aa mutants, including *pSK-GFP-YdomC336A*, *pSK-GFP-YdomC337A* and *pSK-GFP-YdomW413A*, of universally conserved residues within the predicted membrane binding motif were constructed. Replicon constructs *pSK-GFP-WT* and *pSK-GFP-G816V*<sup>[27]</sup> served as positive and negative controls, respectively.

Briefly, polymerase chain reaction (PCR) was carried out in a 50  $\mu$ L reaction volume with appropriate amounts of *pSK-GFP* plasmid, forward and reverse primers, dNTP mix, DNA polymerase and buffer, under thermal conditions as per the manufacturer's ins-

tructions (TaKaRa Bio Inc, Shiga, Japan). The PCR products were gel electrophoresed to confirm full amplification, *DpnI* (Invitrogen, Carlsbad, CA, United States) digested to eliminate residual template, and transformed into XL-blue DH5 $\alpha$  competent cells (Stratagene, San Diego, CA, United States) by heat-shock method. Transformed colonies were selected on ampicillin-agar plates, and isolated DNA (Plasmid Mini-prep Kit; Qiagen, Hilden, Germany) were screened by restriction-digestion. Mutant constructs were confirmed by sequencing (Invitrogen) and stock DNAs were prepared (Plasmid Midi-prep Kit; Qiagen) for *in vitro* transcription and transfection experiments.

### Human hepatoma cell culture

The S10-3 and HepG2/C3A cells, derivatives of human hepatoma lines HuH7 and HepG2, respectively (kind gifts of Dr Suzanne Emerson, NIH) were maintained in DMEM-GlutaMax (Invitrogen) supplemented with 10% heat-inactivated bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) and 1x penicillin-streptomycin mix (Gibco) at 37  $^{\circ}$ C with 5% CO<sub>2</sub> supply<sup>[31]</sup>. For experimental studies, cells were seeded in 12-well ( $1.0 \times 10^6$  cells/well) or 24-well ( $0.5 \times 10^6$  cells/well) flat-bottom culture plates.

### In vitro capped mRNA synthesis and cell transfection

The replicon constructs (cDNA) were linearized with *BglII* (Invitrogen) and *in vitro* transcribed in the presence of anti-reverse cap analog (ARCA; Ambion, Austin, TX, United States) essentially as described elsewhere<sup>[31]</sup>. The transcription mix was gel electrophoresed to check the size, integrity and quality of the capped-mRNA, followed by transfection into S10-3 cells. The transfected cultures were incubated at

34.5 °C for 6 d to allow for optimal production of green fluorescent protein (GFP), indicating virus replication. S10-3 culture transfected with *pSK-GFP-WT* transcript showing green fluorescence served as positive control, while that receiving *pSK-GFP-G816V*, a lethal mutant<sup>[27]</sup>, served as negative control. All transfections were performed in duplicate and repeated for reproducibility.

#### **RNA trans-encapsidation and virion infectivity assay**

The mutant RNA encapsidation into viral ORF2 (capsid) proteins over-expressed by a recombinant baculovirus (vBacORF2) that could produce virus particles in S10-3 cells was performed, and tested for their infectivity on naïve HepG2/C3A cells<sup>[32,33]</sup>. In sum, RNA transfected S10-3 cells (in 12-well plates) were transduced with vBacORF2 on the following day. On day 6 post-transfection (*i.e.*, day 5 post-transduction), lysates were prepared by vigorous vortexing of the cells in 10 × PBS, and normalized with sterile water. Lysates (inoculums) were cleared by centrifugation and overlaid on the HepG2/C3A cells (in 24-well plates), following 2.5 h incubation at 37 °C. The inoculums were aspirated and complete medium was added, and the cells were incubated for 6 d to establish infection and GFP production. The assay was done in duplicate and repeated for reproducibility.

#### **Fluorescence microscopy**

The replication fitness of the mutant RNA was indirectly assessed by careful observation of GFP-positive S10-3 and HepG2/C3A cells under fluorescence microscope. The expression of capsid protein in S10-3 cells was confirmed on day 4 post-transduction<sup>[32]</sup>. Briefly, vBacORF2 transduced cells (in 8-chambered glass slides) were immune-stained with anti-ORF2 chimp sera and Alexa Fluor 488 goat anti-human IgG (Molecular Probes, Eugene, OR, United States). Following mounting with Vectashield (Vector Laboratories, Burlingame, CA, United States), slides were observed under FITC filter-aided indirect fluorescence microscope (H600L; Nikon, Tokyo, Japan).

#### **Flow cytometry**

On day 6, the duplicated transfected S10-3 cultures (24-well plates) were harvested by trypsinization<sup>[32]</sup>. In sum, each culture suspension (about 500 µL in cold PBS) was cleared at 4 °C and cell pellets were re-suspended in 300 µL of cold PBS. The samples were immediately subjected to flow cytometry (10000 cells counted/sample), and data was analyzed for GFP-positive cells.

## **RESULTS**

#### **Mapping of potential palmitoylation-site and $\alpha$ -helix segment in the Y-domain**

Multiple sequence analysis of HEV strains and re-

presentative alphaviruses identified a potential palmitoylation-site homolog CC and an  $\alpha$ -helix counterpart LYSWLFE in the Y-domain, predicted for cytoplasmic membrane binding. The residues C<sub>336</sub>C<sub>337</sub> and their positions were highly conserved across the available sequences of all four human HEV genotypes (Figure 2). In the predicted  $\alpha$ -helix, while residue and positional conservation of L<sub>410</sub>, S<sub>412</sub> and W<sub>413</sub> were found among HEV and SFV, SINV and equine encephalitis virus (EEV) (Figure 3), the segment L<sub>410</sub>Y<sub>411</sub>S<sub>412</sub>W<sub>413</sub>L<sub>414</sub>F<sub>415</sub>E<sub>416</sub> showed a high conservation within the HEV genotypes (Figure 4).

#### **Y-domain nts 788-994 are indispensable for virus replication**

The saturation mutations introduced in the cDNA did not affect the gross yield of *in vitro* synthesized transcripts (Figure 5A, left). Of the 10 consecutive mutant transcripts (*pSK-GFP-Ydom1* to *Ydom10*), mutants of nts 788-994 (*Ydom3*, 4 and 5) drastically affected RNA replication by > 92% in S10-3 cells, whereas those of nts 650-787 (*Ydom1* and 2) and nts 995-1339 (*Ydom6*, 7, 8, 9 and 10) had very mild or insignificant effect on viability compared to the wild-type (*Ydom-WT*) (Figure 5B). This clearly demonstrated the indispensability of the internal sequences but not the up- or downstream sequences of the Y-domain at transcriptional level.

#### **Universally conserved C<sub>336</sub>, C<sub>337</sub> and W<sub>413</sub> are critical for RNA replication**

Similar to the saturation mutations, aa substitutions in the cDNA had no effect on the gross yield of *in vitro* synthesized RNA (Figure 5A, right). Introduction of C<sub>336</sub>A, C<sub>337</sub>A and W<sub>413</sub>A substitutions within the predicted membrane binding motif of Y-domain completely abolished virus replication (Figure 5B). Interestingly, the aa C<sub>336</sub>, C<sub>337</sub> and W<sub>413</sub> corresponded to codons (nts 1031-1033, 1034-1036 and 1213-1215, respectively) that were shown to be dispensable by saturation mutations. This very clearly indicated their post-translational functional/structural essentiality in virus replication, probably through membrane binding in intracellular replication complexes.

#### **Effects of RNA hairpin/stem-loop structures (nts 788-994) on virion infectivity**

As revealed by transfection results, *Ydom3* (nts 788-856) had the most drastic effect on RNA replication, followed by *Ydom5* (nts 926-994) and *Ydom4* (nts 857-925). In line with this, while nts 788-856 formed the most stable RNA hairpin/stem-loop compared to nts 926-994, nts 857-925 presented the least stable structure (Figure 6A). This strongly supported the deleterious effects of saturation mutations that could completely unzip and destabilize the RNA secondary structures, critical for virus replication. Further, the three saturation



Accession no.	a.a. 301	336/337
consensus	LFPSSA----CSTKSTFHAVPVHIWDRMLMFGATLDDQAFCCSRLMTYLRGISYKVTVGAL	
AF028091	...TS.....A.....T.	
AF076239	...TS.....A.....T.	
FJ457024	...TS.....T.....T.	
AF459438	...TS.....A.....A.T.	
JF443720	...TS.....A.....A.T.	
D10330	...TS.....A.....T.	
M73218	...TS.....A.....T.	
DQ459342	...TS.....A.....L.....T.	
JF443718	...TS.....A.....T.	
AF051830	...TS.....A.....T.	
JF443719	...TS.....A.....T.	
X99441	...TS.....A.....T.	
JF443721	...TS.....A.....T.	
JF443722	...TS.....T.....T.	
JF443723	...TS.....T.....T.	
JF443724	...TS.....T.....T.	
JF443725	...TS.....T.....A.T.	
JF443726	...TS.....T.....T.	
AF444002	...TS.....A.....T.	
AF444003	...TS.....A.....T.	
L25547	...TS.....A.....T.	
L25595	...TS.....A.....T.	
D11092	...TS.....A.....T.	
JQ655734	...TS.....S.....A.....T.	
L08816	...TS.....A.....T.	
D11093	...TS.....A.....T.	
JF443717	...TS.....A.....T.	
X98292	...TS.....A.....T.	
AY230202	...TS.....T.....T.	
M74506	...T.....AV.....T.....T.	
AB161718	...T.....A.....T.	
AB161719	...T.....A.....T.	
AB220972	...T.....A.....T.	
AB074917	...T.....A.....T.	
AB220973	...T.....A.....T.	
AB480825	...T.....A.....T.	
AB220975	...T.....A.....T.	
AB220978	...T.....A.....T.	
AB161717	...T.....A.....T.	
AB220976	...T.....A.....T.	
AB220977	...T.....A.....T.	
AB220979	...T.....A.....T.	
AB291965	...T.....A.....T.	
AB291966	...T.....A.....T.	
AB291967	...T.....A.....T.	
AB291968	...T.....A.....T.	
AB074915	...T.....A.....T.	
AB091395	...T.....A.....T.	
AB200239	...T.....A.....T.	
AB099347	...T.....A.....T.	
AB193176	...T.....A.....T.	
AB193177	...T.....A.....T.	
AB193178	...T.....A.....T.	
AB097811	...T.....A.....T.	
AB097812	...T.....A.....T.	
AB220971	...T.....A.....T.	
AB080575	...T.....A.....T.	
AB481227	...T.....A.....T.	
GU119961	...T.....A.....T.	
GU188851	...T.....A.....T.	
JQ655735	...T.....A.....T.	
JQ740781	...T.....I.....L.....T.	
AB220974	...T.....A.....T.	
AB369690	...T.....A.....T.	
JQ993308	...T.....A.....T.	

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EF570133 .....
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AB521805 .....
AB521806 .....
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AB602440 .....
DQ450072 ..... Y ..... P.R. ....
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GU119960 .....
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AB197674 .....
EF077630 .....
JQ655733 .....
FJ763142 .....
AY723745 ..... L .....
AJ272108 .....
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GU206559 .....
HM152568 .....
GU361892 ..... G .....
KC163335 .....
AY594199 .....
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AB253420 .....
AB291964 .....
EU676172 .....
JX855794 .....
DQ279091 ..... T .....
HM439284 ..... F .....
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AB698654 .....
AB573435 .....
AB602441 ..... N .....
AB074918 .....
AB630970 .....
AB074920 .....
AB089824 .....
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## II

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Accession no. a.a. 301 336/337
consensus LFPSA----CSTKSTFHAVPVHIWDRMLFGATLDDQAFCCSRLMTYLRGISYKVTVGAL
AY575858 .....
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AF082843 .....
AF060669 ..... X ..... X .....
FJ426404 .....
HQ389543 .....
JQ679014 .....
HQ389544 .....
HQ709170 .....
JQ679013 .....
AB591734 .....
AB073912 .....
AY115488 .....
AB291963 .....
AB630971 .....
AB222182 .....
AB189070 .....
AB698071 .....
AB189071 .....
AB189072 .....
AB189074 .....
AB189073 .....
AB189075 .....

```

AB369691	.....
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AB291962	.....
AB236320	.....
AB591733	.....
AB222183	..... Y .....
AB301710	.....
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AB091394	.....
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AB291953	.....
AB443625	..... V .
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AB291956	.....
AB443623	.....
AB443624	.....
AB443626	.....
AB291960	.....
AB291957	.....
AB291954	.....
AB291951	.....
AB222184	.....
AB369689	.....
AB740232	.....
FJ527832	.....
AB290312	..... L .....
JQ953664	.....
FJ705359	.....
FJ998008	.....
AB248520	.....
JQ013795	.....
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AB481226	.....
AB248522	.....
JQ953665	.....
JQ026407	.....
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EU375463	.....
AB369687	.....
FJ653660	.....
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EU723516	.....
JQ953666	.....
FJ956757	.....
JN906974	.....
JN906975	.....
JN906976	.....
EU495148	.....
EU360977	.....
EU723512	.....
EU723513	.....
AB290313	.....
AF455784	.....
AB740222	..... C ..... Y ..... T .
FJ906895	..... C ..... Y ..... T .
AB740220	..... C ..... T .





I

Accession no.	a.a.	410	416
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AF028091	...R...D.....		
AF076239	...R...D.....		
FJ457024	...R.....		
AF459438	...R...D.....		
JF443720	...R...D.....		
D10330	...R.....P.....		
M73218	...R.....		
DQ459342	...R.....		
JF443718	...R.....		
AF051830	...R.....		
JF443719	...R.....		
JF443721	...R.....		
JF443722	...R.....		
JF443723	...R.....		
JF443724	...R.....		
JF443725	...R.....		
JF443726	...R.....		
AF444002	...R.....		
AF444003	...R.....		
L25547	...R.....		
L25595	...R.....		
D11092	...R.....		
JQ655734	...R.....		
L08816	...R.....		
D11093	...R.....		
JF443717	...R.....		
X98292	...R.....		
AY230202	...R.....		
M74506	...L.....S.....		
AB161718	K...L.....		
AB161719	K...L.....		
AB220972	K...L.....		
AB074917	K...L.....		
AB220973	K...L.....		
AB480825	K...L.....		
AB220975	K...L.....		
AB220978	K...L.....		
AB161717	K...L.....		
AB220976	K...L.....		
AB220977	K...L.....		
AB220979	K...L.....		
AB291965	K...L.....		
AB291966	K...L.....		
AB291967	K...L.....		
AB291968	K...L.....		
AB074915	K...L.....		
AB091395	K...L.....		
AB200239	K...L.....		
AB099347	K...L.....		
AB193176	K...L.....		
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AB193178	K...L.....		
AB097811	K...L.....		
AB097812	K...L.....		
AB220971	K...L.....		
AB080575	K...L.....		
AB481227	K...L.....		
GU119961	K...LX.....		
GU188851	K...L.....		
JQ655735	K...L.....		
JQ740781	K...L.....		
AB220974	K...L.....		
AB369690	K...L.....		
JQ993308	K...L.....		
EF570133	K...L.....		

II

Accession no.	a.a.	410	416
consensus	RRLEVEHAQKFITRLYSWLFKSG		
AY575858	.....		
AY575859	.....		
AF082843	.....		
AF060669	.....		
JN837481	.....		
AB481228	.....		
FJ426403	.....		
FJ426404	.....		
HQ389543	.....		
JQ679014	.....		
HQ389544	.....		
HQ709170	.....		
JQ679013	.....		
AB591734	.....		
AB073912	.....		
AY115488	.....		
AB291963	.....		
AB630971	.....		
AB222182	.....		
AB189070	.....		
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AB291960	.....		
AB291957	.....		
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AB222184	.....		
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FJ527832	.....		
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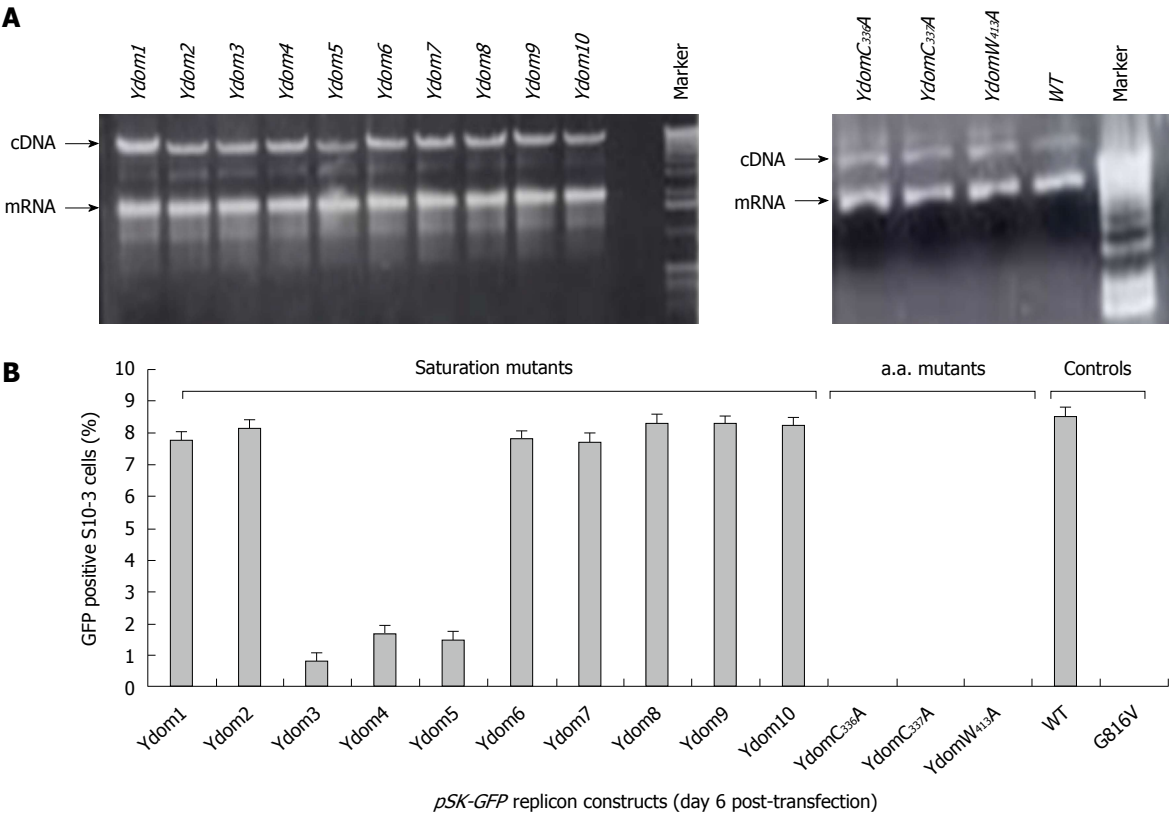
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EU366959	K...L.....	AB248522	.....
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AB197674	K...L.....	EU375463	.....
EF077630	K...L.....	AB369687	.....
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FJ763142	K...L.....	EU723514	.....
AY723745	K...L.....	EU723515	.....
AJ272108	K...L.....	EU723516	.....
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GU206559	K...L.....	FJ956757	.....
HM152568	K...L.....	JN906974	.....
GU361892	K...L.....	JN906975	.....
KC163335	K...L.....	JN906976	.....
AY594199	K...L.....	EU495148	.....
FJ610232	K...L.....	EU360977	.....
AB253420	K...L.....	EU723512	...I.....
AB291964	K...L.....	EU723513	...I.....
EU676172	K...L.....	AB290313	.....
JX855794	K...L.....	AF455784	.....
DQ279091	K...L.....	AB740222	.....V.....
AB108537	K...L.....	FJ906895	.....V.....
AB698654	K...L.....	AB740220	.....
AB573435	K...L.....	JQ768461	...L.....
AB602441	K...Q...R.V.....	JX109834	...L.....
AB074918	.....	JX121233	...L.....
AB630970	.....	GU937805	...L.....
AB089824	.....	AB740221	...L.....
AY575857	.....	FJ906896	...L.....
	*:* * * : * * *		*:* * * : * * *

**Figure 4** Multiple sequence analysis of human hepatitis E virus strains (GenBank;  $n = 206$ ), showing the highly conserved segment (L<sub>310</sub>Y<sub>311</sub>S<sub>312</sub>W<sub>313</sub>L<sub>314</sub>F<sub>315</sub>E<sub>316</sub>) of predicted membrane-binding helix ( $\alpha$ 1) within the ORF1 Y-domain.

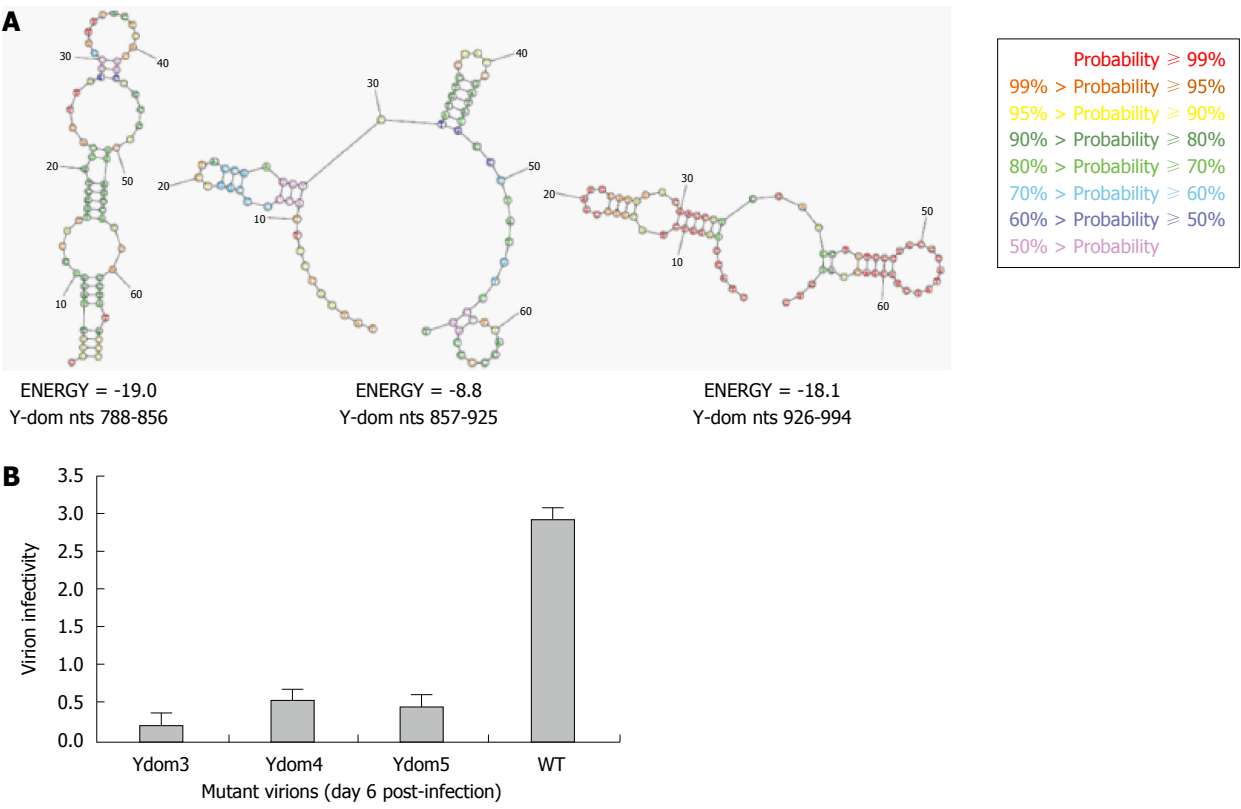
MTase core region, where C→A changes completely abolished palmitoylation<sup>[15,39]</sup>. These mutations were also shown to attenuate virus replication and pathogenicity in infected mice<sup>[8,9]</sup>. Likewise, the C<sub>420</sub>A mutation in SNV-nsP1 also had inhibitory effect on virus replication<sup>[8]</sup>. In ToMV, the palmitoylation-site counterpart contains three conserved but distantly located cysteines (C<sub>179</sub>/C<sub>186</sub>/C<sub>581</sub>), wherein C→S substitution strongly decreased membrane binding, 5' capping and RNA replication<sup>[10]</sup>. Akin to this, mutating the two conserved cysteines (C<sub>179</sub>/C<sub>186</sub>) at the same position aborted replication of BMV<sup>[11]</sup> and AMV<sup>[12]</sup>. Interestingly, mutation of the only C<sub>461</sub> of CMV-1a also abrogated membrane binding and RNA replication<sup>[13]</sup>. In accordance with this, C→A substitution of the highly conserved C<sub>336</sub>C<sub>337</sub> residues in the predicted palmitoylation-site of HEV Y-domain also abolished RNA replication completely.

Moreover, in addition to cysteine palmitoylation, many of the viral polyproteins contain consensus hydrophobic sequences for tight membrane binding<sup>[40]</sup>. In SFV-nsP1, the amphipathic segment GSTLYTESRKLLRSWHLPSV (aa 245-264) that forms an

$\alpha$ -helix, has been implicated in membrane binding<sup>[18,19]</sup> and RNA replication<sup>[21]</sup>. A mutational analysis of BMV and CMV-1a, wherein virus replication was abolished while affecting membrane binding and RNA recruitment, suggested the structural conservation of its amphipathic helix A<sup>[13]</sup>. Similarly, the poliovirus-2C<sup>[41]</sup> and hepatitis C virus (HCV)-NS5A<sup>[42]</sup> have been implicated in amphipathic helix-modulated interactions with intracellular membranes. In this report, a highly conserved segment LYSWLFE (aa 410-416) was mapped as the  $\alpha$ -helix counterpart of the ORF1 Y-domain. Sequence alignment showed the universal conservation of L<sub>410</sub>, S<sub>412</sub> and W<sub>413</sub> among HEV and the alphavirus-like superfamily. In line with this and the previously reported deleterious effect of W<sub>259</sub>A change on SFV replication<sup>[21]</sup>, the specifically selected W<sub>413</sub>A completely abolished HEV RNA replication. Notably, tryptophan is a signature hydrophobic residue that is critical for  $\alpha$ -helical protein folding for protein-protein interactions. Of the several examples, W<sub>630</sub> in the conserved motif KTXXXW of amphipathic helix of the G protein-coupled receptor Frizzled (C-terminal) has been shown crucial in intracellular protein



**Figure 5 Molecular characterization of hepatitis E virus Y-domain sequences.** A: Agarose-gel electropherograms showing the gross RNA yield of *pSK-GFP* saturation mutants: *Ydom1* to *Ydom10* (left panel) and specific amino acid mutants: *YdomC336A*, *YdomC337A* and *YdomW413A* (right panel), compared to wild-type (WT); B: Flow cytometry analysis of GFP-positive S10-3 cells, showing the replication competence of the Y-domain mutant replicons.



**Figure 6 Analysis of Y-domain mutant virions' infectivity.** A: *In silico* prediction of stable RNA hairpin/stem-loop structures (wild-type) of three consecutive regions (*Ydom3*: nts 788-856, *Ydom4*: nts 857-925 and *Ydom5*: nts 926-994); B: Flow cytometry analysis of naïve HepG2/C3A cell infectivity by trans-encapsitated virions harboring the three saturation mutant RNAs (*Ydom3*, *Ydom4* and *Ydom5*).

interactions<sup>[43]</sup>. Such functional/structural homology suggests the essentiality of the Y-domain (C-terminal) predicted  $\alpha$ -helix in HEV replication that may embody common principles of viral nonstructural proteins in membrane interaction.

In conclusion, the present study shows the indispensability of highly conserved sequences (nts 788-994) of ORF1 Y-domain in HEV RNA replication and infectivity. Also, the universally conserved C<sub>336</sub>, C<sub>337</sub> and W<sub>413</sub> residues corresponding to the dispensable codons within the predicted membrane binding motif of Y-domain are critical for virus viability. Taken together, this is the first demonstration of the essentiality of Y-domain in the HEV life cycle, probably through gene regulation and/or membrane binding in replication complexes. Nevertheless, further molecular and biochemical studies are recommended to validate these findings.

## COMMENTS

### Background

Hepatitis E virus (HEV) is the etiological agent of acute and chronic hepatitis in humans, worldwide. While there has been great progress in understanding the virus biology, the function of nonstructural open reading frame 1 "Y-domain" remains completely unexplored.

### Research frontiers

The author has performed *in silico* analysis of closely related single-strand RNA virus sequences and mapped a potential palmitoylation-site and  $\alpha$ -helix segment in the HEV nonstructural Y-domain. Mutational characterization of the viral replicon in S10-3 cells has shown the criticality of "Y-domain" residues C<sub>336</sub>C<sub>337</sub> and W<sub>413</sub> of the putative palmitoylation and helix, respectively. Further introduction of base mutations in the "Y-domain" severely affected RNA replication, revealing their post-transcriptional essentiality. Notably, the universally conserved C<sub>336</sub>C<sub>337</sub> and W<sub>413</sub> corresponded to non-conserved codons, indicating their post-translational indispensability. Moreover, RNA secondary structure prediction showed hairpin formations by the critical bases (nts 788-994) where mutations drastically affected virion infectivity of naïve HepG2 cells.

### Innovations and breakthroughs

This is a novel study showing a critical role of the hitherto undefined Y-domain in HEV genomic RNA replication and infectious virion production in cultured liver cells.

### Applications

The data warrants further biochemical and molecular studies of the Y-domain towards understanding HEV replication.

### Terminology

The putative palmitoylation and  $\alpha$ -helix motifs of the HEV Y-domain may be key to intracellular membrane-binding, essential for RNA replication and production of infectious virions.

### Peer-review

The author showed a critical role of the Y-domain in HEV genomic RNA replication and infectious virion production.

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## Basic Study

# Suppression of colorectal tumorigenesis by recombinant *Bacteroides fragilis* enterotoxin-2 *in vivo*

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## Abstract

### AIM

To evaluate the impact of recombinant *Bacteroides fragilis* enterotoxin-2 (BFT-2, or Fragilysin) on colorectal tumorigenesis in mice induced by azoxymethane/dextran sulfate sodium (AOM/DSS).

### METHODS

Recombinant proBFT-2 was expressed in *Escherichia coli* strain Rosetta (DE3) and BFT-2 was obtained and tested for its biological activity *via* colorectal adenocarcinoma cell strains SW-480. Seventy C57BL/6J mice were randomly divided into a blank (BC;  $n = 10$ ), model (AD;  $n = 20$ ), model + low-dose toxin (ADLT;  $n = 20$ , 10  $\mu$ g), and a model + high-dose toxin (ADHT;  $n = 20$ , 20  $\mu$ g) group. Mice weight, tumor formation and pathology were analyzed. Immunohistochemistry

determined Ki-67 and Caspase-3 expression in normal and tumor tissues of colorectal mucosa.

## RESULTS

Recombinant BFT-2 was successfully obtained, along with its biological activity. The most obvious weight loss occurred in the AD group compared with the ADLT group ( $21.82 \pm 0.68$  vs  $23.23 \pm 0.91$ ,  $P < 0.05$ ) and the ADHT group ( $21.82 \pm 0.68$  vs  $23.57 \pm 1.06$ ,  $P < 0.05$ ). More tumors were found in the AD group than in the ADLT and ADHT groups ( $19.75 \pm 3.30$  vs  $6.50 \pm 1.73$ ,  $P < 0.05$ ;  $19.75 \pm 3.30$  vs  $6.00 \pm 2.16$ ,  $P < 0.05$ ). Pathology showed that 12 mice had adenocarcinoma and 6 cases had adenoma in the AD group. Five mice had adenocarcinoma and 15 had adenoma in the ADLT group. Four mice had adenocarcinoma and 16 had adenoma in the ADHT group. The incidence of colorectal adenocarcinoma in both the ADHT group and the ADHT group was reduced compared to that in the AD group ( $P < 0.05$ ,  $P < 0.05$ ). The positive rate of Ki-67 in the ADLT group and the ADHT group was 50% and 40%, respectively, both of which were lower than that found in the AD group (94.44%,  $P < 0.05$ ,  $P < 0.05$ ). Caspase-3 expression in the ADLT group and the ADHT group was 45% and 55%, both of which were higher than that found in the BC group (16.67%,  $P < 0.05$ ,  $P < 0.05$ ).

## CONCLUSION

Oral administration with lower-dose biologically active recombinant BFT-2 inhibited colorectal tumorigenesis in mice.

**Key words:** Colorectal neoplasms; *Bacteroides fragilis* toxin; Fragilysin; Recombinant proteins; Mice

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**Core tip:** *Bacteroides fragilis* enterotoxin-2 (BFT-2) has been considered to promote the development of colorectal cancer (CRC). In this study, we obtained the biologically active recombinant BFT-2 *in vitro* and found that lower-dose of biologically active BFT-2 could inhibit colorectal tumor formation *via* the route of intra-gastric administration in a mice model of CRC, which manifested to inhibit cell proliferation and promoted apoptosis. The specific mechanism is still unknown but the findings provide some insights into prevention and treatment of CRC as an intestinal mucosal vaccine.

Lv Y, Ye T, Wang HP, Zhao JY, Chen WJ, Wang X, Shen CX, Wu YB, Cai YK. Suppression of colorectal tumorigenesis by recombinant *Bacteroides fragilis* enterotoxin-2 *in vivo*. *World J Gastroenterol* 2017; 23(4): 603-613 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/603.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.603>

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive tract<sup>[1]</sup>. The relationship between intestinal flora and CRC is an intense area of recent research<sup>[2,3]</sup>. Preliminary observations showed that the proportion of *Bacteroides* in patients with CRC increased and was accompanied by reduced diversity<sup>[4]</sup>.

*Bacteroides fragilis* (*B. fragilis*) is an obligate anaerobe that colonizes the lower digestive tract of humans. Enterotoxigenic *B. fragilis* (ETBF) is a subtype of *B. fragilis*, which can specifically secrete an extracellular 20 kDa zinc-dependent metalloproteinase referred to as *B. fragilis* toxin (BFT, or Fragilysin)<sup>[5,6]</sup>. BFT can damage the tight junction of the intestines, increase intestinal permeability, and provoke diarrhea<sup>[7]</sup>. Recent studies have shown that BFT is the major virulence factor of ETBF, and plays an important role in the occurrence and development of CRC<sup>[8-10]</sup>.

The current study aimed to obtain BFT-2 *via* genetic engineering and to evaluate the impact of BFT-2 on the formation of colorectal tumor by utilizing an AOM/DSS-induced mouse model of CRC.

## MATERIALS AND METHODS

### Materials

**Plasmid and strain:** Recombinant plasmid pET-32a containing the target gene<sup>[11]</sup>, pro-*B. fragilis* enterotoxin-2 without the signal peptide nucleotide sequence (proBFT-2), and positive clones of *Escherichia coli* (*E. coli*) strain Rosetta (DE3) were provided by Shanghai Biotechnology Corporation (GenBank ID: AB026626).

**Cell-line:** The human colorectal adenocarcinoma cell-line SW-480 was purchased from Shanghai Cell Bank of the Chinese Academy of Science.

**Animal experiments:** Seventy SPF certified C57BL/6J mice with a 17-g body weight, aged 5 wk were purchased and fed in the Animal Center of the Institute of Biomedical Laboratory of the East China Normal University. The production license was SCXK (Shanghai) 2011-0031, and the usage license was SYXK (Shanghai) 2010-0094. The animal experiment was approved by the 1990 Animal Ethics Committee of the East China Normal University, and the controlled environmental factors for experimentation strictly followed the relevant regulations of the national standard GB14925-2010 of the experimental animals that were released and implemented by the National Administration of Quality Supervision and Quarantine. All animals were permitted free access to food and water.

**Reagents:** Ni-NTA agarose gels were purchased from GE (United States). DMEM medium was purchased from Invitrogen (United States). IPTG was purchased from Sigma (United States). TEV protease was provided by Bioengineering (Shanghai, China). Azomethane (AOM) and dextran sulfate sodium (DSS) that was used to induce a state of CRC in the experimental mice were purchased from Sigma and MP (United States), respectively. Ki-67 and Caspase-3 antibodies were purchased from Abcam (United Kingdom).

## Methods

**Expression and purification of recombinant protein proBFT-2:** The nucleotide sequence coding for proBFT-2 without the signal peptide nucleotide sequence as the target gene was cloned to construct a recombinant plasmid pET-32a to produce the positive transformed clone strain DE3. Then, the positive transformed clone strains were cultured on a small scale, induced by IPTG, screened for the optimal condition of IPTG induction concerning concentration, temperature and time, and the SDS-PAGE method was utilized to select the best protein expression-inducing conditions. Then, the positive clones of DE3 with optimized inducing conditions were induced on a large scale. After IPTG induction of expression and cell sonication, the culture was centrifuged and the supernatants are applied to Ni-agarose affinity chromatography. The eluate was collected and tested by SDS-PAGE to detect recombinant proBFT-2 expression.

**Analysis of the purified proBFT-2:** ProBFT-2 was mixed with the TEV enzyme to remove the His-tag, and then an enzyme-digested protein solution was purified by nickel agarose affinity column chromatography. Finally, SDS-PAGE was used to detect the sample solution and elution before and after enzyme digestion.

**ProBFT-2 digestion and hydrolytic release of BFT-2:** In this procedure, 10 µg/mL trypsin was incubated with proBFT-2 solution at 37 °C for 1 h, leading to release of BFT-2 by proBFT-2 protein hydrolysis, following which it was purified by nickel column affinity chromatography, and the eluted fractions were collected. After the elution was dialyzed and purified, SDS-PAGE was performed. Finally, the elution was preserved at -80 °C.

**Detection of BFT-2 biological activity:** During the log phase of growth,  $5 \times 10^5$  SW-480 cells were seeded into a 24-well plate with a total volume of 50 µL of DMEM per well. The cells were incubated with 5% CO<sub>2</sub> at 37 °C for 2 d. Then, the medium was replaced with FBS-free DMEM, and 2 µg/mL BFT-2 was added to a final volume of 1 mL. After that, morphological

changes were observed by optical microscopy at 20, 40, 60, 90 and 120 min, respectively<sup>[12]</sup>.

**Grouping for the animal experiment:** After 1 wk of adaptive observation, the mice were divided into four groups: the blank control group (BC), the AOM/DSS group (AD), the AOM/DSS + low-dose toxin group (ADLT), and the AOM/DSS + high-dose toxin group (ADHT). Mice in the BC group drank and ate freely, while mice in the AD group were intraperitoneally injected with AOM (0.2 mg/kg) on the first day of the first week. From the second week onwards, the mice in the AD group were given 2% DSS-free drinking water, which lasted 1 wk. At the third week, the water provided for mice in the AD group was switched to normal drinking water. In addition, a 3-wk period was considered to be one cycle and the process lasted for three cycles in total. Based on the AD group, mice in the ADLT group and the ADHT group received intra-gastric administration of 10 µg and 20 µg BFT-2 on the first day of DSS drinking, and mice in the other groups were given an equal volume of saline as the control<sup>[13,14]</sup>.

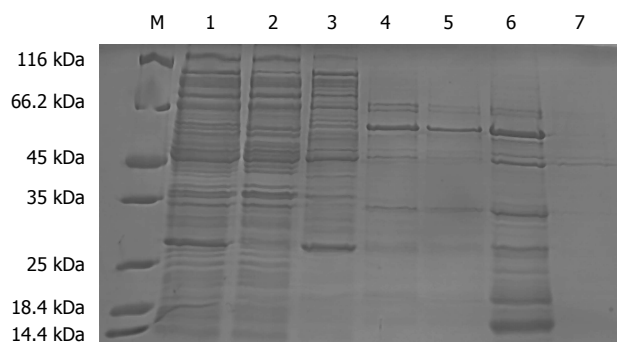
**Observation of the general condition of the animals:** The general conditions of the mice were observed during the experiment, including hair color, mental status, activities, feeding, defecation, nutritional status, presence or absence of anal bleeding, and rectal prolapse. Body weight of the mice was weighed and recoded once a week. At the end of wk 14, the mice were sacrificed by cervical dislocation after fasting for 12 h.

**Status of tumor formation:** The abdominal cavity was fully exposed after the mice were sacrificed, and the large intestine was dissociated and completely removed. Along the longitudinal axis, the intestinal tract was split and flattened. Then, PBS buffer was used to wash the intestinal tract. Finally, the number, location and the size of the tumors, and the length of the large intestine were recorded.

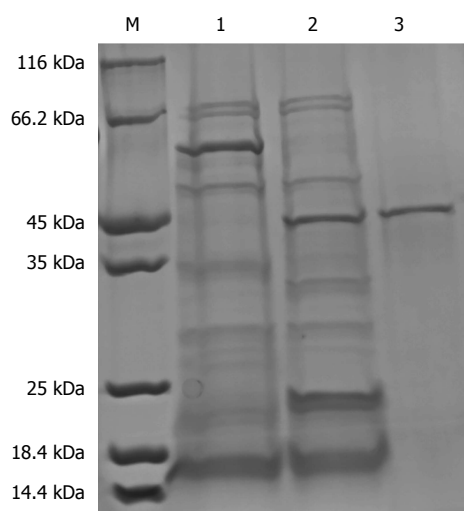
**Histopathological examination:** Normal tissue of the colorectal mucosa and tumor tissues were selected and fixed in 4% paraformaldehyde solution. After fixing, the following steps were performed and included dehydration, transparency, embedding with paraffin, and HE staining. Finally, the lesion level of the induced CRC was evaluated for each group.

**Immunohistochemical examination of normal colorectal mucosal tissues and tumor tissues:** The expressions of Ki-67 and Caspase-3 in the specimens of normal colorectal mucosal tissues and tumor tissues were measured by SP staining. Ki-67 is expressed in the nucleus, and Caspase-3 is expressed in the cytoplasm. Semi-quantitative





**Figure 1** SDS-PAGE (10%) analysis for nickel agarose affinity chromatography purification of fusion protein. A new protein band with a molecular weight of approximately 55 kDa appeared in the 500 mmol/L imidazole elution fractions. M: Protein marker; 1: Sample; 2: Outflow; 3: 20 mmol/L Imidazole elution fractions; 4, 5: 50 mmol/L Imidazole elution fractions; 6, 7: 500 mmol/L Imidazole elution fractions.

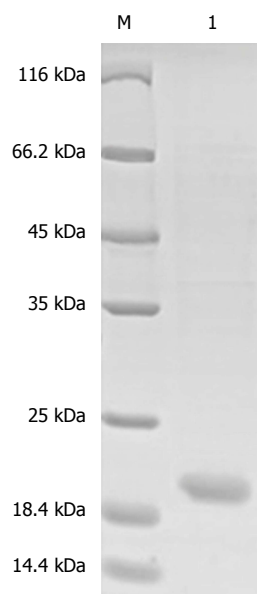


**Figure 2** SDS-PAGE (10%) analysis for nickel agarose affinity chromatography purification of fusion protein after TEV digestion. A protein band of about 42 kDa appeared after the new protein was cut by TEV enzyme. M: Protein marker; 1: Before TEV digestion; 2: After TEV digestion (sample); 3: Outflow.

scoring was used. Based on the staining intensity, the specimen was classified according to the following levels: negative staining was considered as 0 points, weakly positive staining (+, pale yellow) as 1 point, moderately positive staining (++ , brown) as 2 points and strongly positive staining (+++ , tan) as 3 points. Furthermore, the specimen was scored based on the proportion of the number of positive cells. According to the range of positive staining, 5% was considered as 0 points, 5%-25% as 1 point, 26%-50% as 2 points, and 51%-75% as 3 points; greater than 75% was considered as 4 points. Statistics were performed for positive immunoreactivity when the product of the staining intensity and the proportion of the range of positive observations was greater than or equal to 2.

### Statistical analysis

SPSS version 20.0 statistical software was used to



**Figure 3** SDS-PAGE (10%) analysis of proBFT-2 hydrolysis using trypsin. A new protein band with a molecular of 20 kDa appeared after the protein was digested with trypsin. M: Protein marker; 10 µg/mL trypsin hydrolyzes proBFT-2 at 37 °C for 1 h.

perform statistical analysis for the data. Continuous data that showed normal distribution was represented as mean  $\pm$  SD, and the independent sample Student's *t*-test was used to perform comparisons of the data. For comparisons of categorical data of two independent groups, the  $\chi^2$  test was used.  $P < 0.05$  was considered a statistically significant difference.

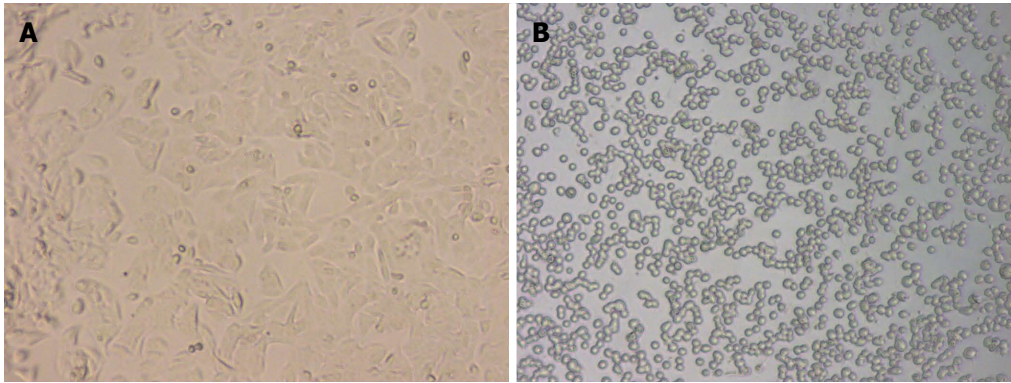
## RESULTS

### Expression and purification of recombinant protein BFT-2

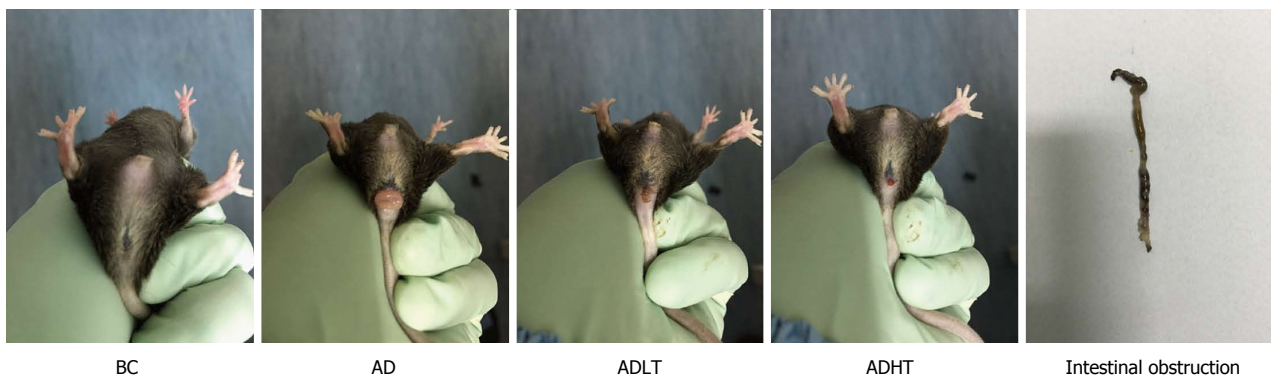
The result showed that a new protein band with a molecular weight of approximately 55 kDa appeared in the 500 mmol/L imidazole elution fractions, which was larger than that of the target protein proBFT-2 (42 kDa) (Figure 1). After the new protein was cut by TEV enzyme, a protein band of about 42 kDa appeared, which was in line with the molecular weight of proBFT-2 (Figure 2). After the protein was digested with trypsin, re-detection found a new protein band with a molecular weight of 20 kDa, which was consistent with the molecular weight of BFT-2 (Figure 3).

### Detection of BFT-2 biological activity

SW-480 is a human colorectal adenocarcinoma cell line, which grows adherently in a fusiform shape under a normal condition. After treatment with 2 µg/mL BFT-2 at 37 °C for 1 h, the SW-480 cells were observed by an optical microscope and found to show obvious morphological changes, such as cell rounding and separating from each other. This demonstrated that BFT-2 had biological activity<sup>[7,15]</sup> (Figure 4).



**Figure 4** Before BFT-2 treatment (A) and after BFT-2 treatment (B). SW-480, which grows adherently in a fusiform shape under a normal condition, showed cell rounding and separating from each other after treatment with BFT-2.



**Figure 5** General status of mice in each group. BC: Normal; AD: Rectal prolapse; ADLT: Tumor prolapse; ADHT: Bloody stools, intestinal obstruction.

### General status of the animals

At the initial 7 wk, the mice in each group had normal diet and defecation, and no red and swollen anus was found. Moreover, the mice were reactive to irritants. At 8 wk, the mice in the AD group began to present with messy and dull hair, lags in response, curled and lack of exercise and reduced diet, and some mice had loose stools accompanied by blood or black mucous secretions, while rectal prolapse occurred in 1 mouse. At 10 wk, the mice in the ADLT group and the ADHT group showed reduced appetite, fatigue, lack of exercise, and loose stools. At 14 wk, all the mice in the AD group had bloody stools accompanied by blood mucous secretions that was loose; in addition, rectal prolapse occurred in these mice, and 2 mice died. However, 8 mice in the ADLT group and 5 mice in the ADHT group showed rectal prolapse; in addition, bloody secretion and black stool appeared, but no death was found (Figure 5).

Body weight of the mice was not statistically significant when they were enrolled in the experiment. Body weight of the mice in the BC group showed a steady growth. At 8 wk, body weight of the mice in the AD group had decreased slowly. Moreover, it decreased slower in the ADLT group and the ADHT group as compared with the AD group. At 10 wk, body weight of the mice between the AD group and either the ADLT

group or the ADHT group was statistically significant. At 14 wk, body weight of the mice in the BC group was  $29.48 \pm 0.88$  g, in the AD group was  $21.82 \pm 0.68$  g, in the ADLT group was  $23.23 \pm 0.91$  g, and in the ADHT group was  $23.57 \pm 1.06$  g. The differences of the changes of average body mass of the mice in the BC group were all statistically significant with those among the AD group, the ADLT group and the ADHT group ( $P = 0.0001$ ,  $P = 0.0001$  and  $P = 0.0001$ , respectively). There was a significant difference in the alteration of the average body weight of the mice in the AD group when compared to the ADLT group and the ADHT group ( $P = 0.006$  and  $P = 0.002$ , respectively), but there was no statistical difference in the alteration of the average body weight of the mice between the ADLT group and the ADHT group ( $P = 0.632$ ) (Figure 6).

### Status of tumor formation

All the mice in the BC group showed no occurrence of formation of tumors, and the length of large intestine was  $8.90 \pm 0.10$  cm. In the AD group, the ADLT group and the ADHT group, multiple tumors with different sizes were found on the mucosal surface, and these tumors were placed into the intestine tract. In addition, most of these tumors were located in the distal site of the large intestine and in the anal canal,

**Table 1** Comparison of the average lengths of the large intestine, the numbers of tumors and the diameters of tumors of mice from each group

	BC group	AD group	ADLT group	ADHT group
Length of the large intestine, cm	8.90 ± 0.10	8.10 ± 0.40 <sup>1</sup>	8.27 ± 0.31 <sup>1</sup>	8.20 ± 0.10 <sup>1</sup>
Number of tumors	-	19.75 ± 3.30	6.50 ± 1.73 <sup>2</sup>	6.00 ± 2.16 <sup>2</sup>
Diameter of tumors, mm	-	1.72 ± 0.40	1.15 ± 0.41 <sup>3</sup>	1.07 ± 0.40 <sup>3</sup>

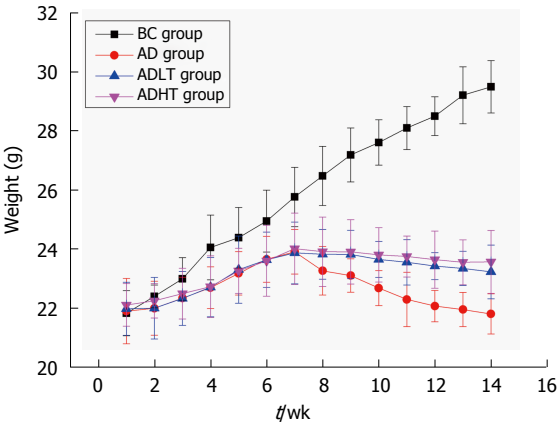
<sup>1</sup>Represents a statistical significance in the length of the large intestine when compared between the BC group and the AD group or the ADLT group or the ADHT group ( $P < 0.05$ , respectively); <sup>2</sup>Represents that the number of tumors in the AD group was statistically different from those in the ADLT group and the ADHT group ( $P < 0.05$ , respectively); <sup>3</sup>Represents that the average diameter of tumors in the AD group was statistically different from those in the ADLT group and the ADHT group ( $P < 0.05$ , respectively).

**Table 2** Expression of Ki-67 and Caspase-3 of mice from each group

Group	n	Ki-67		Caspase-3	
		-	+	-	+
BC	10	8 (80.00)	2 (20.00)	3 (30.00)	7 (70.00)
AD	18	1 (5.56)	17 (94.44) <sup>1</sup>	15 (83.33)	3 (16.67) <sup>3</sup>
ADLT	20	10 (50.00)	10 (50.00) <sup>1,2</sup>	11 (55.00)	9 (45.00) <sup>3,4</sup>
ADHT	20	12 (60.00)	8 (40.00) <sup>1,2</sup>	9 (45.00)	11 (55.00) <sup>3,4</sup>

<sup>1,3</sup>Represents that the expressions of Ki-67 and Caspase-3 in the BC group were statistically different when compared to that in the AD group or the ADLT group or the ADHT group ( $P < 0.05$ , respectively) and ( $P < 0.05$ , respectively); <sup>2,4</sup>Represents that the expressions of Ki-67 and Caspase-3 in the AD group were statistically different with those in the ADLT group and the ADHT group ( $P < 0.05$ , respectively) and ( $P < 0.05$ , respectively).

which were accompanied by different degrees of colon shortening. The length of the large intestine was  $8.10 \pm 0.4$  cm,  $8.27 \pm 0.31$  cm and  $8.20 \pm 0.1$  cm in the AD group, ADLT group and ADHT group, respectively. Furthermore, the number of formed tumors was  $19.75 \pm 3.30$ ,  $6.50 \pm 1.73$  and  $6.00 \pm 2.16$  in the AD group, ADLT group and ADHT group, respectively. In addition, the average diameter was  $1.72 \pm 0.40$  mm,  $1.15 \pm 0.41$  mm and  $1.07 \pm 0.40$  mm in the AD group, ADLT group and ADHT group, respectively. The length of the large intestine was shorter in the AD group, the ADLT group and the ADHT group, compared with that in the BC group ( $P = 0.027$ ,  $P = 0.028$  and  $P = 0.001$ , respectively), and there was no statistical difference among the AD group, the ADLT group and the ADHT group ( $P = 0.793$ ). The number of tumors in the AD group was significantly higher than that in the ADLT group and the ADHT group, and the difference was statistically significant ( $P = 0.0001$  and  $P = 0.0001$ , respectively), but there was no statistical difference between the ADLT group and the ADHT group. The average diameter of tumors in the AD group was larger than that in the ADLT group and the ADHT group ( $P = 0.006$  and  $P = 0.002$ , respectively), but there was



**Figure 6** Alteration of body weight of mice of each group. Body weight of the mice in the BC group showed a steady growth. Significant decline appeared in the AD group since 8 wk, compared with the ADLT and ADHT groups. At 14 wk, the body weight of mice in the BC group was  $29.48 \pm 0.88$  g, in the AD group was  $21.82 \pm 0.68$  g, in the ADLT group was  $23.23 \pm 0.91$  g, and in the ADHT group was  $23.57 \pm 1.06$  g. AD vs ADLT,  $P = 0.006$ ; AD vs ADHT,  $P = 0.002$ ; ADLT vs ADHT,  $P = 0.632$ .

no difference between the ADLT group and the ADHT group ( $P = 0.74$ ; Figure 7, Table 1).

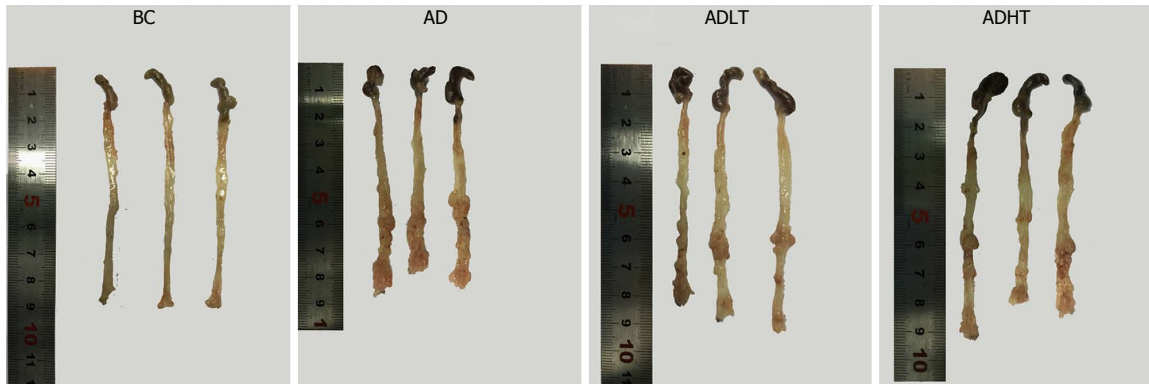
### Histopathologic changes

There were 12 cases of adenocarcinoma and 6 cases of adenoma in the AD group, 5 cases of adenocarcinoma and 15 cases of adenoma in the ADLT group, and 4 cases of adenocarcinoma and 16 cases of adenoma in the ADHT group. The incidence of CRC in both the ADHT group and the ADHT group was reduced compared to that in the AD group ( $P = 0.0001$  and  $P = 0.0001$ , respectively), and there was no significant difference between the ADLT group and the ADHT group ( $P > 0.05$ ). The CRC induced in the experiment were almost exclusively adenocarcinomas, which were well-differentiated tubular adenocarcinomas in terms of histology (Figure 8).

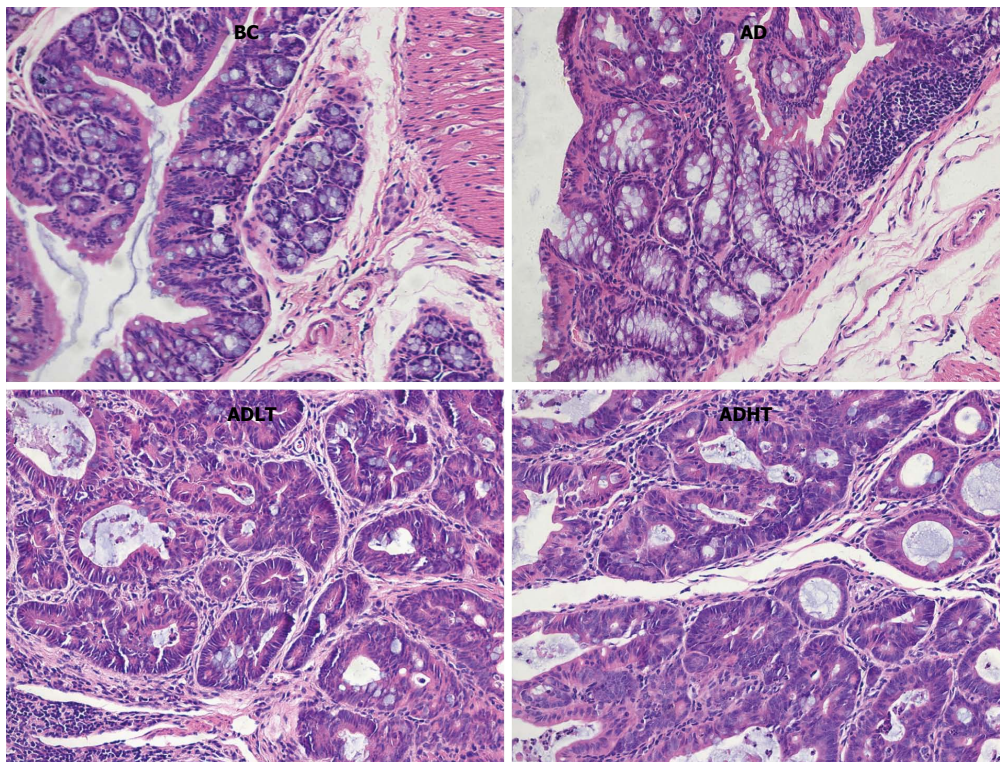
### Expression of Ki-67 and Caspase-3 in the colon

When compared to the BC group, Ki-67 had higher positive expression in the AD group, the ADLT group and the ADHT group, with statistical difference ( $P = 0.0001$ ,  $P = 0.0001$  and  $P = 0.002$ , respectively). Furthermore, there was a statistical significance between the AD group and either the ADLT group or the ADHT group ( $P = 0.0001$  and  $P = 0.0001$ , respectively), but no difference was found between the ADLT group and the ADHT group. There was statistically significant difference in Caspase-3 positive expression in the BC group when compared to the AD group, the ADLT group and the ADHT group ( $P = 0.0001$ ,  $P = 0.0001$  and  $P = 0.028$ , respectively). Moreover, the expression between the AD group and either the ADLT group or the ADHT group was statistically significant ( $P = 0.0001$  and  $P = 0.0001$ , respectively), but there was no difference between the





**Figure 7** Comparison of the samples *in vitro* of the large intestine of mice from each group. All the mice in the BC group showed no occurrence of the formation of tumors. In the AD group, the ADLT group and the ADHT group, multiple tumors of different sizes were found on the mucosal surface and located in the distal site of the large intestine and the anal canal, which were accompanied by different degrees of colon shortening.



**Figure 8** Comparison of the tissue of the large intestine of mice from each group. HE staining, magnification  $\times 200$ .

ADLT group and the ADHT group (Figures 9 and 10; Table 2).

## DISCUSSION

In recent years, the relationship between the intestinal flora and CRC has become a hot topic for research<sup>[16,17]</sup>. Previous studies have demonstrated that ETBF can promote the occurrence and development of CRC. Toprak *et al*<sup>[8]</sup> collected stool specimens of patients with CRC and from normal human subjects, and culture of those tissues and comparative analysis were performed. From those stool specimens, the identified ratio of *B. fragilis*

was 77% and 68%, respectively ( $P > 0.05$ ), and the positive ratio of BFT genes that were detected in the isolated strains of *B. fragilis* was 38% and 12%, respectively ( $P = 0.009$ ). Therefore, it was preliminarily concluded that BFT was closely associated with the occurrence and development of CRC. Boleij *et al*<sup>[9]</sup> compared the presence of BFT genes in the colorectal mucosa between cases with CRC and cases in the blank control group, finding that the detection rates of the left colon were 85.7% and 53.1% ( $P = 0.03$ ), and a detection rate of 91.7% and 55.5% in the right colon ( $P = 0.04$ ). Furthermore, positive rates of BFT genes were 100% and 72.7% in advanced and early CRC ( $P = 0.09$ ),



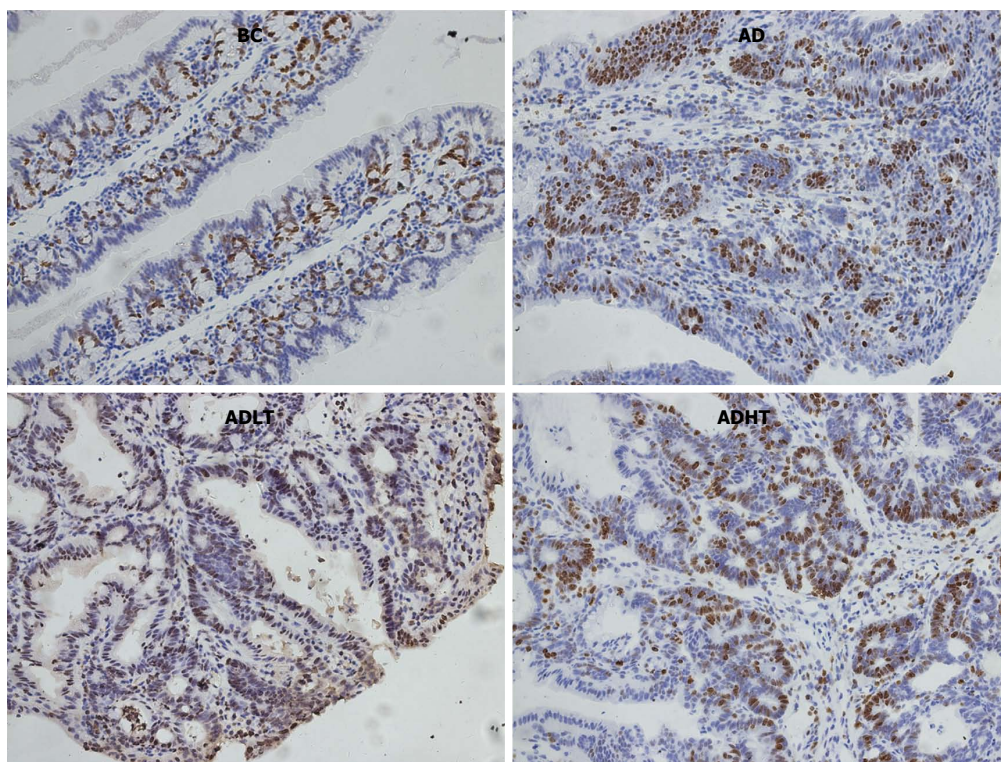


Figure 9 Expression of Ki-67 in the large intestine of mice from each group. SP staining, magnification  $\times 200$ .

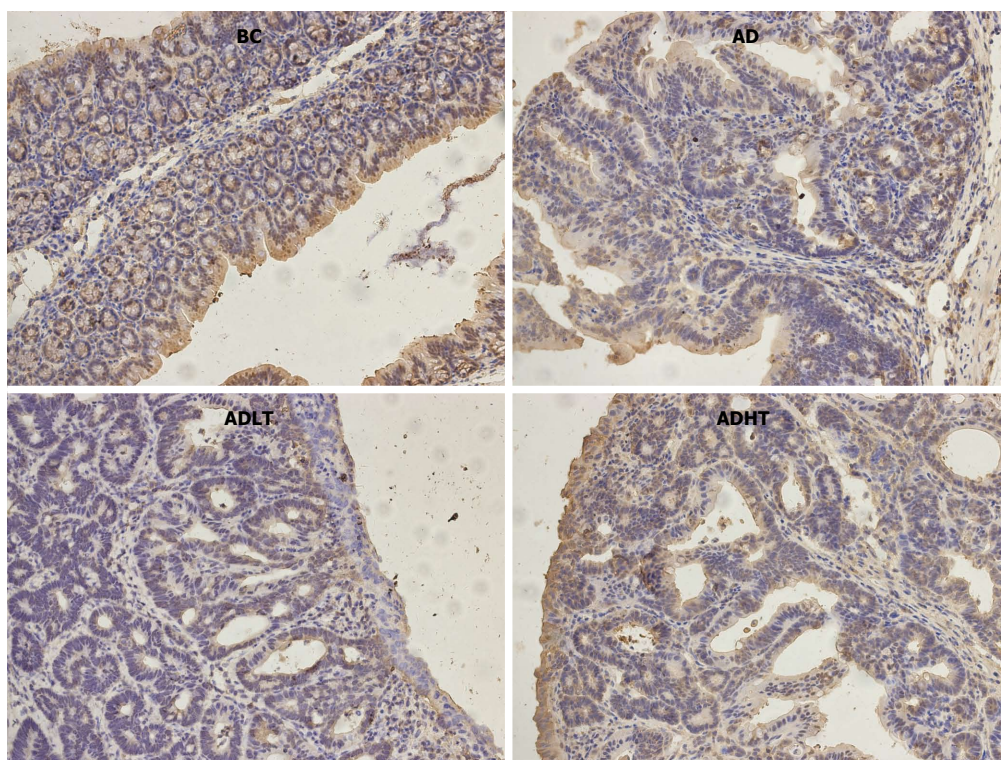


Figure 10 Expression of Caspase-3 in the large intestine of mice from each group. SP staining, magnification  $\times 200$ .

respectively, suggesting that BFT was a risk factor for the formation of CRC. Wu *et al.*<sup>[18]</sup> utilized APC<sup>min</sup> mice [which are multiple intestinal neoplasia (Min) mice that are heterozygous for the adenomatous polyposis coli (*apc*) gene] to perform ETBF single colonization through intra-gastric administration. This experiment indicated that ETBF promotes proliferation of colon cancer through the IL-17/IL-23 pathway. However, it remains largely unknown with respect to how BFT plays a prominent role in human cancer.

The initial design of this study was to verify how BFT-2 promotes the occurrence and development of CRC and to identify the possible mechanisms. However, the result was completely unexpected, and derived for us a completely opposite conclusion. The study found that both the ADLT group and the ADHT group showed the inhibition of colon tumor formation when BFT-2 was used to treat AOM/DSS-induced mice. The performance of the disease burden in both the ADLT and the ADHT group were light and delayed as compared with those in the AD group, and body weight significantly decreased slowly ( $P = 0.006$  and  $P = 0.002$ , respectively). At the end of the experiment, the average number of formed tumors in the ADLT and the ADHT groups was  $6.50 \pm 1.73$  and  $6.00 \pm 2.16$ , respectively, which were reduced significantly when compared to the AD group ( $19.75 \pm 3.30$ ;  $P = 0.0001$  and  $P = 0.0001$  respectively). Meanwhile, pathological results showed that the occurrence rate of CRC was 25% in the ADLT group, while it was 20% in the ADHT group, which were reduced when compared with that found for the AD group (66.7%;  $P = 0.0001$  and  $P = 0.0001$ , respectively).

In addition, Ki-67 had a high rate of positive expression in both the ADLT and the ADHT group, where it was 50% and 40%, respectively. The observations were statistically significant when compared with that found in the AD group (94.44%;  $P = 0.0001$  and  $P = 0.0001$ , respectively). The expression of Caspase-3 was 45% and 55% in the ADLT and ADHT groups, respectively, which were also statistically significant as compared with that found in the BC group (16.67%;  $P = 0.0001$  and  $P = 0.0001$ , respectively); this observation suggested that BFT-2 could inhibit the proliferation of cells and promote apoptosis.

ETBF can produce three different toxins, including BFT-1<sup>[19]</sup>, BFT-2<sup>[20]</sup> and BFT-3<sup>[11]</sup>, of which BFT-2 is the most active<sup>[21]</sup>. In this study, BFT-2 was shown to inhibit the formation of colorectal tumor in mice, and reflected a dual role of intestinal flora in the regulation of intestinal immune function. On one hand, some intestinal flora and their products as antigens can stimulate intestinal inflammation, causing DNA damage and further promoting the occurrence of cancer<sup>[2,22]</sup>. By contrast, they can regulate intestinal immunity, enhance the ability of intestinal immune cells to resist pathogens, improve pathogen tolerance, and down-regulate the release of inflammatory

cytokines that carry with them the risk of promoting tumors; thus, the toxicants have the capacity to inhibit the occurrence of tumors<sup>[23,24]</sup>. This effect also explains why the progression of CRC has an intricate association with inflammatory signaling pathways of the intestinal system.

The conclusions drawn from our study were similar to those described in the study of Doulberis *et al.*<sup>[14]</sup>. In that study, cholera toxin (CT) and a well-established mouse model of colon cancer was used in which tumor formation was initiated by a single dose of the genotoxic agent AOM, which subsequently promoted inflammation that was caused by the colitogenic DSS. Those authors found that a single and low non-pathogenic oral dose of CT administered at the beginning of each DSS treatment cycle down-regulated neutrophils and up-regulated regulatory T cells and IL-10 in the colonic mucosa.

CT-induced disruption of the tumor-promoting character of DSS-induced inflammation led to the reduction of AOM-initiated colonic polypoidogenesis. This result added value to the emerging notion that certain gastrointestinal tract bacteria or their products affect the immune system and render the microenvironment of preneoplastic lesions less favorable for promoting their evolution to cancer. Therefore, it is of great importance to develop specific bacterial antigens to regulate intestinal immunity, thus inhibiting intestinal tumorigenesis. These specific antigens may become safe and effective consolidation agents that can regulate intestinal immune function, which has the potential to control intestinal inflammatory in the future, and reduce the risk of relevant tumors<sup>[25]</sup>.

In summary, this study successfully constructed, expressed and purified recombinant protein BFT-2 through genetic engineering techniques. BFT-2 blocked the formation of CRC with the performance that alleviated disease in a murine model, and reduced the number and size of the formed tumors in mice. This study further elaborated the mechanisms of BFT-2 in inhibiting the formation of CRC, and it also demonstrated that key signaling pathways can provide some insights into the mechanistic underpinnings for the prevention and treatment of CRC by employing an intestinal mucosal vaccine.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The relationship between intestinal flora and colorectal cancer (CRC) is an



intense area of recent research. Their preliminary observations showed that the proportion of *Bacteroides* in patients with CRC increased and was accompanied by reduced diversity. Enterotoxigenic *Bacteroides fragilis* (ETBF), a subtype of *Bacteroides fragilis* (*B. fragilis*) which subordinated to *Bacteroides*, can specifically secrete an extracellular 20-kDa zinc-dependent metalloproteinase referred to *B. fragilis* enterotoxin (BFT). As the major virulence factor of ETBF, BFT plays an important role in the occurrence and development of CRC, but the specific mechanism *in vivo* has not yet been elucidated.

### Research frontiers

Previous experiments have already proved that the *BFT* genes are over-expressed in CRC patients, compared to healthy volunteers, and that intra-gastric administration of ETBF to APC<sup>min</sup> mice can accelerate the formation of CRC through the IL-17/IL-23 pathway.

### Innovations and breakthroughs

This is the first study evaluating the impact of recombinant BFT-2 on colorectal tumorigenesis in mice induced by azoxymethane/dextran sulfate sodium (AOM/DSS). The results showed that BFT-2 could inhibit colorectal tumor formation in mice, mainly reflected by alleviated disease manifestations, reduced colorectal tumor numbers and size, and inhibited formation of colorectal adenocarcinoma. It was also suggested that BFT-2 led to inhibited cell proliferation and promoted apoptosis. As there is no doubt for the outcomes.

### Applications

The authors speculated that oral administration with lower-dose BFT-2 could regulate intestinal immune function, enhance the intestinal disease tolerance and inhibit tumorigenesis similar to intestinal mucosal vaccine. The results reflect the dual role of intestinal flora and its metabolites in the occurrence and development of CRC, but its specific mechanism still needs further exploration.

### Terminology

BFT is the major virulence factor of ETBF, which is a zinc-dependent metalloproteinase essentially. BFT is synthesized in intracellular bacteria and released to the extracellular environment with biological activity which can stimulate E-cadherin cleavage and destruction of tight junctions in intestinal epithelium. Three subtypes of BFT have been isolated, among which BFT-2 is the most active and better studied. A method for obtaining pure BFT-2 is extraction from culture medium using complicated procedures and yielding poor products.

### Peer-review

The authors evaluated the impact caused by BFT-2 after induced oncogenesis of colorectal tumors with AOM/DSS in mice. Though the initial purpose of the study was to verify if BFT-2 was able to further stimulate cancer development, the authors found that BFT could actually inhibit tumor formation. It was also suggested that BFT-2 led to inhibited cell proliferation and promoted apoptosis. Though it would be interesting to see a control group treated only with BFT, its absence should not affect the interpretation of obtained results. Overall, the study was well-designed and the findings appear to be robust.

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## Retrospective Cohort Study

# Assessment of multi-modality evaluations of obscure gastrointestinal bleeding

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## Abstract

### AIM

To determine the frequency of bleeding source detection in patients with obscure gastrointestinal bleeding (OGIB) who underwent double balloon enteroscopy (DBE) after pre-procedure imaging [multiphase computed tomography enterography (MPCTE), video capsule endoscopy (VCE), or both] and assess the impact of imaging on DBE diagnostic yield.

### METHODS

Retrospective cohort study using a prospectively maintained database of all adult patients presenting with OGIB who underwent DBE from September 1<sup>st</sup>, 2002 to June 30<sup>th</sup>, 2013 at a single tertiary center.

## RESULTS

Four hundred and ninety five patients (52% females; median age 68 years) underwent DBE for OGIB. AVCE and/or MPCTE performed within 1 year prior to DBE (in 441 patients) increased the diagnostic yield of DBE (67.1% with preceding imaging *vs* 59.5% without). Using DBE as the gold standard, VCE and MPCTE had a diagnostic yield of 72.7% and 32.5% respectively. There were no increased odds of finding a bleeding site at DBE compared to VCE (OR = 1.3,  $P = 0.150$ ). There were increased odds of finding a bleeding site at DBE compared to MPCTE (OR = 5.9,  $P < 0.001$ ). In inpatients with overt OGIB, diagnostic yield of DBE was not affected by preceding imaging.

## CONCLUSION

DBE is a safe and well-tolerated procedure for the diagnosis and treatment of OGIB, with a diagnostic yield that may be increased after obtaining a preceding VCE or MPCTE. However, inpatients with active ongoing bleeding may benefit from proceeding directly to antegrade DBE.

**Key words:** Double balloon enteroscopy; Computed tomography enterography; Video capsule enteroscopy; Obscure gastrointestinal bleeding

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**Core tip:** The yield of double balloon enteroscopy (DBE) without preceding video capsule endoscopy (VCE) or multiphase computed tomography enterography (MPCTE) was 59.4%, and with preceding imaging was 67.5%. Overall diagnostic yield of antegrade DBE is superior to CTE and equivalent to VCE in the evaluation of obscure gastrointestinal bleeding. The diagnostic yields of DBE for inpatients *vs* outpatients were similar but the highest sensitivity of VCE using DBE as gold standard was in inpatients (84.9%). The incremental diagnostic yield of DBE of all patients with negative preceding VCE and MPCTE was 66% (35/53 patients). An appropriate strategy might be antegrade DBE in inpatients with evidence of ongoing bleeding if DBE is available.

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## INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined as bleeding from the gastrointestinal (GI) tract that

persists or recurs without an obvious etiology after negative upper endoscopy and colonoscopy<sup>[1,2]</sup>. OGIB is further categorized into "obscure overt" or "obscure occult" bleeding based on presence or absence of evident bleeding and accounts for approximately 5% of all GI bleeding<sup>[3]</sup>. Though lesions may be missed in the esophagus, stomach, and colon, the etiology of OGIB is secondary to small bowel pathology in up to 75% of cases<sup>[4-6]</sup> leading some experts to recommend that this term be replaced by the term 'small bowel bleeding'<sup>[7]</sup>. The evaluation of OGIB frequently requires significant utilization of resources and results in patient frustration due to lack of definitive findings and clinical improvement in many cases<sup>[8]</sup>.

The current American Society of Gastrointestinal Endoscopy guidelines recommend a variety of diagnostic options when evaluating OGIB, with slight differences between the "overt" and "occult" GI bleeding algorithms<sup>[9]</sup>. At many referral centers, multiphase computed tomography enterography (MPCTE) and/or video capsule endoscopy (VCE) are performed after a negative routine endoscopic exam but prior to double balloon enteroscopy (DBE) as these diagnostic studies are less invasive and may direct DBE-guided therapies<sup>[10,11]</sup>. MPCTE allows for evaluation of dynamic changes in abnormal enhancement patterns and compares findings across phases in 2-dimensional and 3-dimensional images<sup>[12]</sup>. Images are evaluated in arterial, enteric and delayed phases allowing for evaluation of ongoing or recent bleeding. In contrast, VCE requires ingestion of a small pill-size camera that provides endoluminal photographs of the entire GI tract for evaluation of small bowel mucosal lesions. These technologies are generally considered complimentary as each can provide different but vital information in the evaluation of OGIB. Though generally performed before DBE, there is a paucity of data regarding how often these tests alter subsequent diagnostic evaluation or treatment.

In this study, we aimed to determine the frequency of bleeding source detection in patients with OGIB who underwent DBE (antegrade/retrograde) after pre-procedure imaging (*i.e.* MPCTE, VCE, or both) and to assess the impact of imaging on DBE diagnostic yield. We also aimed to assess the agreement between findings of the pre-procedure imaging and the DBE itself.

## MATERIALS AND METHODS

A retrospective cohort study was conducted following the approval of the Institutional Review Board of Mayo Clinic-Rochester (IRB No. 13-002000 and No. 14-009997). Medical records were reviewed of all adult patients presenting with OGIB who underwent a DBE (antegrade/retrograde) from September 1<sup>st</sup>, 2002 to June 30<sup>th</sup>, 2013 using a prospectively maintained DBE database. Patients who underwent DBE for indications other than OGIB (*i.e.* enteral feeding tube placement,

failed colonoscopy, evaluation for hereditary polyposis syndromes, small bowel mass, strictures, etc.) were excluded. The electronic medical record was utilized to obtain demographic, endoscopic, radiologic, and clinical outcomes data. At our institution, single balloon enteroscopy is not utilized for assessment of OGIB, and hence this procedure was not included in our study.

Demographic features including age at the time of DBE procedure, gender, and gastrointestinal surgeries prior to DBE were recorded. The total number of blood transfusions up to 30 d prior to the date of DBE procedure and the use of anticoagulant/antiplatelet agents at the time of procedure were collected. Details of VCE and MPCTE performed prior to the DBE were also collected. Only VCE and MPCTE performed within 1 year prior to the DBE procedure were included. All VCE were performed using Pillcam or Pillcam2 (Given Imaging, Yoqneam, Israel). When VCE was performed prior to DBE the date of the procedure, positive and negative findings, and time from VCE to DBE were noted. Positive VCE findings were categorized as (1) arteriovenous malformation (AVM); (2) red spot; (3) frank blood; (4) polyp; (5) ulcer; or (6) other<sup>[13]</sup>. Similarly, when MPCTE was performed prior to DBE, positive or negative findings and time from MPCTE to DBE were recorded. Positive MPCTE findings were categorized as (1) vascular malformations; (2) blood; (3) polyp/tumor; (4) ulcer; and (5) other<sup>[14,15]</sup>. Cross-sectional imaging findings were abstracted from the final radiologic report.

DBE procedural details included the approach (antegrade vs retrograde), type of obscure bleeding (overt vs occult), and hospital admission status (inpatient vs outpatient). The OGIB was defined as "overt" when the clinician's note reported it to be overt or when there was clinically-evident bleeding including melena or hematochezia reported in the medical records. OGIB was defined as "occult" when the clinician's review reported it to be occult, or when iron deficiency anemia or positive stool testing for blood loss were the sole indication for DBE. We documented whether total enteroscopy was achieved, defined as complete evaluation of the small bowel using either a single approach or combined antegrade-retrograde approach. Findings from DBE were classified into (1) vascular lesions (angioectasias/AVMs, Dieulafoy's lesion, or ectopic varices); (2) mucosal lesions [erythema, erosions, ulcers, inflammation]; or (3) tumor/polyp<sup>[16]</sup>. If none of the above findings were seen, then the DBE was reported as negative. Therapies performed including argon plasma coagulation (APC), biopsy, hemostatic clip placement, bipolar cauterization, polypectomy and stricture dilation were also recorded. Adverse events including bleeding within 7 d of the procedure, perforation, pancreatitis and re-bleeding within 1 year of the procedure were recorded. Any repeat DBE performed

within 1 year of the index DBE was documented.

### Statistical analysis

The statistical methods of this study were reviewed by Joseph Larson and Felicity Enders, PhD from the Mayo Clinic Division of Health Sciences Research. Continuous measures were summarized using medians and ranges while categorical measures were summarized using counts and percentages. Differences among two groups were assessed using the Kruskal-Wallis test and Chi-square or Fisher's exact test for continuous or categorical measures, respectively.

To evaluate the predictive ability of VCE and MPCTE to identify bleeding sites, DBE was treated as the gold standard and the sensitivity, specificity, diagnosis yield, and accuracy were calculated among patients with VCE and MPCTE within one year of DBE. Ninety-five percent confidence intervals for each of these measures were also determined. This analysis was repeated among the following subgroups; antegrade and retrograde approach, inpatient and outpatient procedure, overt and occult bleeding.

Because the same patients underwent VCE or MPCTE and DBE, to assess the findings from the procedures, matched logistic regression performed with the finding treated as the outcome and the DBE test treated as the predictor. Odds ratios and 95% confident intervals along with *P* values are presented for these tests.

All analyses used an significance level of 5% and were performed using the SAS (v9.3, SAS Institute Inc., Cary, NC, United States).

## RESULTS

During the study period, 495 patients [51.5% females; median age 68.2 (range: 18.1-95.4) years] underwent DBE for OGIB. Overt OGIB was reported in 253 (51.1%) patients, and occult OGIB was reported in 242 (48.9%) patients. The procedure was performed in an outpatient setting in 381 (77.0%) patients and in an inpatient setting in 114 (23.0%) patients. The type of DBE approach was antegrade in 331 (75.1%) patients and retrograde in 110 (24.9%) patients. Total enteroscopy was achieved in a bidirectional manner in 19 (4.3%). Additional demographic data including DBE cases with surgically-altered anatomy and the use anticoagulant/antiplatelet agents is noted in Table 1.

Of the 495 patients, 458 patients had had VCE and/or MPCTE performed prior to DBE (441 patients within 1 year prior to DBE). Of the 441 patients, 296 had a positive DBE finding (yield of 67.1%). The findings noted on DBE in these patients are outlined in Table 2. The remaining 37 patients underwent a DBE without a preceding VCE or MPCTE. Of these 37 patients, 22 had a positive finding (yield of 59.5%, *P* = 0.36).

Among the 441 patients with VCE and/or MPCTE

**Table 1 Patient characteristics *n* (%)**

	DBE negative ( <i>n</i> = 164)	DBE positive ( <i>n</i> = 331)	Total ( <i>n</i> = 495)	<i>P</i> value
Age at DBE, median (range)	65.0 (18.1-95.4)	69.5 (18.3-91.8)	68.2 (18.1-95.4)	0.005 <sup>1</sup>
Gender				0.104 <sup>2</sup>
Male	71 (43.3)	169 (51.1)	240 (48.5)	
Female	93 (56.7)	162 (48.9)	255 (51.5)	
Altered anatomy				0.736 <sup>2</sup>
None	125 (76.2)	249 (75.2)	374 (75.6)	
Roux-en-Y	13 (7.9)	21 (6.3)	34 (6.9)	
Billroth/Ileo-colonic/IPAA	7 (4.3)	21 (6.3)	28 (5.7)	
Other	19 (11.6)	40 (12.1)	59 (11.9)	
On warfarin	19 (11.6)	36 (10.9)	55 (11.1)	0.813 <sup>2</sup>
On clopidogrel	7 (4.3)	20 (6.0)	27 (5.5)	0.413 <sup>2</sup>
On ASA 325	49 (29.9)	118 (35.6)	167 (33.7)	0.201 <sup>2</sup>
VCE performed within 1 yr prior to DBE	47 (29.4)	130 (40.9)	177 (36.9)	
MPCTE performed within 1 yr prior to DBE	31 (19.4)	53 (16.7)	84 (17.5)	
VCE and MPCTE performed within 1 yr prior to DBE	67 (41.9)	113 (35.5)	180 (37.8)	
DBE performed without VCE and MPCTE done	15 (9.4)	22 (6.9)	37 (7.7)	
Type of OGIB				0.728 <sup>2</sup>
Overt	82 (50.0)	171 (51.7)	253 (51.1)	
Occult	82 (50.0)	160 (48.3)	242 (48.9)	
Type of approach of DBE				< 0.001 <sup>2</sup>
Anterograde	103 (62.8)	268 (81.0)	371 (74.9)	
Retrograde	61 (37.2)	63 (19.0)	124 (25.1)	
Total enteroscopy done	9 (5.5)	15 (4.5)	24 (4.8)	0.641 <sup>2</sup>
Procedure location				0.393 <sup>2</sup>
Inpatient	34 (20.7)	80 (24.2)	114 (23.0)	
Outpatient	130 (79.3)	251 (75.8)	381 (77.0)	

<sup>1</sup>Kruskal Wallis; <sup>2</sup> $\chi^2$ . VCE: Video capsule endoscopy; MPCTE: Multiphase computed tomography enterography; DBE: Double balloon enteroscopy; IPAA: Ileal pouch-anal anastomosis; OGIB: Obscure gastrointestinal bleeding.

**Table 2 Double balloon enteroscopy findings, therapy and complications *n* (%)**

	DBE negative ( <i>n</i> = 145)	DBE positive ( <i>n</i> = 296)	Total ( <i>n</i> = 441)
DBE findings			
Angiectasia/arterio venous malformation	0 (0.0)	212 (71.6)	212 (48.1)
Dieulafoy lesion	0	8 (2.7)	8 (1.8)
Varix	0	3 (1.0)	3 (0.7)
Evidence of Crohn's disease	0	5 (1.7)	5 (1.1)
Erythema	0	19 (6.4)	19 (4.3)
Erosion	0	31 (10.1)	31 (6.8)
Ulcer	0	56 (18.9)	56 (12.7)
Polyp identified	0	50 (16.9)	50 (11.3)
Other findings	40 (27.6)	64 (21.6)	104 (23.6)
Therapy and complications			
Any therapy done?	4 (2.8)	296 (100)	300 (68.0)
Epinephrine injection	1 (0.7)	2 (0.7)	3 (0.7)
Biopsy	3 (2.1)	89 (30.1)	92 (20.9)
Clipping	0	67 (22.6)	67 (15.2)
Argon plasma coagulation	0	213 (72.0)	213 (48.3)
Bipolar cauterization	0	10 (3.4)	10 (2.3)
Early re-bleeding (< 24 h)	0	7 (2.4)	7 (1.6)
Late re-bleeding (24 h-1 yr)	4 (2.8)	14 (4.7)	18 (4.1)
Pancreatitis	0	1 (0.3)	1 (0.2)

VCE: Video capsule endoscopy; MPCTE: Multiphase computed tomography enterography; DBE: Double balloon enteroscopy.

prior to DBE, therapeutic or diagnostic applications were performed in 300 (68.0%) patients including APC in 213 (48.3%) patients, biopsy in 92 (20.9%) patients, hemostatic clip placement in 67 (15.2%) patients, and bipolar cauterization in 10 (2.3%) patients. Early

rebleeding (< 24 h from the time of procedure) was reported in 7 (2.4%) patients when the DBE was positive. Late rebleeding (24 h - 1 year from the time of index DBE) was reported in 14 (4.7%) patients when DBE findings were positive and 4 (2.8%) patients when



**Table 3** Video capsule endoscopy and multiphase computed tomography enterography findings of all patients who had a video capsule endoscopy and multiphase computed tomography enterography performed within 1 year prior to double balloon enteroscopy *n* (%)

	Capsule endoscopy				Computed tomography enterography			
	DBE negative ( <i>n</i> = 104)	DBE positive ( <i>n</i> = 233)	Total ( <i>n</i> = 337)	<i>P</i> value	DBE negative ( <i>n</i> = 95)	DBE positive ( <i>n</i> = 157)	Total ( <i>n</i> = 252)	<i>P</i> value
Capsule endoscopy positive	74 (71.2)	171 (73.4)	245 (72.7)	0.692 <sup>2</sup>	27 (28.4)	55 (35.0)	82 (32.5)	0.332 <sup>2</sup>
MPCTE positive								
Days from VCE to DBE, median (range)	42 (0-356)	35 (0-351)	37 (0-356)	0.924 <sup>1</sup>	19 (0-338)	29 (0-351)	23.5 (0-351)	0.162 <sup>1</sup>
Days from MPCTE to DBE, median (range)								
VCE and DBE within 30 d of each other	41 (39.4)	105 (45.1)	146 (43.3)	0.344 <sup>2</sup>	59 (62.1)	84 (53.5)	143 (56.7)	0.192 <sup>2</sup>
MPCTE and DBE within 30 d of each other								
Arterio-venous malformation	27 (26.0)	94 (40.3)	121 (35.9)	0.014 <sup>2</sup>	13 (48.1)	32 (58.2)	45 (54.9)	0.108 <sup>2</sup>
Vascular lesion								
Blood	23 (22.1)	51 (21.9)	74 (22.0)	0.999 <sup>2</sup>				
Red spot	13 (12.5)	18 (7.7)	31 (9.2)	0.220 <sup>2</sup>				
Polyp	4 (3.8)	12 (5.2)	16 (4.7)	0.784 <sup>2</sup>				
Ulcer	17 (16.3)	20 (8.6)	37 (11.0)	0.040 <sup>2</sup>	2 (7.4)	1 (1.8)	3 (3.7)	
Other					2 (7.4)	0 (0.0)	2 (2.4)	

<sup>1</sup>Kruskal Wallis; <sup>2</sup>Fisher exact. VCE: Video capsule endoscopy; MPCTE: Multiphase computed tomography enterography; DBE: Double balloon enteroscopy.

**Table 4** Comparison of video capsule endoscopy and multiphase computed tomography enterography in all patients with capsule endoscopy and computed tomography enterography performed within 1 year of double balloon enteroscopy

Comparison test	Statistic	Count summary	%	95%CI	
				Lower limit (%)	Upper limit (%)
VCE	Total, <i>n</i>	337			
	Sensitivity	171/233	73.4	67.7	79.1
	Specificity	30/104	28.8	20.1	37.6
	DX yield	245/337	72.7	67.9	77.5
	Accuracy	201/337	59.6	54.4	64.9
MPCTE	Total, <i>n</i>	252			
	Sensitivity	55/157	35.0	27.6	42.5
	Specificity	68/95	71.6	62.5	80.6
	DX yield	82/252	32.5	26.8	38.3
	Accuracy	123/252	48.8	42.6	55.0

VCE: Video capsule endoscopy; MPCTE: Multiphase computed tomography enterography.

DBE findings were negative (Table 2). A single patient developed pancreatitis and there were no perforations as complications.

Among 337 patients who had a VCE performed within the year preceding DBE, a bleeding site was identified at VCE in 171 (73.4%) patients when the DBE was positive and in 74 (71.2%) patients when the DBE was negative ( $P = 0.692$ ). The median number of days between VCE and DBE was not significantly different when the DBE was positive (42; range: 0-356) or negative (35; range: 0-351) ( $P = 0.924$ ) (Table 3). Using DBE as the gold standard, VCE had a sensitivity of 73.4% (95%CI: 67.7%-79.1%), specificity of 28.8% (95%CI: 20.1%-37.6%), and diagnostic yield of 72.7% (95%CI: 67.9%-77.5%) (Table 4). Among the patients with negative DBE, the commonest VCE findings were AVM (26.0%), blood (22.1%) and ulcer (16.3%).

In 252 patients who had MPCTE in the year preceding DBE, a bleeding site was identified in 55 (35.0%)

patients when the DBE was positive and in 27 (28.4%) patients when the DBE was negative. The median number of days between MPCTE to DBE was not significantly different when the DBE was positive (19.0; range: 0-338) or negative (29; range: 0-351) ( $P = 0.162$ ) (Table 3). Using DBE as the gold standard, MPCTE had a sensitivity of 35.0% (95%CI: 27.6%-42.5%), specificity of 71.6% (95%CI: 62.5%-80.6%), and diagnostic yield of 32.5% (95%CI: 26.8%-38.3%) (Table 4). Among patients with negative DBE, the most common MPCTE findings were vascular lesions (48.1%), blood (33.3%), and ulcer (7.4%).

Of 53 patients who had preceding negative test(s), 35 (66.0%) had a positive DBE. Of these with positive DBE, 28 (80.0%) were antegrade DBE, 16 (45.7%) were for overt bleeding, and only 4 (11.4%) were in inpatients. AVMs were the commonest finding, found in 23 (65.7%) patients and treated with APC.

Of the 37 patients who went straight to DBE without preceding CE or MPCTE, the DBE was done

antegrade in 26 (70.3%) patients, in 13 (35.1%) inpatients, and for overt bleeding in 26 (70.3%) patients. The commonest findings at DBE were AVM in 17 (45.9%) and ulcer in 8 (21.6%).

In order to compare findings on DBE to VCE and MPCTE, matched odds ratios were examined. There were no increased odds of finding a bleeding site at DBE compared to VCE (OR = 1.3, 95%CI: 0.9-1.7,  $P = 0.150$ ). There were increased odds of finding a bleeding site at DBE compared to MPCTE (OR = 5.9, 95%CI: 3.5-9.7,  $P < 0.001$ ).

When comparing by DBE approach, VCE had a diagnostic yield of 75.6% (95%CI: 70.3%-80.9%) with an antegrade approach; and a diagnostic yield of 63.9% (95%CI: 53.5%-74.2%) with a retrograde approach. MPCTE had a diagnostic yield of 31.6% (95%CI: 24.9%-38.2% with the antegrade approach and diagnostic yield of 35.4% (95%CI: 23.8%-47.0%) with a retrograde approach (Supplementary Table 1).

When comparing by procedure setting, VCE had a diagnostic yield of 76.7% (95%CI: 67.0%-86.4%) in the inpatient setting and a diagnostic yield of 71.6% (95%CI: 66.2%-77.0%) in the outpatient setting. With the inpatient setting, MPCTE had a diagnostic yield of 40.8% (95%CI: 27.1%-54.6%) and a diagnostic yield of 30.5% (95%CI: 24.2%-36.9%) with the outpatient setting (Supplementary Table 2). Thus, in the inpatient setting, VCE had higher diagnostic yield than MPCTE.

When comparing overt to occult bleeding, VCE had a diagnostic yield of 72.9% (95%CI: 66.3%-79.6%) for overt bleeds and a diagnostic yield of 72.5% (95%CI: 65.7%-79.2%) for occult bleeds. With overt bleeds, MPCTE had a diagnostic yield of 34.4% (95%CI: 26.0%-42.9%) and a diagnostic yield of 30.8% (95%CI: 22.8%-38.7%) with occult bleeds (Supplementary Table 3).

## DISCUSSION

Small bowel bleeding is the commonest cause of OGIB, seen in 75% of cases<sup>[17]</sup>. Identifying the site of bleeding and its therapy remain challenging due to this anatomic location. DBE is an effective way to address these challenges but is costly and not readily available at all centers. Our study aimed to look at the diagnostic yield of DBE and of preceding VCE and MPCTE. This would allow us to analyze the need for imaging prior to DBE. In our large single center cohort of 495 patients with OGIB, the yield of DBE without preceding VCE or MPCTE was 59.4%, and with preceding imaging was 67.5%. Thus, although the diagnostic yield of DBE is higher when pre-DBE imaging is positive, a source lesion is frequently identified when pre-DBE imaging is negative or not performed.

Using direct visualization by DBE as the gold standard, VCE had a diagnostic yield of 72.7% but a relatively low specificity of 28.8%. This is similar to prior studies<sup>[18]</sup>. The commonest findings at VCE with

negative DBE were AVMs and blood; it is possible that these abnormalities had subsided by the time of the DBE since the time interval between the tests in our study could be up to 1 year<sup>[11,19]</sup>. This would be characteristic of AVMs which often bleed intermittently, and could artificially increase the apparent false positive rate.

A preceding MPCTE was done in fewer patients compared to VCE and had a lower diagnostic yield of 32.5%. However, the specificity was higher at 71.6%, also similar to previous studies<sup>[14,20,21]</sup>. The vascular lesions seen in nearly half the patients with positive MPCTE and negative DBE were likely deep in the bowel wall and hence not seen endoscopically.

Antegrade DBEs overall had higher diagnostic yields than retrograde DBEs (72.51% vs 50.91%,  $P < 0.001$ ). This has been shown in one other smaller series<sup>[22]</sup>. Thus, the overall diagnostic yield of antegrade DBE is superior to CTE and roughly equivalent to VCE in the evaluation of OGIB. This is an important finding because it suggests that almost all patients should undergo antegrade DBE before retrograde, unless otherwise dictated by abnormal MPCTE suggesting ileal tumors or polyps.

The diagnostic yields of DBE for inpatients vs outpatients were similar in our data but the highest sensitivity of VCE using DBE as gold standard was in inpatients (84.9%). This group also showed the highest specificity (45.0%). Interestingly the incremental diagnostic yield of DBE of all patients with negative preceding VCE and MPCTE was 66% (35/53 patients). Thus, this raises the question of whether an appropriate strategy might be to directly proceed to antegrade DBE in inpatients with evidence of ongoing bleeding if DBE is available. This is also reflected by the matched odds ratios comparing VCE and MPCTE to DBE where there were no increased odds of finding a bleeding site at DBE compared to VCE and increased odds at DBE compared to MPCTE. In our data, none of the tests had a significantly higher yield in patients with overt bleeding compared to occult bleeding, which is unlike prior studies<sup>[23-25]</sup>.

In conclusion, our data suggest that DBE is a generally safe and well tolerated procedure for the diagnosis and treatment of OGIB, with a diagnostic yield that may be increased after obtaining a preceding VCE or MPCTE. However, inpatients with active ongoing bleeding may benefit from proceeding directly to antegrade DBE, which has the benefits of improved diagnostic yield in these patients, ability to intervene therapeutically, and avoidance of an additional diagnostic test. A prospective evaluation and cost-effectiveness analysis of this clinical algorithm would be warranted.

## COMMENTS

### Background

The etiology of obscure gastrointestinal bleeding (OGIB) is secondary to small

bowel pathology in up to 75% of cases. The authors sought to determine the frequency of bleeding source detection in patients with OGIB who underwent double balloon enteroscopy (DBE) after pre-procedure imaging (multiphase computed tomography enterography, video capsule endoscopy, or both) and assess the impact of imaging on DBE diagnostic yield.

### Research frontiers

Diagnostic yields of DBE, computed tomography enterography and video capsule endoscopy in obscure gastrointestinal (GI) bleeding.

### Innovations and breakthroughs

This is the one of the largest cohort of patients with occult GI bleeding undergoing DBE for occult GI bleeding. A large proportion of patients also had preceding imaging, allowing for comparison of the various techniques.

### Applications

Inpatients with active ongoing bleeding may benefit from proceeding directly to antegrade DBE without preceding testing, which has the benefits of improved diagnostic yield in these patients, ability to intervene therapeutically, and avoidance of an additional diagnostic test.

### Terminology

Obscure GI bleeding - GI bleeding where etiology is not in the esophagus, stomach, or colon.

### Peer-review

Even though the theme has been studied for years all over the world, it is still necessary to update and clarify the best approach to a challenging clinical entity such as OGIB. This study is up to date and follows the most recent guidelines and studies.

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## Retrospective Cohort Study

# Complications and management of forgotten long-term biliary stents

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## Abstract

### AIM

To evaluate complications and management outcomes of retained long-term plastic biliary stents.

### METHODS

Endoscopic plastic biliary stent placement was performed in 802 patients at Yeungnam University Hospital between January 2000 and December 2014. Follow-up loss with a subsequently forgotten stent for more than 12 mo occurred in 38 patients. We retrospectively examined the cause of biliary stent insertion, status of stents, complications associated with biliary stents and management outcomes of long-term plastic biliary stents. Continuous variables were analyzed using the *t* test. Observed frequencies in subsets of the study population were compared using Fisher's exact test and  $\chi^2$  tests. Statistical significance was defined as  $P < 0.05$  (two-tailed).

### RESULTS

Mean age of patients was  $73.7 \pm 12$  years and male-to-female ratio was 2.2:1. Indications of plastic biliary stent insertion were bile duct stones (63.2%, 24/38) and benign bile duct stricture (52.6%, 20/38). Mean duration of retained plastic stent was  $22.6 \pm 12.2$  mo, and in 10 cases (26.3%), stents were retained for more than 24 mo. Common bile duct (CBD) stones or sludge

were found in most cases (92.1%, 35/38). The most common complication was acute cholangitis (94.7%, 36/38). Stent removal by endoscopic approach was successfully performed in 92.1% (35/38) of the cases. In 3 cases, an additional plastic stent was inserted alongside the previous stent due to failure of the stent removal. Endoscopic removal of bile duct stones was successful in 73.7% (28/38) of the cases. When patients were divided into two groups by duration of stent placement (12 to 24 mo *vs* over 24 mo), there were no differences in the development of cholangitis, presence of biliary stones, and success rate of endoscopic removal of stones and biliary stents.

## CONCLUSION

The most common complication of retained long-term plastic biliary stents was acute cholangitis associated with CBD stones. Endoscopic management was successfully performed in most cases.

**Key words:** Biliary stent; Long-term complications; Forgotten stents; Acute cholangitis; Biliary stone

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**Core tip:** There is little information on the consequence of retained long-term biliary stents and management of complications. In this study, follow-up loss with a subsequently forgotten stent for more than 12 mo occurred in 38 patients and cholangitis due to common bile duct stones or sludge developed in most cases (94.7%, 36/38). Stent removal by endoscopic approach was successfully performed in 92.1% (35/38) of the cases.

Sohn SH, Park JH, Kim KH, Kim TN. Complications and management of forgotten long-term biliary stents. *World J Gastroenterol* 2017; 23(4): 622-628 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/622.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.622>

## INTRODUCTION

Endoscopic retrograde cholangiopancreatogram (ERCP) with endoscopic sphincterotomy (EST) and stone extraction is widely accepted as the treatment of choice for a patient of any age with choledocholithiasis. This technique has been reported to be successful in 80%-95% of the cases<sup>[1-3]</sup>. Endoscopic removal of biliary stones may infrequently be impossible despite improved ERCP techniques, especially when large or impacted stones are present, or in cases of a coexisting narrowing of the distal common bile duct (CBD). Surgical procedures could be options for patients who failed endoscopic restoration of bile drainage. Endoscopic insertion of biliary endoprosthesis has been proposed as an alternative for elderly patients or those

with high surgical risks<sup>[4-7]</sup>.

Biliary stents are tubular devices made of plastic or metal, and are used primarily to establish patency of an obstructed bile duct caused by malignancy, benign biliary strictures, or bile duct stones. Early complications of biliary stents are infection, pancreatitis, and bleeding; most late complications are stent dysfunction, and much less frequently cholecystitis, duodenal perforation, and bleeding<sup>[8]</sup>. The value of short-term biliary stenting has been proven, but the benefit of long-term stenting is less clear. Several studies have shown the effectiveness of long-term biliary endoprosthesis in the management of irretrievable CBD stones in high-risk elderly patients<sup>[4-7]</sup>. In these studies, the long-term complications, such as occlusion, stent migration or cholangitis, increased with time and replacement or removal of the biliary stents was recommended after 3-6 mo<sup>[9]</sup>. In a study with 58 patients who underwent biliary endoprosthesis as a permanent treatment, complications (most commonly cholangitis) occurred in 40% of patients, and 16% of patients died because of biliary-related causes<sup>[10]</sup>. In these studies, clinical symptoms and laboratory tests were followed-up regularly by physicians.

Although there are several case reports, little is known about what happens when biliary stents were forgotten or omitted by patients and consequently retained for a period of time greater than 12 mo. The aim of this study was to evaluate complications of retained long-term biliary stents and management outcomes in patients with a plastic biliary stent left unnoticed for longer than 12 mo.

## MATERIALS AND METHODS

Endoscopic plastic biliary stent placement was performed in 802 patients at Yeungnam University Hospital between January 2000 and December 2014. The indications of tubal catheterization were failure of stone removal and coexistence of benign bile duct narrowing. Among them, follow-up loss with a subsequently forgotten stent for more than 12 mo happened in 38 patients.

Patient characteristics, plastic biliary stent indication, status and complications of retained long-term biliary stents, and management outcomes were reviewed retrospectively. Patients were divided into two groups by stent placement duration: a 12-mo to 24-mo group, and an over 24-mo group. Various factors were compared between the two groups.

All patients with retained long-term plastic biliary stents were evaluated with ERCP followed by a computed tomography scan. ERCP was performed with a standard side-viewing duodenoscope (TJF-140; Olympus, Tokyo, Japan) by experienced endoscopists. After taking a cholangiogram, removal of biliary endoprosthesis was attempted using a stone retrieval basket. In cases of coexisting biliary stones, endoscopic stone removal was performed using conventional

**Table 1** Baseline patient characteristics *n* (%)

	Total ( <i>n</i> = 38)
Age, mean, yr	73.7 ± 12.1
Sex, male/female	26(68.4)/12(31.6)
Indication of biliary stent	
CBD stone	19 (50.0)
CBD stricture	14 (36.8)
CBD stone with CBD stricture	5 (13.2)
Types of stents	
Diameter, Fr: 7/10/11.5	2(5.3)/21(55.3)/15(39.5)
Length, cm: 5/7/9	13(34.2)/22(57.9)/3(7.9)
Underlying diseases	
Chronic pancreatitis	4 (10.5)
Liver cirrhosis	1 (2.6)
STG BII	4 (10.5)
Chronic renal failure	2 (5.3)
Cerebrovascular accident	5 (13.2)

CBD: Common bile duct; STG BII: Subtotal gastrectomy with Billroth II.

**Table 2** Presenting symptoms and laboratory findings at admission

Variable	Total ( <i>n</i> = 38)
Symptoms	
Abdominal pain	36 (94.7)
Fever	20 (52.6)
Jaundice	13 (34.2)
Without symptom	2 (5.3)
Laboratory findings	
WBC, cells/mcL	10270.20 ± 4835.67
ALT, IU/L	122.50 ± 238.72
GGT, IU/L	236 ± 209.18
Total bilirubin, mg/dL	2.57 ± 2.19
Amylase, U/L	213.74 ± 494.75

Values are presented as mean ± SD or *n* (%). WBC: White blood cell; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase.

methods. When stent removal failed, an additional plastic biliary stent (Cotton-Leung biliary stents; Wilson-Cook Medical, Winston-Salem, NC, United States) was inserted alongside the previous stent to treat cholangitis. When complete stone removal failed or biliary stricture was found, a plastic biliary stent was inserted to prevent cholangitis. Complete stone removal was confirmed by cholangiogram at the end of each procedure.

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL, United States). Continuous variables were analyzed using the *t* test. Observed frequencies in subsets of the study population were compared using Fisher's exact test and  $\chi^2$  tests. Statistical significance was defined as *P* < 0.05 (two-tailed).

## RESULTS

Clinical characteristics of patients are summarized in Table 1. Mean patient age was 73.7 ± 12.1 years

(range: 38-92 years), and the male-to-female ratio was 2.2:1. Endoscopic retrograde biliary drainage (ERBD) was performed in 19 patients (50.0%) with CBD stones, 14 patients (36.8%) with bile duct strictures, and 5 patients (13.2%) with CBD stones combined with bile duct strictures. The mean duration of stent-stay was 22.6 ± 12.2 mo (range: 12-58 mo), and in 10 cases (26.3%) stents were in place for more than 24 mo. Most patients visited the hospital due to development of clinical symptoms (92.1%, 36/38), and 2 cases of retained biliary stents were found incidentally. Patients presented with abdominal pain (36/38), fever (20/38), jaundice (13/38), and abnormal liver function tests (13/38) (Table 2).

Complications and clinical outcomes of retained long-term biliary stents are described in Table 3. The most common complication was acute cholangitis (94.7%), followed by obstructive jaundice (34.2%), internal stent migration (13.2%), and pancreatitis (5.3%). In 36 patients with cholangitis, 35 cases were caused by CBD stones and 1 case by stent occlusion. Stent removal by endoscopic approach was performed successfully in 92.1% of the cases (35/38). All stent lumens were occluded with sludge and brown pigment stones, or sludge was adherent to the stent. An additional plastic stent was inserted alongside the previous stent in 3 patients with failed stent removal. Surgical management was considered for these patients, but was not performed due to comorbidities and generally poor patient conditions.

CBD stones were found in 35 patients (92.1%). Of the 14 patients who underwent ERBD due to CBD strictures and had no stones initially, CBD stones or sludge developed in 11 patients (78.6%) (Figure 1). Endoscopic CBD stone removal was successful in 73.7% of patients (28/38). Stone removal was performed by a stone retrieval basket and brown stones were stuck tightly to stents in most cases (Figure 2).

Retrieved CBD stones were brown pigment stones in all cases. In some patients, large stones formed around the plastic biliary stent and impacted at the CBD, which were impossible to remove endoscopically (Figure 3). For 10 patients with failed endoscopic stone removal or biliary strictures, biliary stents were reinserted to prevent cholangitis.

When patients were divided into two groups based on the duration of stent-stay, there were no significant differences between the 12-to-24-mo group and the over 24-mo group in development of cholangitis, pancreatitis, internal stent migration, CBD stone development, and overall success rates of endoscopic stone removal and biliary stent.

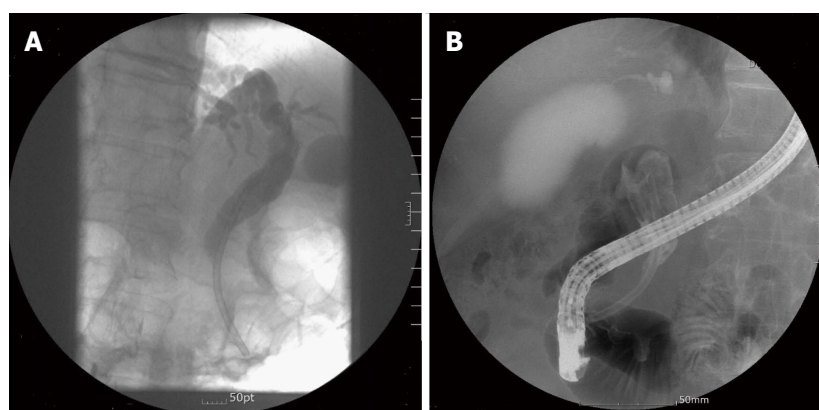
## DISCUSSION

The current standard treatment of bile duct stones is EST and stone extraction, followed by laparoscopic cholecystectomy if indicated. Surgical procedures such

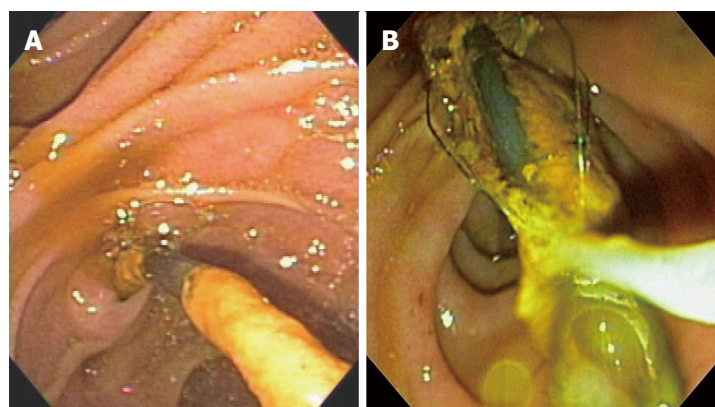
**Table 3** Complications and outcomes according to biliary stent duration *n* (%)

Variable	Total ( <i>n</i> = 38)	1-2 yr ( <i>n</i> = 28)	Over 2 yr ( <i>n</i> = 10)	<i>P</i> value
Age, yr	73.7 ± 12.1	75.6 ± 10.0	68.3 ± 16.1	0.104
Sex, male/female	26/14(68.4/31.6)	19/9(67.9/32.1)	7/3 (70/30)	1.000
Duration of stent-stay, mo	22.6 ± 12.2	16.4 ± 3.0	39.7 ± 11.7	0.001
Complications of stents				
Jaundice	13 (34.2)	11 (39.3)	2 (20.0)	0.441
Cholangitis	36 (94.7)	26 (92.9)	10 (100.0)	1.000
Pancreatitis	2 (5.3)	1 (3.6)	1 (10.0)	0.462
Internal migration	5 (13.2)	3 (10.7)	2 (20.0)	0.592
Presence of CBD stone	35 (92.1)	25 (89.3)	10 (100.0)	0.552
Endoscopic treatment				
Stone removal	28 (73.7)	21 (75.0)	7 (70.0)	1.000
Stent removal	35 (92.1)	26 (92.9)	9 (90.0)	1.000
Stent reinsertion	19 (50.0)	16 (57.1)	3 (15.8)	0.269
Additional stent insertion	3 (7.9)	2 (7.1)	1 (10.0)	1.000

OR: Odds ratio; CI: Confidence interval; CBD: Common bile duct.



**Figure 1** Stone formation 29 mo after stent insertion. A: Plastic biliary stent inserted for common bile duct stricture without stone; B: Large brown stone filled the bile duct.



**Figure 2** Brown stone stuck tightly to stent was removed by stone retrieval basket (A and B).

as bile duct exploration and sphincteroplasty, and drainage procedures such as choledochoduodenostomy and hepaticojejunostomy, are options for patients who fail endoscopic therapy<sup>[1-3]</sup>.

Biliary endoprosthesis has been used for biliary drainage as a temporary measure or alternative for patients who are unfit for surgery. Biliary drainage is

of great importance in maintaining bile flow in cases of stone impaction in the CBD. Placement of a biliary stent is essential for the treatment or prevention of acute cholangitis in this clinical setting, with a success rate of > 95%<sup>[1-3]</sup>. This procedure stabilizes the patient so that elective surgery or subsequent endoscopic treatment can be carried out when the patient's status



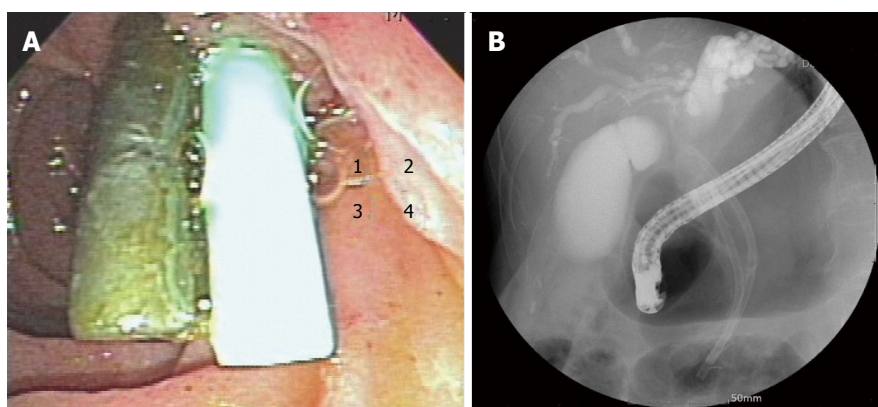


Figure 3 Additional stent inserted alongside previous stent (A) and large stones formed around the plastic biliary stent (B).

has improved, thus reducing procedural risks. In elderly patients or patients with severe comorbidities who are poor candidates for further endoscopic or surgical treatments, endobiliary stenting may serve as a permanent therapy; studies report that complication rates associated with surgery in elderly patients range from 12% to 28%<sup>[11-15]</sup>. In this study, poor surgical candidates who underwent endoscopic biliary stenting showed symptom relief and a low complication rate for more than 1 year, although most patients ultimately developed complications associated with the retained biliary stent itself.

Early outcomes of biliary stenting are well established and include good drainage and a low complication rate, but late outcomes remain uncertain. Most reports revealed that the success rate of endoscopic biliary stenting was nearly 100%, and that early morbidity was low and could be controlled well<sup>[16-18]</sup>. The major disadvantage of biliary stenting is clogging of the endoprosthesis. It is widely known that the mean patency duration of the plastic biliary stent is about 6 mo to 12 mo for benign diseases<sup>[19,20]</sup>. Replacement of plastic stents is therefore recommended every 3-6 mo to prevent cholangitis<sup>[8,9]</sup>. Biliary passage patency is maintained longer when stents are inserted for benign diseases such as biliary stone or benign biliary stricture, as compared to metal stents of malignancies. In this study, biliary stenting was performed for benign diseases, and the mean duration of stent placement was  $22.55 \pm 12.16$  mo before developing symptoms or complications. The plastic biliary stent does not serve as the sole conduit for bile duct flow when used for benign diseases. As a passage between CBD and stent was retained in benign diseases, this passage may provide a conduit for bile flow even when the stent is completely obstructed<sup>[10,21,22]</sup>. Stent occlusion is the main cause of malignant biliary obstruction, and symptoms and complications occur earlier.

Although biliary patency may be maintained for a longer period after plastic biliary stent insertion, long-standing biliary stents consequentially increase

the risk of cholangitis due to formation of biliary stones, because the biliary stent itself may serve as a nidus for stone formation. Lost sphincter of Oddi function, a mechanical barrier preventing regurgitation of duodenal contents into the bile duct, results in bacterial growth in the bile duct by ascending infection and plays an important role in the formation of brown pigment stones<sup>[23-25]</sup>. All stones in this study appeared to be brown pigment stones. Brown stones were distinguishable by their reddish brown to dark brown color and muddy appearance in macroscopic findings seen at endoscopy.

In this study, 92.1% of patients presented with symptoms of cholangitis, which most often occurred due to development of a CBD stone rather than stent occlusion. Of the 19 patients who underwent stenting for biliary stricture and had no stones initially, 16 patients (84.2%) developed a biliary stone or sludge. In this study, biliary stones were found stuck to biliary stents. Based on this information, the biliary stent may serve as a nidus for stone formation and development, and stent occlusion could develop because of biliary stasis.

Several previous studies showed that biliary patency restoration was successfully achieved in more than 95% of patients with stent obstruction<sup>[26]</sup>. The follow-up duration for development of complications and clinical symptoms was relatively short in most studies.

Little is known about the complications and management of long-term biliary stents retained more than 1 year. A recent study of 5 cases of long-standing forgotten biliary stents with 45.5 mo (range: 23-84 mo) of stent-stay reported that 4 of them required surgery for treatment<sup>[27]</sup>. Another recently published article documented that 2 of 3 patients (75%) underwent surgery for treatment of long-stayed forgotten stents<sup>[28-30]</sup>. In this study, however, 35 of 38 patients (92.1%) with retained long-term biliary stents were successfully treated by endoscopic approach. In 3 cases of endoscopic stent removal failure in patients who were unfit for surgery, cholangitis could be

controlled by additional biliary stent placement without surgery.

In conclusion, the most common complication of retained long-term plastic biliary stents was acute cholangitis associated with CBD stones. Endoscopic management was successfully performed in most cases. Biliary patency was likely to be maintained more than 1 year. The rate of complications, such as cholangitis or stent impaction, might be increased as the stent was in place for a longer duration, and CBD stone development ultimately occurred in most cases of long-standing biliary stent. All patients should be informed of the possibility of complications related to retained long-term endoprosthesis placement, and stent change or definite treatment should be considered within 1 year of stent placement. Endoscopic management should be the primary option for the long-term biliary stent, especially in patients with comorbidities or who are unfit for surgery.

## COMMENTS

### Background

Biliary endoprosthesis has been used for biliary drainage as a temporary measure or alternative for patients who are unfit for surgery. Biliary drainage is of great importance in maintaining bile flow in cases of stone impaction in the biliary duct. In elderly patients or patients with severe comorbidities who are poor candidates for further endoscopic or surgical treatments, endobiliary stenting may serve as a permanent therapy.

### Research frontiers

There is little information on the consequence of retained long-term stayed forgotten biliary stents and management of complications, except a few case reports. In this study, the authors aimed to evaluate complications and management outcomes of retained long-term plastic biliary stents.

### Innovations and breakthroughs

The most common complication of retained long-term plastic biliary stents was acute cholangitis associated with common bile duct stones. Endoscopic management was successfully performed in most cases.

### Applications

In this study, long-term biliary stents were successfully treated by endoscopic approach. This study emphasizes the importance of patient follow-up and programmed withdrawal of stents. Endoscopic management could be the primary option for the long-term biliary stent, especially in patients with comorbidities or who are unfit for surgery.

### Terminology

Biliary stents are tubular devices made of plastic or metal, and are used primarily to establish patency of an obstructed bile duct caused by malignancy, benign biliary strictures, or bile duct stones.

### Peer-review

The authors performed a good retrospective study of a cohort of patients hospitalized for choledocholithiasis. The authors are interested in the management and the complications related to the presence of forgotten long-term biliary stents of more than 1 year. The authors collected a good amount of cases and the results can be useful information for the gastroenterologist.

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## Retrospective Cohort Study

# Validation of prognostic indices in Egyptian Budd-Chiari syndrome patients: A single-center study

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## Abstract

### AIM

To compare predictive ability of Budd-Chiari syndrome (BCS) prognostic indices (PIs) for one-year survival and Transjugular intrahepatic portosystemic shunt (TIPS) patency.

### METHODS

This retrospective study enrolled 194 Egyptian patients with primary BCS who presented to the Budd-Chiari Study Group of Ain Shams University Hospital. Calculation of the available PIs was performed using Child-Pugh and model for end-stage liver disease scores, BCS-specific PIs (Clichy, New Clichy and



Rotterdam) for all patients, and BCS-TIPS PI only for patients who underwent TIPS. The overall one-year survival rate and the one-year shunt patency rate for TIPS were reported.

## RESULTS

The overall one-year survival rate was 69.6%, and the New Clichy PI revealed the best validity for its prediction at a cut-off value of 3.75, with sensitivity and specificity of 78% and 73.3%, respectively [area under receiver operating characteristic curve (AUC) = 0.806]. The one-year survival rate post-TIPS was 89.7%, and the BCS-TIPS score demonstrated validity for its prediction at a cut-off value of 3.92 (sensitivity and specificity were 71.4% and 64.5%, respectively) (AUC = 0.715). Logistic regression analysis revealed that the New Clichy PI ( $P = 0.030$ ), high serum total bilirubin ( $P = 0.047$ ) and low albumin ( $P < 0.001$ ) were independent factors for predicting mortality within one year. The one-year shunt patency rate in TIPS was 80.2%, and none of the PIs exhibited significant validity for its prediction.

## CONCLUSION

The New Clichy score could independently predict the one-year survival in Egyptian BCS patients.

**Key words:** Budd-Chiari syndrome; Prognostic indices; New Clichy score; One-year survival; Transjugular intrahepatic portosystemic shunt

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**Core tip:** We analyzed the predictive ability of Budd-Chiari syndrome (BCS) prognostic indices (PIs) for one-year overall survival and transjugular intrahepatic portosystemic shunt (TIPS) patency rate in 194 Egyptian patients. Calculation of the available PIs was performed using Child-Pugh and model for end-stage liver disease scores, BCS-specific PIs (Clichy, New Clichy and Rotterdam) for all patients, and BCS-TIPS PI only for patients who underwent TIPS. We found that the New Clichy score independently predicted one-year survival in Egyptian BCS patients.

Sakr M, Abdelhakam SM, Elsayed SA, Allam EH, Farid AM, Abdelmoaty W, Hassan AM, Shaker M, El-Gharib M, Eldorriy A. Validation of prognostic indices in Egyptian Budd-Chiari syndrome patients: A single-center study. *World J Gastroenterol* 2017; 23(4): 629-637 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/629.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.629>

## INTRODUCTION

Budd-Chiari syndrome (BCS) is caused by hepatic venous outflow obstruction from the small hepatic

veins (HVs) to the site of entry of the inferior vena cava (IVC) into the right atrium<sup>[1]</sup>.

It is difficult to predict the prognosis of BCS patients because of the large variability in clinical presentation and disease course<sup>[2]</sup>. Little is known about factors that may help predict the survival of BCS patients<sup>[3]</sup>, and various trials were done to determine parameters that might predict the prognosis in these patients<sup>[4]</sup>.

Several scores were evaluated in BCS, including the Child-Pugh score, the model for end-stage liver disease (MELD) score and several BCS-specific prognostic indices (PIs), including the Clichy PI, the Rotterdam score, the New Clichy PI and the BCS-TIPS score<sup>[5]</sup>. These scores contain clinical and laboratory parameters and can be used to stratify BCS patients, however, their use for the management of an individual patient is still controversial<sup>[6]</sup>.

Patient characteristics, etiological factors, and treatment outcomes have changed since these indices were elaborated, and comparability between studies from different centers is crucial for rapid advances in BCS<sup>[7]</sup>. Comparability relies on adjustments for baseline characteristics and requires the availability of a single, validated and widely accepted PI. However, an accurate PI to make therapeutic decisions in individual patients has not been established<sup>[8,9]</sup>.

The aim of the present study was to compare the predictive ability of the available PIs for BCS for the one-year overall survival and the one-year shunt patency rate of transjugular intrahepatic portosystemic shunt (TIPS) in Egyptian patients.

## MATERIALS AND METHODS

This retrospective cohort study enrolled 194 Egyptian patients with primary BCS who presented to the Budd-Chiari Study Group (BCSG), Tropical Medicine Department of Ain Shams University Hospital (Cairo, Egypt) between November 2005 and December 2014. The study protocol was approved by the Research Ethical Committee of Faculty of Medicine, Ain Shams University according to the ethical guidelines of the 1975 Declaration of Helsinki.

Patients with any other concomitant cause of liver disease (e.g., viral, autoimmune or metabolic), secondary BCS or hepatocellular carcinoma were excluded.

The following patient medical records and databases were reviewed: (1) clinical data; (2) laboratory investigations: CBC, liver profile, coagulation profile, viral markers (HBsAg, HBcAb, HCV Ab) using the enzyme-linked immunosorbent assay (ELISA) technique; (3) thrombophilia workup to clarify the underlying etiology of BCS as follows: Antinuclear antibodies, anti-β2 glycoprotein-1, anticardiolipin antibodies IgM and IgG were measured by ELISA technique and Lupus anticoagulant was measured by coagulation-based functional assay to diagnose antiphospholipid

syndrome (APS). Protein C, S and antithrombin III were measured by coagulation-based functional assay to diagnose protein C, S, or antithrombin III deficiency. Genotyping of factor V Leiden G1691A, prothrombin G20210A, and methylene tetrahydrofolate reductase (MTHFR) C677 were performed *via* real-time PCR and fluorescence melting curve detection analysis to diagnose mutations. Janus tyrosine kinase-2 (JAK II) V617F mutation was detected by PCR and/or a bone marrow biopsy to diagnose myeloproliferative disorder (MPD). Flow cytometry for CD55 and CD59 was done to diagnose paroxysmal nocturnal hemoglobinuria<sup>[10]</sup>; and (4) radiological assessment using abdominal duplex ultrasonography (US) to assess the patency of the HVs, the portal vein (PV), and the IVC. Abdominal multi-slice computed tomography, magnetic resonance imaging and/or MR venography were performed when indicated to confirm all diagnoses and assess vascular anatomy.

Calculation of available BCS PIs was performed for all patients; from their data at initial presentation; as follows: (1) Modified Child-Pugh score: The sum of the scoring points from the five parameters [ascites (none = 1 point, moderate = 2 points, severe = 3 points), serum bilirubin (< 2 mg/dL = 1 point, 2-3 mg/dL = 2 points, > 3 mg/dL = 3 points), albumin (> 3.5 g/dL = 1 point, 2.8-3.5 g/dL = 2 points, < 2.8 g/dL = 3 points), hepatic encephalopathy (absent = 1 point, grades 1 and 2 = 2 points, grades 3 and 4 = 3 points), and prothrombin time International Normalized Ratio "PT INR" (< 1.7 = 1 point, 1.71-2.30 = 2 points, > 2.30 = 3 points)] corresponds to one of three groups (Child A = 5-6 points, Child B = 7-9 points, Child C = 10 or more points)<sup>[11]</sup>; (2) MELD score:  $3.8 \times (\ln \text{ serum bilirubin mg/dL}) + 11.2 \times (\ln \text{ INR}) + 9.6 \times (\ln \text{ serum creatinine mg/dL}) + 6.4$ <sup>[12]</sup>; (3) Clichy PI: (ascites score  $\times 0.75$ ) + (Pugh score  $\times 0.28$ ) + (age  $\times 0.037$ ) + (creatinine  $\times 0.0036$ ), where ascites was scored as absent, controlled with sodium restriction or diuretics or resistant to medical treatment (scored as 1, 2 or 3, respectively)<sup>[8]</sup>; (4) Rotterdam BCS index:  $1.27 \times \text{encephalopathy} + 1.04 \times \text{ascites} + 0.72 \times \text{prothrombin time} + 0.004 \times \text{bilirubin}$ , where ascites and hepatic encephalopathy were scored as present (1) or absent (0) and prothrombin time as higher (1) or equal/lower (0) than an INR of 2.3<sup>[3]</sup>; (5) New Clichy PI:  $0.95 \times \text{ascites score} + 0.35 \times \text{Pugh score} + 0.047 \times \text{age} + 0.0045 \times \text{serum creatinine} + (2.2 \times \text{form III}) - 2.6$ , where ascites was scored as in Clichy PI, and clinic-pathological form III (acute on top of chronic) was defined by the presence of at least one acute and one chronic feature and coded as 1 for patients with form III and 0 for the other patients<sup>[9]</sup>; and (6) BCS-TIPS PI (only for patients who underwent TIPS procedure):  $\text{age} \times 0.08 + \text{bilirubin} \times 0.16 + \text{INR} \times 0.63$ <sup>[5]</sup>.

The patterns of management were reported. All enrolled patients received anticoagulant therapy as early as possible after securing risky esophago-gastric varices in an attempt to reduce the risk of clot extension and new thrombotic episodes. Treatment of the underlying

prothrombotic cause was also initiated concomitantly, *e.g.*, folic acid supplementation for MTHFR mutation and diuretic therapy when indicated. Angioplasty and/or stenting were used in patients with partial or short segment occlusion of HVs and IVC to re-establish the physiological drainage of portal and sinusoidal blood. Patients with BCS who were non-responsive to medical treatment or who were not candidates for angioplasty/stenting (*i.e.*, complete occlusion of all HVs with patent IVC and PV) were treated using TIPS to transform the portal system into an outflow tract. TIPS was also performed in patients with failed trials of HV stenting. Living donor liver transplantation (LDLT) was performed for patients with liver decompensation (because they would not benefit from TIPS) and for patients with failed TIPS. A mesoatrial shunt was performed to decompress the liver as a bridge to liver transplantation in patients who were not fit for radiological intervention<sup>[13,14]</sup>.

The overall one-year survival rate was reported for all included patients. The one-year shunt patency rate was reported for patients who underwent the TIPS procedure.

### Statistical analysis

Data analysis was performed using SPSS (SPSS Inc. 2009. PASW Statistics for Windows, Version 18.0, Chicago, IL, United States). Quantitative variables are presented as the mean and standard deviation to describe the studied patients. Qualitative variables are presented as counts and percentages. Student's *t*-test was used to compare quantitative variables between two independent groups. The Chi-square test was used to compare qualitative data between groups. The receiver operating characteristic (ROC) curve was used to measure the prognostic ability and determine the best cut-off value for different PIs, and logistic regression analysis was used to measure the independent effect of some variables on one-year patient survival. Kaplan-Meier survival analysis was performed to assess one-year survival and one-year shunt patency for patients. *P* value < 0.05 was considered statistically significant.

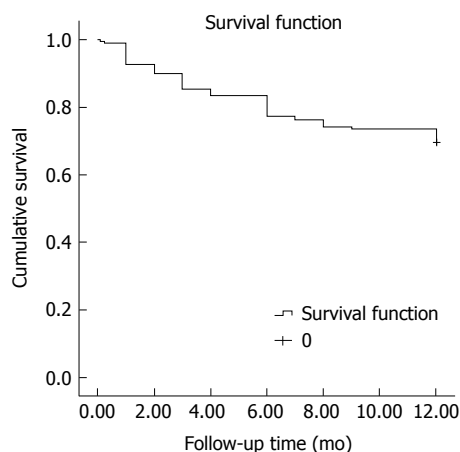
The statistical methods of this study were reviewed by Azza M Hassan, Lecturer of Community, Environmental and Occupational Medicine, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

## RESULTS

### Characteristics of the study population, clinical and investigational data

The current study included 194 Egyptian patients with primary BCS. Their mean age was  $28.79 \pm 8.94$  years, with female predominance (111/194, 57.2%).

The most common etiology of BCS in the current study was FVLM, which was found in 57 patients (29.4%), followed by MTHFR mutation in 48 patients (24.7%) and MPD in 43 patients (22.2%); twenty-nine patients of them (67.4%) were overt and 14 patients



**Figure 1** One-year survival function (time to death) of the included patients (Kaplan-Meier).

(32.6%) were occult. Forty patients (20.6%) had primary APS, and 19 patients (9.8%) had secondary APS. PC deficiency was present in 25 patients (12.9%); PS deficiency was present in 6 patients (3.1%), and anti-thrombin III deficiency was present in 18 patients (9.3%). Seven patients (3.6%) were idiopathic. Multiple etiologies were present in 73 patients (37.6%). Twenty-six patients (13.4%) did not complete the etiological panel.

The most common clinical presentations were hepatomegaly in 181 patients (93.3%) followed by ascites in 166 patients (85.6%), and abdominal pain in 163 patients (84%). Jaundice was present in 85 patients (43.8%). The chronic form of presentation was found in 135 patients (69.6%) and the acute/subacute form was found in 59 patients (30.4%).

Single HV occlusion was diagnosed in 7 patients (3.6%). Two HVs were occluded in 32 patients (16.5%), and three HVs were occluded in 155 patients (79.9%). The IVC was involved (occluded/attenuated/web) in 32 patients (16.5%), and PV thrombosis was diagnosed in 8 patients (4.1%).

Most patients were Child B (45.9%), followed by Child C (28.9%) and Child A (25.3%). We found that Rotterdam class III was the most common (72.2%), followed by class II and class I (15.5% and 12.4%, respectively). The following mean values of different prognostic scores in the included patients were observed: Child score:  $8.34 \pm 2.29$ , MELD:  $12.25 \pm 7.03$ , Clichy:  $5.02 \pm 1.14$ , New Clichy:  $3.72 \pm 1.44$ , Rotterdam:  $2.27 \pm 1.58$  and BCS-TIPS score (calculated only for the 107 patients who underwent TIPS):  $3.70 \pm 0.88$ .

#### Patterns of management and one-year survival

Patients were classified into two groups according to the patterns of management of BCS in the current study. (1) the interventional group included 131 patients (67.5%) who underwent interventional management and medical treatment. A total of 107 patients in this group underwent the TIPS procedure, 20 patients had HV

angioplasty with stenting, one patient had angioplasty without stenting, one patient had LDLT, and two patients underwent mesoatrial shunt; and (2) the non-interventional group included 63 patients (32.5%) who were not suitable for any intervention and were treated only medically with anticoagulation and treatment for the underlying etiology with or without diuretics.

The overall one-year survival rate of the studied cohort was 69.6% (Total number of deaths by the end of first year: 59/194 patients). The estimated mean survival time was 9.84 mo (95%CI: 9.29-10.38) (Figure 1). The interventional group had a significantly better one-year survival rate than the non-interventional group. The one-year survival rates for both groups were 87.8% and 31.7%, respectively (number of deaths in the two groups was 16/131 and 43/63 patients, respectively) ( $P < 0.001$ ).

Eleven (10.3%) of the 107 patients who underwent the TIPS procedure died by the end of the first year, and the one-year survival rate post-TIPS was 89.7%.

#### Factors affecting overall one-year survival

The overall one-year survival rate was not significantly related to either age or gender. However, univariate analysis revealed that many factors significantly affected the one-year survival: the presence of genital and oral ulcers, history of DVT, use of hormonal therapy in females, acute and subacute presentations, presence of jaundice, hepatic encephalopathy, ascites and advanced ascites score. The non-survivor group exhibited significantly higher serum bilirubin with lower serum albumin compared to the survivor group. Poor prognosis and shortened survival was related to the presence of PV thrombosis and/or IVC occlusion (Table 1).

#### PIs and one-year survival

All prognostic scores were significantly related to overall one-year survival, with significantly higher scores in patients who died (Table 2). Their area under ROC curves (AUC) exceeded 0.5. However, only three PIs exhibited significant validity and predictive ability regarding the overall one-year survival; which makes them useful for individual decisions in day-to-day practice because their AUC exceeded 0.8; these scores were New Clichy, Clichy and Child-Pugh scores. The New Clichy PI was the best factor (AUC = 0.806) at a cut-off value of 3.75, with sensitivity and specificity of 78% and 73.3%, respectively (Table 3 and Figure 2).

The BCS-TIPS score exhibited validity for the prediction of one-year survival post-TIPS at a cut-off value of 3.92 (sensitivity and specificity were 71.4% and 64.5%, respectively) (AUC = 0.715) (Table 3 and Figure 3).

#### Logistic regression analysis for factors affecting one-year survival

Multivariate logistic regression analysis for factors affecting the one-year survival for all studied patients revealed that New Clichy PI ( $P = 0.030$ ), high serum

**Table 1** Factors related to the one-year survival

	Alive ( <i>n</i> = 135) <i>n</i> (%)	Dead ( <i>n</i> = 59) <i>n</i> (%)	$\chi^2/t$	<i>P</i> value
Age in years, mean $\pm$ SD	28.6 $\pm$ 8.47	29.22 $\pm$ 10.01	0.44	0.66
Gender				
Male	53 (39.3)	30 (50.8)	2.25	0.13
Female	82 (60.7)	29 (49.2)		
Genital/oral ulcer	2 (1.5)	6 (10.2)	7.84	0.01
History of DVT	13 (9.6)	17 (28.8)	11.56	0.001
Use of OCP <sup>1</sup>	15 (18.3)	11 (37.9)	5.25	0.02
Presentation				
Acute/subacute	31 (23)	28 (47.5)	11.64	0.001
Chronic	104 (77)	31 (52.5)		
Jaundice	45 (33.3)	40 (67.8)	19.81	< 0.001
Hepatic encephalopathy	14 (10.4)	24 (40.7)	23.94	< 0.001
Ascites	110 (81.5)	56 (94.9)	6.00	0.01
Ascites score <sup>2</sup>				
1	26 (19.3)	3 (5.1)	51.38	< 0.001
2	89 (65.9)	17 (28.8)		
3	20 (14.8)	39 (66.1)		
IVC (occluded/attenuated/web)	16 (11.9)	16 (27.1)	6.95	0.01
PV thrombosis	1 (0.7)	7 (11.9)	12.85	< 0.001
Total bilirubin, mean $\pm$ SD	2.15 $\pm$ 1.40	5.05 $\pm$ 4.93	4.44	< 0.001
Direct bilirubin, mean $\pm$ SD	0.90 $\pm$ 0.80	2.59 $\pm$ 2.94	4.34	< 0.001
Albumin, mean $\pm$ SD	3.46 $\pm$ 0.63	2.89 $\pm$ 0.65	5.75	< 0.001

<sup>1</sup>Percentage was calculated among female patients; <sup>2</sup>Ascites score was calculated as (1): absent, (2): controlled with sodium restriction or diuretics, or (3): resistant to medical treatment. DVT: Deep venous thrombosis; OCP: Oral contraceptive pills; IVC: Inferior vena cava; PV: Portal vein.

**Table 2** Relation between different prognostic indices and the one-year survival rate

	One-year survival		<i>t</i>	<i>P</i> value
	Alive ( <i>n</i> = 135) mean $\pm$ SD	Dead ( <i>n</i> = 59) mean $\pm$ SD		
Child score	7.54 $\pm$ 1.79	10.17 $\pm$ 2.28	7.85	< 0.001
MELD score	10.42 $\pm$ 5.44	16.42 $\pm$ 8.40	5.05	< 0.001
Clichy PI	4.64 $\pm$ 0.94	5.89 $\pm$ 1.09	8.12	< 0.001
New clichy PI	3.24 $\pm$ 1.18	4.82 $\pm$ 1.37	8.11	< 0.001
Rotterdam index	1.78 $\pm$ 0.88	3.41 $\pm$ 2.15	5.64	< 0.001
BCS-TIPS score <sup>1</sup>	3.61 $\pm$ 0.83	4.33 $\pm$ 0.97	2.99	0.003

<sup>1</sup>Calculated only for 107 patients who underwent TIPS. MELD: Model for end-stage liver disease; PI: Prognostic index; BCS-TIPS score: Budd-Chiari syndrome-transjugular intrahepatic portosystemic shunt score.

total bilirubin ( $P = 0.047$ ) and low serum albumin levels ( $P < 0.001$ ) were independent factors for predicting mortality within one year (Table 4).

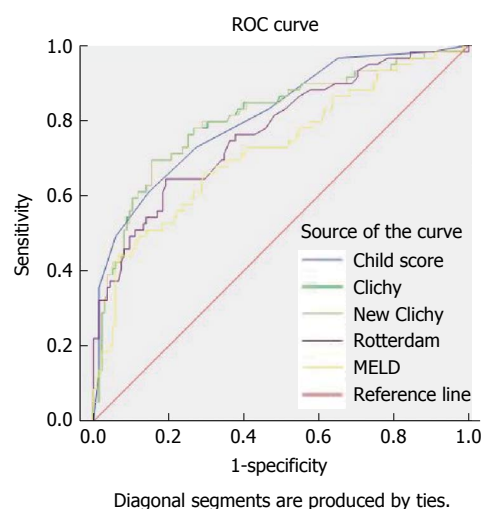
### Factors affecting one-year shunt patency in TIPS

Nineteen (19.8%) of the 96 TIPS patients who completed the one-year follow-up had occluded shunts at the end of the first year, and the one-year shunt patency rate of TIPS was 80.2%.

The one-year shunt patency was not related to any of the studied factors (demographic, clinico-laboratory, etiologic or PIs) (Table 5).

## DISCUSSION

Several BCS-specific PIs and numerous clinical and



**Figure 2** Receiver operating characteristic curve showing the validity of different prognostic indices (Child score, model for end-stage liver disease score, Clichy prognostic index, New Clichy prognostic index and Rotterdam index) for prediction of the one-year overall survival of the studied patients. MELD: Model for end-stage liver disease; PI: Prognostic index; ROC: Receiver operating characteristic.

laboratory parameters have been previously reported<sup>[15,16]</sup>. However, the predictive accuracy of these PIs remains insufficient for predicting the survival of BCS patients<sup>[5,6]</sup>.

The current study compared the predictive ability of the available PIs for BCS for the overall one-year survival and the one-year shunt patency rate of TIPS in Egyptian patients.

The overall one-year survival rate was 69.6% in this



**Table 3** Diagnostic performance of prognostic indices for prediction of one-year survival among the studied patients

Prognostic indices	Cut-off	Sensitivity	Specificity	AUC	SE	P value	95%CI
Child score	≥ 8.55	72.90%	72.60%	0.811	0.034	< 0.001	0.743-0.878
MELD score	≥ 11.59	69.50%	64.40%	0.723	0.042	< 0.001	0.641-0.805
Clichy PI	≥ 4.95	78.00%	70.40%	0.807	0.036	< 0.001	0.735-0.878
New clichy PI	≥ 3.75	78.00%	73.30%	0.806	0.036	< 0.001	0.735-0.878
Rotterdam index	≥ 1.94	71.20%	65.20%	0.771	0.038	< 0.001	0.696-0.845
BCS-TIPS score <sup>1</sup>	≥ 3.92	71.40%	64.50%	0.715	0.072	0.010	0.574-0.857

<sup>1</sup>Calculated only for 107 patients who underwent TIPS. AUC: Area under the receiver operating characteristic (ROC) curve; MELD: Model for end-stage liver disease; PI: Prognostic index; BCS-TIPS score: Budd-Chiari syndrome-transjugular intrahepatic portosystemic shunt score; SE: Standard error.

**Table 4** Logistic regression analysis for factors affecting the one-year survival

	B	P value	OR (95%CI)
New Clichy PI	0.291	0.030	1.338 (1.029-1.741)
Hepatic encephalopathy	0.731	0.118	2.077 (0.830-5.199)
Total bilirubin	0.194	0.047	1.214 (1.003-1.470)
Albumin	-0.857	< 0.001	0.424 (0.323-0.558)

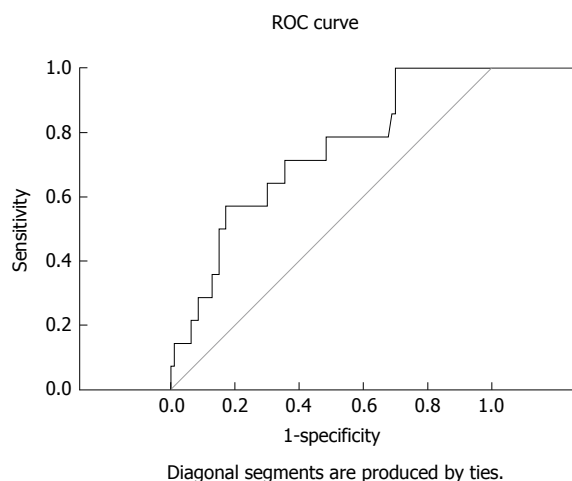
B: Regression coefficient; PI: Prognostic index.

**Table 5** Relation between different prognostic indices and the one-year shunt patency in transjugular intrahepatic portosystemic shunt patients

	One-year shunt patency <sup>1</sup>		$\chi^2$	P value
	Patent (n = 77) mean ± SD	Occluded (n = 19) mean ± SD		
Child score	7.67 ± 1.69	7.74 ± 2.00	0.15	0.89
MELD score	10.26 ± 5.32	10.56 ± 7.17	0.21	0.84
Clichy PI	4.70 ± 0.92	4.86 ± 1.06	0.65	0.52
New Clichy PI	3.32 ± 1.16	3.52 ± 1.34	0.65	0.52
Rotterdam index	1.79 ± 0.80	1.99 ± 1.05	0.91	0.37
BCS-TIPS score	3.57 ± 0.80	3.73 ± 1.03	0.69	0.49

<sup>1</sup>Total transjugular intrahepatic portosystemic shunt (TIPS) patients was 107, among them eleven patients died and 96 patients completed the one-year follow up. MELD: Model for end-stage liver disease; PI: Prognostic index; BCS-TIPS score: Budd-Chiari syndrome-transjugular intrahepatic portosystemic shunt score.

study. We found a striking difference in survival at one year, which was higher in the group of patients who underwent intervention than patients who were unfit for intervention and managed only medically (87.8% vs 31.7%, respectively). This result is consistent with a systematic review of 79 studies discussing BCS survival by Qi *et al.*<sup>[15]</sup> in 2015. The authors found that the median one-year survival rate was 93% (range: 80%-100%) in 9 previous studies performed on patients receiving interventional radiological treatments, and 68.1% (range: 14%-92%) in 6 studies that included patients receiving medical therapy alone. The survival figures may have been affected by differences in the selection criteria of the included BCS patients as well as their underlying disease etiologies which led to a strong influence on the expected natural history and outcome of the disease. In fact, BCS patients



**Figure 3** Receiver operating characteristic curve showing the validity of Budd-Chiari syndrome - transjugular intrahepatic portosystemic shunt score for prediction of the one-year survival among patients who underwent transjugular intrahepatic portosystemic shunt procedure (n = 107). ROC: Receiver operating characteristic.

from different geographic regions tend to show distinct disease etiologies<sup>[17]</sup>. In particular, thromboses are more common in Western, whereas venous webs are more frequent in Eastern and Japanese BCS patients<sup>[18]</sup>. Our study of Egyptian BCS patients in 2011<sup>[19]</sup> as well as the current study indicated that FVLM and MTHFR mutation are the most commonly detected prothrombotic disorders in Egyptian BCS patients.

The survival of BCS patients demonstrated gradual improvement over time and a favorable prognosis<sup>[20,21]</sup>. The dramatic improvement in survival over years is easily explained by the increasing recognition of BCS, establishment of an effective treatment strategy, improvement in interventional radiological techniques, and advances in the management of portal hypertension-related complications<sup>[22,23]</sup>. Actually, the first year after diagnosis of BCS is a critical period and is related to longer term prognosis in those patients<sup>[15,20]</sup>.

Neither age nor gender were related to prognosis in the univariate analysis in the current study, which is similar to previous reports<sup>[15]</sup>. We found that the presence of oral and genital ulcers and use of hormonal therapy were related to poor prognosis. This result is also consistent with previous reports<sup>[24,25]</sup>. The current study also revealed that acute and subacute

presentations were linked to higher mortality rates compared to chronic presentation because the acute form may lead to fulminant hepatitis and acute liver cell failure, with a subsequent poor prognosis<sup>[26]</sup>.

Patients who presented with PVT and/or IVC thrombosis exhibited higher mortality rates in the current study, which is consistent with DeLeve *et al*<sup>[10]</sup>.

Univariate analysis demonstrated that all studied PIs (Child, MELD, Rotterdam, Clichy, New Clichy and BCS-TIPS scores) were significantly related to one-year survival in the current study and distinguished survivors from non-survivors. The survivor group of our patients exhibited lower values for all PIs than the non-survivor group. This result is consistent with the studies performed by Zhang *et al*<sup>[27]</sup> and Rautou *et al*<sup>[6]</sup> for all PIs except the BCS-TIPS score, which exhibited lower predictive ability in their studies. This discrepancy may be attributed to the different durations of follow-up and the different baseline criteria of the enrolled patients.

The Child, Clichy and New Clichy scores exhibited significant validity and predictive ability in the current study, which makes these scores useful for individual decisions in day-to-day practice (their AUC exceeded 0.8). This result is consistent with Rautou *et al*<sup>[6]</sup>.

The New Clichy score in our patients exhibited the highest sensitivity (78%) and specificity (73.3%) at a cut-off value of  $> 3.75$  for the prediction of one-year survival (AUC = 0.806), followed by the Clichy score, the Child score, the Rotterdam score, and the MELD score. In Chinese patients included in the study of Zhang *et al*<sup>[27]</sup>, New Clichy score exhibited the highest specificity but lowest sensitivity (93.9% and 50%, respectively), and the Clichy score exhibited the highest sensitivity but lowest specificity (87.5% and 53.5%, respectively). These differences may be attributed to the fact that BCS exhibits characteristics that differ according to ethnic and geographical considerations<sup>[17]</sup>. In the study of Zhang *et al*<sup>[27]</sup>, the authors didn't mention any cut-off value for their PIs. However; through their ROC curve analysis, the New Clichy score AUC was the largest (0.776), and its Youden index was 0.44, indicating a high predictive value.

Multivariate logistic regression analysis in the current study revealed that the New Clichy PI ( $P = 0.030$ ), high serum total bilirubin ( $P = 0.047$ ) and low serum albumin levels ( $P < 0.001$ ) were independent factors for predicting mortality within one year. Therefore, these factors were related to poor prognosis and outcome. Pavri *et al*<sup>[28]</sup> performed a multivariate analysis and demonstrated that increasing age, presence of cirrhosis at diagnosis and chronic kidney disease were significantly associated with poor prognosis, in contrast to bilirubin or other markers of liver disease severity, which were not related to prognosis. Fitsiori *et al*<sup>[29]</sup> found that the presence of ascites, elevated creatinine, Child-Pugh score and MELD score were predictors of prognosis. Different study designs, variable clinical

presentations and laboratory parameters at diagnosis may explain the variability in the identification of prognostic factors for BCS.

The PIs, except the BCS-TIPS score, were developed in the pre-TIPS era, and these factors remain useful for the identification of patients with a poor prognosis on anticoagulation and supportive care who should be considered for TIPS. The introduction of TIPS dramatically improved prognosis<sup>[10]</sup>. The TIPS procedure remains an extremely effective therapy for eligible BCS patients with good survival rate<sup>[30]</sup>. The one-year survival rate post-TIPS in the current study was 89.7% and the BCS-TIPS score exhibited a good validity for its prediction. This is comparable to the study of Qi *et al*<sup>[31]</sup> and the meta-analysis of Zhang *et al*<sup>[21]</sup> which revealed a one-year survival rate of 83.8% and 87.3%, respectively.

The one-year shunt patency rate following TIPS was 80.2% in the current study. The patency rate was not related to any of the studied factors (demographic, clinico-laboratory, etiologic or PIs).

In conclusion, the New Clichy score could independently predict one-year survival in Egyptian BCS patients at a cut-off value of 3.75. None of the PIs exhibited significant validity for the prediction of one-year shunt patency of the TIPS procedure. Because BCS patients have different characteristics according to ethnic and geographical distribution; all PIs could be more or less good in stratifying patients in clinical trials. However, further extended studies are needed to clarify the possibility of using a single PI in the management of an individual patient.

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## COMMENTS

### Background

Budd-Chiari syndrome (BCS) is a rare potentially life-threatening hepatic disorder caused by obstruction of hepatic venous outflow at any level from the hepatic venules to the right atrium. Little is known about factors that may predict the survival of BCS patients, and various trials were performed to determine parameters that may predict prognosis in these patients.

### Research frontiers

The authors analyzed the predictive ability of BCS prognostic indices (PIs) for the overall one-year survival and transjugular intrahepatic portosystemic shunt (TIPS) patency rate for 194 Egyptian patients. Calculation of the available PIs was performed which included Child-Pugh and model for end-stage liver disease scores, BCS specific PIs (Clichy, New Clichy and Rotterdam) for all patients, and BCS-TIPS prognostic index only for patients who underwent TIPS. They found that the New Clichy score could independently predict one-year survival in Egyptian BCS patients. The one-year shunt patency rate in TIPS was 80.2%, and none of the PIs exhibited significant validity for its prediction.

### Innovations and breakthroughs

This is the largest Egyptian study that addresses the predictive ability of BCS PIs for one-year overall survival and TIPS patency rate.

## Applications

This study may represent a future strategy for the use of the New Clichy score for predicting the one-year survival and making individual decisions in BCS.

## Terminology

New Clichy score =  $0.95 \times \text{ascites score} + 0.35 \times \text{Pugh score} + 0.047 \times \text{age} + 0.0045 \times \text{serum creatinine} + (2.2 \times \text{form III}) - 2.6$ , where ascites was scored as being absent, controlled with sodium restriction or diuretics or resistant to medical treatment (as scores 1, 2 or 3, respectively), and clinic-pathological form III (acute on top of chronic) was defined by the presence of at least one acute and one chronic feature and was coded as 1 for patients with form III and 0 for the other patients.

## Peer-review

This paper retrospectively evaluates whether various PIs are related to the one year survival of Egyptian patients with BCS in a single centre. The findings will be useful to clinicians treating these patients in Egypt.

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## Retrospective Study

# Predictors of vitamin D deficiency in inflammatory bowel disease and health: A Mississippi perspective

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## Abstract

### AIM

To identify the predictors of vitamin D deficiency in patients with and without inflammatory bowel disease (IBD).

### METHODS

Patients with ulcerative colitis (UC) or Crohn's disease (CD) related diagnostic codes who received medical care at University of Mississippi Medical Center between July 2012 and 2015 were identified. After thorough chart review, we identified patients with biopsy proven IBD who had also been tested for serum 25-hydroxyvitamin D [25(OH)D] concentration. We compared these patients to a previously studied cohort of healthy controls who also had vitamin D concentration checked. Logistic regression analysis was performed to determine the association between vitamin d deficiency and UC, CD, race, age, gender and body mass index (BMI).

### RESULTS

We identified 237 patients with confirmed IBD. Of these, only 211 had a serum 25(OH)D concentrations available in the medical record. The group of healthy controls consisted of 98 individuals with available serum 25(OH)D concentration. 43% of IBD patients were African American (AA). Patients with CD were more likely to have vitamin D concentration checked. Bivariate analysis showed that AA (51% vs 21%, *P*

= 0.00001), subjects with BMI > 30 kg/m<sup>2</sup> (39% *vs* 23%  $P = 0.01$ ) and CD (40% *vs* 26%,  $P = 0.04$ ) were more likely to be vitamin D deficient than vitamin D sufficient. Those with Age > 65 were more likely to be vitamin D sufficient (46% *vs* 15%,  $P = 0.04$ ). Multiple regression showed that only BMI > 30 kg/m<sup>2</sup> and AA race are associated with vitamin D deficiency.

### CONCLUSION

BMI > 30 kg/m<sup>2</sup> and AA race are predictive of vitamin D deficiency. Gender, age and diagnosis of IBD are not predictive of vitamin D deficiency.

**Key words:** Vitamin D deficiency; Inflammatory bowel disease; Body mass index; Ulcerative colitis; Crohn's disease; African American

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**Core tip:** The studies evaluating the relationship between vitamin D deficiency and inflammatory bowel disease (IBD) have shown heterogeneity perhaps due to multiple overlapping risk factors that need to be accounted for. We performed a retrospective study to identify the risk factors for vitamin D deficiency in a population with a large African American (AA) component. Using logistic regression analysis we studied the effect of diagnosis, race, age, gender and body mass index (BMI) on prevalence of vitamin D deficiency. In subjects with and without IBD, BMI > 30 kg/m<sup>2</sup> and AA race are predictive of vitamin D deficiency. Gender, age and diagnosis of IBD were not predictive of vitamin D deficiency in our population.

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## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic idiopathic conditions of the gastrointestinal tract that manifest as inflammation with distinct, yet often overlapping clinical features<sup>[1]</sup>. The Etiology of IBD is thought to reflect innate and adaptive immune-mediated responses to luminal bacterial antigens leading to enhanced intestinal permeability and dysregulated intestinal immunity<sup>[1-3]</sup>. There has been an increasing interest in the regulatory effects of vitamin D on the immune system in IBD and colorectal cancer<sup>[4,5]</sup>. Numerous studies have demonstrated a link between low serum 25-hydroxyvitamin D [25(OH)D] concentrations and IBD in both CD<sup>[6-10]</sup> and UC patients<sup>[10,11]</sup>. Vitamin D deficiency may predispose

IBD patients to a higher disease activity<sup>[6]</sup>, suboptimal response to treatment<sup>[12]</sup>, and higher incidence of surgery and hospitalization<sup>[13]</sup>. On the other hand, as pointed out by Tajika *et al*<sup>[14]</sup>, IBD patients may be at an increased risk for low serum 25(OH)D concentrations due to one or more of the following: Faulty conversion of vitamin D to active metabolic forms; failure to conserve an adequate functional pool of vitamin D; Insufficient dietary intake and inadequate sun exposure<sup>[15,16]</sup>; malabsorption of dietary and biliary vitamin D and its metabolites<sup>[17,18]</sup>; and loss of protein-bound 25hydroxy vitamin D due to a protein losing enteropathy<sup>[19]</sup>.

Studies aimed at delineating this complex relationship are confounded by factors such as age, BMI, and race leading to inconsistent conclusions<sup>[20-25]</sup>. Furthermore, African Americans (AA) are understudied in most of the IBD literature, and data representing this population is scarce<sup>[26]</sup>. According to the 2011 United States Census Bureau, 40% of Mississippians are AA<sup>[27]</sup> thereby presenting a unique opportunity to study this population.

We aim to determine the vitamin D status in an understudied cohort consisting of IBD and non-IBD patients and Investigate the association between serum 25(OH)D concentrations and IBD diagnosis (UC and CD). In addition, this study aimed to investigate risk factors for vitamin D deficiency namely race, gender, age, and BMI; as well as to compare vitamin D status with that of healthy controls.

## MATERIALS AND METHODS

This retrospective study was conducted at University of Mississippi Medical Center (UMMC), which is a tertiary care center and the only academic medical institution in the state of Mississippi. Over half a million patient encounters are reported every year. While UMMC caters to both high and low acuity patients, being a referral center more patients tend to be sicker. There are no reports for who sees most of the IBD patients in the state but we see over 500 patients annually. We get patients through word of mouth and community referrals. Less than 15% of the patients are uninsured and the majority has either public or private insurance. All patient visits are in the main campus in Jackson. Patients were identified using various diagnosis codes for UC, CD and IBD. Electronic medical records for all patients with IBD associated diagnostic codes seen between July 2012 and July 2015 were reviewed. Demographic, biometric, and clinical information was collected through review of electronic medical records. A standard document was used to collect the information however a pilot study was not conducted. Diagnosis of IBD was based on endoscopic, clinical and histologic data. IBD Patients with available plasma 25(OH)D concentration were included in this study. We excluded patients with history of malignancy. The control group consisted of patients without IBD or any

**Table 1** Comparison between inflammatory bowel disease patients with and without available vitamin D concentration *n* (%)

	IBD patients without available vitamin D concentration ( <i>n</i> = 26)	IBD patients with available vitamin D concentration ( <i>n</i> = 211)	<i>P</i> value
CD	10 (38.5)	129 (61.1)	0.034
Age (yr), median (IQR)	32 (26)	41 (25)	0.030
Female	12 (46.2)	125 (59.2)	0.213
AA	11 (42.3)	91 (43.1)	0.391
BMI (kg/m <sup>2</sup> ), median (IQR)	25.6 (9.9)	29.3 (7.5)	0.179
Patients on vitamin D supplementation	2 (8.33)	36 (17.06)	0.271

CD: Crohn's disease; AA: African American; BMI: Body mass index.

**Table 2** Comparison between inflammatory bowel disease and non-inflammatory bowel disease patients *n* (%)

	Controls ( <i>n</i> = 98)	IBD patients ( <i>n</i> = 211)	<i>P</i> value
Patients with vitamin D deficiency	56 (57.1)	143 (61.6)	0.0694
Age at vitamin D testing (yr), median (IQR)	60.5 (14.5)	41 (25)	< 0.0001
Female	86 (87.8)	125 (59.2)	< 0.0001
AA	23 (23.9)	91 (43.1)	0.0009
BMI (kg/m <sup>2</sup> ), median (IQR)	29.3 (7.5)	27 (8.9)	0.0438
Patients receiving vitamin D supplementation	Not available	37 (17.5)	Not Applicable

AA: African American; BMI: Body mass index.

active systemic disease that presented to UMMC and had plasma 25(OH)D concentrations obtained during routine follow up.

### Vitamin D status assessment

All vitamin D concentrations were assessed using The ARCHITECT 25-OH vitamin D assay (Abbott diagnostics, Germany). There is no absolute consensus on Vitamin D deficiency and sufficiency. Vitamin D was operationalized into clinically meaningful categories for analysis. Plasma 25(OH) D concentrations < 20 ng/mL (50 nmol/L) indicate vitamin D deficiency. Plasma 25(OH) D concentrations between 21 and 29 ng/mL (52.5 and 72.5 nmol/L) represent vitamin D insufficiency while concentrations > 30 ng/mL (75 nmol/L) represent vitamin D sufficiency<sup>[22,28,29]</sup>.

### Statistical analysis

A biomedical statistician performed statistical analysis. We used a generalized logistic regression model to estimate odds ratio (OR). The generalized logistic regression extends the traditional model and in this instance, our outcome of interest was ordinal and has three levels for vitamin D: Deficient, insufficient and sufficient.

**Table 3** Distribution of vitamin D concentration across various diagnosis, demographics (age, race, gender) and body mass index (modifiable risk factor) *n* (%)

		Vitamin D			<i>P</i> value
		Deficient	Insufficient	Sufficient	
Total	309	100 (32.4)	99 (32.0)	110 (35.6)	
Diagnosis					
Controls	98 (31.7)	27.6%	29.6%	42.8%	0.0407
CD	129 (41.7)	40.3%	33.3%	26.4%	
UC	82 (26.5)	25.6%	32.9%	41.5%	
Age (yr)					
< 35	72 (23.3)	38.9%	34.7%	26.4%	0.0415
35-49	73 (23.6)	34.2%	28.8%	37.0%	
50-64	99 (32.0)	37.4%	28.3%	34.3%	
> 65	65 (21.0)	15.4%	38.5%	46.2%	
Race					
White	189 (61.2)	21.7%	34.4%	43.9%	< 0.0001
AA	114 (36.9)	50.9%	28.1%	21.0%	
Other	6 (1.9)	16.7%	33.3%	50.0%	
Gender					
Female	211 (68.3)	33.7%	32.2%	34.1%	0.6857
Male	98 (31.7)	29.6%	31.6%	38.8%	
BMI (kg/m <sup>2</sup> )					
< 25	97 (31.4)	29.9%	25.8%	44.3%	0.0110
25-30	102 (33.0)	27.5%	31.4%	41.2%	
> 30	110 (35.6)	39.1%	38.2%	22.7%	

CD: Crohn's disease; AA: African American; BMI: Body mass index; UC: Ulcerative colitis.

Comparisons of the distributions for demographic characteristics were made with Pearson's  $\chi^2$  statistic. Higher than expected Pearson's residual (*i.e.*,  $|Z| > 2.0$ ) was considered evidence of departure from independence. We considered  $P < 0.05$  evidence of statistical significance.

Data were reported as frequencies and proportions for the marginal distributions of the categorical variables and proportions for the joint distributions of the cross-classification tables. The institutional review board at UMMC approved this study.

## RESULTS

Two hundred and thirty seven IBD patients (139 CD, 98 UC) and 98 controls were identified. Amongst the IBD patients, 211 had 25(OH)D concentration checked on 257 occasions. Those with CD were more likely to have a 25(OH)D concentration measured in our facility. Also those tested for vitamin D concentration tended to be slightly older. Otherwise there were no major differences between IBD patients with and without measured 25(OH)D concentration (Table 1).

Of 309 patients included in final analysis, 98 (31.7%) were controls, 129 (41.7%) were CD patients and 82 (26.5%) were UC patients. Compared to IBD patients, the controls had higher mean age and female preponderance. IBD patients were more likely to be AA and had lower mean body mass index (BMI) (Table 2).

Demographics of the study population as a whole

**Table 4 Associations of body mass index with diagnosis and demographic variables**

	<i>n</i> (%)	BMI < 25 kg/m <sup>2</sup>	BMI 25-30 kg/m <sup>2</sup>	BMI > 30 kg/m <sup>2</sup>	<i>P</i> value
Total	309	97 (31.4)	102 (33.0)	110 (35.6)	
Diagnosis					
Controls	98 (31.7)	20.4%	34.7%	44.9%	0.0048
CD	129 (41.7)	42.6%	29.5%	27.9%	
UC	82 (26.5)	26.8%	36.6%	36.6%	
Age (yr)					
< 35	72 (23.3)	52.8%	29.2%	18.1%	0.0007
35-49	73 (23.6)	26.0%	30.1%	43.8%	
50-64	99 (32.0)	23.2%	37.4%	39.4%	
> 65	65 (21.0)	26.2%	33.9%	40.0%	
Race					
White	189 (61.2)	28.6%	36.5%	34.9%	0.5253
AA	114 (36.9)	36.0%	27.2%	36.8%	
Other	6 (1.9)	33.3%	33.3%	33.3%	
Gender					
Female	211 (68.3)	27.5%	30.3%	42.2%	0.0017
Male	98 (31.7)	39.8%	38.8%	21.4%	

CD: Crohn's disease; AA: African American; BMI: Body mass index; UC: Ulcerative colitis.

are shown in Table 3. Overall, there was a 2:1 female-to-male ratio. Within the IBD cohort, 115 (54.5%) subjects were White, 91 (43.1%) were AA and 5 (2.3%) were of other races. BMI was categorized into normal, overweight, and obese, with similar proportion of individuals in each category.

Vitamin D as the outcome is also presented in Table 3 and divided into clinically meaningful categories. The marginal distribution of vitamin D given in the first row of Table 3 indicates that the sample is approximately evenly distributed with about one-third in each category.

### Bivariate analysis

Table 3 gives the results of a chi-square contingency table analysis to determine the association of vitamin D with each of the demographic variables.

Disease status (CD vs UC vs Control) and plasma vitamin D concentrations were significantly associated ( $P = 0.04$ ). The proportion of controls with sufficient vitamin D was higher as compared to the other two groups. For the CD group, there were many more with deficient vitamin D than expected and fewer with sufficient vitamin D than expected.

Age and vitamin D were significantly associated ( $P = 0.041$ ). The Pearson's residuals indicated that the youngest age group (less than 35), had a higher proportion with deficient vitamin D than expected and a lower proportion of sufficient vitamin D than expected. The opposite was true for the age greater than 65 group where the proportion of those with deficient vitamin D was lower than expected, while the proportion in the sufficient group was higher than expected.

Race and vitamin D were significantly associated

( $P < 0.0001$ ). The proportion of AA with deficient vitamin D was much higher than expected and the proportion with sufficient vitamin D was much lower than expected. Whites and others showed the opposite trend with lower than expected proportions with deficient vitamin D and higher than expected proportions with sufficient vitamin D.

Gender was not significantly associated with vitamin D sufficiency ( $P = 0.6$ ).

BMI and vitamin D were significantly associated ( $P = 0.0110$ ). For BMI < 25 kg/m<sup>2</sup>, the proportion of sufficient vitamin D subjects was higher than expected. Subjects with BMI > 30 kg/m<sup>2</sup> had a higher proportion with deficient vitamin D than expected and a lower proportion with sufficient vitamin D than expected.

For all demographic variables, the insufficient group did not appear to differ significantly from the marginal of approximately one-third. The differences were in the sufficient and deficient vitamin D concentrations for diagnosis, Age, race and BMI.

Of the four factors that appeared to be associated with plasma vitamin D concentrations, BMI is the only modifiable risk factor. Therefore, we investigated the potential for confounding factors for the relationship of BMI with vitamin D by statistically testing the associations between BMI and non-modifiable risk factors: age, race and gender (Table 4).

BMI was associated with diagnosis ( $P = 0.0048$ ), age ( $P = 0.0007$ ) and gender ( $P = 0.0017$ ). BMI was not significantly associated with race (Table 4).

### Distribution of vitamin D across stratified levels of BMI and diagnosis

**BMI < 25 kg/m<sup>2</sup>:** Those < 35 years old are more likely to have vitamin D deficiency. Curiously, the 50-64 year age group is less likely to exhibit vitamin D deficiency compared to the other groups. The CD patients are more likely to have deficient vitamin D than the other groups. There is a significant association between diagnosis and vitamin D only in the BMI < 25 kg/m<sup>2</sup> group ( $P = 0.0026$ ) (Table 5).

**BMI 25-30 kg/m<sup>2</sup>:** No association was found in the BMI 25-30 kg/m<sup>2</sup> group ( $P = 0.389$ ) (Table 5).

**BMI > 30 kg/m<sup>2</sup>:** The BMI > 30 kg/m<sup>2</sup> group is the most homogeneous, and there is no statistical evidence of an association ( $P = 0.88$ ).

That is, the BMI > 30 kg/m<sup>2</sup> group is more likely to be vitamin D deficient, but there is no further evidence of a relationship between diagnosis and vitamin D once BMI > 30 kg/m<sup>2</sup> is considered. On the other hand, the BMI < 25 kg/m<sup>2</sup> group is more likely to have sufficient vitamin D, but the presence of CD may alter the effect on vitamin D. We find this group stands out as being vitamin D deficient compared to the others (Table 5).

### Seasonal variation in vitamin D concentrations

We compared mean vitamin D concentrations in



**Table 5** Distribution of vitamin D concentration across stratified levels of body mass index and diagnosis

	<i>n</i> (%)	Vitamin D deficient	Vitamin D insufficient	Vitamin D sufficient	<i>P</i> value
BMI < 25 (kg/m <sup>2</sup> )					
Total	97	29 (29.9)	25 (25.8)	43 (44.3)	
Diagnosis					
Controls	20 (20.6)	15.0%	15.0%	70.0%	0.0026
CD	55 (56.7)	43.6%	27.3%	29.1%	
UC	22 (22.7)	9.1%	31.8%	59.1%	
BMI = 25-30 (kg/m <sup>2</sup> )					
Total	102	28 (27.5)	32 (31.4)	42 (41.2)	
Diagnosis					
Controls	34 (33.3)	26.5%	26.5%	47.1%	0.3894
CD	38 (37.3)	34.2%	36.8%	29.0%	
UC	30 (29.4)	20.0%	30.0%	50.0%	
BMI > 30 (kg/m <sup>2</sup> )					
Total	110	43 (39.1)	42 (38.2)	25 (22.7)	
Diagnosis					
Controls	44 (40.0)	34.1%	38.6%	27.3%	0.8823
CD	36 (32.7)	41.7%	38.9%	19.4%	
UC	30 (27.3)	43.3%	36.7%	20.0%	

CD: Crohn's disease; AA: African American; BMI: Body mass index; UC: Ulcerative colitis.

traditional summer vs winter months and while the summertime vitamin D concentrations were marginally higher (26.9 vs 23.1,  $P = 0.064$ ), this is not statistically significant. Admittedly, it was close enough to consider adding into the regression analysis, however this p-value is only using a partial dataset (based on seasonal analysis many patients are excluded) - therefore not included into the regression analysis.

### Regression model

We also investigated a cumulative logistic regression model that included age and race as covariates and the nine BMI categories. We did not include gender since it was not significantly associated with outcome or predictor. The model found race remained significant ( $P = 0.0006$ ), age was not significant ( $P = 0.15$ ), and there were significant differences between the nine BMI groups. To adjust for the multiple testing, we considered the proportions different only if  $P < 0.002$ , a conservative Bonferroni approach. Differences identified were the same as the stratified analysis in Table 6.

### Reduced model

We performed a manual backward elimination procedure to reduce the model. For the procedure, we retained variables with  $P < 0.10$ . The reduced model includes race, BMI and diagnosis only. Table 6 gives the results of the analyses, both full and reduced model for comparison. The results are similar for both models; therefore, we interpret the odds ratios from

the final model, only.

Although there was no significant effect for diagnosis at the  $P < 0.05$  level, we did find a global difference ( $P = 0.085$ ) and retained it in the model. Using a simple confidence interval for the odds ratio, the odds of deficiency compared to sufficiency are higher for the CD group compared to controls [odds ratio (OR) = 2.22; 95%CI: 1.07-4.63]. There was also a similar result for insufficiency compared to sufficiency comparing CD to controls (OR = 2.16; 95%CI: 1.07-4.36).

Race was a significant predictor of vitamin D concentrations based on the global test ( $P < 0.0001$ ). Whites are about one-fourth less likely than AA to exhibit deficiency compared to sufficiency (OR = 0.23; 95%CI: 0.12-0.43). No other results are significant and the sample size is very small for the "Other" group, leading to loss of power.

Finally, BMI was a significant predictor of vitamin D concentrations based on a significant global test ( $P = 0.003$ ). Using the normal weight group as a reference (*i.e.*, 25-30 kg/m<sup>2</sup>), we estimated odds ratios for underweight and overweight. Although there were no significant effects for underweight, there was a significant effect for the overweight (BMI > 30 kg/m<sup>2</sup>) group. The overweight group is much more likely to develop vitamin D deficiency (OR = 2.61; 95%CI 1.26-5.42), as well as insufficiency compared to sufficiency (OR = 2.27; 95%CI: 1.14-4.52).

## DISCUSSION

Ergocalciferol (vitamin D<sub>2</sub>), the predominant circulating and storage form of vitamin D<sup>[30]</sup> and cholecalciferol (vitamin D<sub>3</sub>) are obtained from diet or supplementation. Vitamin D<sub>3</sub> is also formed in the skin *via* ultraviolet B (UVB) light exposure<sup>[31]</sup>. There are accumulating epidemiological, clinical, and basic data that support an immune-modulatory role for vitamin D in IBD<sup>[20]</sup>. On the other hand, IBD patients may be at a higher risk for vitamin D deficiency, thereby making this relationship a bidirectional one.

Bivariate analysis identified the following risk factors for vitamin D deficiency: (1) CD; (2) BMI > 30 kg/m<sup>2</sup>; (3) Age < 35 years; and (4) AA race. However, regression analysis showed that only AA race and BMI > 30 kg/m<sup>2</sup> were significantly associated with vitamin D deficiency. While CD and vitamin D deficiency showed correlation, the relationship was not statistically significant likely due to insufficient numbers ( $P = 0.085$ ). Similar findings have also been reported previously<sup>[15,32]</sup>.

The prevalence of Obesity is increasing in the United States. According to the most recent obesity prevalence survey conducted by the Centers for Disease Control, greater than 35.1% United States adults and 35.5% of adults in Mississippi fall in the BMI > 35 kg/m<sup>2</sup> category<sup>[33]</sup>. This is a potentially

**Table 6** Results of multivariate modelling with age, race, gender, body mass index and diagnosis as predictors of deficient, insufficient and sufficient vitamin D

	Full model		Global <i>P</i> value (full)	Reduced model		Global <i>P</i> value (reduced)
	OR (95%CI), deficient <i>vs</i> sufficient	OR (95%CI), insufficient <i>vs</i> sufficient		OR (95%CI), deficient <i>vs</i> sufficient	OR (95%CI), insufficient <i>vs</i> sufficient	
Diagnosis			0.1482			0.0852
Controls	Ref.	Ref.		Ref.	Ref.	
CD	1.71 (0.74, 3.94)	2.11 (0.95, 4.69)		2.22 (1.07, 4.63)	2.16 (1.07, 4.36)	
UC	0.73 (0.30, 1.76)	1.13 (0.50, 2.53)		0.92 (0.42, 2.02)	1.2 (0.59, 2.48)	
Gender			0.9584			
Female	Ref.	Ref.				
Male	0.9 (0.46, 1.79)	0.95 (0.50, 1.81)				
Age (yr)			0.1578			
< 35	3.62 (1.18, 11.12)	1.47 (0.56, 3.83)				
35-49	1.9 (0.68, 5.30)	0.68 (0.28, 1.63)				
50-64	2.61 (1.04, 6.58)	0.86 (0.40, 1.86)				
> 65	Ref.	Ref.				
Race			0.0006 <sup>1</sup>			< 0.0001 <sup>1</sup>
AA	Ref.	Ref.		Ref.	Ref.	
White	0.25 <sup>1</sup> (0.13, 0.48)	0.67 (0.34, 1.29)		0.23 <sup>1</sup> (0.12, 0.43)	0.64 (0.33, 1.22)	
Other	0.3 (0.03, 3.38)	0.68 (0.09, 4.89)		0.18 (0.02, 1.95)	0.57 (0.08, 3.96)	
BMI (kg/m <sup>2</sup> )			0.0017 <sup>1</sup>			0.0030 <sup>1</sup>
< 25	0.68 (0.32, 1.43)	0.57 (0.28, 1.17)		0.71 (0.34, 1.48)	0.63 (0.31, 1.26)	
25-30	Ref.	Ref.		Ref.	Ref.	
> 30	2.71 <sup>1</sup> (1.28, 5.73)	2.36 <sup>1</sup> (1.17, 4.75)		2.61 <sup>1</sup> (1.26, 5.42)	2.27 <sup>1</sup> (1.14, 4.52)	

<sup>1</sup>Statistically significant. CD: Crohn's disease; AA: African American; BMI: Body mass index; UC: Ulcerative colitis.

modifiable risk factor and may affect disease severity in IBD patients due to a pro-inflammatory effect<sup>[34]</sup> and through sequestration and/or volumetric dilution of vitamin D by adipose tissue<sup>[32,35]</sup>. Recently, Vimalaswaran *et al.*<sup>[25]</sup> performed a bidirectional mendelian randomization analysis providing evidence for the role of obesity as a causal risk factor for the development of vitamin D deficiency.

Melanin in skin absorbs UVB light slowing the absorption and conversion of vitamin D3<sup>[36]</sup>. Therefore, African Americans are considered to be at increased risk for Vitamin D deficiency<sup>[37]</sup>. If vitamin D deficiency is a cause of IBD, then it could be theorized that African Americans would be at enhanced risk for IBD as well. Traditionally, African American risk for IBD is considered to be lower, not higher. This may in part be reflective of under-diagnosis within this population<sup>[26]</sup>. We found that race was a significant predictor of vitamin D concentrations based on the global test ( $P < 0.0001$ ) with African American patients having a higher proportion of deficiency, while Whites and other races were four times less likely to have vitamin D deficiency.

Traditionally increasing age has been linked to vitamin D deficiency. This is related to multiple factors including: decreased metabolic activity of aging skin<sup>[23]</sup>, reduced muscle mass that normally serves as a reservoir of vitamin D<sup>[30]</sup> and decreased sun exposure associated with residing in assisted living facilities<sup>[21]</sup>.

Contrary to traditional belief, our initial analysis suggested that older age is protective against vitamin D deficiency ( $P = 0.0415$ ). Future prospective studies are needed to help delineate the role of dietary, environmental and socio-economic factors that contribute to these findings.

While we feel that our study is well conducted and methodologically sound, we do recognize certain limitations. Our study is retrospective and data regarding all factors that affect vitamin D concentrations including: detailed dietary records, unreported supplement use, and cumulative sun exposure were not available for analysis. In many patients we struggled to find exact dates of symptom onset, history regarding smoking and alcohol use, objective assessment of symptoms including mayo clinical score or CDAI. Based on these issues we did

not collect data regarding disease severity/need for surgery/complications/exact medication use etc. We do believe that the lack of this data does not undermine the validity of the presented data. This study includes patients from a single center and results may not be applicable to a different geographic area. Some of our findings may have achieved significance if we had studied a larger number of individuals. Despite these limitations, we are confident that this analysis accurately assesses the characteristics of vitamin D deficiency in a previously understudied population.

In summary, we hereby present data from a unique population in which disease state and diagnosis is significantly affected by dietary and socioeconomic status. Specifically, we show that BMI > 30 kg/m<sup>2</sup> and AA race are associated with vitamin D deficiency in IBD and non-IBD patients. Future studies aimed at better understanding these differences may lead to improved disease outcomes.

## COMMENTS

### Background

The diagnosis and management of inflammatory bowel disease has made remarkable progress over the past decade. Both diagnostic as well as therapeutic paradigms are being constantly evaluated and improved. Despite the improved ability to identify and treat inflammatory bowel disease (IBD), it remains a significant source of morbidity and financial burden. The current treatment of IBD is also fraught with severe complications and adverse events. Therefore identification of preventable risk factors and development of risk-free novel agents is imperative to the progress of this field. In the recent past vitamin D has come to limelight for its immune modulating properties and has attracted the attention of IBD researchers worldwide, on one hand vitamin D deficiency is thought to play a role in pathogenesis of IBD and on the other hand it may be a consequence of IBD. There is significant disagreement amongst studies evaluating the relationship between IBD and vitamin D deficiency. This is at least partly due to confounding by factors that may affect both plasma vitamin D concentration and IBD. These factors may include age, race, gender, sex and body mass index (BMI). Additionally such data is quite sparse on African American patients. The population in Mississippi is unique in having a high percentage of African Americans as well as obese subjects. In this study we identified that a BMI > 30 and African American race are independent risk factors for vitamin D deficiency.

### Research frontiers

The association between vitamin D deficiency and pathogenesis of IBD is under investigation. Prior studies have significant disagreement. Better identification of confounding factors can help streamline future research and obtain concordant results. A more precise understanding of this relationship may lead to novel insights in to prevention and management.

### Innovations and breakthroughs

In this study the authors studied multiple possible risk factors for vitamin D deficiency in patients with and without IBD. The authors applied multiple regression to establish that BMI > 30 and African American race are associated with vitamin D deficiency in those with and without IBD. The authors also confirmed that age and gender did not significantly effect serum 25(OH)D concentrations in the studied population. This study confirms the findings of a large bi-directional Mendelian analysis. However literature is lacking in such data in African American patients and that makes our population unique.

### Applications

This study suggests that in future studies assessing the role of vitamin D deficiency in development of IBD as well as that of vitamin D in management of

IBD should take into account the effects of race and BMI.

### Terminology

IBD or inflammatory bowel disease is a group of conditions of the small and large bowel characterized by chronic inflammation and a remitting and relapsing course. Crohn's disease and ulcerative colitis are the two main forms of inflammatory bowel disease. While the mechanism of tissue destruction is autoimmune the exact etiology of these conditions is unknown. Over the past decade significant research has lead to better understanding and treatment options for these conditions however these treatments are associated with significant adverse events with average efficacy. The role of preventable risk factors such as vitamin D deficiency and obesity is currently under investigation.

### Peer-review

This is a well-written paper addressing the relevant scientific question; predictors of vitamin D deficiency in especially African American IBD patients. BMI > 30 and race and not diagnosis are found as predictors for vitamin D insufficiency, which is interesting and relevant to clinic practise.

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## Retrospective Study

# Prediction of esophageal and gastric histology by macroscopic diagnosis during upper endoscopy in pediatric celiac disease

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## Abstract

### AIM

To determine the sensitivity of macroscopic appearance for predicting histological diagnosis at sites other than duodenum in pediatric celiac disease (CD).

### METHODS

Endoscopic and histologic findings in pediatric patients undergoing upper endoscopy for first-time diagnosis of CD at Stollery Children's Hospital from 2010-2012 were retrospectively reviewed.

### RESULTS

Clinical charts from 140 patients were reviewed. Esophageal and gastric biopsies were taken in 54.3% and 77.9% of patients, respectively. Endoscopic appearance was normal in the esophagus and stomach in 75% and 86.2%. Endoscopic esophageal diagnoses were eosinophilic esophagitis (EE) (11.8%), esophagitis (7.9%), glycogenic acanthosis (1.3%) and non-specific abnormalities (3.9%). Endoscopic gastric diagnoses were gastritis (8.3%), pancreatic rest (0.9%), and non-specific abnormalities (4.6%). Histology was normal in 76.3% of esophageal and 87.2% of gastric speci-

mens. Abnormal esophageal histology was EE (10.5%), esophagitis (10.5%), glycogenic acanthosis (1.3%) and non-specific (1.3%). Gastritis was reported in 12.8% of specimens. Sensitivity and specificity of normal endoscopy for predicting normal esophageal histology was 86.2% and 61.1%, and for normal gastric histology was 87.4% and 21.4%.

## CONCLUSION

In the absence of macroscopic abnormalities, routine esophageal and gastric biopsy during endoscopy for pediatric CD does not identify major pathologies. These findings have cost and time saving implications for clinical practice.

**Key words:** Endoscopy; Histology; Esophagus; Gastric biopsy; Celiac disease

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**Core tip:** We performed a retrospective chart review of esophageal and gastric endoscopic and histologic findings in pediatric patients diagnosed with celiac disease (CD). Our findings suggest that, in the absence of macroscopic abnormalities, routine esophageal and gastric biopsy during upper endoscopy for pediatric CD does not identify major pathologies. The implication of limiting biopsies to the duodenum and duodenal bulb may be both cost and time-saving. Overall, the results of this study may have the ability to promote standardization and optimal resource allocation for routine diagnostic practices for pediatric CD.

Boschee ED, Yap JYK, Turner JM. Prediction of esophageal and gastric histology by macroscopic diagnosis during upper endoscopy in pediatric celiac disease. *World J Gastroenterol* 2017; 23(4): 646-652 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/646.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.646>

## INTRODUCTION

Celiac disease (CD) is an autoimmune gluten-dependent enteropathy characterized by small intestinal inflammation and villous atrophy in genetically susceptible individuals<sup>[1]</sup>. Intestinal inflammation and activation of the immune system leads to production of autoantibodies such as anti-tissue transglutaminase (tTG) and endomysial antibodies (EMA), which are now widely used as a key component of screening and diagnosis<sup>[2]</sup>. The preferred diagnostic approach in North America involves both serology and confirmatory upper endoscopy with small bowel biopsy. While the diagnosis of CD has increased dramatically in recent decades, with an estimated prevalence in North America of 1%, CD is still felt to be significantly underdiagnosed<sup>[1,3]</sup>.

Accurate diagnosis of celiac disease requires a

minimum of six duodenal biopsies; a minimum of four biopsies from the distal duodenum and one or two biopsies from the duodenal bulb are recommended<sup>[4]</sup>. Current guidelines for endoscopic and histologic diagnostic practices for CD focus on duodenal biopsies; there is no consensus over a standard approach to the upper endoscopy as a whole. Therefore, in the absence of specific guidelines practice is likely to vary regarding additional biopsies taken during endoscopy, which may be taken routinely from sites other than the duodenum even in the face of normal microscopic findings. Additional biopsies have the potential to add considerable additional cost for the pathological assessment of this common duodenal disorder. Furthermore, accumulating evidence suggests that a biopsy-avoiding approach may be accurate in select pediatric patients with CD<sup>[5-10]</sup>. This approach has not been endorsed for North America, in part given concerns over the potential for missing alternate tissue diagnoses<sup>[11]</sup>. Increasing demands and resource allocation pressure on existing endoscopy resources in Canada may mean that such an approach, if proven low risk, could be considered. However, there is a paucity of studies investigating the frequency of endoscopic and histological abnormalities in intestinal sites apart from the duodenum in pediatric patients with CD and the utility of endoscopic diagnosis in predicting tissue histology in the stomach and esophagus in CD has yet to be studied.

We therefore aimed to study the sensitivity of normal esophageal and gastric macroscopic appearance in predicting normal tissue histology at these sites in pediatric patients with CD. Our secondary goal was to report the prevalence of coexistent esophageal and gastric diagnoses in pediatric CD. We hypothesized that normal endoscopic appearance is highly predictive of normal histology in the esophagus and stomach in pediatric patients with CD, thereby obviating the need to routinely biopsy these areas in the absence of gross macroscopic abnormalities. Additionally, we hypothesized that few pediatric patients with CD would have coexistent gastrointestinal diagnoses of clinical significance, suggesting that few alternate diagnoses would be missed by a biopsy avoiding approach in intestinal sites other than the duodenum.

## MATERIALS AND METHODS

The research study was approved by the University of Alberta Human Research Ethics Board. A single researcher (Boschee ED) performed chart review and data collection. Patients were identified retrospectively from the Stollery Children's Hospital Celiac Disease Clinic database, 2010-2012, and included following review of patient clinic charts. Criteria for inclusion were: age between 0 and 18 years at the time of biopsy; completion of an assessment by a pediatric gastroenterologist at the Stollery Children's Hospital following referral to consider a diagnosis of celiac disease; completion of first-time diagnostic upper endos-

**Table 1** Characteristics of pediatric celiac disease patients reviewed for study inclusion at the time of upper endoscopy and biopsy ( $n = 140$ )  $n$  (%)

	Result
Demographics	
Female gender	86/140 (61.4)
Age at biopsy (yr) (mean $\pm$ SD)	9.1 $\pm$ 4.3
Weight at biopsy (kg) (mean $\pm$ SD)	34.7 $\pm$ 18.9
Height at biopsy (cm) (mean $\pm$ SD)	134.4 $\pm$ 25.1
Presenting symptoms	
Abdominal pain	87/140 (62.1)
Constipation	41/140 (29.3)
Bloating	40/140 (28.6)
Fatigue	37/140 (26.4)
Irritability	36/140 (25.7)
Poor weight gain	35/140 (25.0)
Diarrhea	33/140 (23.6)
Vomiting	13/140 (9.3)
Asymptomatic	14/140 (10.0)
Serology	
aTTG ( $n = 137$ ) (mean $\pm$ SD)	393.9 $\pm$ 634.1 (0.9-3550)
IgA ( $n = 75$ ) (mean $\pm$ SD)	1.3 $\pm$ 0.7 (0.1-3.1)

aTTG: Anti-tissue transglutaminase; IgA: Immunoglobulin A.

copy and duodenal biopsy; and subsequent proven histological diagnosis of CD. All patients diagnosed with CD had duodenal histological classification of Marsh grade 2 or 3, as per current NASPGHAN guidelines<sup>[12]</sup>. Patients following a gluten free diet at the time of endoscopy and biopsy were excluded, as were those with a prior diagnosis of CD or with non-confirmatory histology (Marsh grade 0 or 1).

Patient demographic information collected included age at the time of biopsy, as well as gender, growth parameters and presenting symptoms (if any). When available, serological test results including serum aTTG, IgA and EMA prior to endoscopy and biopsy were recorded. Endoscopy and histology reports were reviewed for the reported endoscopic and histologic diagnoses in the esophagus and stomach.

The statistical analyses of this study were performed by biostatisticians, Dr. Maryna Yaskina and Sung Hyun Kang, of the Women and Children's Health Research Institute. Statistical analyses were performed using SPSS 23 and R version 3.2.3 software. Continuous variables were expressed as means, and statistical significance was defined by alpha less than 0.05. Sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios were calculated using a 2  $\times$  2 table in the standard manner. Agreement between macroscopic and histologic diagnoses at each tissue site was measured using the Cohen's kappa coefficient, a measure of agreement for categorical variables.

## RESULTS

One-hundred forty patients were identified and reviewed for possible inclusion in the study; 61.4% were female and mean age at biopsy was 9.1 years

**Table 2** Macroscopic and histologic findings in the esophagus of pediatric patients undergoing upper endoscopy for investigation of celiac disease  $n$  (%)

Diagnosis	Endoscopic	Histologic
Normal	57/76 (75.0)	58/76 (76.3)
Eosinophilic esophagitis	9/76 (11.8)	8/76 (10.5)
Reflux esophagitis	6/76 (7.9)	8/76 (10.5)
Glycogenic acanthosis	1/76 (1.3)	1/76 (1.3)
Non-specific abnormalities	3/76 (3.9)	1/76 (1.3)

(Table 1). The most frequent presenting symptoms were abdominal pain, constipation, bloating, fatigue, and irritability; 10% of patients were asymptomatic (Table 1). The mean aTTG prior to biopsy was 393.9 with a range of 0.9 to 3550 (Table 1). In this population, esophageal biopsies were taken in 54.3% of patients ( $n = 76$ ), and gastric biopsies were taken in 77.9% ( $n = 109$ ). Of the patients with esophageal or gastric biopsies, five pediatric gastroenterologists performed upper endoscopy (gastroenterologist #1 - 40 patients, #2 - 33 patients, #3 - 19 patients, #4 - 13 patients, #5 - 9 patients) and three pathologists interpreted the specimens (pathologist #1 - 70 patients, #2 - 43 patients, #3 - 1 patient).

A normal macroscopic appearing esophagus was reported in 75.0% (57/76) of patients (Table 2). All these 57 patients with normal macroscopic esophageal appearance had normal histology, except for two with eosinophilic esophagitis and five with reflux esophagitis. Macroscopic abnormalities visible at upper endoscopy included: eosinophilic esophagitis (9/76, 11.8%), reflux esophagitis (6/76, 7.9%), glycogenic acanthosis (1/76, 1.3%) and non-specific abnormalities (3/76, 3.9%) (Figure 1). These non-specific macroscopic abnormalities were nodularity and a prominent gastro-esophageal Z-line. Endoscopic signs of eosinophilic esophagitis included exudates, trachealization and linear furrowing. Macroscopic signs of reflux esophagitis were erythema and friability, erosions and ulceration. Esophageal biopsies revealed normal histology in 76.3% (58/76) of patients. Histologic esophageal abnormalities reported included: eosinophilic esophagitis (8/76, 10.5%), reflux esophagitis (8/76, 10.5%), glycogenic acanthosis (1/76, 1.3%) and non-specific abnormalities (1/76, 1.3%). The latter was increased eosinophils at the gastro-esophageal junction.

In the stomach, 86.2% (94/109) of patients had a normal macroscopic appearance (Table 3), while macroscopic abnormalities included gastritis (9/109, 8.3%), pancreatic rest (1/109, 0.9%) and non-specific abnormalities (5/109, 4.6%) (Figure 2). Endoscopic signs of gastritis described included edema, hyperemia and erosions. The non-specific gastric abnormalities reported were non-specific erythema, edema and hyperemia. Biopsies of the stomach were normal in 87.2% (95/109) of patients. Gastritis was found in 12.8% (14/109) of gastric specimens, with only one child having proven *H. pylori* infection. Only 11/94

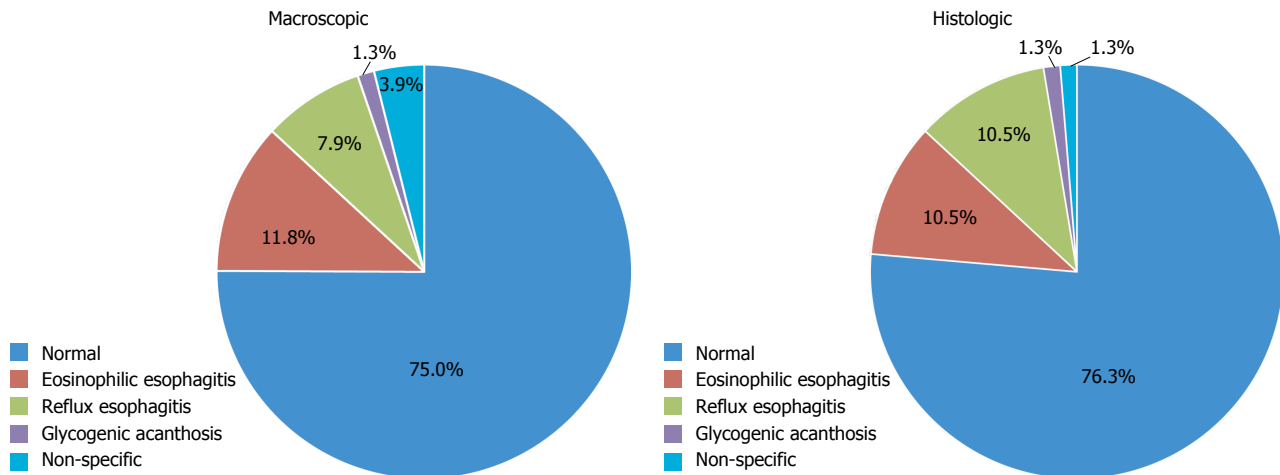


Figure 1 Macroscopic and histologic findings in the esophagus of pediatric patients undergoing upper endoscopy for investigation of celiac disease.

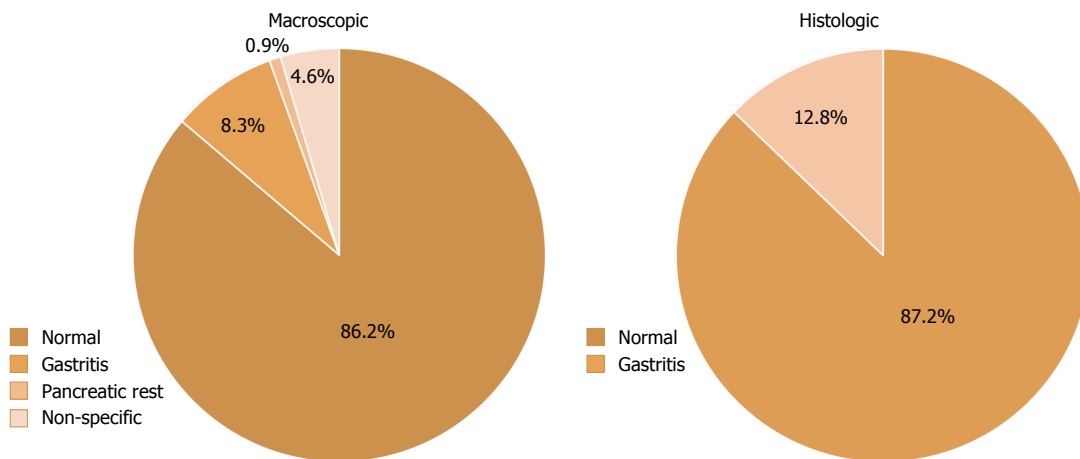


Figure 2 Macroscopic and histologic findings in the stomach of pediatric patients undergoing upper endoscopy for investigation of celiac disease.

**Table 3** Macroscopic and histologic findings in the stomach of pediatric patients undergoing upper endoscopy for investigation of celiac disease *n* (%)

Diagnosis	Endoscopic	Histologic
Normal	94/109 (86.2)	95/109 (87.2)
Gastritis	9/109 (8.3)	14/109 (12.8)
Pancreatic rest	1/109 (0.9)	-
Non-specific abnormalities	5/109 (4.6)	-

patients with a normal endoscopic gastric appearance had a histological diagnosis of gastritis. The patient with *H. pylori* infection had a macroscopic diagnosis of gastritis.

Of the 87 patients who reported abdominal pain as a presenting symptom, 75.9% had normal macroscopic appearance of both the esophagus and stomach. Seventy-two of these 87 patients had biopsies taken from at least one of the esophagus or stomach, and 70.8% of these patients had normal histologic results. Eosinophilic esophagitis was found in 4 patients reporting abdominal pain, by both macroscopic and histo-

logic diagnosis. Esophagitis was found histologically in 6 of these patients, 1 of which was associated with macroscopic changes. Gastritis was found histologically in 8 patients with abdominal pain, 1 of which was associated with macroscopic abnormalities. Sixteen of the patients with abdominal pain had a normal appearing esophagus and stomach by endoscopy and had no biopsy taken from either site due to gastroenterologist practice. The other gastrointestinal presenting symptoms relevant to the esophagus and stomach reported in the study population were bloating and vomiting. Of the 40 patients who reported bloating, 82.5% received a normal macroscopic diagnosis in the esophagus and stomach; 71.9% of this group who had biopsies taken from at least one of these sites (*n* = 32) also had normal histologic results. In the 13 patients with vomiting, 61.5% had normal esophageal and gastric macroscopic diagnoses and 63.6% of the 11 patients who had biopsies taken also had normal histology.

The sensitivity of normal macroscopic appearance for predicting normal esophageal histology was 86.2% (97.5%CI: 74.6%-93.9%). The sensitivity of normal macroscopic appearance for predicting normal gastric



histology was 87.4% (97.5%CI: 79.0%-93.3%). The positive predictive value of normal macroscopic diagnosis for normal histology in the esophagus and stomach were 87.7% and 88.3%, respectively. The Cohen's kappa coefficient representing agreement between esophageal macroscopic and histologic diagnoses for all of the gastroenterologists as a group was 0.464 (95%CI: 0.233-0.695,  $P < 0.001$ ), and in the stomach was 0.085 (95%CI: -0.133-0.303,  $P = 0.372$ ).

## DISCUSSION

The findings of this study suggest that a normal macroscopic appearance in the esophagus and stomach is adequate to predict normal tissue histology in patients undergoing endoscopy for the diagnosis of pediatric celiac disease, obviating the need for routine biopsies from these additional gastrointestinal sites. Limiting the number of tissue biopsies may potentially reduce endoscopy time and costs, as well as endoscopy-associated risks such as bleeding or perforation. In Alberta, based on estimates from the Northern Alberta Clinical Trials and Research Centre, the estimated costs associated with processing each gastrointestinal biopsy specimen is \$48, plus \$55 for pathologist reporting<sup>[13]</sup>. Reducing the number of total routine biopsies during endoscopy for CD, and hence costs as well as shortened total endoscopy times, could have important system-level implications.

There is a relative paucity of recent studies in the literature investigating the sensitivity of macroscopic diagnosis for predicting tissue histology in pediatrics, particularly in patients with celiac disease. Several adult studies done prior to 1990 reported the correlation between abnormal gastric macroscopic and histologic findings in adults with dyspepsia to be about 50%<sup>[14,15]</sup>. Black *et al*<sup>[16]</sup> studied the sensitivity of endoscopic appearance for predicting abnormal gastric and duodenal histology in pediatric patients with a variety of gastrointestinal complaints in 1988, finding higher severity of reported endoscopic disease as compared to histologic results. Dahshan and Rabah<sup>[17]</sup> published a similar study in 2000 evaluating the correlation between esophageal and gastric endoscopic and histologic findings in 204 children. They reported sensitivities of 81% in the esophagus and 86% in the stomach, and esophageal and gastric specificities of 41% and 43%, respectively<sup>[17]</sup>. Long *et al*<sup>[18]</sup> reported a sensitivity of 54% and specificity of 92% for prediction of duodenal histology by macroscopic findings in pediatric biopsy-proven duodenitis. A recent article investigated duodenitis in children with multiple gastrointestinal diagnoses, including CD, and found a sensitivity of 34% for endoscopic diagnosis in predicting duodenal histology<sup>[19]</sup>. Lastly, Sheiko *et al*<sup>[20]</sup> reported the correlation of macroscopic and histologic findings in 1000 pediatric patients with a variety of gastrointestinal concerns, 6.6% of who had histology consistent with celiac disease. This group reported a

kappa coefficient of 0.45 for endoscopic and histologic concordance in the esophagus, and 0.18 for gastric concordance, recommending routine collection of esophageal, gastric and duodenal specimens in their broad, mostly non-celiac population<sup>[20]</sup>. Significant improvements in endoscopic imaging capabilities and detail have been made over the past few decades, and this remains the first pediatric study to specifically investigate the ability of normal macroscopic findings to predict normal esophageal and gastric histology in patients with celiac disease.

While literature focused on coexistent gastrointestinal diagnoses at endoscopy in pediatric CD is limited, the coexistent esophageal diagnoses found in this study, specifically eosinophilic esophagitis and gastritis, are similar to that which has been previously described. Stewart *et al*<sup>[21]</sup> reported concomitant eosinophilic esophagitis in 3/245 patients diagnosed with CD, with a standardized incidence ratio of 48.4 in their population. In a case series of 17 children with eosinophilic esophagitis, 6/17 were also found to have CD though three of these patients had subsequent histologic remission on a gluten free diet<sup>[22]</sup>. The authors concluded that the eosinophilic infiltration in these patients may have been directly related to the CD itself<sup>[20]</sup>. A large Italian study of 230 pediatric CD patients found esophagitis in 12.6% of their study population, which is a very similar prevalence to the current study<sup>[23]</sup>. Both chronic superficial and lymphocytic gastritis have been described in pediatric CD, and it has been suggested that mucosal involvement in CD is not limited to the duodenum<sup>[24]</sup>. Oderda *et al*<sup>[23]</sup> reported chronic superficial gastritis in 40% of their patients. Another recent group described gastritis in a group of children with CD, finding chronic superficial gastritis in 21% of patients, lymphocytic gastritis in 7%, interstitial gastritis in 0.5% and *H. pylori*-related gastritis in 2.7%<sup>[24]</sup>. In this study, lymphocytic gastritis was seen predominantly in the children with the most advanced CD on duodenal biopsy (Marsh grade 3C) and seemed to correlate with longer exposure to gluten<sup>[24]</sup>. The authors concluded that "gastric intraepithelial lymphocytosis may represent a concurrent manifestation of CD rather than a separate entity in the pediatric population<sup>[24]</sup>". Chronic superficial gastritis was more prevalent in children with gastrointestinal symptoms as opposed to an atypical presentation of CD and showed improvement with gluten free diet; it was theorized to be due to CD-related delayed gastric emptying<sup>[24]</sup>. Interestingly, only 20.8% of the children with chronic superficial gastritis had macroscopic abnormalities<sup>[24]</sup>.

Regardless, in this study we found few additional diagnoses that would alter clinical management and were not identified macroscopically. Two patients with histologic eosinophilic esophagitis and five with reflux esophagitis had normal appearing endoscopy, however the clinical significance of these incidental microscopic changes is unclear and may not necessitate any

changes in patient management. In the stomach, 11 out of 94 patients with histologic gastritis had a normal endoscopic diagnosis. The singular case of *H. pylori*-related gastritis was correctly identified by endoscopy. Clinical presenting symptoms were not reliable predictors of macroscopic or histologic abnormalities. Overall, this study supports the conclusion that, in the absence of macroscopic abnormalities, few clinically significant gastric and esophageal diagnoses would be missed without biopsies from these sites.

Advances in serological testing have shifted the focus of celiac disease diagnosis largely to serology results, and biopsy is being thought of increasingly as confirmatory. Current European guidelines propose that pediatric patients with classic symptoms of celiac disease, at risk HLA genes and markedly elevated aTTG antibodies (greater than ten times the upper limit of normal) may not require intestinal biopsy for diagnosis. Yet this approach has not been widely adopted in North America. There are likely to be multiple reasons for lack of acceptability of a serological diagnostic approach in North America, including fear of inaccurately committing patients to a lifelong gluten free diet and lack of standardization of celiac serology tests between laboratories<sup>[1]</sup>. The concern has also been expressed that coexistent diagnoses might well be missed by a biopsy avoiding approach<sup>[7]</sup>. This study would suggest that this a minimal concern and should not preclude considering a serological diagnostic approach in North America, which would have significant resource and system implications<sup>[5]</sup>. Hence, this study is important as it serves as the first pediatric study to focus specifically on esophageal and gastric coexistent diagnoses in CD, helping to address one barrier to the adoption of a serological approach to CD diagnosis in Canada.

This study has a few limitations. First, as this was a retrospective review, multiple practitioners were involved in performing endoscopy and in interpreting histological specimens, so variations in interpretation and diagnosis between individuals may have occurred. The gastroenterologists and pathologists were also not blinded to the patients' clinical history, which could also have influenced their interpretation of endoscopic and histologic findings. Additionally, there were an unequal number of patients studied who had esophageal biopsies taken as compared to those with gastric biopsies (76 vs 109 patients, respectively). This of itself suggests that endoscopists may already attempt to reduce the number of biopsies taken based on macroscopic findings and their own perceptions of the relevance of any biopsy findings in that setting. Unfortunately, this also biases our comparison between groups in terms of whether the diagnostic performance of macroscopic appearance for histology is more accurate in one intestinal site compared to the other. Nevertheless, this study provides evidence to support the current approach of those endoscopists who do not take routine gastric and/

or esophageal biopsies in the setting of CD diagnosis. It also provides evidence to improve efficiency and reduce cost in current endoscopic and biopsy practices for pediatric CD by avoiding routine esophageal and gastric biopsy approach in the absence of endoscopic findings.

In conclusion, the findings of this study suggest that normal macroscopic diagnosis is strongly predictive of normal histology in the esophagus and stomach in pediatric patients with CD. Therefore, routine esophageal and gastric biopsies during endoscopy for pediatric CD are not required, in the absence of gross macroscopic findings. The implication of limiting biopsies to the duodenum and duodenal bulb may be both cost and time-saving. Additionally, we report a prevalence of coexistent gastrointestinal findings to CD in our patient population of 21% in the esophagus (including eosinophilic esophagitis and reflux esophagitis) and 13% with gastritis. However, the prevalence of clinically relevant diagnoses that may be missed by a biopsy-avoiding approach in the absence of macroscopic abnormalities is very low. Overall, the results of this study have the ability to promote standardization and optimal resource allocation for routine diagnostic practices for pediatric CD.

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## COMMENTS

### Background

Celiac disease (CD) is the most common autoimmune enteropathy in children. In North America, diagnosis requires upper endoscopy and duodenal biopsies. Recent guidelines support diagnosis without biopsy in select pediatric patients, yet concerns exist over the potential for missing alternate tissue diagnoses. Additionally, in the absence of specific guidelines, practice is likely to vary regarding additional biopsies taken during endoscopy, which may be taken routinely from sites other than the duodenum even in the face of normal microscopic findings. Additional biopsies have the potential to add considerable additional cost for the pathological assessment of this common duodenal disorder.

### Research frontiers

There is a paucity of studies investigating the frequency of endoscopic and histological abnormalities in intestinal sites apart from the duodenum in pediatric patients with CD. Additionally, the utility of endoscopic diagnosis in predicting tissue histology in the stomach and esophagus in CD has yet to be studied.

### Innovations and breakthroughs

The findings of this study suggest that normal macroscopic diagnosis is strongly predictive of normal histology in the esophagus and stomach in pediatric patients with celiac disease. Therefore, routine esophageal and gastric biopsies during endoscopy for pediatric celiac disease are not required in the absence of gross macroscopic findings.

## Applications

The implication of limiting biopsies to the duodenum and duodenal bulb may be both cost and time-saving. Overall, the results of this study have the ability to promote standardization and optimal resource allocation for routine diagnostic practices for pediatric celiac disease.

## Terminology

Macroscopic - visual appearance of gastrointestinal mucosa at the time of endoscopy. Histologic - microscopic appearance of gastrointestinal mucosa cells.

## Peer-review

This is a well designed, performed and written clinical retrospective study for the determination of the sensitivity of normal esophageal and gastric macroscopic appearance in predicting normal tissue histology at sites other than the duodenum in pediatric CD patients. They concluded that routine esophageal and gastric biopsies during endoscopy for pediatric CD are not required, in the absence of macroscopic abnormalities.

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## Retrospective Study

# Laparoscopic resection vs laparoscopic radiofrequency ablation for the treatment of small hepatocellular carcinomas: A single-center analysis

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**Author contributions:** Casaccia M designed the research providing intellectual content of critical importance to the work, and drafted the article; Santori G designed the research, performed the analysis, and revised the article; Bottino G collected data and drafted part of the article; Diviacco P drafted part of the article and revised it; Andorno E provided intellectual content of critical importance and made the final revision.

**Institutional review board statement:** This study didn't require the approval by the Ethics Committee of the IRCCS - Azienda Ospedaliera Universitaria San Martino-IST of Genoa.

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

**Conflict-of-interest statement:** We have no financial relationships to disclose.

**Data sharing statement:** No additional data are available.

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## Abstract

### AIM

To compare survival and recurrence after laparoscopic liver resection (LLR) and laparoscopic radiofrequency ablation (LRFA) for the treatment of small hepatocellular carcinoma (HCC).

### METHODS

Between June 1, 2005 and November 30, 2010, 46 patients ( $62.26 \pm 8.55$  years old; female/male: 12/34) treated for small HCC were enrolled following strict criteria. Patients with better liver function and larger tumors were referred for LLR ( $n = 24$ ), while those with poorer liver function and multiple tumors were referred for LRFA ( $n = 22$ ), and they were then followed for similar durations ( $44.74 \pm 21.3$  mo for LLR vs  $40.27 \pm 30.8$  mo for LRFA).

### RESULTS

The LLR and LRFA groups were homogeneous with regard to age, sex, etiology of liver cirrhosis, and AFP levels. The overall survival (OS) and disease-



free survival (DFS) probability was 0.354 and 0.260, respectively. A significantly higher OS was observed in the LLR group (LLR: 0.442; LRFA: 0.261;  $P = 0.048$ ), whereas no statistical difference was found for DFS (LLR: 0.206; LRFA: 0.286;  $P = 0.205$ ). In the LRFA group was treated a greater number of nodules (LLR:  $1.41 \pm 0.77$ ; LRFA:  $2.72 \pm 1.54$ ;  $P < 0.001$ ). Cox regression analysis found the number of intraoperative HCC nodules as the unique variable statistically significant for OS (hazard ratio: 2.225;  $P < 0.001$ ). The rank-hazard plot showed a steeper increase of relative hazard for intraoperative nodules  $> 2$ .

## CONCLUSION

Our preliminary results confirm the superiority of hepatic resection on thermoablation in the treatment of small HCC in selected patients, when both approaches are made laparoscopically. LLR showed better results compared to LRFA in terms of OS. These data need to be confirmed by further studies on a larger number of patients.

**Key words:** Hepatocellular carcinoma; Laparoscopic liver resection; Laparoscopic radiofrequency ablation; Survival; Disease-free survival

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**Core tip:** This is a retrospective study to evaluate and compare the oncological results of hepatic resection and thermoablation in the treatment of small hepatocellular carcinoma in selected patients, when both approaches are made laparoscopically. Our preliminary results confirm the superiority of laparoscopic liver resection compared to laparoscopic radiofrequency ablation in terms of overall survival. These data need to be confirmed by further studies on a larger number of patients.

Casaccia M, Santori G, Bottino G, Diviacco P, Andorno E. Laparoscopic resection vs laparoscopic radiofrequency ablation for the treatment of small hepatocellular carcinomas: A single-center analysis. *World J Gastroenterol* 2017; 23(4): 653-660 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/653.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.653>

## INTRODUCTION

Both liver resection and ablative therapies are able to modify the natural history of hepatocellular carcinoma (HCC)<sup>[1-3]</sup>. Indeed, it is demonstrated that they allow an increase in survival in patients with small nodules, although relapse after these two treatments remains a questionable topic. However, studies in the literature comparing radiofrequency ablation to liver resection seem to confirm the superiority of the latter in terms of overall survival and disease-free survival<sup>[4-6]</sup>. To

date, interstitial therapies were always compared to hepatic resection performed through an open approach. However, the fact of comparing a minimally invasive technique to a quite invasive one has some limits. Currently we are witnessing an increasing use of laparoscopy in liver surgery, extending indications to patients with cirrhosis and HCC<sup>[7,8]</sup>. Laparoscopic surgery of the liver represents a viable alternative to open surgery retaining its characteristics of a minimally invasive approach and providing better results and a similar postoperative complication rate<sup>[9,10]</sup>. Moreover, laparoscopic liver resection (LLR) in selected cirrhotic patients with HCC has shown advantages in terms of survival and recurrence similar to those after open surgery<sup>[11,12]</sup>.

With the aim to compare LLR and laparoscopic radiofrequency ablation (LRFA) in terms of postoperative survival and HCC recurrence, we evaluated two groups of cirrhotic patients with similar clinical characteristics.

## MATERIALS AND METHODS

### Patient characteristics

All patients ( $n = 46$ ) referred to our unit between June 1, 2005 and November 30, 2010 underwent an established protocol to define HCC staging. In case of severe liver impairment, the possibility of liver transplantation was considered as first option. Patient eligibility to liver transplantation did not preclude LLR or LRFA as a bridge treatment. To be included in the study patients were assessed for the severity of the liver disease following the Child-Pugh classification<sup>[13]</sup>. Plasma levels of alfa-fetoprotein (AFP) were measured. In case of presence of severe coagulation disorders or very low platelet levels (platelet count  $< 40 \times 10^9/L$ ), patients were excluded from the procedure. A spiral computed tomography (CT) scan and/or gadolinium-enhanced magnetic resonance imaging (MRI) was performed in all patients to document the number, size, and segmental location of all liver lesions. Only patients with "small" HCC were taken into account. According to Yao *et al*<sup>[14]</sup>, "small" HCC is defined by a single HCC nodule  $\leq 6.5$  cm, or with  $\leq 3$  lesions when the largest of which is  $\leq 4.5$  cm. The choice of LLR or LRFA was based on the tumor position: if it was located in a resectable segment, a LLR was realized; in case of deep-sited lesions requiring major hepatic resection, LRFA was indicated. Furthermore, LRFA permitted to treat lesions otherwise difficult or impossible to reach by a percutaneous approach. Each patient agreed to treatment by written consent.

### LLR group

Twenty-four HCC patients were selected to undergo LLR. These patients fell into the following conditions: (1) well compensated Child's class A/B cirrhosis; (2) esophageal varices  $\leq$  grade 2, platelet count  $\geq 40 \times 10^9/L$  and/or international normalized ratio  $> 1.5$ ;

and (3) “small” lesion accessible to the laparoscopic approach and treatable by limited resection (< 3 segments). Only one patient was operated in emergency situation for an hemoperitoneum from a ruptured HCC located in segment III. Most resections were anatomic (*i.e.*, resection of 1 or more anatomic segments). Non-anatomic resections consisted of resection of less than 1 segment including the tumor and an intended 1-cm tumor-free margin. Details of the technique adopted were described elsewhere<sup>[15]</sup>.

### LRFA group

Twenty-two HCC patients were selected for LRFA. These patients fulfilled at least one of the following criteria: (1) large tumours (but with a diameter < 5 cm, or multiple lesions requiring repeated punctures); (2) superficial lesions adjacent to visceral structures which could be displaced by laparoscopic maneuvers; and (3) deep-sited lesions with a very difficult or impossible percutaneous approach. Exclusion criteria were the same as for the LLR group. However, main portal branches thrombosis or a severe liver disease (Child-Pugh class C) did not contraindicated the procedure.

The surgical technique adopted for LRFA was described elsewhere<sup>[16]</sup>.

The liver was investigated by intraoperative laparoscopic ultrasonography (LUS) to confirm the number and size of the lesions and define their relations with the intrahepatic vascular structures. Furthermore, a definitive histological diagnosis of both HCC and liver cirrhosis has been obtained by an intraoperative biopsy of all patients undergoing LRFA or hepatic resection.

### Patient follow-up

To assess the response to LRFA or LLR, liver US and CT scan (and/or MRI) were performed within 1 month after treatment. When no enhancement or a thin peripheral enhancement rim (representative of an inflammatory response) was observed, a complete response to LRFA was achieved. An incomplete response to LRFA was defined as persistent nodular enhancement. Post-treatment recurrence, evaluated by spiral CT scans at 3 mo and every 6 mo after treatment, was defined as the new appearance of a contrast-enhanced lesion area within 2 cm from the thermoablated nodule, or the surgical margin (local HCC recurrence), or > 2 cm from the same sites (distant HCC recurrence).

### Statistical analysis

The results were expressed as mean  $\pm$  standard deviations, counts and percentages. Continuous variables were preliminarily evaluated for normal distribution with the Shapiro-Wilk test, and then compared by using the Wilcoxon-Mann-Whitney test. Categorical variables were compared with the Fisher's

exact test.

Patient overall survival (OS) and disease-free survival (DFS) were calculated with the Kaplan-Meier product-limit estimator. In the OS evaluation, the entry/final time-point corresponded to date of treatment/date of death (uncensored observations) or date of last follow-up for patients alive at the end of the study (censored observations). The final time-point for DFS was the date of the first recurrence, by censoring survival/dead patients without any recurrence. The log-rank test was applied to evaluate the differences in OS and DFS after grouping for treatments.

Cox proportional-hazard models were performed for both patient survival and DFS. In each Cox model, the Efron approximation was used for its accuracy and computational efficiency. To determine whether the fitted Cox regression models adequately described the data, the proportional-hazards assumption was tested as previously described<sup>[17,18]</sup>. Only univariate Cox regression models that did not infringe the proportional hazard assumption for OS were presented. A rank-hazard plot for the covariates of significant Cox models was created as previously described<sup>[19]</sup>. Statistical significance was assumed with a two-tailed *P* value < 0.05.

The statistical analysis was performed by using the R software/environment (version 3.3.1; R Foundation for Statistical Computing, Vienna, Austria)<sup>[20]</sup>.

## RESULTS

Between June 1, 2005 and November 30, 2010, 46 patients ( $62.26 \pm 8.55$  years old; Female/Male: 12/34) treated for small HCC were enrolled following strict criteria. Patients with similar baseline characteristics underwent LLR ( $n = 24$ ) or LRFA ( $n = 22$ ). Patients with better liver function and larger tumors were referred for resection, while those with poorer liver function and multiple tumors were referred for ablation. Characteristics of patients grouped for laparoscopic procedure are summarized in Table 1. The LLR and LRFA groups were homogeneous with regard to age, sex, etiology of liver cirrhosis, and AFP levels. Mean size of dominant nodule was larger and almost statistically significant in the LLR group (33 mm vs 26.2 mm;  $P = 0.097$ ), whereas number of tumors was higher in the LRFA group, since 16 (72.7%) patients presented with multiple lesions in the LRFA group vs 4 (16.6%) patients in the LLR group ( $P < 0.001$ ). Consequently, the mean number of treated nodules per patient was greater in the LRFA group when compared with the LLR group ( $2.72 \pm 1.54$  vs  $1.41 \pm 0.77$ ,  $P < 0.001$ ). Liver function impairment was more severe in the LRFA group ( $P = 0.007$ ), where the patients classified in the Child class A were 54.5%, whereas in the LLR group they exceeded 90%. Mean follow-up duration for the LLR and LRFA groups was

**Table 1** Patient characteristics, etiology of liver cirrhosis, Child-Pugh class, and post-operative parameters grouped for laparoscopic procedure *n* (%)

Variables	LLR ( <i>n</i> = 24)	LRFA ( <i>n</i> = 22)	<i>P</i> value
Patient-related characteristics			
Sex (F/M)	8/16	4/18	NS
Age (yr)	63.58 ± 9.55	60.82 ± 7.25	NS
Etiology of liver cirrhosis			
HCV-related	11 (45.8)	8 (36.4)	NS
HBV-related	6 (25)	8 (36.4)	NS
Alcoholic	6 (25)	3 (13.6)	NS
Mixte	1 (4.2)	0 (0)	NS
Other	0 (0)	2 (9.1)	NS
Child-Pugh class			
Child A	22 (91.7)	12 (54.5)	0.007
Child B	2 (8.3)	6 (27.3)	NS
Child C	0 (0)	2 (9.1)	NS
Unknown	0 (0)	2 (9.1)	NS
Post-operative parameters			
Single tumour	20 (83.3)	6 (27.3)	< 0.001
Multiple tumours	4 (16.6)	16 (72.7)	< 0.001
Treated nodules/patient	1.41 ± 0.77	2.72 ± 1.54	< 0.001
Tumour diameter (mm)	33.0 ± 13.83	26.25 ± 13.13	NS
Follow-up (mo)	44.74 ± 21.30	40.27 ± 30.89	NS
Intrahepatic recurrence	16 (66.7)	15 (68.2)	NS
Local recurrence	5 (20.9)	8 (36.4)	NS
Recurrence IT (mo)	25.53 ± 19.11	22.88 ± 30.62	NS

LLR: Laparoscopic liver resection; LRFA: Laparoscopic radiofrequency ablation; HCV: Hepatitis C virus; HBV: Hepatitis B virus; IT: Interval time; NS: Not significant.

similar (44.74 ± 21.30 mo vs 40.27 ± 30.89 mo, *P* = 0.273).

Disease recurrence was observed in a shorter time for the LLR group when compared to LRFA (25.53 ± 19.11 mo vs 22.88 ± 30.62 mo), even if the difference did not reach statistical significance (*P* = 0.084). In both groups, HCC recurred mainly at distance from the previous site of treatment; when considering the local recurrence rate, this was more favorable for the LLR (20.9%) than for LRFA (36.4%), although without reaching statistical significance (*P* = 0.330). Treatments for liver recurrence after LLR included chemoembolization in 9 cases, orthotopic liver transplantation in 3 cases, percutaneous radiofrequency ablation in 2 cases, a repeat resection in 2 cases (5 patients receiving 2 treatments). Recurrences after LRFA were treated with orthotopic liver transplantation in 6 cases, chemoembolization in 5 cases and liver resection in 2 cases (2 patients receiving 2 treatments).

The OS probability was 0.354 (1-year OS: 0.891; 3-year OS: 0.660; 5-year OS: 0.512) (Figure 1A). The cumulative hazard plot for the corresponding OS curve is shown in Figure 1B. The DFS probability was 0.260 (1-year DFS: 0.591; 3-year DFS: 0.346; 5-year DFS: 0.260) (Figure 1C). The cumulative hazard plot for the corresponding DFS curve is shown in Figure 1D. By stratifying for laparoscopic treatment, a significantly

**Table 2** Cox proportional hazard regression for overall survival and disease-free survival

Variables	β	HR (95%CI)	<i>P</i> value
Overall survival			
Treatment (LRFA)	0.812	2.252 (0.983-5.156)	NS
Sex (Male)	0.073	1.076 (0.426-2.717)	NS
Age	0.008	1.008 (0.961-1.059)	NS
Diagnosis (HCV)	0.828	2.288 (0.624-8.382)	NS
Preop TACE (Yes)	0.500	1.649 (0.737-3.689)	NS
No. intraop. nodules	0.800	2.225 (1.594-3.108)	< 0.001
Local recurrence (Yes)	-0.014	0.985 (0.414-2.345)	NS
Dist. recurrence (Yes)	1.543	4.680 (0.618-35.440)	NS
Disease-free survival			
Treatment (LRFA)	0.464	1.590 (0.771-3.282)	NS
Sex (Male)	0.587	1.798 (0.732-4.422)	NS
Age	-0.019	0.980 (0.939-1.024)	NS
Diagnosis (HCV)	-0.300	0.740 (0.275-1.993)	NS
Preop TACE (Yes)	0.125	1.133 (0.549-2.340)	NS
No. intraop. nodules	0.216	1.241 (0.946-1.628)	NS

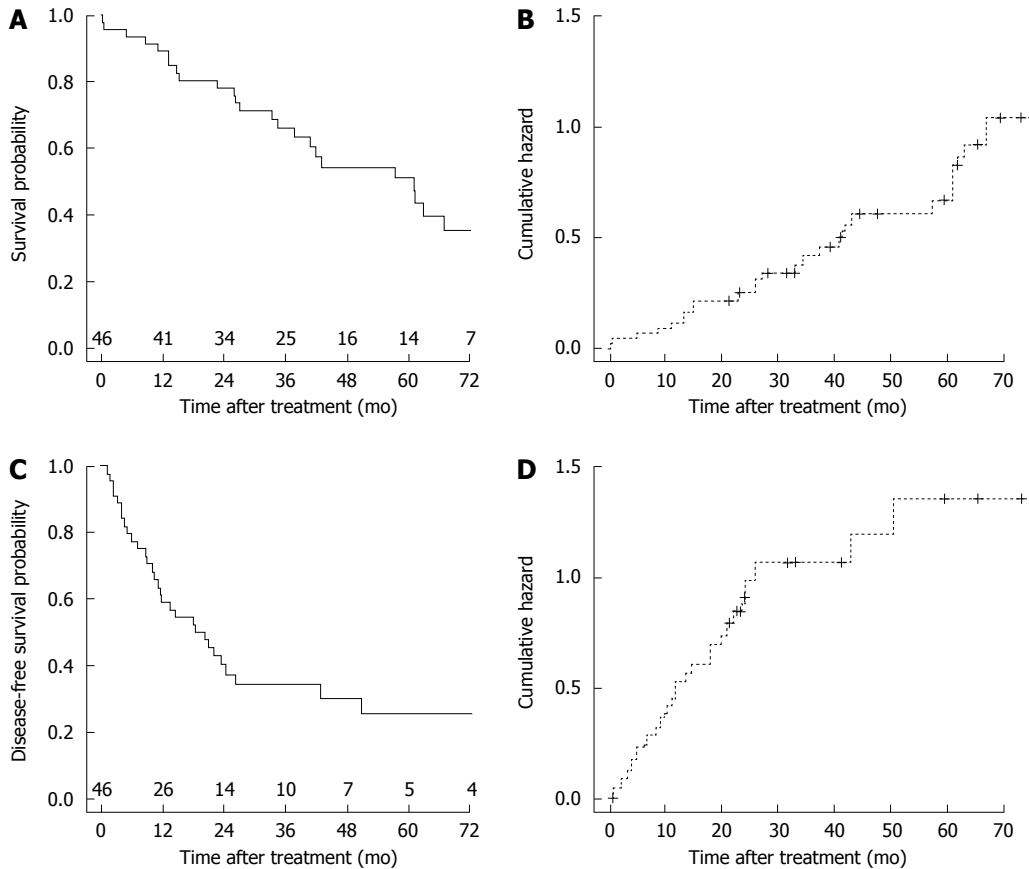
The square brackets show the target code selected for each categorical variable. β: Regression coefficient; LRFA: Laparoscopic radiofrequency ablation; HCV: Hepatitis C virus; TACE: Transarterial chemoembolization; NS: Not significant.

higher OS (*P* = 0.048) was observed in the LLR group (Figure 2A), whereas no difference for DFS (*P* = 0.205) occurred (Figure 2B).

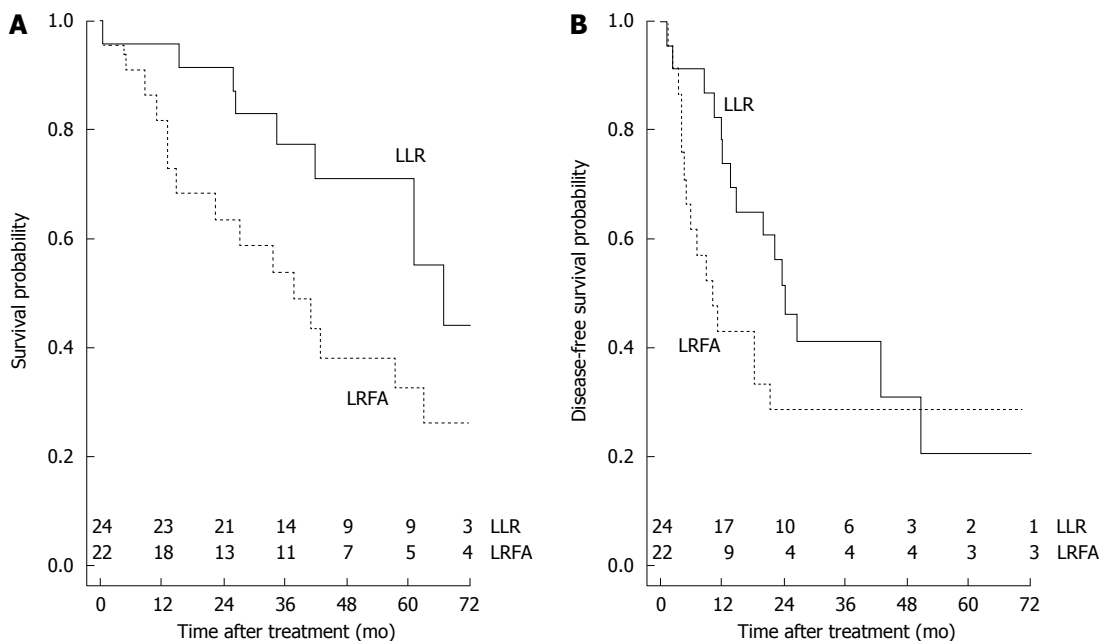
The main variables were entered into univariate Cox models for OS and DFS. Only the univariate Cox regression models that did not infringe the proportional hazard assumption for OS were reported in Table 2. The number of intraoperative HCC nodules was the unique variable statistically significant for OS (HR = 2.225; *P* < 0.001). The corresponding rank-hazard plot showed a steeper increase of relative hazard for intraoperative nodules > 2 (Figure 3). Univariate Cox analysis for DFS did not find any significant predictor (Table 2).

## DISCUSSION

There is some dispute whether survival benefits of RFA compared with liver resection exist for patients with HCC conforming to the Milan criteria. Prospective randomized trials comparing these two procedures for HCC conforming to the Milan criteria showed controversial results<sup>[4,21,22]</sup>. A recent meta-analysis by Yi *et al*<sup>[23]</sup> showed that liver resection was superior to RFA in the treatment of patients with HCC conforming to the Milan criteria in terms of 3 and 5-year survival rates and local recurrence rate, suggesting that liver resection remains the better choice of treatment for small HCC. The laparoscopic approach to RFA has proved to be superior to the percutaneous approach in lesions that are difficult or impossible to be treated in such a way or in severe liver disease, thus extending its indications. Despite good results, LRFA yielded inferior OS and DFS rates when compared to hepatic resection for small HCC<sup>[2,24,25]</sup>.

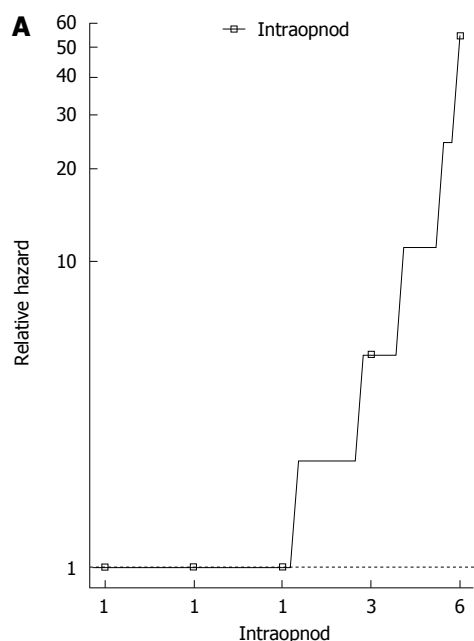


**Figure 1** Overall and disease-free survival curves of the laparoscopically-treated hepatocellular carcinoma patients and their corresponding cumulative hazard curves. A: Overall survival curve of the laparoscopically-treated hepatocellular carcinoma (HCC) patients. Number of patients at risk at each time point is shown at top of the x-axis; B: Cumulative hazard for the corresponding overall survival curve; C: Disease-free survival curve of the laparoscopically-treated HCC patients. Number of patients at risk at each time point is shown at top of the X-axis; D: Cumulative hazard for the corresponding disease-free survival curve.



**Figure 2** Comparison of the overall and disease-free survival probability for hepatocellular carcinoma patients by stratifying for laparoscopic treatment. A: Comparison of the overall survival probability for hepatocellular carcinoma (HCC) patients by stratifying for laparoscopic treatment (LLR: 0.442; LRFA: 0.261;  $P = 0.048$ ). Number of patients at risk at each time point is shown at top of the x-axis; B: Comparison of the disease-free survival probability for HCC patients by stratifying for laparoscopic treatment (LLR: 0.206; LRFA: 0.286;  $P = 0.205$ ). Number of patients at risk at each time point is shown at top of the X-axis. LLR: Laparoscopic liver resection; LRFA: Laparoscopic radiofrequency ablation.





**Figure 3** Rank-hazard plot for the number of intraoperative hepatocellular carcinoma nodules. Based on the univariate Cox model for overall survival.

In the lights of comparable oncological results following liver resection regardless the type of surgical approach, laparoscopic or “open”<sup>[12]</sup>, we wanted to verify that the superiority of liver resection on thermoablation in terms of OS and DFS persisted even when both approaches were laparoscopic. In our study, different indications to LRFA and LLR were responsible for the slightly different patients characteristics of the two groups. In fact, patients with normal liver function and larger tumors were resected, whereas those with liver dysfunction, multiple tumors, and portal hypertension were ablated. Another limitation are the various therapies used to treat tumor recurrences in both LRFA and LLR groups, including repeat RFA, transarterial chemoembolization (TACE), second resection, liver transplantation, systemic chemotherapy, and supportive treatment. Different therapeutic schedules for tumor recurrences could affect these findings. High rate of intrahepatic recurrence after ablation therapies and/or surgical resection is the main cause of late death of patients with HCC. In current study, local recurrence was found to be more frequent after LRFA than LLR. Local recurrences after LRFA may be attributable to insufficient ablation of the primary tumor and/or the presence of tumor venous invasion in the adjacent liver. Surgical resection could remove the primary tumor and venous tumor thrombi. This may explain the better outcomes following LLR. A significantly higher OS was observed in the LLR group in comparison with LRFA group. Our results are comparable to literature data<sup>[21,26]</sup>, reflecting the superiority of the resection over thermoablation, thus confirming that this relation is maintained also if a laparoscopic approach is used.

Similarly, better results in 1- and 3-year DFS were observed in the LLR group in comparison with LRFA group, but this difference disappeared after 5 years.

In literature, factors that are reported to be responsible for influencing the OS and DFS are tumor size, number of tumors, Child class, AFP levels and treatment modality<sup>[21-23]</sup>. Besides the underlying liver function, the most important parameter affecting the overall survival is the number of tumor nodules, as testified by many studies comparing open liver resection to thermoablation<sup>[21,22,27]</sup>. This statement is also confirmed in our study. In fact, Cox regression analysis demonstrated that the only independent risk factor associated with OS was the intraoperative number of HCC nodules. Moreover, the corresponding rank-hazard plot showed a steeper increase of relative hazard for intraoperative nodules > 2, confirming the finding of Liu *et al.*<sup>[27]</sup>. Thanks to these results, many centers are adopting a more aggressive approach to resection when possible as opposed to RFA<sup>[28,29]</sup>. Laparoscopic liver resection, if feasible, provides many of the advantages of a less invasive procedure with complete tumor extirpation and assessment of resection margins, thus preserving better oncological results over thermoablation.

Our preliminary results suggest that LLR for HCC is feasible with good oncologic results. Laparoscopy should be routinely considered in selected patients in centers experienced in liver surgery and in advanced laparoscopy. Better survival of hepatic resection on thermoablation in the treatment of small HCC are confirmed also when both approaches are made laparoscopically. These data need to be confirmed by further studies on a larger number of patients.

## COMMENTS

### Background

Both liver resection and ablative therapies are able to modify the natural history of hepatocellular carcinoma (HCC). Indeed, it is demonstrated that they allow an increase in survival in patients with small nodules, although relapse after these two treatments remains a questionable topic. However, studies in the literature comparing radiofrequency ablation to liver resection seem to confirm the superiority of the latter in terms of overall survival and disease-free survival. To date, interstitial therapies were always compared to hepatic resection performed through an open approach. However, the fact of comparing a minimally invasive technique to a quite invasive one has some limits. Currently the authors are witnessing an increasing use of laparoscopy in liver surgery, extending indications to patients with cirrhosis and HCC. Laparoscopic surgery of the liver represents a viable alternative to open surgery retaining its characteristics of a minimally invasive approach and providing better results and a similar postoperative complication rate. Moreover, laparoscopic liver resection in selected cirrhotic patients with HCC, has shown advantages in terms of survival and recurrence similar to those after open surgery. With the aim to compare laparoscopic liver resection (LLR) and laparoscopic radiofrequency ablation (LRFA) in terms of postoperative survival and HCC recurrence, we evaluated two groups of cirrhotic patients with similar clinical characteristics.

### Research frontiers

In the lights of comparable oncological results following liver resection regardless the type of surgical approach, laparoscopic or “open”, the authors wanted to verify that the superiority of liver resection on thermoablation in terms

of overall survival (OS) and disease-free survival (DFS) persisted even when both approaches were laparoscopic.

### Innovations and breakthroughs

In this study, better outcomes are obtained following LLR. A significantly higher OS was observed in the LLR group in comparison with LRFA group. These results are comparable to literature data, reflecting the superiority of the resection over thermoablation, thus confirming that this relation is maintained also if a laparoscopic approach is used.

### Applications

Current preliminary results suggest that LLR for HCC is feasible with good oncologic results. Laparoscopy should be routinely considered in selected patients in centers experienced in liver surgery and in advanced laparoscopy. Better survival of hepatic resection on thermoablation in the treatment of small HCC are confirmed also when both approaches are made laparoscopically. These data need to be confirmed by further studies on a larger number of patients.

### Peer-review

It's a good study, with a good design and a robust results however the groups in relation to lesions (number and localization) were different that results, maybe, in different survival.

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## Retrospective Study

# Endoscopic ultrasound-guided gallbladder drainage for acute cholecystitis: Long-term outcomes after removal of a self-expandable metal stent

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**Author contributions:** All authors helped to perform the research; Kamata K and Takenaka M wrote the manuscript and analyzed the data; Kamata K performed the procedures; Takenaka M drafted the conception and design; Kitano M, Omoto S, Miyata T, Minaga K, Yamao K, Imai H, Sakurai T, Watanabe T, Nishida N and Kudo M contributed to writing the manuscript; Kitano M and Kudo M also contributed to drafting conception and design.

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**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of the Kindai University Hospital.

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

**Conflict-of-interest statement:** All authors declare no conflicts-of-interest related to this article.

**Data sharing statement:** No additional data are available.

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## Abstract

### AIM

To assess the long-term outcomes of this procedure after removal of self-expandable metal stent (SEMS). The efficacy and safety of endoscopic ultrasound-guided gallbladder drainage (EUS-GBD) with SEMS were also assessed.

### METHODS

Between January 2010 and April 2015, 12 patients with acute calculous cholecystitis, who were deemed unsuitable for cholecystectomy, underwent EUS-GBD with a SEMS. EUS-GBD was performed under the guidance of EUS and fluoroscopy, by puncturing the gallbladder with a needle, inserting a guidewire, dilating the puncture hole, and placing a SEMS. The



SEMS was removed and/or replaced with a 7-Fr plastic pigtail stent after cholecystitis improved. The technical and clinical success rates, adverse event rate, and recurrence rate were all measured.

## RESULTS

The rates of technical success, clinical success, and adverse events were 100%, 100%, and 0%, respectively. After cholecystitis improved, the SEMS was removed without replacement in eight patients, whereas it was replaced with a 7-Fr pigtail stent in four patients. Recurrence was seen in one patient (8.3%) who did not receive a replacement pigtail stent. The median follow-up period after EUS-GBD was 304 d (78-1492).

## CONCLUSION

EUS-GBD with a SEMS is a possible alternative treatment for acute cholecystitis. Long-term outcomes after removal of the SEMS were excellent. Removal of the SEMS at 4-wk after SEMS placement and improvement of symptoms might avoid migration of the stent and recurrence of cholecystitis due to food impaction.

**Key words:** Endoscopic ultrasound-guided gallbladder drainage; Cholecystitis; Endoscopic ultrasound-guided biliary drainage

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**Core tip:** Endoscopic ultrasound-guided gallbladder drainage (EUS-GBD) was recently used to treat acute cholecystitis. The aim of this study was to assess the utility of removal of self-expandable metal stent (SEMS) at 4-wk after EUS-GBD. Twelve patients with acute calculous cholecystitis underwent EUS-GBD with a SEMS. The rates of technical success, clinical success, and adverse events were 100%, 100%, and 0%, respectively. Recurrence was seen in one patient (8.3%). The median follow-up period after EUS-GBD was 304 d. Removal of the SEMS at 4-wk after SEMS placement might avoid migration of the stent and recurrence of cholecystitis due to food impaction.

Kamata K, Takenaka M, Kitano M, Omoto S, Miyata T, Minaga K, Yamao K, Imai H, Sakurai T, Watanabe T, Nishida N, Kudo M. Endoscopic ultrasound-guided gallbladder drainage for acute cholecystitis: Long-term outcomes after removal of a self-expandable metal stent. *World J Gastroenterol* 2017; 23(4): 661-667 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/661.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.661>

## INTRODUCTION

Laparoscopic cholecystectomy is the standard

treatment for acute cholecystitis caused by cholecystolithiasis<sup>[1,2]</sup>. For patients at high surgical risk, percutaneous transhepatic gallbladder aspiration (PTGBA) or percutaneous transhepatic gallbladder drainage (PTGBD) can be selected for treatment of cholecystitis. However, the efficacy rate of PTGBA is insufficient (61%-77%), and PTGBD involves an external drainage tube, which decreases the ability of the patient to carry out their normal daily activities<sup>[3,4]</sup>. Recently, endoscopic ultrasound-guided gallbladder drainage (EUS-GBD) was developed for acute cholecystitis<sup>[5-17]</sup>. Jang *et al*<sup>[14]</sup> showed that EUS-GBD was comparable with PTGBD in terms of its technical feasibility, efficacy, and procedural safety.

The aim of this study was to evaluate the outcomes of EUS-GBD in patients with acute calculous cholecystitis deemed unsuitable for cholecystectomy. The examined procedure used a self-expandable metal stent (SEMS), and we also assessed the long-term outcomes of the procedure following removal of the SEMS.

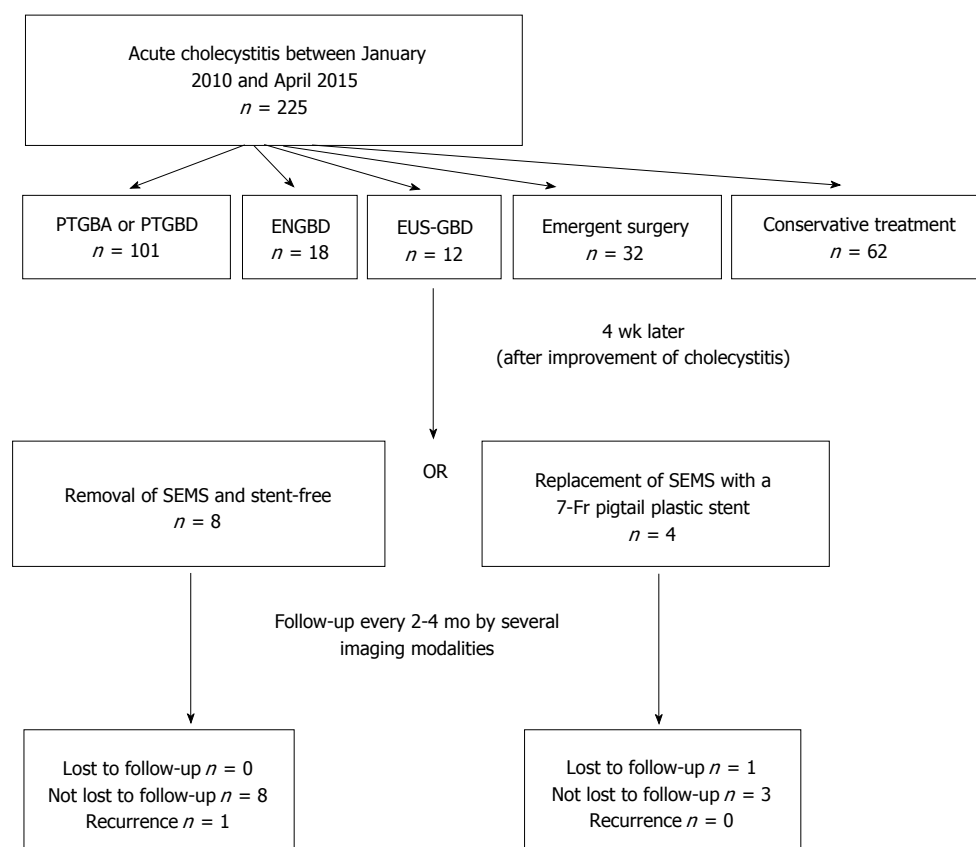
## MATERIALS AND METHODS

### Patients

Between January 2006 and October 2014, 225 patients with acute cholecystitis due to gallstones visited our hospital. Among these, 101, 18, 32, and 62 patients underwent PTGBA and/or PTGBD, endoscopic naso-gallbladder drainage, emergent surgery and conservative treatment, respectively. The remaining 12 patients with acute calculous cholecystitis, who were deemed unsuitable for cholecystectomy because of poor surgical performance indications and had a risk of self-removal of drainage tube, underwent EUS-GBD. Cases of cholecystitis due to deployment of the metal stent and the cases that cystic duct was obstructed due to advanced cancer were excluded from this study. The surgical performance indications for all patients were poor (class III or IV on the American Society of Anesthesiologists (ASA) Physical Status classification system). These patients were identified by retrospective review of the medical database of our hospital. Acute calculous cholecystitis was diagnosed in all patients on the basis of the characteristic clinical features (abdominal pain and fever), laboratory data (high level of serum C-reactive protein; CRP), and imaging studies. The study was approved by the institutional review board of the Kinki University Faculty of Medicine, and informed consent was obtained from the patients after explaining to them that we could perform PTGBA, PTGBD, or EUS-GBD.

### EUS-GBD technique

An echoendoscope (GF-UCT240-AL5, Olympus, Tokyo, Japan) was introduced into the stomach or duodenum. The echoendoscope images were used to ensure that gallstones were present in the swollen



**Figure 1 Strategy of endoscopic ultrasound-guided gallbladder drainage procedure.** ENGBD: Endoscopic naso-gallbladder drainage; EUS-GBD: Endoscopic ultrasound-guided gallbladder drainage; PTGBA: Percutaneous transhepatic gallbladder aspiration; PTGBD: Percutaneous transhepatic gallbladder drainage; SEMS: Self-expandable metal stent.

gallbladder before EUS-GBD was performed. After visualization of the swollen gallbladder adjacent to the antrum or duodenal bulb, the echoendoscope was manipulated until an appropriate puncture route, free from interposing vessels, was identified. The puncture site was selected as the region where the distance between the gastrointestinal tract and the gallbladder was smallest (1 cm or less). When both the stomach and duodenum provided equally good access, the duodenum was selected as the puncture site because it was easier to maintain the scope position at the duodenum than at the stomach.

The neck or body of the gallbladder was generally chosen as the ideal target, and was then punctured with a 19G needle (EchoTip Ultra, Cook Medical, Limerick, Ireland) under endosonographic guidance. The gallbladder was then irrigated with a saline solution through the 19G needle, using a 20 mL syringe. Irrigation was performed at least ten times, and was continued until the color of the bile became weak. This was performed to prevent peritonitis due to bile leakage immediately after the gallbladder was punctured. Thereafter, a sufficient length of 0.035 inch guidewire (Revowave, Piolax, Kanagawa, Japan) was inserted into the gallbladder lumen until there were more than two coils present. The puncture tract was then serially dilated using either biliary

dilation catheters (6F-7F-9F, Soehendra Biliary Dilation Catheter, Cook, Bloomington, IN, United States) or a balloon dilator (Max Pass 4 mm, Olympus, Tokyo, Japan) over the guidewire. If passing dilators or balloons proved difficult, electrocautery was planned to be used. A SEMS (10 mm in diameter, 6 cm in length, Wallflex partially covered stent, Boston Scientific, Natick, MA, United States) was deployed between the gallbladder and the stomach or duodenum. If functional success was obtained, the SEMS was removed and/or replaced with a 7-Fr plastic pigtail stent (4 or 6 cm in length) 4 wk after the original EUS-GBD (Figure 1). Where possible, the stent was replaced after removal of the SEMS in order to keep the fistula considering the possibility of the recurrence. This technique was approved by the institutional review board of the Kinki University Faculty of Medicine.

#### Follow-up after EUS-GBD

Several imaging modalities including ultrasonography, computed tomography (CT), fistulography, and/or EUS were performed to determine if gallstones remained in the gallbladder before removal of the SEMS. CT (looking for air images in the gallbladder) and/or fistulography were performed to determine if the fistula remained open 1 wk after removal of the SEMS. After removal of the SEMS, patients were continually followed up by

**Table 1 Patient characteristics**

Characteristics	
Age, mean $\pm$ SD, yr	76.3 $\pm$ 12.1
Sex, male/female	9/3
Underlying condition	
III	66.7% (8/12)
IV	33.3% (4/12)
Advanced malignancy	8.3% (1/12)
White blood cell count (mean, range)	14525 (9100-21300) per $\mu$ L
C-reactive protein (mean, range)	15.7 (2.0-32.7) mg/dL

**Table 2 Outcomes of endoscopic ultrasound-guided gallbladder drainage**

Technical success rate	100% (12/12)
Functional success rate	100% (12/12)
Rate of removal	67% (8/12)
Rate of replacement	33% (4/12)
Adverse events	0% (0/12)
Recurrence of cholecystitis	8.3% (1/12)
Follow-up period, days [median, range]	304 (78-1492)
Patient status on follow-up	
Alive	91.7% (11/12)
Dead	8.3% (1/12)

SEMS: Self-expandable metal stent.

blood tests and imaging modalities every 2-4 mo. It was determined whether the cystic duct was patent before and after removal of the SEMS by performing fistulography and/or EUS.

### Assessment of outcomes

The long-term outcomes of EUS-GBD after removal of SEMS was the primary outcomes in this study. The outcomes assessed were technical and clinical success rates, adverse events rate, and recurrence rate. Technical success was defined as successful stent deployment between the gallbladder lumen and the stomach or duodenum. Clinical success was defined as improvement of typical clinical symptoms within 3 d, with confirmatory laboratory tests, with or without improved radiologic findings<sup>[14]</sup>. The incidence of the following adverse events was assessed: peritonitis, bile leakage, bleeding, stent migration, and stent occlusion. Recurrence of acute cholecystitis after EUS-GBD was defined on the basis of the characteristic clinical features, laboratory data, and imaging studies.

### Statistical analysis

Continuous variables are expressed as median or mean values with standard deviation or range. All statistical analyses were performed using SAS software version 9.1 (SAS Institute, Cary, NC, United States).

## RESULTS

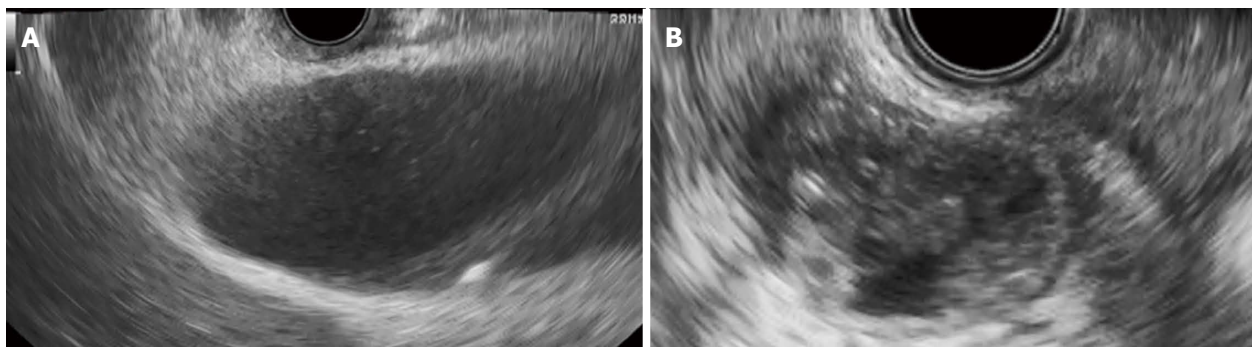
Table 1 shows the patients' characteristics. In total, 12 patients (mean age 76 years, 9 men and 3 women)

underwent EUS-GBD. Eight patients were ASA class III, and the others were ASA class IV. One patient had advanced ovarian cancer which expected long-term survival and there was no influence of the tumor on the cystic duct. Blood examination revealed a mean white blood cell (WBC) count of 14525 cells per  $\mu$ L and a mean CRP level of 15.7 mg/dL. All cases were moderate cholecystitis. The diameter of gallstones was less than 10 mm in all patients. The EUS-GBD procedure was performed *via* the stomach or duodenum in three and nine cases, respectively. The distance between the gastrointestinal tract and the gallbladder was 1 cm or less in all cases. Dilation of the puncture site was performed by biliary dilation and/or balloon catheters without using electrocautery. Table 2 shows the outcomes of EUS-GBD. The technical success and clinical success rates were both 100% (12/12), with no adverse events recorded. At day 3 post-EUS-GBD, the mean WBC count and mean CRP were 7075 cells per  $\mu$ L and 2.37 mg/dL, respectively. The SEMS was removed from eight patients 4 wk after the EUS-GBD. In these eight patients, the plastic pigtail stent was not deployed after removal of the SEMS because the guidewire could not be sufficiently inserted due to shrinkage of the gallbladder by the EUS-GBD treatment. In the remaining four patients, the SEMS was replaced with a 7-Fr plastic double pigtail stent 4 wk after EUS-GBD. The median post-EUS-GBD follow-up period for these 12 patients was 304 d. During the follow-up period, one of the patients (8.3%) died due to advanced cancer. At the time the records were subjected to retrospective evaluation (April 1, 2016), recurrence was present in one of the patients (8.3%) who did not receive a replacement pigtail stent (Figure 2). In four patients received replacement of SEMS with a 7-Fr plastic double pigtail stent, the stent was kept permanently in all of those patients.

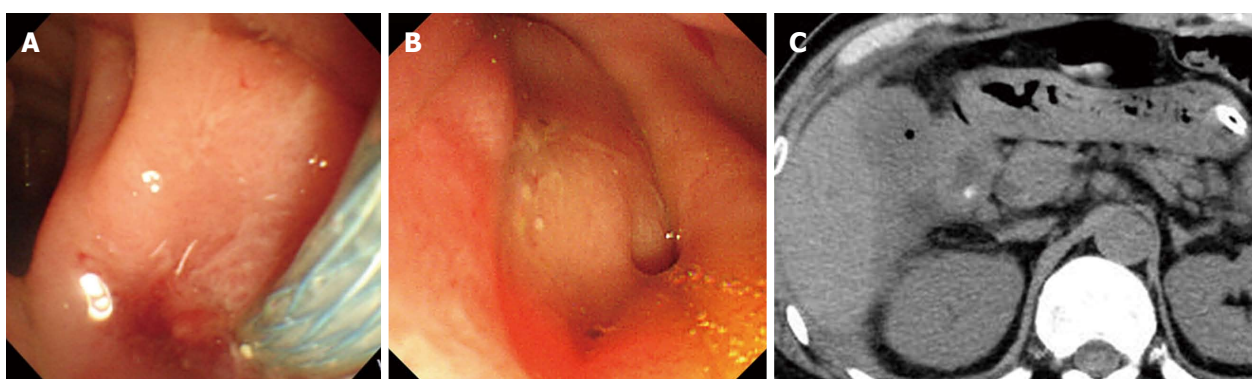
Before removal of the SEMS, gallstones did not remain in the gallbladder in all cases. One week after removal of the SEMS, air in the gallbladder was imaged by CT in nine cases (Figure 3). The other three cases that did not show air images in the gallbladder were cases in which the double pigtail plastic stents were not deployed. Fistulography was performed in eight cases that did not undergo replacement of the stent. Among these, fistulography images of the gallbladder were obtained in three cases. In total, the fistula was confirmed by CT and/or fistulography in 9 of 12 cases. Cystic duct patency was confirmed by fistulography and/or EUS before as well as 1 week after removal of the SEMS in all cases.

## DISCUSSION

The aim of the current study was to evaluate the feasibility of EUS-GBD for patients with acute calculous cholecystitis, who were deemed unsuitable for cholecystectomy. In this study, both technical and



**Figure 2 Endosonographic image.** A: Endosonographic image before gallbladder drainage. Contents in the gallbladder were mainly sludge without apparent gallstones; B: Endosonographic image at the time of recurrence of cholecystitis. Sludge volume in the gallbladder was more increased than when gallbladder drainage was performed.



**Figure 3 Esophagogastroduodenoscopy and computed tomography.** A: Esophagogastroduodenoscopy image of deployment of the metal stent in the duodenum; B: Esophagogastroduodenoscopy image of the fistula 1 wk after removal of the metal stent; C: Computed tomography after removal of the metal stent showing air image in the gallbladder.

clinical success was achieved in the treatment of acute cholecystitis in all 12 patients. One of the risks of EUS-GBD is bile leakage into the peritoneal space, which can cause bile peritonitis. The bile leakage is caused by migration of the stent exposing the gap between the puncture tract and the stent<sup>[5,6,8]</sup>. In the present study, several techniques were used to avoid such bile leakage. Firstly, the guidewire was inserted until at least two full coils were in the lumen. The gallbladder lumen has more space for coiling than the bile duct, and yields better stability. Secondly, we irrigated the gallbladder lumen with saline solution after puncturing the gallbladder and before proceeding to the next step. This irrigation procedure may reduce the chance of peritonitis due to bile leakage during dilation. We also used SEMSs in our study, and, compared with plastic stents, SEMSs are better at sealing the gap between the stent and the needle tracts in the gallbladder wall, thus preventing bile leakage<sup>[8]</sup>. As a result, no adverse events occurred in this study. In a systematic review of EUS-guided biliary drainage by Wang *et al.*<sup>[18]</sup> in 2016, the rate of adverse events was 38.46% in the group in which cystotomes were used during dilation of the puncture site, which was higher than that in the group in which dilators or balloons were used. Dilation of the puncture site was performed by biliary dilation and/

or balloon catheters without using electrocautery in this study. This might be another reason why there were no adverse events in this study.

During a long-term follow-up with a median period of 275 d, Choi *et al.*<sup>[19]</sup> reported that stent distal migration was noted in two patients (3.6%), one at 170 d and the other at 303 d post-EUS-GBD. They also reported recurrence of acute cholecystitis due to food impaction. To avoid stent migration and food impaction into the gallbladder, we either removed the SEMS, or replaced it with a pigtail plastic stent, 4 wk after EUS-GBD. In our study, there was neither stent migration nor food impaction. Performance of these additional procedures after EUS-GBD may prevent such complications.

Recently, the use of lumen-apposing metal stents (LAMS) with anchor flanges and flares for EUS-GBD resulted in excellent outcomes<sup>[13,15,20]</sup>. With a LAMS, the distance between the gastrointestinal tract and the gallbladder needs to be 1 cm or less<sup>[19]</sup>. In terms of this, a conventional biliary SEMS may have allowed us more freedom in selecting the puncture site, although this is not certain because the distance was 1 cm or less in all cases in this study.

In a study examining the use of LAMS for high-risk surgical patients with acute cholecystitis<sup>[20]</sup>, Walter



*et al.*<sup>[20]</sup> reported that technical success was 90%, and clinical success was 96%, and that no migration was seen in any patients. In 15 of the 27 patients with technical success, LAMS were removed approximately 3 mo after EUS-GBD, whereas they were left in place in the other 12 patients. Removal of the LAMS was not achieved due to tissue overgrowth in two patients. Two patients also developed a LAMS obstruction. Thus, long-term deployment of metal stents in EUS-GBD could cause adverse events, including food impaction. Therefore, early removal of the metal stent after EUS-GBD, at a time of around 4 wk (as in the present study), may be considered desirable. However, we did observe a recurrence of acute cholecystitis in one patient (8.3%), where the SEMS was not replaced with a pigtail stent. There is a possibility that this patient recurred cholecystitis due to uncertain small gallstones or sludge remaining after EUS-GBD. Therefore, replacement with a pigtail plastic stent may be helpful for avoiding recurrence. Another reason for the low recurrence rate in this study might be that no gallstones remained in the gallbladder before removal of the SEMS in all cases.

Moon *et al.*<sup>[21]</sup> reported that gross pathology showed adherence of the gallbladder to the stomach wall around the site of cholecystogastrostomy 4 wk after LAMS removal in an animal study. We also performed a preliminary examination of EUS-guided biliary drainage using a conventional biliary SEMS in an animal study using five pigs. We found that at autopsy 1 wk after the procedure, fistulas were created between the bile duct and duodenum in all pigs<sup>[22]</sup>. Thus, a strong fistula might develop between the gallbladder and the gastrointestinal tract within 4 wk using a conventional biliary SEMS as well as a LAMS. This study has a few limitations. Firstly, the number of EUS-GBD cases was low, and all cases were from a single institute. Secondly, the indications for EUS-GBD were limited to those patients deemed unsuitable for cholecystectomy. A larger study comparing the efficacy and safety of EUS-GBD with and without early SEMS removal is warranted. However, a large number of institutions are needed to obtain the required number of patients, otherwise the criteria used for patient selection should be less strict.

In a systematic review of EUS-GBD, LAMS seemed to have a high potential in terms of efficacy and safety; however, the technical success of LAMS (91.5%) was lower than that of conventional biliary SEMS (98.6%)<sup>[23]</sup>. Further studies including long-term results are required to investigate whether SEMS or LAMS are better for EUS-GBD. EUS-GBD with SEMS is a possible alternative treatment for acute cholecystitis. Long-term outcomes after removal of SEMS were promising. Removal of the SEMS after SEMS placement and improvement of symptoms might avoid migration of the stent and recurrence of cholecystitis due to food impaction.

## COMMENTS

### Background

Laparoscopic cholecystectomy is the standard treatment for acute cholecystitis caused by cholelithiasis. For patients at high surgical risk, percutaneous transhepatic gallbladder aspiration (PTGBA) or percutaneous transhepatic gallbladder drainage (PTGBD) can be selected for treatment of cholecystitis. However, the efficacy rate of PTGBA is insufficient (61%-77%), and PTGBD involves an external drainage tube, which decreases the ability of the patient to carry out their normal daily activities. Recently, endoscopic ultrasound-guided gallbladder drainage (EUS-GBD) was developed for acute cholecystitis.

### Research frontiers

There were few reports on long term outcomes of EUS-GBD. This study, the Long-term outcomes after removal of self-expandable metal stent (SEMS), was first report and the results of this study contribute to clarifying the potential of this procedure for acute cholecystitis.

### Innovations and breakthroughs

In this study, EUS-GBD using SEMS was a useful for removal of gallstones in the gallbladder. Gallstones disappeared after EUS-GBD in all cases. During long-term follow-up period after the removal of the SEMS, the recurrence of the cholecystitis was seen in only one patient (8.3%) and there were no complications.

### Applications

This study suggests that EUS-GBD using SEMS and removal of the SEMS 4 wk after the procedure are useful for patients with cholecystitis who were deemed unsuitable for cholecystectomy.

### Peer-review

This study described the use of EUS-GBD for the treatment of acute cholecystitis in patients deemed unsuitable for surgical procedures. Long-term outcomes after removal of SEMS were promising. Removal of the SEMS after SEMS placement and improvement of symptoms might avoid migration of the stent and recurrence of cholecystitis due to food impaction. A larger study comparing the efficacy and safety of EUS-GBD with and without early SEMS removal is warranted.

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**P- Reviewer:** Cariati A, Tsuyuguchi T, Tuncyurek O **S- Editor:** Qi Y  
**L- Editor:** A **E- Editor:** Wang CH



## Retrospective Study

# Comparative study: Vonoprazan and proton pump inhibitors in *Helicobacter pylori* eradication therapy

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## Abstract

### AIM

To compare the effectiveness and safety of vonoprazan-based therapy with proton pump inhibitor (PPI)-based therapies to treat *Helicobacter pylori* (*H. pylori*).

### METHODS

We retrospectively analysed data from first-line (vonoprazan or PPI with 200 mg clarithromycin and 750 mg amoxicillin twice daily for 7 d) ( $n = 1353$ ) and second-line (vonoprazan or PPI with 250 mg metronidazole and 750 mg amoxicillin twice daily for 7 d) ( $n = 261$ ) eradication treatments for *H. pylori*-positive patients with associated gastrointestinal diseases from April 2014 to December 2015 at Hattori Clinic, Japan. The primary endpoint was the eradication rate, which was assessed with a full analysis set. The secondary endpoints were adverse events and related factors.

### RESULTS

After the first-line treatments, the eradication rates for vonoprazan, esomeprazol, rabeprazole, and lansoprazole were 87.9% (95%CI: 84.9%-90.5%), 71.6% (95%CI: 67.5%-75.5%), 62.9% (95%CI: 52.0%-72.9%), and 57.3% (95%CI: 50.4%-64.1%), respectively. The vonoprazan eradication rate was significantly higher than that of the PPIs ( $P < 0.01$ ). Interestingly, smoking did not affect the *H. pylori* eradication rate in the vonoprazan group ( $P = 0.34$ ), whereas it decreased the rates in the PPI groups ( $P = 0.013$ ). The incidence of adverse events in the vonop-

razan group was not different from the PPI group ( $P = 0.054$ ), although the vonoprazan group exhibited a wider range of adverse events. Vonoprazan-based triple therapy was highly effective as a second-line treatment, with an eradication rate similar to that of PPI-based therapy.

### CONCLUSION

Vonoprazan might be superior to PPIs in first-line *H. pylori* therapy, particularly for smokers. However, caution is required due to possible adverse events.

**Key words:** *Helicobacter pylori*; Eradication treatment; Vonoprazan; Proton pump inhibitors; Adverse event; Smoking

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**Core tip:** Because the *Helicobacter pylori* (*H. pylori*) eradication rate of conventional proton pump inhibitor (PPI)-based treatment has decreased because clarithromycin-resistant strains have appeared in recent years, a new treatment strategy is required. Vonoprazan is a novel potassium-competitive acid blocker that has strong, long-lasting effects, but few studies have investigated its efficacy against *H. pylori*. We compared vonoprazan-based therapy and PPI-based therapy as first-line and second-line treatments. Vonoprazan-based therapy was superior to PPI-based therapy, particularly for smokers, but adverse events (AEs) due to vonoprazan occurred more frequently. Vonoprazan-based therapy is a potentially efficacious treatment, but it should be used with caution due to possible AEs.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) contributes to upper gastrointestinal diseases, such as chronic gastritis, peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer<sup>[1-3]</sup>. Proton pump inhibitor (PPI)-based *H. pylori* eradication therapy has been shown to be effective for treatment of *H. pylori*-related diseases<sup>[4]</sup>.

In Japan, the first-line regimen consists of triple therapy with a PPI (omeprazole, rabeprazole, lansoprazole, or esomeprazole), amoxicillin (AMPC), and clarithromycin (CAM) for 7 d. As of 2000, the cost of this therapy for patients with gastric or duodenal ulcers has been covered under Japan's national

health insurance<sup>[5]</sup>. After the approval of the first regimen, the second-line treatment, in which CAM is replaced by metronidazole (MNZ), was also approved in 2007. Dating from February 2013, *H. pylori* eradication therapy was expanded to include patients with *H. pylori* infection-associated gastritis to prevent gastric cancer.

However, the eradication rate with the first-line treatment has reportedly decreased due to the increase of CAM-resistant strains in recent years<sup>[5-7]</sup>. Therefore, a more effective strategy is required for CAM-resistant patients.

Vonoprazan is a novel potassium-competitive acid blocker (P-CAB) and to a new class of gastric acid-suppressive agents<sup>[8]</sup>. P-CABs, which block  $H^+$ ,  $K^+$  ATPase in a competitive and reversible manner, result in stronger and more sustained acid suppression than PPIs<sup>[9]</sup>. Alteration of the intragastric pH, to a higher pH with a lower percentage of time spent  $< pH\ 4$ , is crucial in *H. pylori* eradication therapy<sup>[10]</sup>. Therefore, P-CAB-based triple therapy should be more efficient than PPI-based therapy for *H. pylori*-infected patients, including CAM-resistant patients. A phase III randomized, double-blind study showed that vonoprazan (P-CAB)-based treatment was effective in both first- and second-line *H. pylori* eradication therapy compared to treatment with lansoprazole<sup>[11]</sup>.

In this study, we evaluated the clinical effectiveness and safety of vonoprazan-based *H. pylori* eradication therapy and compared it to that of conventional PPI-based therapy in clinical practice.

## MATERIALS AND METHODS

### Patients and study design

This study was conducted in a single institution (Hattori Clinic). We retrospectively examined data from patients administered first- and/or second-line eradication therapy.

*H. pylori*-positive patients diagnosed via a high-resolution endoscope (GIF260, 290 series; Olympus, Tokyo, Japan) with gastric ulcer and/or ulcer scar (GU/GUs), duodenal ulcer and/or ulcer scar (DU/DUs), gastroduodenal ulcer and/or ulcer scar (GDU/GDUs), gastric MALT lymphoma, post-endoscopic submucosal dissection (post ESD) for early gastric cancer or atrophic gastritis from April 2014 to December 2015 at Hattori Clinic were enrolled in this study.

The exclusion criteria were as follows: less than 20 years of age; past history of total gastrectomy; history of drug allergy to PPIs, AMPC, CAM, or MNZ; clinically significant disease (hepatic, renal or cardiac disease); and pregnancy.

The presence of *H. pylori* at admission and after first- and second-line eradication therapy was confirmed with the  $^{13}C$ -urea breath test (UBT). The cut-off value was 2.5‰. Confirmation of eradication by UBT was performed no less than 8 wk after eradication



**Table 1** Baseline and demographic characteristics of patients in this study

	First-line eradication therapy					Second-line eradication therapy				
	VPZ group <i>n</i> = 546	PPI group <i>n</i> = 807	EPZ group <i>n</i> = 507	RPZ group <i>n</i> = 89	LPZ group <i>n</i> = 211	VPZ group <i>n</i> = 76	PPI group <i>n</i> = 185	EPZ group <i>n</i> = 104	RPZ group <i>n</i> = 24	LPZ group <i>n</i> = 57
Age, mean ± SD, yr	57.4 ± 11.8	56.7 ± 12.8	56.9 ± 11.6	60.7 ± 11.2	56.1 ± 12.1	56.9 ± 12.8	56.0 ± 12.6	57.5 ± 12.5	58.2 ± 12.2	58.1 ± 12.3
Sex, <i>n</i> (%)										
Male	225 (41.2)	318 (39.4)	193 (38.1)	35 (39.3)	90 (42.7)	30 (39.5)	71 (38.4)	39 (37.5)	9 (39.3)	23 (40.4)
Female	321 (58.8)	489 (60.6)	314 (61.9)	54 (60.7)	121 (57.3)	46 (60.5)	114 (61.6)	65 (62.5)	15 (60.7)	34 (59.6)
Indication										
GU(s)	32	33	18	4	11	3	7	6	0	1
DU(s)	37	68	36	8	24	5	13	5	2	6
GDU(s)	4	4	2	1	1	1	0	0	0	0
MALT lymphoma	0	2	0	0	2	0	1	0	0	1
Post ESD	1	3	2	0	1	1	0	0	0	0
Atrophic gastritis	472	697	449	76	172	66	164	93	22	49

GU/GUs: Gastric ulcer and/or ulcer scar; DU/DUs: Duodenal ulcer and/or ulcer scar; GDU/GDUs: Gastroduodenal ulcer and/or ulcer scar; MALT: Mucosa-associated lymphoid tissue; ESD: Endoscopic submucosal dissection; VPZ: Vonoprazan; PPI: Proton pump inhibitor; EPZ: Esomeprazole; RPZ: Rabeprazole; LPZ: Lansoprazole.

treatment was completed. UBT-negative patients whose endoscopic findings showed gastric atrophy received an additional stool antigen test.

*H. pylori*-positive patients received one of the following first-line treatments: (1) vonoprazan (VPZ) group; 20 mg vonoprazan, 200 mg CAM, and 750 mg AMPC twice daily for 7 d; (2) esomeprazole (EPZ) group; 20 mg esomeprazole, 200 mg CAM, and 750 mg AMPC twice daily for 7 d; (3) rabeprazole (RPZ) group; 10 mg rabeprazole, 200 mg CAM, and 750 mg AMPC twice daily for 7 d; (4) lansoprazole (LPZ) group; 30 mg lansoprazole, 200 mg CAM, and 750 mg AMPC twice daily for 7 d. For the second-line treatment, 200 mg CAM was replaced with 250 mg MNZ and a selected acid blocker medication depended on the attending doctor's discretion.

Adverse events (AEs) were defined as undesirable medical symptoms or conditions after the beginning of the treatment, which were interrogated directly by each investigator.

The primary endpoint was eradication rate. The secondary endpoints were AEs and related factors.

Subgroup analyses of demographic and clinical characteristics, including presence of endoscopic gastric atrophy, age, gender, alcohol consumption, body mass index (BMI), and smoking, were also conducted. The Kimura-Takemoto classification was used to classify atrophic gastritis<sup>[12]</sup>.

This study was conducted in accordance with the Declaration of Helsinki, and the research protocol was approved by the institutional review board of the Hattori Clinic.

### Statistical analysis

The eradication rate was evaluated with a full analysis set (FAS) and calculated with 95%CI. In the FAS analysis, patients who were lost during follow-up and who did not comply with the protocol were excluded.

Statistical analysis of eradication rate among the

four regimens was assessed using the  $\chi^2$  test. Patient characteristics among the four groups were assessed via Fisher's exact test and the  $\chi^2$  test. Factors associated with treatment failure were assessed by logistic regression analysis. *P* values < 0.05 were considered to be statistically significant.

## RESULTS

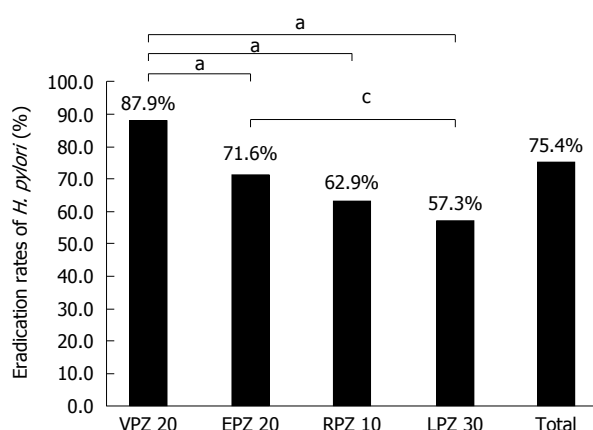
### Patient characteristics

In total, 1353 patients completed the first-line treatment protocol. The baseline characteristics and demographics of patients in this study were presented in Table 1. Most patients (*n* = 1169) were diagnosed with *H. pylori*-infected gastritis without any other lesions or diseases. Others diagnoses were GU/GUs (*n* = 65), DU/DUs (*n* = 105), GDU/GDUs (*n* = 8), MALT lymphoma (*n* = 2), and post ESD for early gastric cancer (*n* = 4). The patients were treated with VPZ (*n* = 546), EPZ (*n* = 507), RPZ (*n* = 89), or LPZ (*n* = 211). Demographic and other baseline characteristics for all the patients receiving the four regimens were not significantly different with regard to age, sex, and upper gastrointestinal diseases. In total, 261 patients completed the second-line treatment protocol. Demographic and other baseline characteristics in the second-line treatment were also shown in Table 1 and there were not significant differences in all of them.

### Eradication rates

FAS analysis indicated that the first-line treatment eradication rate was 87.9% (95%CI: 84.9%-90.5%) in the VPZ group, 71.6% (95%CI: 67.5%-75.5%) in the EPZ group, 62.9% (95%CI: 52.0%-72.9%) in the RPZ group, and 57.3% (95%CI: 50.4%-64.1%) in the LPZ group (Figure 1). The eradication rate achieved in the VPZ group was significantly higher than that in the other three groups (Table 2).

The eradication rates for the second-line treatment



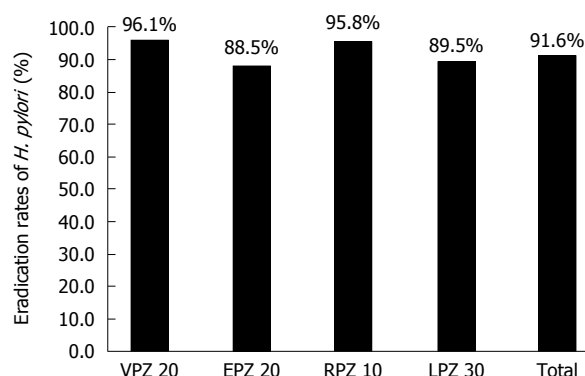
**Figure 1** *Helicobacter pylori* eradication rates (full analysis set) for vonoprazan, esomeprazole, rabeprazole, and lansoprazole in first-line triple therapy. The eradication rate was significantly higher in the VPZ group than that in the EPZ, RPZ, and LPZ groups (<sup>a</sup> $P < 0.05$ ); <sup>c</sup> $P < 0.05$  vs lansoprazole. VPZ 20: 20 mg VPZ, 200 mg CAM, and 750 mg AMPC twice a day for 1 wk. EPZ 20: 20 mg EPZ, 200 mg CAM, and 750 mg AMPC twice a day for 1 wk. RPZ 10: 10 mg RPZ, 200 mg CAM, and 750 mg AMPC twice a day for 1 wk. LPZ 30: 30 mg LPZ, 200 mg CAM, and 750 mg AMPC twice a day for 1 wk. VPZ: Vonoprazan; EPZ: Esomeprazole; CAM: Clarithromycin; AMPC: Amoxicillin; RPZ: Rabeprazole; LPZ: Lansoprazole; *H. pylori*: *Helicobacter pylori*.

were 96.1% (95%CI: 88.9%-99.2%) in the VPZ group, 88.5% (95%CI: 80.7%-93.9%) in the EPZ group, 95.8% (95%CI: 78.9%-99.9%) in the RPZ group, and 89.5% (95%CI: 78.5%-96.0%) in the LPZ group (Figure 2), and there were no significant differences among the four groups.

We evaluated age, extent of atrophic gastritis, sex, BMI, alcohol abuse, and smoking habits as predictive factors for successful *H. pylori* eradication in the first- and second-line treatments among the four groups. For the first-line treatment, smoking alone showed a significant difference between the four groups by univariate analyses. Thus, smoking habits decreased *H. pylori* eradication rates in the three PPI groups, whereas eradication was unaffected in the VPZ group (Table 2). There were no predictive factors observed for second-line treatment and there were no predictive factors demonstrated in both the first-line treatment and the second-line treatment by multivariate analyses (data not shown).

## AEs

The AE incidence during first-line eradication therapy was 11.2% in the VPZ group compared with 7.7% in the EPZ group, 10.1% in the RPZ group, and 5.7% in the LPZ group. The most frequently observed AE was diarrhoea/soft stool. There was no significant difference in AE incidence between the VPZ group and the PPI groups ( $P = 0.054$ ). However, the VPZ group showed a wider range of AEs including appetite loss, headache, fever and haematuria. The AE incidence for second-line eradication therapy was also not significantly different, and diarrhoea/soft stool occurred



**Figure 2** *Helicobacter pylori* eradication rates (full analysis set) for vonoprazan, esomeprazole, rabeprazole, and lansoprazole in second-line triple therapy. The eradication rates of the four second-line therapies were not significantly different. VPZ 20: 20 mg VPZ, 250 mg MNZ, and 750 mg AMPC twice a day for 1 wk; EPZ 20: 20 mg EPZ, 250 mg MNZ, and 750 mg AMPC twice a day for 1 wk; RPZ 10: 10 mg RPZ, 250 mg MNZ, and 750 mg AMPC twice a day for 1 wk; LPZ 30: 30 mg LPZ, 250 mg MNZ, and 750 mg AMPC twice a day for 1 wk. VPZ: Vonoprazan; MNZ: Metronidazole; AMPC: Amoxicillin; EPZ: Esomeprazole; RPZ: Rabeprazole; LPZ: Lansoprazole; *H. pylori*: *Helicobacter pylori*.

most frequently, similar to first-line eradication therapy (Table 3). None of the patients showed serious and critical AEs in during either the first or second-line therapy and none of the patients discontinued *H. pylori* eradication treatment because of AEs.

## DISCUSSION

We compared the efficacies and safety profiles of vonoprazan and three PPIs (esomeprazole, rabeprazole, and lansoprazole) used for *H. pylori* eradication and found vonoprazan to be superior to PPIs as a first-line therapy ( $P < 0.01$ ).

Several factors, such as cytochrome P450 2C19 (CYP2C19) polymorphisms, antibiotic susceptibility, smoking habits, and patient compliance, are known to cause *H. pylori* eradication failure<sup>[13]</sup>. Insufficient gastric acid inhibition and *H. pylori* antibiotic resistance are major factors underlying *H. pylori* eradication failure<sup>[13]</sup>.

*H. pylori* eradication is dependent on the maintenance of a near neutral gastric pH throughout the day<sup>[11]</sup>. Maintenance of a gastric pH  $> 5$  is necessary for *H. pylori* to replicate. Additionally, acid suppression has been reported to enhance antibiotic stability and bacterial sensitivity<sup>[14]</sup>. Therefore, adequate acid suppression is essential for *H. pylori* eradication therapy.

Vonoprazan is a highly effective anti-acid drug compared to PPIs. Sakurai *et al*<sup>[15]</sup> showed that vonoprazan suppressed acid secretion more rapidly and persistently than two PPIs, esomeprazole and rabeprazole. The authors showed that even on the first day of administration, the mean pH for vonoprazan was above 5, in contrast to the pH induced by PPIs, and the 24 h pH 4 holding-time (acid-inhibitory effect)

**Table 2** Univariate analysis of predictors for successful *Helicobacter pylori* first-line eradication

	VPZ group				PPI group			
	Eradication rate	OR	95%CI	P value	Eradication rate	OR	95%CI	P value
Sex								
Male	90.2%	1			67.0%	1		
Female	86.3%	0.682	0.396-1.174	0.17	66.9%	0.995	0.737-1.343	0.97
Age (yr)								
< 60	86.6%	1			74.1%	1		
≥ 60	87.9%	1	0.598-1.673	1	76.7%	1.155	0.860-1.551	0.34
BMI (kg/m <sup>2</sup> )								
< 25	88.4%	1			69.1%	1		
≥ 25	86.5%	0.843	0.464-1.530	0.57	68.8%	0.987	0.683-1.435	0.94
Smoking								
No	87.3%	1			68.1%	1		
Yes	91.3%	1.412	0.583-3.420	0.44	56.6%	0.611	0.412-0.906	0.01
Alcohol								
Non-drinker	88.1%	1			67.7%	1		
Drinker	87.4%	0.937	0.555-1.580	0.81	67.3%	0.984	0.716-1.352	0.92
Atrophy								
Closed type	87.9%	1			65.6%	1		
Open type	89.1%	1.130	0.619-2.069	0.69	65.6%	0.998	0.708-1.408	0.99

VPZ: Vonoprazan; PPI: Proton pump inhibitor.

**Table 3** Adverse events in first- and second-line eradication therapies *n* (%)

	First-line eradication therapy				Second-line eradication therapy			
	VPZ	EPZ	RPZ	LPZ	VPZ	EPZ	RPZ	LPZ
Adverse events	61 (11.2)	39 (7.7)	9 (10.1)	12 (5.7)	6 (7.9)	14 (13.5)	3 (12.5)	7 (12.3)
Diarrhoea/soft stool	29 (47.5)	25 (64.1)	5 (55.6)	8 (66.7)	4 (66.7)	6 (43.0)	3 (100)	5 (71.4)
Eruption	7 (11.5)	3 (7.7)	2 (22.2)	2 (16.7)	0	3 (21.4)	0	0
Constipation	6 (10.0)	3 (7.7)	0	0				
Dysgeusia	3 (5.0)	3 (7.7)	1 (11.1)	0	0	1 (7.1)	0	1 (14.3)
Nausea and vomiting	2 (3.3)	2 (5.1)	0	1 (8.3)	0	1 (7.1)	0	0
Abdominal pain	5 (8.2)	2 (5.1)	0	0	1 (16.7)	2 (14.3)	0	0
Appetite loss	3 (5.0)	0	0	0				
General fatigue	2 (3.3)	1 (2.6)	0	0	1 (16.7)	0	0	0
Heartburn	1 (1.6)	0	0	1 (8.3)				
Headache	1 (1.6)	0	0	0	0	0	0	1 (14.3)
Fever	1 (1.6)	0	0	0				
Flatulence	0	0	1 (11.1)	0				
Haematuria	1 (1.6)	0	0	0				
Vertigo					0	1 (7.1)	0	0

VPZ: Vonoprazan group; EPZ: Esomeprazole group; RPZ: Rabeprazole group; LPZ: Lansoprazole group.

of vonoprazan greatly exceeded that of the PPIs<sup>[15]</sup>. Considering the significance of acid suppression for *H. pylori* eradication therapy, vonoprazan could be effective for resolving *H. pylori* eradication failure.

The acid-inhibitory effect of PPIs is affected by CYP2C19 polymorphisms, and for extensive CYP2C19 metabolizers, the acid-inhibitory effect of PPIs is decreased. The population frequency of poor, intermediate, extensive metabolizers in Japan is 18.8%, 43.8% and 35.5%, respectively<sup>[16]</sup>.

Esomeprazole is less susceptible to CYP2C19 polymorphisms<sup>[17,18]</sup>. Indeed, the eradication rate of esomeprazole was significantly higher than that of lansoprazole in our study. We suggest that the eradication rates observed in our results correspond to the population frequency of genetic polymorphisms

affecting the metabolism of the PPI. Vonoprazan is also unaffected by CYP2C19 polymorphisms because it is poorly metabolized by CYP2C19<sup>[19-21]</sup>.

We hypothesized that the significantly higher eradication rate for vonoprazan compared to the other PPIs was caused by tolerance of CYP2C19 polymorphisms.

Drug interactions with antibiotics may also affect eradication rate. Comparing the pharmacokinetics of co-administration of vonoprazan, CAM plus AMPC and single administration of the drugs, co-administered vonoprazan and CAM resulted in a higher mean C<sub>max</sub> and area under the curve (AUC) compared with those of single administration. However, AMPC had no effect on C<sub>max</sub> and AUC<sup>[19-21]</sup>. Both CAM and vonoprazan are predominantly metabolized by CYP3A4 and have also been reported to be possible CYP3A4 inhibitors

when used in combination. Thus, vonoprazan and CAM might mutually inhibit their metabolism, increasing the plasma concentrations of both CAM and vonoprazan. A phase III, randomized, double-blind, multicentre study on vonoprazan showed higher eradication rates with vonoprazan (82.0%) against CAM resistance compared to that of lansoprazole (40.0%)<sup>[10]</sup>; thus, we hypothesize that these pharmacological characteristics of vonoprazan will be advantageous for *H. pylori* eradication therapy.

Although vonoprazan might be effective in *H. pylori* eradication therapy, AEs are still possible with this new drug. However, in our study, there was no significant difference in the incidence of AEs between vonoprazan and PPIs ( $P = 0.054$ ). Notably, vonoprazan caused a wider variety of AEs compared to PPIs, although every AE was relatively mild and completely resolved after discontinuation of *H. pylori* eradication therapy. As we mentioned above, the plasma concentrations of CAM and vonoprazan might be increased during first-line treatment, making it likely that unexpected and more frequent AEs happen. Thus, vonoprazan-associated AEs need to be carefully monitored in the future.

A phase III study also showed an extremely high eradication rate (98%) for second-line triple therapy with vonoprazan, AMPC, and MNZ<sup>[8]</sup>. Correspondingly, our second-line eradication rate was 96.1% overall, and there were no significant differences in the second-line eradication rates and AEs between the three PPIs and vonoprazan. In the second-line treatment, CAM was replaced by MNZ, which is believed to be unaffected by CYP3A and CYP2C19 polymorphisms. Due to the original high eradication rates of second-line therapy with PPIs, addition of MNZ did not result in differences in the second-line eradication rates.

Tobacco smoking was also demonstrated to be a factor in *H. pylori* eradication failure; this is because smoking stimulates acid secretion<sup>[22]</sup>, affects the metabolism of cytochrome P450<sup>[23]</sup>, and decreases gastric blood flow and mucous secretion<sup>[24]</sup>. Hence, smoking reduces the delivery of antibiotics to the gastric mucosa. Most significantly, smoking stimulates acid secretion, which results in decreased intragastric pH. Indeed, Suzuki *et al.*<sup>[25]</sup> reported that smoking increased the risk of *H. pylori* eradication failure. Interestingly, *H. pylori* eradication rates among patients in the VPZ group who smoked were unaffected in our study, whereas the eradication rates in the PPI groups decreased significantly. Thus, *H. pylori* therapy with vonoprazan was effective even for smoking patients; this was most likely because of strong acid suppression. Although smoking alone showed a significant difference between the four groups by univariate analyses, it was not a significant factor by multivariate analyses. We believe that this result might have arisen because we did not investigate drug sensitivity, and *H. pylori* resistance to CAM might be a major reason for treatment failure. It is necessary

to investigate factors contributing to the success of *H. pylori* eradication treatment, including drug sensitivity, in the future.

This study was limited because it was retrospective and only performed at a single centre; moreover, we did not investigate drug sensitivity and CYP2C19 polymorphism. Triple therapy combining a PPI with AMPC and CAM generates an unacceptably low eradication rate in most of the world. Sequential therapy, quadruple therapy, concomitant therapy and high dose dual therapy are recommended in the era of increased CAM resistance<sup>[13]</sup>. However, although the resistance of *H. pylori* to CAM is increasing in Japan, only triple therapy with PPI, AMPC and CAM has been recognized under Japan's national health insurance. Vonoprazan-based triple therapy has been available in Japan dating from February 2015. In our results, the vonoprazan eradication rate was high (87.9%), but it is not a satisfactory result regarding the efficacy of *H. pylori* eradication throughout the world. If vonoprazan-based triple therapy was provided to CAM-sensitive patients, the eradication rate might increase to over 90%. Therefore, it is necessary to investigate drug sensitivity before treatment in Japan. If the patients have CAM-resistant strains of *H. pylori*, they will require regimens of vonoprazan and different types and doses of antibiotics as well as different periods instead of CAM.

*H. pylori* eradication therapy is an effective treatment to help prevent gastric cancer. However, indiscriminate use of *H. pylori* eradication therapy might result in the expansion of antibiotic resistance instead of promoting gastric cancer prevention. Therefore, we need to select appropriate *H. pylori* eradication regimens by taking into consideration individual variations, such as antibiotic resistance and CYP2C19 polymorphism; however, the cost of these tests is not covered under Japan's national health insurance scheme.

In conclusion, triple therapy with vonoprazan, AMPC, and CAM is superior to PPI-based therapy for first-line eradication, however possible AEs should be monitored.

## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) eradication therapy is an effective treatment to help prevent gastric cancer. Triple therapy combining a proton pump inhibitor (PPI) with amoxicillin (AMPC), and clarithromycin (CAM) for *H. pylori* eradication is standard first-line therapy in Japan. However, the *H. pylori* eradication rate has decreased due to the increased prevalence of CAM resistance, thus a more effective strategy is required. Vonoprazan is a novel potassium-competitive acid blocker that has strong, long-lasting effects, but few studies have investigated its efficacy against *H. pylori* thus far. In this study, we retrospectively examined the effectiveness and safety of vonoprazan-based therapy compared with PPI-based therapies to treat *H. pylori*.

### Research frontiers

Triple therapy combining a PPI with AMPC and CAM provides unacceptably low eradication rates in most regions of the world. A good *H. pylori* therapy regimen



with an eradication rate above 90% is needed. A more effective regimen is necessary in Japan.

### Innovations and breakthroughs

In this study, vonoprazan resulted in a significantly higher eradication rate (87.9%) than that of the three PPIs in the first-line treatment. There were no significant differences in the second-line eradication rates. Interestingly, *H. pylori* eradication rate for vonoprazan in smoking patients was similar to that of non-smoking patients, whereas the eradication rates with PPIs for smokers were decreased.

### Applications

It is necessary to investigate drug sensitivity before treatment because *H. pylori* CAM resistance is increasing in Japan. If the patients have CAM-resistant strains of *H. pylori*, treatment will require regimens of vonoprazan and different types and doses of antibiotics. Vonoprazan-containing triple therapy, quadruple therapy, sequential therapy and dual therapy that considers drug sensitivity will produce an excellent eradication rate.

### Peer-review

This manuscript is the retrospective study of *Helicobacter pylori* eradication using vonoprazan and conventional PPIs.

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## Retrospective Study

# Negative oncologic impact of poor postoperative pain control in left-sided pancreatic cancer

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**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board of Yonsei University College of Medicine, Seoul 03722, South Korea.

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. Individuals can't be identified according to the data presented.

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**Data sharing statement:** No additional data are available.

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## Abstract

### AIM

To investigate the association between postoperative pain control and oncologic outcomes in resected pancreatic ductal adenocarcinoma (PDAC).

### METHODS

From January 2009 to December 2014, 221 patients were diagnosed with PDAC and underwent resection with curative intent. Retrospective review of the patients was performed based on electronic medical records system. One patient without records of numerical rating scale (NRS) pain intensity scores was excluded and eight patients who underwent total pancreatectomy were also excluded. NRS scores during 7 postoperative days following resection of PDAC were

reviewed along with clinicopathologic characteristics. Patients were stratified into a good pain control group and a poor pain control group according to the difference in average pain intensity between the early (POD 1, 2, 3) and late (POD 5, 7) postoperative periods. Cox-proportional hazards multivariate analysis was performed to determine association between postoperative pain control and oncologic outcomes.

### RESULTS

A total of 212 patients were dichotomized into good pain control group ( $n = 162$ ) and poor pain control group ( $n = 66$ ). Median follow-up period was 17 mo. A negative impact of poor postoperative pain control on overall survival (OS) was observed in the group of patients receiving distal pancreatectomy (DP group; 42.0 mo *vs* 5.0 mo,  $P = 0.001$ ). Poor postoperative pain control was also associated with poor disease-free survival (DFS) in the DP group (18.0 mo *vs* 8.0 mo,  $P = 0.001$ ). Patients undergoing pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy (PD group) did not show associations between postoperative pain control and oncologic outcomes. Poor patients' perceived pain control was revealed as an independent risk factor of both DFS (HR = 4.157; 95%CI: 1.938-8.915;  $P < 0.001$ ) and OS (HR = 4.741; 95%CI: 2.214-10.153;  $P < 0.001$ ) in resected left-sided pancreatic cancer.

### CONCLUSION

Adequate postoperative pain relief during the early postoperative period has important clinical implications for oncologic outcomes after resection of left-sided pancreatic cancer.

**Key words:** Pancreatic cancer; Pancreatectomy; Survival; Postoperative pain; Recurrence

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**Core tip:** This is a retrospective review to evaluate the association between postoperative pain control and oncologic outcomes in resected pancreatic ductal adenocarcinoma. In multivariate analysis, poor patients' perceived pain control was an independent risk factor for both disease-free survival (HR = 4.157; 95%CI: 1.938-8.915;  $P < 0.001$ ) and overall survival (HR = 4.741; 95%CI: 2.214-10.153;  $P < 0.001$ ) in resected left-sided pancreatic cancer. Adequate postoperative pain control to reduce patients' perceived pain during immediate postoperative period may be as important as adjuvant therapy in resected left-sided pancreatic cancer.

## INTRODUCTION

Pancreas cancer is one of the most fatal malignancies in the world and is currently the fourth leading cause of cancer death in the United States<sup>[1]</sup>. Surgical excision remains the only curative therapy for pancreatic cancer. However, the resection rate is less than 20% at the time of initial diagnosis, and the rate of recurrence is extremely high even after surgery, occurring in up to 65% to 95% of patients<sup>[2-4]</sup>. To overcome the high incidence of micrometastatic disease, margin-negative resection<sup>[5]</sup> and the use of adjuvant treatment<sup>[2,3,6]</sup> have been considered as important prognostic factors of long-term survival. Nonetheless, 5-year overall survival remains less than 25% even after receiving adjuvant chemotherapy following resection<sup>[2,3]</sup>.

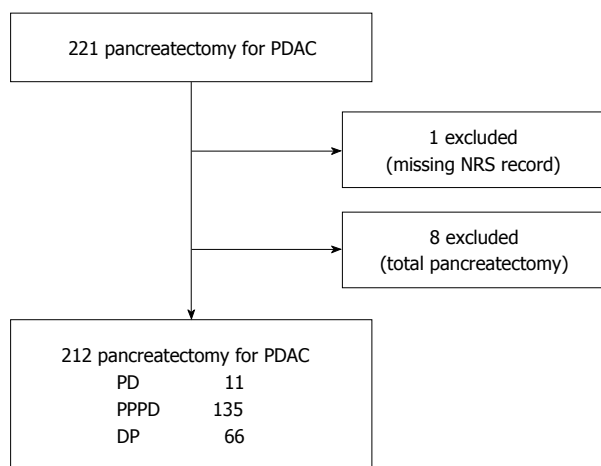
Recently, the importance of the perioperative period on oncologic outcome after cancer surgery has been emphasized in several review studies<sup>[7-10]</sup>. These studies underlined that the paracrine and neuroendocrine responses caused by surgical stress could promote tumor metastasis through direct action on residual malignant cells and by suppressing natural killer (NK) cell activity, thus compromising antimetastatic cell-mediated immunity (CMI)<sup>[8,11,12]</sup>. Downregulation of immunity after surgery is known to peak at postoperative day (POD) 3<sup>[13]</sup>, and the decline in NK cell cytotoxicity has been documented to last until POD 7 to 9, depending on the surgical procedure<sup>[14-16]</sup>. A decrease in NK cell cytotoxicity following pancreaticoduodenectomy (PD) at POD 7 was also recently reported<sup>[17]</sup>. These results indicate that the early postoperative period harbors potential for the initiation of cancer metastasis, either *de novo* or from pre-existing micrometastasis.

Even when surgeons achieve R0 resection, various factors of this disproportionately pivotal perioperative period can facilitate growth of potential residual cancer beyond a critical immunological threshold, leading to cancer recurrence. Suggested perioperative risk factors that modulate surgery-induced immunosuppression include anesthetic technique, analgesic agents, blood transfusion, hypothermia, and pain<sup>[7-10]</sup>.

Among these factors, acute pain is known to suppress NK cell activity<sup>[18]</sup>, and its immunosuppressive properties have been shown to promote tumor growth in animal models<sup>[19-22]</sup>. Postsurgical pain activates the sympathetic nervous system (SNS), leading to catecholamine secretion<sup>[23]</sup>, which directly inhibits NK cells. Furthermore, postoperative pain is not only a result of surgical tissue damage and nociception, but also reflects psychological stress, which has been reported as a risk factor of metastatic progression in

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**Figure 1 Patient eligibility.** PDAC: Pancreatic ductal adenocarcinoma; NRS: Numerical rating scale; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; DP: Distal pancreatectomy with or without spleen preservation.

some clinical trials<sup>[24,25]</sup>.

In spite of its potential role as an immunomodulator promoting tumor growth and metastasis, there has been no study to evaluate the oncologic significance of postoperative pain following resection of pancreas cancer. In this study, we investigated the association between postoperative pain control and oncologic outcomes in resected pancreatic ductal adenocarcinoma (PDAC).

## MATERIALS AND METHODS

### Patients and study design

From January 2009 to December 2014, 221 patients with PDAC underwent pancreatectomy with curative intent in our center. We retrospectively reviewed clinicopathologic characteristics and numerical rating scale (NRS) pain intensity score recorded in the nursing records system. One patient was excluded because of missing NRS data for an unknown reason and eight patients who required total pancreatectomy were also excluded (Figure 1). The study was reviewed and approved by the Institutional Review Board of Yonsei University College of Medicine.

### Data collection and analysis

NRS score from the nursing records system was available from 2009. Nurses administered the 11-point NRS, with the score ranging from 0 to 10, to evaluate pain intensity whenever the patients reported pain. Patients were instructed to rate 0 as “no pain at all” and 10 as “the worst possible pain”. NRS scores during 7 postoperative days following resection of PDAC were reviewed.

We defined early pain score as the average of all pain scores reported on POD 1, 2, and 3 and late pain score as the average of scores reported on POD 5 and 7. In consideration of the subjective nature of pain

and the importance of “perceived control”, we applied the concept of pain control expressed as difference in pain intensity between the two periods rather than objective pain intensity value. We defined the “good pain control group” as the group of patients whose late pain intensity was lower than that of early pain intensity and the “poor pain control group” as the group of patients whose late pain intensity was the same or higher than the early pain intensity.

Postoperative complications were defined using the Clavien-Dindo classification of surgical complications<sup>[26]</sup>. Major complications were defined as complications with a Clavien-Dindo score of III or higher, which require additional interventional and/or medical treatment associated with prolonged hospital stay. TNM stages were classified according to the American Joint Committee on Cancer (AJCC; 7<sup>th</sup> edition) staging system<sup>[27]</sup>. Multivisceral resection was defined as resection of any organ or a part of an organ other than the pancreas and spleen. Combined resection was defined as any multivisceral resection or vascular resection.

### Statistical analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, United States) and MedCalc 16.8.4 for Windows (MedCalc Inc., Mariakerke, Belgium). For continuous variables, *t*-test was performed and reported as mean and standard deviation. For matched data analysis, paired *t*-test was performed. Categorical variables were compared using the Chi-Square test or Fisher’s exact test and reported as number (*n*) and percentage (%). Overall survival (OS) rates and disease-free survival (DFS) rates were estimated using the Kaplan-Meier method. Log-rank test was performed to compare the categorical groups in univariate analysis. A multivariable Cox proportional hazards regression model was used to determine independent risk factors associated with OS and DFS. This model included all of the categorized patient, resection, and tumor characteristics with log-rank *P* values  $\leq 0.150$ . Exponential ( $\beta$ ) measures were reported with 95%CI to evaluate the risks associated with each factor. Statistical significance was achieved at *P* < 0.05.

## RESULTS

### Patients characteristics

A total of 212 patients who underwent pancreatectomy for PDAC were retrospectively reviewed. The clinicopathological characteristics are summarized in Table 1. Median follow-up period was 17 mo. Sixty-six patients (31.3%) received neoadjuvant concurrent chemoradiotherapy (CCRT) before pancreas resection, and 154 patients (72.6%) received adjuvant treatment of chemotherapy, radiotherapy, or CCRT according to their general condition. R0 resection

**Table 1 Clinicopathologic characteristics of the patients *n* (%)**

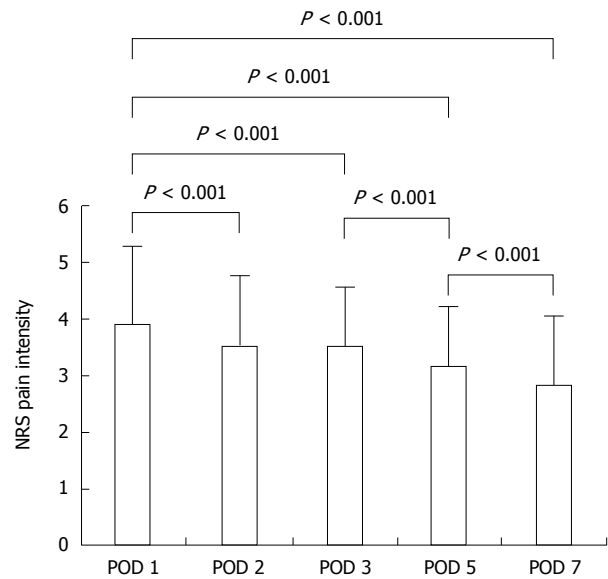
Characteristic ( <i>n</i> = 212)	Frequency, mean $\pm$ SD
Age (yr)	62.8 $\pm$ 9.5
Male gender	125 (59.0)
BMI (kg/m <sup>2</sup> )	22.8 $\pm$ 2.9
Diabetes	73 (34.4)
ASA	
1	66 (31.1)
2	92 (43.4)
3	49 (23.1)
4	2 (0.9)
Operative time (min)	402.2 $\pm$ 129.3
Intraoperative bleeding (mL)	626.0 $\pm$ 482.8
Intraoperative transfusion	47 (22.2)
pT stage	
T0	9 (4.2)
T1	16 (7.5)
T2	3 (1.4)
T3	182 (85.8)
T4	2 (0.9)
pN stage	
N0	106 (50.0)
N1	106 (50.0)
pTNM staging	
I	18 (8.5)
II	182 (85.8)
III	2 (0.9)
IV	1 (0.5)
R status	
R0	187 (88.2)
R1	23 (10.8)
R2	2 (0.9)
Cell differentiation	
Well	24 (11.3)
Moderate	152 (71.7)
Poor	17 (8.0)
Undifferentiated	1 (0.5)
Retrieved lymph nodes	17.3 $\pm$ 9.9
Vascular resection	55 (25.9)
Mutivisceral resection	36 (17.5)
Combined resection	71 (33.5)
Lymphovascular invasion	71 (33.5)
Perineural invasion	146 (68.9)
Neoadjuvant CCRT	66 (31.3)
Adjuvant treatment	154 (72.6)
Complications	
Minor	112 (52.8)
Major( $\geq$ G3)	20 (9.4)
Length of hospital stay (d)	21.1 $\pm$ 15.0
Recurrence	137 (64.6)

ASA: American Society of Anesthesiologists; CCRT: Concurrent chemoradiotherapy.

was achieved in 187 patients (88.2%). In terms of resection methods, 146 patients (68.9%) underwent pancreaticoduodenectomy (PD) or pylorus-preserving pancreatoduodenectomy (PPPD), and 66 patients (31.1%) underwent distal pancreatectomy (DP) with or without spleen preservation.

#### Changes in postoperative pain intensity following pancreatectomy

For the overall patient population, postoperative pain intensity decreased significantly at POD 2, 3, 5, and



**Figure 2 Overall changes in postoperative numerical rating scale pain intensity following pancreatectomy.** POD: Postoperative day; NRS: Numerical rating scale.

7 compared with POD 1 ( $P < 0.001$  for each; Figure 2). There was a significant decrease in pain intensity between each two successive days ( $P < 0.001$ ), except between POD 2 and 3 ( $P = 0.916$ ).

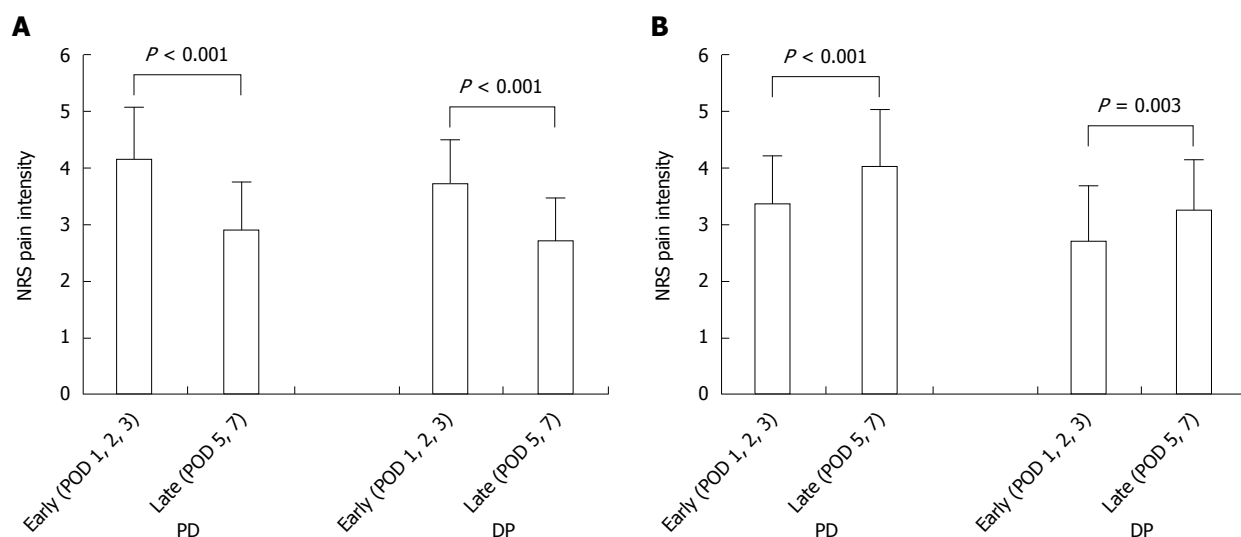
Patients were divided into a good pain control group ( $n = 162$ ) and poor pain control group ( $n = 50$ ). The good pain control group showed a reduction of pain intensity from  $4.13 \pm 0.93$  to  $2.87 \pm 0.86$  for the PD group ( $n = 109$ ,  $P < 0.001$ ) and from  $3.71 \pm 0.77$  to  $2.69 \pm 0.78$  for the DP group ( $n = 53$ ,  $P < 0.001$ , Figure 3A). The poor pain control group showed an increase of pain intensity from  $3.35 \pm 0.87$  to  $4.03 \pm 1.02$  for the PD group ( $n = 37$ ,  $P < 0.001$ ) and from  $2.71 \pm 0.99$  to  $3.26 \pm 0.88$  for the DP group ( $n = 13$ ,  $P = 0.003$ , Figure 3B).

#### Oncologic impact of postoperative pain intensity following pancreatectomy

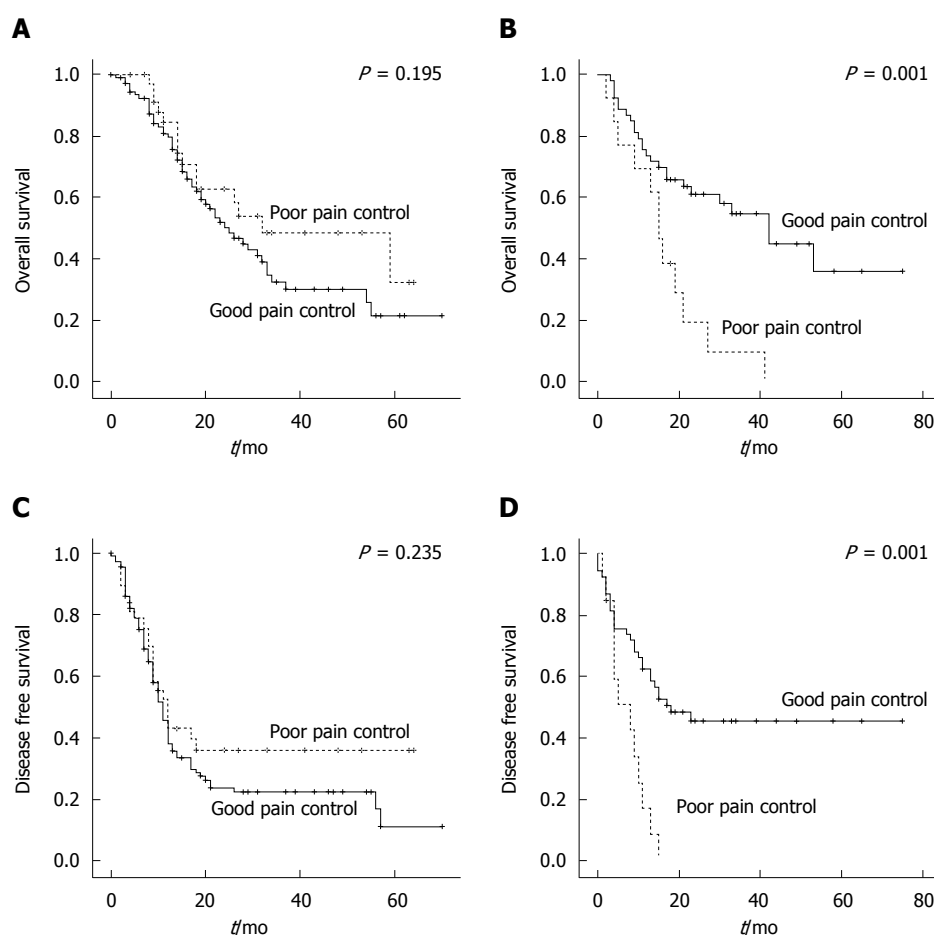
A negative impact of poor postoperative pain control on OS was observed in the DP group [good pain control vs poor pain control, median survival 42.0 mo (95%CI: 26.2-57.8) vs 15.0 mo (95%CI: 11.5-18.5),  $P = 0.001$ , Figure 4B]. Also, poor pain control exerted a negative effect on DFS in the DP group (good pain control vs poor pain control, median 18.0 mo vs 8.0 mo,  $P = 0.001$ , Figure 4D). Patients undergoing pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy (PD group) did not show associations between postoperative pain control and oncologic outcomes.

#### Comparison between the good pain control group and the poor pain control group among patients undergoing distal pancreatectomy

There were no significant differences in preoperative,



**Figure 3** Change in numerical rating scale pain intensity following pancreatectomy stratified by quality of pain control. A: Good pain control group ( $n = 109$ , PD;  $n = 53$ , DP); B: Poor pain control group ( $n = 37$ , PD;  $n = 13$ , DP). PD: Patients underwent pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy; DP: Patients underwent distal pancreatectomy; POD: Postoperative day; NRS: Numerical rating scale.



**Figure 4** Oncologic outcomes. Comparison of overall survival between the good pain control group (solid line) and poor pain control group (dotted curve) after pancreaticoduodenectomy (PD) (A) and distal pancreatectomy (DP) (B); Comparison of disease-free survival between the good pain control group (solid line) and poor pain control group (dotted curve) after PD (C) and DP (D).

**Table 2** Clinicopathologic differences between the good pain control group and poor pain control group undergoing distal pancreatectomy *n* (%)

	Pain control group		<i>P</i> value
	Good ( <i>n</i> = 53)	Poor ( <i>n</i> = 13)	
Age (yr)	65.6 ± 8.2	63.2 ± 11.3	0.378
Male gender	30 (56.6)	7 (53.8)	0.858
BMI (kg/m <sup>2</sup> )	23.1 ± 2.7	23.1 ± 2.4	0.993
Diabetes	17 (32.1)	3 (23.1)	0.739
ASA			
1	16 (30.2)	5 (41.7)	0.342
2	22 (41.5)	6 (50.0)	
3	15 (28.3)	1 (8.3)	
Surgical approach			
Open	35 (66.0)	7 (53.8)	0.523
MIS	18 (34.0%)	6 (46.2)	
Spleen preservation	7 (13.2)	2 (15.4)	> 0.999
Operation time (min)	254.1 ± 95.3	304.6 ± 89.0	0.088
Intraoperative bleeding (mL)	364.2 ± 296.0	470.38 ± 624.8	0.373
Intraoperative transfusion	5 (9.4)	3 (23.1)	0.185
Tumor size (cm)			0.407
< 3	30 (56.6)	9 (69.2)	0.148
≥ 3	23 (43.4)	4 (30.8)	
pT stage			
T0	3 (5.7)	0 (0)	
T1	3 (5.7)	2 (15.4)	0.851
T2	2 (3.8)	1 (7.7)	
T3	45 (84.9)	9 (69.2)	
T4	0 (0.0)	1 (7.7)	
pN stage			0.429
N0	27 (50.9)	7 (53.8)	
N1	26 (49.1)	6 (46.2)	0.121
pTNM staging			
I	5 (9.5)	2 (15.4)	
II	44 (83.0)	10 (77.0)	
III	0 (0.0)	1 (7.7)	0.735
IV	1 (1.9)	0 (0.0)	
R status			
R0	47 (88.7)	11 (84.6)	0.718
R1	6 (11.3)	1 (7.7)	
R2	0 (0.0)	1 (7.7)	
Retrieved lymph nodes	14.8 ± 11.0	14.2 ± 6.6	0.496
Multivisceral resection	12 (22.6)	2 (15.4)	0.729
Combined resection	14 (26.4)	2 (15.4)	0.185
Lymphovascular invasion	14 (27.5)	4 (33.3)	0.499
Perineural invasion	34 (66.7)	5 (41.7)	
Grade			
Well	3 (6.4)	2 (16.7)	0.719
Moderate	38 (80.9)	9 (75.0)	
Poor	6 (12.8)	1 (8.3)	
Neoadjuvant CCRT	12 (22.6)	4 (30.8)	0.154
Preoperative CA19-9			
< 300	34 (69.4)	12 (92.3)	0.739
≥ 300	15 (30.6)	1 (7.7)	
Adjuvant treatment	39 (73.6)	9 (69.2)	0.520
Time to adjuvant treatment (d)	53.7 ± 36.8	63.0 ± 47.0	
Complications			
Minor	33 (62.3)	7 (53.8)	0.578
Major (≥ G3)	3 (7.3)	2 (28.6)	0.148
Use of PCA			0.445
IV PCA	34 (64.2)	10 (76.9)	0.446
Epidural PCA	17 (32.1)	2 (15.4)	
None	2 (3.8)	1 (7.7)	
Length of hospital stay (d)	17.1 ± 11.0	27.2 ± 45.9	

ASA: American Society of Anesthesiologists; CCRT: Concurrent chemo-radiotherapy; MIS: Minimal invasive surgery; PCA: Patient-controlled analgesia.

intraoperative, or postoperative outcomes between the good pain control group and the poor pain control group ( $P > 0.05$ , Table 2). Other interesting finding was that surgical approach, such as open or minimally invasive, did not influence categorization of pain response ( $P = 0.523$ ). Whether the patients received neoadjuvant CCRT or not also did not affect grouping of pain control ( $P = 0.719$ ). There were also no differences in method of postoperative pain management techniques between the group ( $P = 0.445$ ).

### Independent prognostic factors in resected left-sided pancreatic cancer

In univariate analysis, intraoperative transfusion, positive lymph node status, greater tumor diameter ( $\geq 3$  cm), and poor pain control were identified as prognostic factors for predicting DFS in resected left-sided pancreatic cancer ( $P = 0.005$ ,  $P = 0.011$ ,  $P = 0.028$ ,  $P = 0.001$ , respectively; Table 3). For OS, longer operation time ( $\geq 300$  min), positive lymph node status, greater tumor diameter ( $\geq 3$  cm), multivisceral resection, not receiving adjuvant treatment, and poor pain control were significant prognostic factors in univariate analysis ( $P = 0.035$ ,  $P = 0.020$ ,  $P = 0.023$ ,  $P = 0.043$ ,  $P = 0.017$ ,  $P = 0.001$ , respectively; Table 3). Subsequent multivariate analysis revealed positive lymph node status, greater tumor diameter ( $\geq 3$  cm), not receiving adjuvant treatment, and poor pain control as independent risk factors for both DFS and OS in resected left-sided pancreatic cancer (Table 4).

## DISCUSSION

Evidence showing the association of pain relief and reduced surgery-induced tumor growth was first documented by Yeager *et al.*<sup>[28]</sup> in colon carcinoma. Since then, the protective effect of various analgesics on surgery-induced metastasis has been reported by many studies<sup>[19,20,29-31]</sup>. However, there has been no study to evaluate the oncologic significance of postoperative pain control following resection of pancreatic cancer. To the best of our knowledge, the current retrospective study is the first to suggest that early postoperative pain control can influence patient survival after DP for PDAC, regardless of the biology of the tumor, surgical approach, or treatment modality.

A possible mechanism explaining the association of poor pain control and negative oncologic outcome could include interaction of inflammation, pain, and suppressed NK cell activity in the early postsurgical period, resulting in immunosuppression, which is known to peak on POD 3. The immunosuppressive property of pain is attributed to the direct inhibition of NK cell cytotoxicity by catecholamine secreted upon SNS activation<sup>[11,12]</sup>. Also, postoperative pain is associated with increased secretion of proinflammatory



**Table 3** Univariate analysis of factors affecting disease-free survival and overall survival after distal pancreatectomy

	n	DFS		OS	
		Median survival (mo)	P value <sup>1</sup>	Median survival (mo)	P value <sup>1</sup>
Age					
< 65	30	15	0.216	42	0.150
≥ 65	36	11		23	
Sex					
Female	29	11	0.136	27	0.829
Male	37	18		33	
BMI (kg/m <sup>2</sup> )					
< 25	52	11	0.209	30	0.384
≥ 25	14			27	
Diabetes					
No	46	13	0.674	27	0.654
Yes	20	15			
ASA					
1/2	49	13	0.569	30	0.903
3/4	16	15			
Surgical approach					
Open	42	14	0.971	30	0.645
MIS	24	11		41	
Spleen preservation					
Yes	9	15	0.931	27	0.619
No	57	13		33	
Operation time (min)					
< 300	39	17	0.254	41	0.035
≥ 300	27	13		15	
Bleeding (mL)					
< 500	42	11	0.632	30	0.881
≥ 500	23	15		33	
Intraoperative transfusion					
No	58	15	0.005	33	0.056
Yes	8	4		13	
Resection status					
R0	58	13	0.689	30	0.382
R1/R2	8	13		21	
Lymph node status					
N0	34		0.011	41	0.020
N1	32	9		17	
Tumor size (cm)					
< 3	39	18	0.028	42	0.023
≥ 3	27	8		15	
pT stage					
≤ 2	11		0.293	21	0.949
≥ 3	55	13		33	
Multivisceral resection					
No	52	15	0.111	33	0.043
Yes	14	10		13	
Combined resection					
No	50	15	0.474	33	0.303
Yes	16	10		15	
Lymphovascular invasion					
No	45	11	0.259	23	0.762
Yes	18	18		30	
Perineural invasion					
No	24	11	0.947	21	0.621
Yes	39	15		27	
Neoadjuvant CCRT					
Yes	16	15	0.351	33	0.433
No	50	11		21	
Preop CA19-9 (U/mL)					
< 300	46	15	0.782	27	0.540
≥ 300	16	13		42	
Adjuvant treatment					
Yes	48	15	0.094	41	0.017
No	18	4		8	

Major complications (≥ G3)					
No	43	15	0.813	42	0.916
Yes	5	4		21	
Use of PCA					
Epidural	19	13	0.757	27	0.943
IV	44	15		33	
Pain control					
Good	53	18	0.001	42	0.001
Poor	13	8		15	

<sup>1</sup>P values were obtained using a log-rank test. DFS: Disease-free survival; OS: Overall survival; ASA: American Society of Anesthesiologists; MIS: Minimal invasive surgery; CCRT: Concurrent chemoradiotherapy; PCA: Patient-controlled analgesia.

cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$ . Sommer *et al*<sup>[32]</sup> indicated that pain and proinflammatory cytokines interact reciprocally. Pain affects the production and secretion of cytokines, and those cytokines reduce the activation threshold of peripheral nociceptors, resulting in pain augmentation. Several studies<sup>[33-35]</sup> have shown the association of pain relief in the immediate postoperative period and attenuated production of proinflammatory cytokines. The correlation between changes in proinflammatory cytokine levels and decreased NK cell response was also reported by Baxeianis *et al*<sup>[15]</sup>. Considering these findings, unrelieved or elevated pain intensity at the late postsurgical period (POD 5, 7) might reflect prolonged inflammation and a state of immunosuppression and thus a higher chance of tumor metastasis and recurrence, explaining the negative oncologic outcome.

Poor pain control is basically attributed to a failure of appropriate and adequate postoperative analgesic care. However, it has also been suggested that poor pain response can be a result of the patient-specific immune state before surgery<sup>[36]</sup>, a sign of ongoing or forthcoming complications<sup>[37,38]</sup>, or a contributory effect of perioperative psychological factors<sup>[39,40]</sup>. Since there were no significant differences in any clinicopathologic factors between the good pain control group and poor pain control group undergoing DP (Table 2), we can only speculate that inadequate postoperative pain control was the major reason for poor postoperative pain control in left-sided pancreatic cancer. Further study should include establishment of appropriate pain control protocol to minimize influence of inadequate pain control and evaluate whether there are other possible reasons for poor postoperative pain control in left-sided pancreatic cancer.

Currently, it is routine for patients undergoing pancreas cancer surgery to receive adjuvant treatment irrespective of whether R0 resection is achieved. Therefore, the postsurgical period has been viewed as a time for managing complications and improving the general condition of the patient in order to meet the physiological requirements for receiving adjuvant treatment. During this approximately two-months

**Table 4** Univariate and multivariate Cox regression analysis of factors affecting disease-free survival and overall survival after distal pancreatectomy

Variables	Disease-free survival						Overall survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	Exp (β)	95%CI	P value	Exp (β)	95%CI	P value	Exp (β)	95%CI	P value	Exp (β)	95%CI	P value
Positive lymph node status	2.183	1.162-4.101	0.011	2.259	1.150-4.437	0.018	2.105	1.094-4.053	0.02	2.501	1.218-5.134	0.012
Tumor size (≥ 3 cm)	1.943	0.999-3.781	0.028	2.215	1.130-4.341	0.021	2.030	1.016-4.055	0.023	2.662	1.282-5.529	0.009
No adjuvant treatment	1.742	0.800-3.794	0.094	2.468	1.196-5.093	0.015	2.205	0.981-4.955	0.017	4.649	2.124-10.172	< 0.001
Poor pain control	2.934	1.158-7.430	0.001	4.157	1.938-8.915	< 0.001	2.915	1.156-7.350	0.001	4.741	2.214-10.153	< 0.001
Age (≥ 65 yr)	ND			ND			1.608	0.844-3.064	0.150	1.706	0.799-3.640	0.167
Sex (Male)	0.632	0.338-1.181	0.136	0.614	0.318-1.186	0.146	ND			ND		
Operation time (≥ 300 min)	ND			ND			1.949	0.981-3.873	0.035	1.890	0.923-3.868	0.082
Intraoperative transfusion	2.788	0.903-8.612	0.005	1.745	0.688-4.425	0.241	2.159	0.729-6.396	0.056	1.986	0.750-5.257	0.167
Multivisceral resection	1.720	0.769-3.849	0.111	1.166	0.532-2.557	0.701	2.046	0.837-5.006	0.043	1.273	0.563-2.876	0.562

ND: Not determined due to lack of significance.

period, patients do not receive anticancer treatment or intervention. However, this period - especially the immediate early period - harbors a therapeutic window of anticancer treatment, as surgery-induced immunosuppression is still in its recovery phase, and the tumor burden could start to increase again. Although, present study is based on a small sample size and retrospective observation, our data suggest that pain management after DP could be more than a matter of patient recovery to receive adjuvant treatment at the appropriate time. Rather, adequate and appropriate pain control during the early post-operative period might exert a direct curative effect on left-sided pancreatic cancer.

Interestingly, the negative oncologic impact of postoperative pain control was not observed following PD. This may have been influenced by wider surgical extent involved with PD and the impact of postoperative pain control during the postoperative 7 days may be rendered ineffective. The operation time, intraoperative blood loss, and rate of intraoperative transfusion were all higher after PD compared to DP [448.3 min vs 264.1 min,  $P < 0.001$ ; 717.9 cc vs 385.5 cc,  $P < 0.001$ ; 39 (26.9%) vs 8 (12.1%),  $P = 0.017$ , respectively, data not shown], reflecting greater surgical stress. Increased surgical extent has been shown to be associated with higher rates of tumor metastasis<sup>[41]</sup> and delayed recovery of NK cell cytotoxicity<sup>[15]</sup>. Also, intraoperative transfusion has been repeatedly reported to modulate the postoperative immune response<sup>[42,43]</sup>. These factors may overcome the potential immune modulation and oncologic effect of pain control during the period of assessment.

Our study has several limitations. It is a retrospective study, and the number of patients in the poor pain control group after DP was small, making it difficult to reach sound conclusions. We grouped patients into good and poor pain control groups according to differences in NRS pain intensity between

early (POD 1, 2, 3) and late (POD 5, 7) periods, because inflammation and immunosuppression peak on around POD 3. However, this time frame might not fit all cases and may vary according to surgical extent or approach. Future studies are needed to test various analytic approaches targeting the critical time point when postoperative pain most significantly mediates immunomodulation.

In addition, our definition of pain control groups may not fully represent pain control state. There have been reports of using satisfaction score along with pain score in fully assessing adequate pain control<sup>[44,45]</sup>. In determining pain control state, we were limited to the use of NRS pain intensity. Further studies with assessment of satisfaction score and refined definitions for pain control groups should be undertaken.

Lastly, for pain control, patients received intravenous patient-controlled analgesia (PCA) or epidural PCA (both based on fentanyl) or opiates on demand. However, disconnect timing of PCA, as well as the type and amount of analgesics used after clamping, were not investigated in this study. Opioids are believed to exert an immunosuppressive effect when they are used in the absence of pain<sup>[22]</sup>. Also, certain types of opioids, such as tramadol (but not all opioids) can overcome the immunosuppressive effects of pain, reversing the capacity of surgical stress to suppress NK cell cytotoxicity and promote tumor metastasis in animal models<sup>[20,46]</sup>. The complex interaction of pain, opioids, non-opioid analgesics, and their net effect on immunosuppression, which might have impacted oncologic outcome, was not assessed in this study. However, relationship between pain control method and postoperative pain control should be investigated further with a well-designed pain control protocol.

This study suggests that a change in patients' perceived pain intensity in the postoperative period could influence survival outcome in resected left-sided pancreatic cancer. Unlike other prognostic factors, such

as tumor size, lymph node metastasis, differentiation, lymphovascular invasion, and perineural invasion, postoperative pain is a controllable factor. Surgeons play a leading role in controlling pain during the postoperative period. In spite of compelling evidence supporting the immunologic and oncologic importance of the perioperative period, its application to the clinical field is still in its infancy. More research on underutilized modulators of this period - not only postoperative pain, but also intraoperative hypothermia, transfusion, nutritional support, and psychological intervention - is needed for the development of patient-oriented perioperative therapy against pancreatic cancer.

In conclusion, adequate postoperative pain relief during the early postoperative period has important clinical implications for oncologic outcomes after resection of left-sided pancreatic cancer.

## COMMENTS

### Background

Acute pain is known to suppress natural killer (NK) cell cytotoxicity and promote tumor growth and metastasis in preclinical models. Therefore, postoperative pain has been suggested as one of the risk factors for cancer metastasis and recurrence after oncologic surgery. Surgery-induced immunosuppression and inflammation are known to peak around postoperative day (POD) 3, and suppression of NK cell cytotoxicity lasts from approximately POD 7 to 9. The clinical significance of postoperative pain control during this critical period in patients undergoing pancreatotomy for pancreatic ductal adenocarcinoma (PDAC) has yet to be investigated.

### Research frontiers

This study contributes in discovering associations between postoperative pain control and oncologic outcomes in resected pancreatic cancer.

### Innovations and breakthroughs

In this study, patients undergoing distal pancreatectomy for left-sided pancreatic cancer were found to have poor oncologic outcomes under poor postoperative pain control. Adequate postoperative pain relief during the early postoperative period has important clinical implications for oncologic outcomes after resection of left-sided pancreatic cancer.

### Applications

Surgeons play a leading role in controlling pain during the postoperative period. Patients' perceived postoperative pain should be actively relieved after distal pancreatectomy for pancreatic cancer to improve oncologic outcomes.

### Terminology

PD group: Patients underwent pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy. DP group: Patients underwent distal pancreatectomy. Good pain control group: Patients whose late pain intensity was lower than that of early pain intensity. Poor pain control group: Patients whose late pain intensity was the same or higher than the early pain intensity. Early pain intensity: Mean of all pain scores reported on POD 1, 2, and 3. Late pain intensity: Mean of pain scores reported on POD 5 and 7. Pain scores: Measurement of numerical rating scale (NRS) pain intensity score. 11-point NRS with the score ranging from 0 to 10 was used to evaluate pain intensity whenever the patients reported pain. Patients were instructed to rate 0 as "no pain at all" and 10 as "the worst possible pain".

### Peer-review

The study evaluated the association between postoperative pain control and oncologic outcomes in resected PDAC. The results showed that poor pain

control was an independent risk factor for both DFS and OS in resected left-sided pancreatic cancer, but not in patients received PD. This is very interesting.

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## Retrospective Study

# Correlation of transient elastography with hepatic venous pressure gradient in patients with cirrhotic portal hypertension: A study of 326 patients from India

Ashish Kumar, Noor Muhammad Khan, Shrihari Anil Anikhindi, Praveen Sharma, Naresh Bansal, Vikas Singla, Anil Arora

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**Author contributions:** Kumar A designed and performed the research, performed statistical analysis and wrote the paper; Khan NM collected the data; Anikhindi SA contributed to the analysis; Sharma P, Bansal N, Singla V provided clinical advice; Arora A supervised the study.

**Institutional review board statement:** This retrospective study was submitted to the Ethics Committee of Sir Ganga Ram Hospital, New Delhi for review.

**Informed consent statement:** Informed consent from the included patients was not obtained because the study was retrospective, and only anonymous clinical data of the patients were obtained retrospectively from the records. The data were anonymous, and the identities of patients were not disclosed. Each patient provided written informed consent for undergoing hepatic venous pressure gradient (HVPG).

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## Abstract

### AIM

To study the diagnostic accuracy of transient elastography (TE) for detecting clinically significant portal hypertension (CSPH) in Indian patients with cirrhotic portal hypertension.

### METHODS

This retrospective study was conducted at the Institute of Liver, Gastroenterology, and Pancreatico-Biliary Sciences, Sir Ganga Ram Hospital, New Delhi, on consecutive patients with cirrhosis greater than 15 years of age who underwent hepatic venous pressure gradient (HVPG) and TE from July 2011 to May 2016. Correlation between HVPG and TE was analyzed using the Spearman's correlation test. Receiver operating characteristic (ROC) curves were prepared for determining the utility of TE in predicting various stages of portal hypertension. The best cut-off value of TE for

the diagnosis of CSPH was obtained using the Youden index.

## RESULTS

The study included 326 patients [median age 52 (range 16-90) years; 81% males]. The most common etiology of cirrhosis was cryptogenic (45%) followed by alcohol (34%). The median HVPG was 16.0 (range 1.5 to 30.5) mmHg. Eighty-five percent of patients had CSPH. A significant positive correlation was noted between TE and HVPG ( $\rho$  0.361,  $P < 0.001$ ). The area under ROC curve for TE in predicting CSPH was 0.740 (95%CI: 0.662-0.818) ( $P < 0.01$ ). A cut-off value of TE of 21.6 kPa best predicted CSPH with a positive predictive value (PPV) of 93%.

## CONCLUSION

TE has a fair positive correlation with HVPG; thus, TE can be used as a non-invasive modality to assess the degree of portal hypertension. A cut-off TE value of 21.6 kPa identifies CSPH with a PPV of 93%.

**Key words:** Portal hypertension; Cirrhosis; Clinically significant portal hypertension; Liver stiffness; Transient elastography; FibroScan

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**Core tip:** Clinically significant portal hypertension (CSPH), which is defined as hepatic venous pressure gradient (HVPG)  $\geq 10$  mmHg, causes major complications of cirrhosis. HVPG is invasive, so a non-invasive tool to diagnose CSPH is needed. This study of 326 Indian patients tested the diagnostic accuracy of transient elastography (TE) for detecting CSPH. We observed a significant positive correlation between TE and HVPG ( $\rho$  0.361,  $P < 0.001$ ). The area under the receiver operating characteristic curve for TE in predicting CSPH was 0.740. A cut-off value of TE of 21.6 kPa best predicted CSPH with a positive predictive value of 93%. Thus, TE can be used as a non-invasive modality to assess the degree of portal hypertension.

Kumar A, Khan NM, Anikhindi SA, Sharma P, Bansal N, Singla V, Arora A. Correlation of transient elastography with hepatic venous pressure gradient in patients with cirrhotic portal hypertension: A study of 326 patients from India. *World J Gastroenterol* 2017; 23(4): 687-696 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/687.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.687>

## INTRODUCTION

The end result of ongoing injury to the liver due to any cause is hepatic fibrosis, leading to architectural changes and often cirrhosis<sup>[1]</sup>. Progressive hepatic fibrosis is the most important factor leading to

parenchymal dysfunction and the development of portal hypertension. The measurement of portal hypertension is important, as a progressive increase in portal pressure is believed to predict various complications of cirrhosis<sup>[2-4]</sup>. Hepatic venous pressure gradient (HVPG) is the ideal method for the measurement of portal hypertension and the prediction of complications<sup>[5]</sup>. Porto-systemic collaterals develop at HVPG greater than 10 mmHg<sup>[6]</sup>, and variceal bleeding could occur from varices when the pressure increases to greater than 12 mmHg<sup>[7]</sup>. An HVPG greater than 10 mmHg is used as the cut-off for "clinically significant portal hypertension" (CSPH)<sup>[8]</sup>. However, HVPG is an invasive procedure that requires care and training.

Many non-invasive direct and indirect tests have been reported that are able to predict the presence of CSPH in patients with cirrhosis with considerable accuracy. The ideal non-invasive diagnostic test for portal hypertension should be simple, inexpensive, widely accessible and reliable in measurement and interpretation and provide clinically reliable and relevant information about the degree of portal hypertension. Transient elastography (TE) is a novel, non-invasive, ultrasound-based technology that allows the measurement of liver stiffness. Established evidence indicates that TE has good sensitivity and specificity for diagnosing liver fibrosis and cirrhosis and has been popular over the past few years<sup>[9,10]</sup>. Recently, many European studies have reported a fairly good correlation between liver stiffness and portal hypertension, suggesting that it could be a good non-invasive tool for evaluation of portal hypertension<sup>[11]</sup>. However, none of the studies are from South Asia where the etiological profile of cirrhosis is different from other regions of the world.

In the present study, we aimed to identify a possible correlation between TE and HVPG in Indian cirrhosis patients and to investigate whether TE can serve as a non-invasive diagnostic test to identify patients who have CSPH with a reliable cut-off TE value.

## MATERIALS AND METHODS

### Patients

The study was conducted at the Institute of Liver, Gastroenterology, and Pancreatic-Biliary Sciences, Sir Ganga Ram Hospital, New Delhi, on patients who underwent HVPG and TE from July 2011 to May 2016. The study conformed to the Helsinki declaration of 1975 as revised in 1983. The study was retrospective on prospectively enrolled patients during this period.

**Inclusion criteria:** Consecutive patients with cirrhosis greater than 15 years of age who underwent HVPG and TE were included in the study. Both these procedures should have been performed within an

interval of one week.

**Exclusion criteria:** The following patients were excluded from the study: (1) patients with non-cirrhotic cause of portal hypertension; (2) patients with acute-on-chronic liver failure; (3) patients with an invalid reading of TE or whose HVPG was not possible due to technical reasons; (4) patients who received beta blocker therapy in the past two weeks; and (5) concomitant extrahepatic malignancy.

### Evaluation

Each included patient underwent a detailed evaluation in terms of demographic parameters, etiology of cirrhosis, assessment of severity of liver disease (CTP, MELD), and assessment of severity of portal hypertension. A standard methodology was followed for the measurement of liver stiffness by TE and measurement of HVPG.

### Liver stiffness measurement by TE

Liver stiffness measurement was performed using a FIBROSCAN® (Echosens, Paris, France) device in accordance with the manufacturer's recommendations. Measurements were performed on the right lobe of the liver through intercostal spaces with the patient lying in a supine position with the right arm in maximal abduction. The tip of the transducer probe was covered with coupling gel and placed on the skin between the rib bones at the level of the right lobe of the liver. When the target area was located, the operator pressed the probe button to commence the measurements. The measurement depth was between 25 and 65 mm. Ten successful measurements were performed on each patient. The results were expressed in kilopascals (kPa). The median value was considered as the liver stiffness. Interquartile range/median < 30% and success rate > 60% were considered as good quality criteria for TE. Patients with significant ascites underwent large volume paracentesis before liver stiffness measurement. All liver stiffness measurements were performed by a single operator.

### Hepatic venous pressure gradient

HVPG was measured by introducing a 7-French Swan-Ganz catheter via the transfemoral or transjugular approach into a major hepatic vein as previously described<sup>[12]</sup>. The catheter was advanced until it was wedged into the hepatic vein. The occluded position of the catheter was assessed by the absence of reflux after the injection of 2 mL of contrast medium and the appearance of a sinusoidogram. A mean of three HVPG readings was obtained. If there was a difference of greater than 1 mm Hg between the readings, all the recordings were discarded, and fresh readings were obtained.

### Statistical analysis

Continuous variables are expressed as the median

with ranges, and discrete variables are expressed as numbers (%). Correlations between variables were analyzed using Spearman's correlation test. Comparisons of continuous variables between two groups were performed by Mann-Whitney *U* test, and comparisons between multiple groups were performed by Kruskal-Wallis test. Fisher's exact test or  $\chi^2$  test was used to compare categorical variables. Receiver operating characteristic (ROC) curves were prepared to determine the utility of TE in predicting various stages of portal hypertension. The best cut-off value of TE for the diagnosis of CSPH was obtained by using the Youden index. SPSS 17 (Chicago, IL, United States) software was used for analysis.

## RESULTS

### Patients

From January 2014 to June 2016, three hundred and seventy-nine patients were screened for enrolment in the study. Of these, 326 patients were included in the study, and the remaining 53 were excluded due to following reasons: (1) patients with non-cirrhotic cause of portal hypertension ( $n = 16$ ); (2) patients with acute-on-chronic liver failure ( $n = 27$ ); (3) patients with invalid TE reading or whose HVPG was not possible due to technical reasons ( $n = 5$ ); (4) patients who received beta blocker therapy in the past two weeks ( $n = 3$ ); and (5) concomitant extrahepatic malignancy ( $n = 2$ ).

Table 1 presents the demographic profile of patients studied. The median age was 52 years (range 16-90 years), and the majority (81%) were males. The most common etiology of cirrhosis was cryptogenic (45%) followed by alcohol (34%). Ascites was present in 51% of patients. Sixty-four percent of patients were non-bleeders, whereas the remaining had bled from varices in the past. The median CTP score was 7 (range 5-12), and the median MELD score was 12 (range 6-37). The median liver stiffness was 36 kPa with a range of 3 to 75 kPa.

### HVPG

The median HVPG of all patients was 16.0 (range 1.5 to 30.5) mmHg. Table 2 shows patients categorized according to HVPG stages. Four percent patients had normal HVPG ( $\leq 5$  mmHg), while the remaining 96% had portal hypertension (HVPG > 5 mmHg). Eighty-five percent of patients had clinically significant portal hypertension (HVPG  $\geq 10$  mmHg). Seventy-six percent patients had HVPG greater than 12 mmHg (severe portal hypertension, SPH), which is the threshold for variceal bleeding. In addition, 18% patients had very high HVPG (> 20 mmHg, very severe portal hypertension, VSPH).

### Correlation of TE with HVPG

A significant positive correlation was noted between liver stiffness and HVPG levels (Spearman's rho 0.361,



**Table 1** Demographic profile of the study population

Parameter	Value (n = 326)
Gender	
Males	263 (81)
Females	63 (19)
Age, yr	52 (16-90)
BMI, kg/m <sup>2</sup>	23 (17-41)
Etiology	
NASH/cryptogenic	148 (45)
Alcohol	110 (34)
Viral (HBV/HCV)	48 (15)
Others (including mixed etiology)	20 (6)
Ascites	
None	161 (49)
Mild	135 (42)
Moderate to tense	30 (9)
Bleeding status	
Bleeder	118 (36)
Non-bleeder	208 (64)
Hemoglobin, g/dL	10.3 (4.5-17.0)
Platelets, × 10 <sup>3</sup> /cumm	90 (13-422)
Bilirubin, mg/dL	1.6 (0.2-11.2)
AST, IU/dL	53 (16-209)
INR	1.3 (0.9-3.2)
Serum albumin, g/dL	3.0 (1.2-4.6)
CTP score	7 (5-12)
MELD score	12 (6-37)
Varices present	293 (90)
Esophageal varices	280 (86)
Small varices	170/280 (61)
Large varices	110/280 (39)
Gastric varices	79 (24)
Small varices	52/79 (66)
Large varices	27/79 (34)
HVPG, mmHg	16.0 (1.5-30.5)
Transient elastography, kPa	36 (3-75)

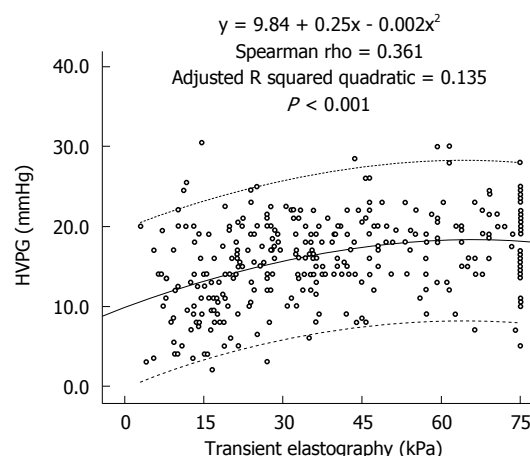
All values are expressed as the median (range) or *n* (%). NASH: Non-alcoholic steatohepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AST: Aspartate aminotransferase; INR: International normalized ratio; CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease; HVPG: Hepatic venous pressure gradient.

$P < 0.001$ ). Figure 1 presents the scatterplot of TE and HVPG values. The HVPG value could be predicted by the following formula:

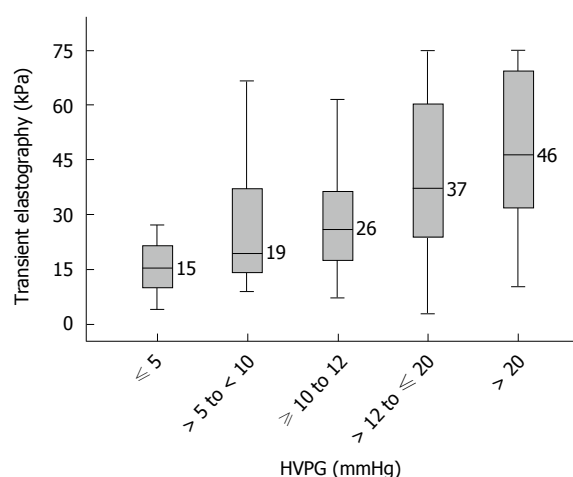
$HVPG = 9.84 + 0.25 \times TE - 0.002 \times TE^2$ . The adjusted R squared value was 0.135.

### TE values in patients with various stages of portal hypertension

Figure 2 presents the median TE values in various stages of portal hypertension. Cirrhotic patients with no portal hypertension (HVPG  $\leq 5$  mmHg) had a median TE value of 15.4 (range 4.1 to 75.0) kPa. Patients with HVPG  $> 5$  to  $< 10$  mmHg (sub-clinical portal hypertension, SCPH) had a median TE value of 19.4 (range 8.8 to 74.0) kPa. Patients with HVPG  $\geq 10$  to 12 mmHg had a median TE value of 25.8 (range 7.3 to 75.0) kPa. Patients with HVPG  $> 12$  to  $\leq 20$  mmHg had a median TE value of 37.1 (range 2.95 to 75.0) kPa. Patients with HVPG  $> 20$  mmHg had median TE value of 46.4 (range 10.1 to 75.0) kPa. Although these



**Figure 1** Scatterplot of transient elastography and hepatic venous pressure gradient values. HVPG: Hepatic venous pressure gradient.



**Figure 2** Median transient elastography values in patients with various stages of portal hypertension. HVPG: Hepatic venous pressure gradient.

TE values were significantly different across the groups ( $P < 0.05$ ), there was considerable overlap in the interquartile ranges between various groups (Figure 2).

### Performance of TE in predicting various stages of portal hypertension

Figure 3 presents the ROC curves of TE for predicting the various stages of portal hypertension. Ninety-six percent of patients had portal hypertension (HVPG  $> 5$  mmHg). TE proved to be an excellent non-invasive modality in predicting portal hypertension with an area under the ROC curve of 0.786 (95%CI: 0.645-0.926) and a  $P$ -value  $< 0.01$  (Figure 3A). Eighty-five percent patients had clinically significant portal hypertension (HVPG  $\geq 10$  mmHg). The area under the ROC curve for TE in predicting CSPH was 0.740 (95%CI: 0.662-0.818) ( $P < 0.01$ ) (Figure 3B). Seventy-six percent patients had HVPG  $> 12$  mmHg, which is the threshold for variceal bleeding. The area under the ROC curve for TE in predicting HVPG  $> 12$  mmHg was 0.721 (95%CI: 0.654-0.788) ( $P < 0.01$ ) (Figure

**Table 2** Groups according to hepatic venous pressure gradient

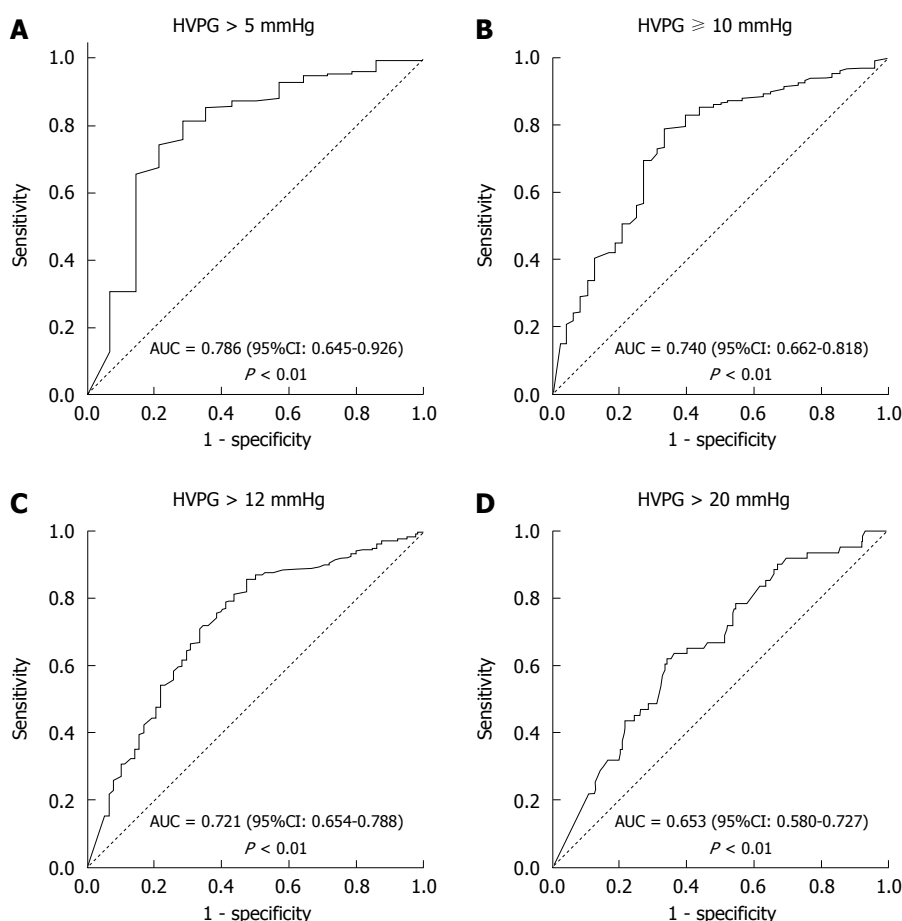
HVPG (mmHg)	n (%)	Portal hypertension (> 5 mmHg)	CSPH ( $\geq 10$ mmHg)	SPH (> 12 mmHg)	VSPH (> 20 mmHg)
$\leq 5$	14 (4)	No (14, 4%)	No (48, 15%)	No (78, 24%)	No (266, 82%)
> 5 to < 10	34 (10)	Yes (312, 96%)	Yes (278, 85%)	Yes (248, 76%)	Yes (60, 18%)
$\geq 10$ to 12	30 (9)				
> 12 to $\leq 20$	188 (58)				
> 20	60 (18)				

CSPH: Clinically significant portal hypertension; SPH: Severe portal hypertension; VSPH: Very severe portal hypertension.

**Table 3** Predictive values of transient elastography for the prediction of clinically significant portal hypertension (HVPG  $\geq 10$  mmHg)

TE cut-off value (mmHg)	CSPH (n)	No CSPH (n)	Total (n)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Accuracy (95%CI)	LR+ (95%CI)	LR- (95%CI)
$\geq 21.6$	219	16	235	79%	67%	93%	35%	77%	2.4 (1.6-3.5)	0.3 (0.2-0.4)
< 21.6	59	32	91	(74%-83%)	(52%-80%)	(89%-96%)	(25%-46%)	(72%-82%)		
Total	278	48	326							

TE: Transient elastography; CSPH: Clinically significant portal hypertension; PPV: Positive predictive value; NPV: Negative predictive value; LR+: Likelihood ratio positive; LR-: Likelihood ratio negative.

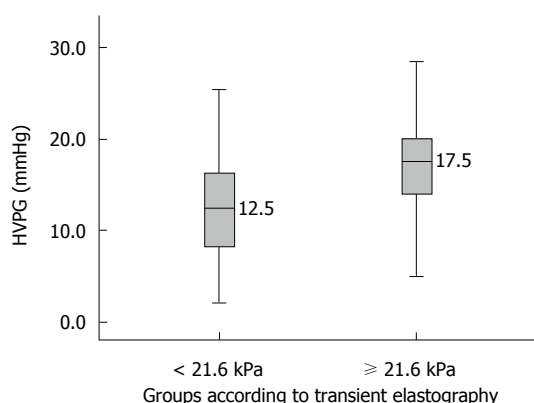


**Figure 3** Receiver operating characteristic curves of transient elastography for predicting various stages of portal hypertension. HVPG: Hepatic venous pressure gradient.

**Table 4** Various studies of the diagnostic performance of transient elastography for clinically significant portal hypertension

Ref.	Place	Year	Number of patients	Correlation coefficient (r) or r <sup>2</sup> of TE with HVPG	AUROC (95%CI)	Best cut-off of TE for CSPH
Carrión <i>et al</i> <sup>[33]</sup>	Spain	2006	124	0.840	0.94	-
Vizzutti <i>et al</i> <sup>[14]</sup>	Italy	2007	61	0.810	0.99 (0.92-0.99)	13.6 (PPV 97%)
Lemoine <i>et al</i> <sup>[34]</sup>	France	2008	92	0.530	0.84 (0.80-0.88)	34.9 for alcohol (PPV 98%) 20.5 for HCV (PPV 88%)
Bureau <i>et al</i> <sup>[35]</sup>	France	2008	150	0.858	0.945 (0.904-0.987)	21 (PPV 92%)
Sánchez-Conde <i>et al</i> <sup>[36]</sup>	Spain	2011	38	0.460	0.80 (0.64-0.97)	14 (PPV 84%)
Reiberger <i>et al</i> <sup>[13]</sup>	Austria	2012	502	0.794	0.817 (0.752-0.891)	18 (PPV 86%)
Llop <i>et al</i> <sup>[20]</sup>	Spain	2012	97	0.552	0.840 (0.748-0.933)	21 (PPV 81%)
Berzigotti <i>et al</i> <sup>[37]</sup>	Spain	2013	117	-	0.883 (0.824-0.943)	21.1 (sensitivity 65%)
Hong <i>et al</i> <sup>[38]</sup>	South Korea	2013	59	0.496	0.851	21.95 (PPV 87%)
Salzl <i>et al</i> <sup>[28]</sup>	Austria	2014	88	0.765	0.87	16.8 (sensitivity 90%)
Augustin <i>et al</i> <sup>[39]</sup>	Spain	2014	40	-	-	25
Zyklus <i>et al</i> <sup>[31]</sup>	Lithuania	2015	107	0.750	0.949	17.4 (accuracy 89%)
Procopet <i>et al</i> <sup>[32]</sup>	Europe (Multicentric)	2015	202	-	0.94 (0.89-0.99)	21.1 (accuracy 90%)
Kitson <i>et al</i> <sup>[40]</sup>	Australia	2015	95	0.380	0.90 (0.83-0.97)	29.0 (PPV 100%)
Elkrief <i>et al</i> <sup>[25]</sup>	France	2015	79	-	0.78 (0.58-0.98)	65.3 (PPV 100%)
Schwabl <i>et al</i> <sup>[41]</sup>	Austria	2015	226	0.836 and 0.846	0.957 & 0.962	16.1 (accuracy 89% & 90%)
Hametner <i>et al</i> <sup>[29]</sup>	Austria	2016	236	-	0.92 (0.86-0.96)	24.8 (PPV 98%)
This study	India	2016	326	0.361	0.740 (0.662-0.818)	21.6 (PPV 93%)
Total			2515			Weighted mean: 21.8

TE: Transient elastography; HVPG: Hepatic venous pressure gradient; AUROC: Area under receiver operating characteristic; CSPH: Clinically significant portal hypertension; PPV: Positive predictive value.



**Figure 4** Median hepatic venous pressure gradient values in patients with transient elastography values less than and greater than 21.6 kPa. HVPG: Hepatic venous pressure gradient.

3C). Eighteen percent of patients had very high portal hypertension (HVPG > 20 mmHg). The area under the ROC curve for TE in predicting HVPG > 20 mmHg was 0.653 (95%CI: 0.580-0.727) ( $P < 0.01$ ) (Figure 3D).

#### Cut-off TE value for predicting CSPH

When HVPG is  $\geq 10$  mmHg, it is known as clinically significant portal hypertension (CSPH). Most complications of portal hypertension, such as varices, ascites, encephalopathy, and bleeding, occur at or above this value. The area under the ROC curve for TE to diagnose CSPH was 0.740 (95%CI: 0.662-0.818). A cut-off value of TE of 21.6 kPa was obtained by using Youden index to best predict CSPH. The sensitivity,

specificity, positive predictive value, negative predictive value, and accuracy of a TE value  $\geq 21.6$  to diagnose CSPH were 79%, 67%, 93%, 35%, and 77%, respectively (Table 3). The median HVPG in patients with a TE values  $\geq 21.6$  was 17.5 mmHg (Figure 4).

## DISCUSSION

In the present study, we showed that in patients with cirrhosis, TE has a fair positive correlation with HVPG, and TE can thus be used as a non-invasive modality to assess the degree of portal hypertension. The TE values increase progressively as portal pressure increases from normal through SCPH, CSPH, SPH and VSPH. We also found that a cut-off TE value of 21.6 kPa has 93% positive predictive value in diagnosing CSPH.

Numerous previous studies have correlated TE with HVPG (Table 4). In these studies, the AUROC curve for prediction of CSPH varied between 0.78 and 0.99. The optimal cut-offs ranged between 13.6 and 65.3 kPa with PPV typically greater than 80%. However, most of these studies used a small number of patients, and almost all of these studies were performed in Europe with none from South Asia where the etiological profile of cirrhosis is different from the West. The largest of these studies was by Reiberger *et al*<sup>[13]</sup> from Austria, who retrospectively correlated TE and HVPG in 502 patients. They identified a very high correlation of TE with HVPG ( $r = 0.799$ ;  $P < 0.0001$ ). Compared with their study, our correlation coefficient was lower

( $r = 0.361$ ). Two possible reasons could explain this difference in the strength of correlation between their study and ours. The first reason is the difference in the etiological profiles of patients in the two studies. Their study had more patients with a viral etiology (56%) that typically exhibits a better correlation, whereas our study had more patients with a NASH/cryptogenic and alcohol etiology, which typically has poorer correlation. The second possible reason for the difference in the strength of correlation between their study and ours was that their patients had less severe liver disease compared with our patients. The mean HVPG of their cohort was  $12.6 (\pm 7.6)$  mmHg, whereas the median HVPG in our patients was 16.0 mmHg. Their median liver stiffness value was 26.4 kPa, whereas that in our study was 36 kPa. TE and HVPG correlate better when liver disease is less advanced. As observed from the scatterplot of our study (Figure 1) and also from the scatterplot of Reiberger's study<sup>[13]</sup>, the slope of the trend line on the left side of the graph when the disease is less severe is steeper, indicating a better correlation compared with the right side when the trend line is flatter, indicating a poorer correlation at higher TE values. Vizzutti *et al.*<sup>[14]</sup> also found that the correlation was excellent for HVPG values less than 10 or 12 mmHg ( $r = 0.81$  and  $r = 0.91$ , respectively); however, the linear regression analysis was not optimal for HVPG values  $\geq 10$  mmHg ( $r = 0.59$ ) or  $\geq 12$  mmHg ( $r = 0.37$ ). In advanced portal hypertension, it is not only the liver fibrosis but also the extrahepatic factors, such as the increase in blood flow due to hyperdynamic circulation, that contribute to portal hypertension<sup>[15]</sup>.

There was an urgent need for a South Asian study on the correlation of TE - HVPG because the results of Western studies may not apply to the South Asian population where the etiological and the clinical profile of chronic liver disease differs from the West. Alcohol consumption and the prevalence of diabetes [a major risk factor for nonalcoholic fatty liver disease (NAFLD)] has been steadily increasing in South Asia<sup>[16]</sup>. The International Diabetes Federation has revised its estimates of the number of people with the diabetes in South-East Asia from 50 million in 2009 to 78.3 million in 2015, with a projection of 140 million by 2040<sup>[17,18]</sup>. Although cirrhosis mortality has been steadily decreasing globally and especially in the West, it has been steadily increasing in India since 1980. With 188,575 liver cirrhosis deaths in 2010, India ranks number one in the world in liver cirrhosis deaths, accounting for almost one-fifth (18%) of global liver cirrhosis deaths<sup>[19]</sup>. With such a huge population of liver cirrhosis patients in India whose etiology differs from the West, an easy and non-invasive modality for portal hypertension estimation is urgently needed for treating these patients. Our study attempts to address this need for Indian and South Asian populations.

Most of the complications of cirrhosis are typically related more to CSPH compared with any other factor.

Although HVPG measurement is the gold standard for diagnosing CSPH, it is not in common use because it is invasive, not widely available, and expensive, thus hindering its broad use in diagnostic and therapeutic algorithms in patients with cirrhosis with CSPH. Our study found that a TE value of 21.6 kPa is a good cut-off for predicting CSPH with a PPV of 93%. Seventeen additional studies have calculated the cut-off TE values for diagnosing CSPH (Table 4). These studies were performed in patients with different disease severities and different proportions of viral cirrhosis, and their cut-off values ranged from 13.6 kPa to 65.3 kPa. The weighted mean of cut-off from all these studies was 21.9 kPa, which is very similar to what we obtained in our study. Adding our study to the pool of studies, the weighted mean for the best cut-off TE value for diagnosing CSPH is 21.8 kPa.

Notably, Llop *et al.*<sup>[20]</sup> from Spain provided two cut-offs of TE instead of one to predict CSPH. They showed that a cut-off of 13.6 kPa was sufficiently sensitive to exclude CSPH, and a cut-off of 21 kPa was sufficiently specific to include CSPH. They suggested that values in between these limits (which were found in 35% of their patients) were not useful. Some authors believe that the use of a single TE cut-off, although simple, limits the value of TE to predict CSPH. The use of at least two cut-offs reproduces the clinical thinking in which a diagnostic test commonly provides three outputs: the condition is included, excluded or "further tests are needed"<sup>[15]</sup>. Thus, TE, using these three outputs, might be useful to select these suspicious patients with cirrhosis for HVPG measurement. However, we believe that multiple cut-offs may lead to confusion, and the single cut-off with a high PPV is best for guiding primary physicians in the community to make treatment decisions.

A recent meta-analysis<sup>[11]</sup>, which included 5 studies of the diagnostic accuracy of TE for significant portal hypertension, also indicated that TE had a high accuracy for the detection of significant portal hypertension. The hierarchical summary receiver-operating characteristic (HSROC) for the diagnosis of significant portal hypertension by TE was 0.93 (95%CI: 0.90-0.95). The Fagan plot analysis showed that TE could be used to diagnose significant portal hypertension (when pre-test probability = 50%), with 81% probability of correctly diagnosing significant portal hypertension following the "positive" measurement. Furthermore, a "negative" measurement was also informative, as significant portal hypertension was present in only 11% of patients. However, when the pre-test probability of significant portal hypertension was as low as 25%, the probability of correctly identifying significant portal hypertension decreased markedly. This finding suggests that an accurate selection of patients is necessary to exploit the performance of TE at its best<sup>[11]</sup>.

Other newer and promising noninvasive modalities are being developed for diagnosing portal



hypertension, such as two-dimensional shear wave elastography (2D-SWE)<sup>[21-25]</sup>; acoustic radiation force impulse (ARFI)<sup>[26-28]</sup>; VITRO Score (Von Willebrand Factor Antigen/Thrombocyte Ratio)<sup>[29]</sup>; aspartate aminotransferase/platelet ratio index (APRI)<sup>[30]</sup>; spleen elastography<sup>[23-27,31]</sup>, and serum tests, such as Fibrosis-4, and Lok score<sup>[32]</sup>. These tests have their own advantages and disadvantages. However, to date, very few studies have been performed on them for correlation with HVPG, so their routine use cannot be recommended outside of clinical trials.

There could be a few limitations in our study. First, it is a retrospective study, so the study may suffer from selection bias. We included only those patients who underwent HVPG and TE during the study period; hence, our patients may not represent the entire population of patients with cirrhosis, as most included patients have moderate to severe portal hypertension. A prospective study design, which includes all consecutive patients of cirrhosis, regardless of degree of portal hypertension, would have been a better study design and more representative of the cirrhotic population of the community. A second limitation could be the lack of follow-up. Follow-up data on complications of portal hypertension would have further validated our results of TE cut-off for CSPH.

In conclusions, our study demonstrated that in patients with cirrhosis, TE has a fair positive correlation with HVPG; thus, TE can be used as a non-invasive modality to assess the degree of portal hypertension. The TE values increase progressively as portal pressure increases from normal through SCPH, CSPH, SPH and VSPH. A cut-off TE value of 21.6 kPa has 93% positive predictive value in diagnosing CSPH. This cut-off will be very useful in diagnosing CSPH and making appropriate treatment decisions in places where HVPG is not available or when patients are unwilling to undergo HVPG due to its invasiveness. As a reliable and non-invasive procedure, TE is a promising and worthy tool to translate into routine clinical practice for detecting CSPH. TE could be integrated in the detection of CSPH in untreated patients for portal hypertension. Further large prospective studies are needed to prospectively validate the findings of our study and also to determine whether TE can be used in monitoring the hemodynamic response and the effect of drugs reducing portal pressure.

## COMMENTS

### Background

Clinically significant portal hypertension (CSPH), which is defined as hepatic venous pressure gradient (HVPG)  $\geq 10$  mmHg, causes major complications of cirrhosis. HVPG is invasive and not always available, so a noninvasive tool to diagnose CSPH is needed. Many studies have correlated transient elastography (TE) with HVPG, but none of them are from South Asia where the etiological profile of cirrhosis differs from other regions of the world.

### Research frontiers

TE is a novel, noninvasive, ultrasound-based technology that allows

measurements of liver stiffness. Established evidence indicates that TE has good sensitivity and specificity for diagnosing liver fibrosis and cirrhosis and has been popular over the past few years. The present study tested the diagnostic accuracy of TE for detecting CSPH in Indian patients.

### Innovations and breakthroughs

The present study showed that in patients with cirrhosis, TE has a fair positive correlation with HVPG; thus, TE can be used as a non-invasive modality to assess the degree of portal hypertension. The TE values increase progressively as portal pressure increases from normal through subclinical portal hypertension (SCPH), CSPH, severe portal hypertension (SPH) and very severe portal hypertension (VSPH). In addition, a cut-off TE value of 21.6 kPa has 93% positive predictive value in diagnosing CSPH.

### Applications

This study suggests that TE, which is a reliable and non-invasive procedure, is a promising and worthy tool to translate into routine clinical practice for detecting CSPH. A TE cut-off value of 21.6 kPa is very useful in diagnosing CSPH and making appropriate treatment decisions in places where HVPG is not available or when patients are unwilling to undergo HVPG due to its invasiveness. Thus, TE could be integrated in the detection of CSPH in untreated patients of portal hypertension.

### Terminology

HVPG represents the approximate gradient between portal vein and intra-abdominal vena cava pressure. Measurement of the hepatic venous pressure gradient HVPG is currently the best available method to evaluate the presence and severity of portal hypertension. TE, known by the brandname FibroScan, is a non-invasive test to quantify liver stiffness. Liver stiffness increases with increasing liver fibrosis.

### Peer-review

The authors of this paper have demonstrated that in patients with cirrhosis, TE has a fair positive correlation with HVPG; thus, TE can be used as a non-invasive modality to assess the degree of portal hypertension. Further large prospective studies are needed to prospectively validate the findings of this study and also to determine whether TE can be used in monitoring the hemodynamic response and the effect of drugs reducing portal pressure.

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## Observational Study

# Implications of small-bowel transit time in the detection rate of capsule endoscopy: A multivariable multicenter study of patients with obscure gastrointestinal bleeding

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**Author contributions:** All authors provided substantial contributions to the conception, design, and/or acquisition of data, and/or the analysis and interpretation of data; Girelli CM performed the statistical analysis and wrote the manuscript; Soncini M implemented and updated the database; Rondonotti E provided intellectual content in the interpretation of study results; all authors critically revised and approved the final draft of the manuscript.

**Institutional review board statement:** Permission to review patient records was granted by the Local Ethics Committee. Further specific ethical review and approval were not required because the study was considered an evaluation of previously collected SBCE records, using anonymous data previously obtained as part of routine clinical care.

**Informed consent statement:** All enrolled patients gave their written informed consent to participate.

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**Data sharing statement:** No additional data are available.

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## Abstract

### AIM

To define the role of small-bowel transit time in the detection rate of significant small-bowel lesions.

### METHODS

Small-bowel capsule endoscopy records, prospectively collected from 30 participating centers in the Lombardy Registry from October 2011 to December 2013, were included in the study if the clinical indication was obscure gastrointestinal bleeding and the capsule reached the cecum. Based on capsule findings, we created two groups: P2 (significant findings) and P0-1 (normal/negligible findings). Groups were compared for age, gender, small-bowel transit time, type of instrument, modality of capsule performance (outpatients vs inpatients), bowel cleanliness, and center volume.



## RESULTS

We retrieved and scrutinized 1,433 out of 2,295 capsule endoscopy records (62.4%) fulfilling the inclusion criteria. Patients were  $67 \pm 15$  years old, and 815 (57%) were males. In comparison with patients in the P0-1 group, those in the P2 group ( $n = 776$ , 54%) were older ( $P < 0.0001$ ), had a longer small-bowel transit time ( $P = 0.0015$ ), and were more frequently examined in low-volume centers ( $P < 0.001$ ). Age and small-bowel transit time were correlated ( $P < 0.001$ ), with age as the sole independent predictor on multivariable analysis. Findings of the P2 group were artero-venous malformations (54.5%), inflammatory (23.6%) and protruding (10.4%) lesions, and luminal blood (11.5%).

## CONCLUSION

In this selected, prospectively collected cohort of small-bowel capsule endoscopy performed for obscure gastrointestinal bleeding, a longer small-bowel transit time was associated with a higher detection rate of significant lesions, along with age and a low center volume, with age serving as an independent predictor.

**Key words:** Capsule endoscopy; Small-bowel transit time; Detection rate; Diagnostic yield; Small bowel; Obscure gastrointestinal bleeding; Prokinetics; Suspect small-bowel bleeding

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**Core tip:** There is growing evidence that a slower small-bowel transit time (SBTT) increases the diagnostic yield of small-bowel capsule endoscopy (SBCE). The present study-an analysis of a large database of consecutive, prospectively collected, complete SBCE performed for obscure gastrointestinal bleeding-confirms this finding. However, we found a correlation between SBTT and age, with age serving as an independent predictor on multivariable analysis. Prokinetics, used to increase the completion rate of SBCE, may hamper the detection rate of significant lesions and should only be used in selected patients.

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## INTRODUCTION

Obscure gastrointestinal bleeding (OGB) is defined by persistent or recurrent bleeding from the gastrointestinal tract after an unremarkable esophagogastroduodenoscopy and colonoscopy. In a recent clinical

guideline, the European Society of Gastrointestinal Endoscopy (ESGE) strongly recommended small-bowel capsule endoscopy (SBCE) as a first-line investigation in patients with OGB<sup>[1]</sup>. In this setting, the diagnostic yield (DY) of SBCE is highly variable, ranging from 40%-80%, depending by the clinical significance of the endoscopic finding, the degree of bowel cleanliness, and the completion rate (CR) of the small-bowel examination. Hospitalization, previous surgery or radiation, diabetes mellitus and very old age have been identified as risk factors for incomplete SBCE evaluation<sup>[2-5]</sup> that, at least theoretically, may impair the DY. To improve the CR, some clinicians administer prokinetics before capsule ingestion. However, two recent retrospective studies suggested that a prolonged small-bowel transit time (SBTT) is associated with an improved DY<sup>[6,7]</sup>. Furthermore, newer-generation devices have longer battery life, possibly minimizing the role of CR in the detection rate (DR) of significant findings.

To evaluate the role of SBTT along with other variables on the DR of significant lesions of SBCE in patients with OGB, we undertook an analysis of the Lombardy Registry, a database collecting the records of nearly all SBCEs performed for clinical purpose in a well-defined Northern Italian region.

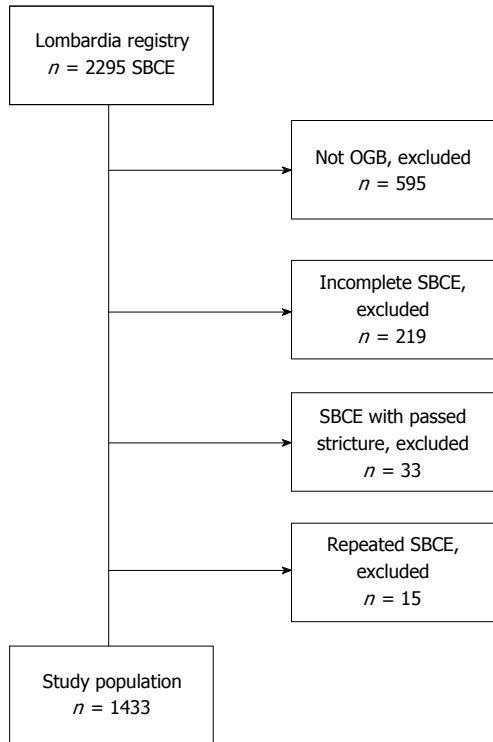
## MATERIALS AND METHODS

### Lombardy registry

Lombardy is a highly populated region of Northern Italy, harboring approximately 10 million inhabitants. In October 2011, we implemented an SBCE database, asking every center in Lombardy performing SBCE to complete an electronic case report form (CRF) of all consecutive patients submitted to SBCE for any clinical indication. At the end of reading and video interpretation, the referring physician of the adhering center uploaded the completed CRF onto a shared Dropbox folder (Dropbox Inc. San Francisco, CA, United States). Thirty of 32 centers (see Supplementary materials) agreed to participate to data collection, which was terminated in December 2013. Centers were mostly primary or secondary care hospitals (21/30, 70%). The CRF collected demographic and clinical data, such as indication to SBCE, capsule operative system, inpatient/outpatient status, risk factors for capsule retention, previous investigations, agile patency-capsule administration, capsule retention, any complication, gastric transit time, SBTT, CR, bowel cleanliness, findings and further workup/treatment of patients with positive findings.

### Inclusion and exclusion criteria

For the aim of the present study, we included all consecutive patients submitted to SBCE for OGB of each participating center in which the capsule reached the cecum (complete small-bowel examination) without the prior administration of prokinetics. Patients with a



**Figure 1 Study design and inclusion criteria.** SBCE: Small-bowel capsule endoscopy; OGB: Obscure gastrointestinal bleeding.

passed small-bowel stricture, or in whom the procedure was repeated, were excluded. We adopted these selection criteria to evaluate the genuine role of SBTT on the SBCE detection rate and to avoid duplicate cases (Figure 1).

### Findings

Findings were classified using the Capsule Endoscopy Standard Terminology<sup>[8]</sup>. They were categorized in two groups, namely P0-1 and P2. P0 and P1 refer to patients with normal or negligible findings (*i.e.*, lesions carrying a very low probability of bleeding), respectively, whereas P2 refers to patients with clinically significant lesions and/or luminal blood (Table 1).

### Bowel cleanliness

Bowel cleanliness was deemed adequate by the single operator immediately after reading of the record. We adopted this subjective criterion, since - at the time of study implementation - the proposed scales of small-bowel cleansing did not undergo external validation and had poor inter- and intra-observer agreement<sup>[9]</sup>.

### Center volume

The center volume was established by the number of SBCEs performed annually and was stratified into three classes: low, middle and high ( $\leq 20$ ; 20-50,  $\geq 50$  procedures/year, respectively). These cutoff values were selected arbitrarily, in order to obtain the most balanced distribution of cases among classes.

**Table 1 Classification of the findings of small-bowel capsule endoscopy**

P0-1 Group	P2 Group
Normal	AVM
Lymphangectasia	Hemangioma
Small isolated phlebectasia	Mass
Lymphatic cyst	Erosion(s)
Isolated tiny red spots	Ulcer(s)
	Blood

AVM: Artero-venous malformations.

### SBCE device and procedural protocol

Most SBCE procedures (86%) were performed by the Pillcam system (Covidien plc, Dublin, Ireland). Others (Endocapsule, Olympus Optical Co, Tokyo, Japan; Miro-Cam, IntroMedic, Seoul, Korea; OMOM capsule, Jinshan Science and Technology Group, Chongqing, China) were used on a minority of patients and were considered together in data analysis. All patients ingested the capsule in the morning in a fasting state, after a standard preparation with two liters of polyethylene glycol consumed 12-18 h before the capsule ingestion. A light snack was allowed four hours after capsule ingestion.

### SBTT

SBTT was calculated in minutes, as the time elapsed from the first frame of the duodenal bulb to the first frame of the cecum. Because of the aforementioned inclusion criteria (*i.e.*, complete SBCE), gastric transit time (GTT) was not included in our data analysis. An inverse relationship between GTT and SBTT is indeed foreseeable for selection bias.

### Ethical considerations

All patients provided their written informed consent before capsule ingestion. This study was conducted in accordance with established research ethics guidelines. Permission to review patient records was granted by the Local Ethics Committee. Further specific ethical review and approval were not required because the study was considered an evaluation of previously collected SBCE records, using anonymous data previously obtained as part of routine clinical care.

### Statistical analysis

Continuous variables are presented as the mean  $\pm$  SD and dichotomous variables are presented as percentages. If variable distributions were not normal (Kurtosis outside the interval between -1 and 1), variables were ranked in their interquartile ranges (IQR) and analyzed by nonparametric Mann-Whitney *U* and Spearman's  $\rho$  tests.  $\chi^2$  ( $2 \times 2$  and  $2 \times 3$  contingency tables), Student's *t*-test, Pearson's R test, and multivariate stepwise regression analysis were used when appropriate. A *P* value  $< 0.05$  was considered statistically significant. The SPSS

**Table 2** Comparison between groups with significant (P2) and normal/negligible (P0-1) findings of small bowel capsule endoscopy in patients with obscure gastrointestinal bleeding

	P2 group	P0-1 group	P value
n (%)	776 (54)	657 (46)	
Age (yr), mean $\pm$ SD	69 $\pm$ 13	64 $\pm$ 16	< 0.0001
Male gender (%)	59.0	54.5	0.09
SBTT (min), mean $\pm$ SD	283 $\pm$ 105	269 $\pm$ 98	0.0015
Pillcam SB (%)	86.3	85.3	0.61
Outpatients (%)	56.6	61	0.1
Center's volume (%), low/mid/high	7/33/60	3/27/70	< 0.001
Adequate cleanliness (%)	97	95	0.06

SBTT: Small-bowel transit time.

**Table 3** Product-moment correlation matrix in the final model of multiple stepwise regression

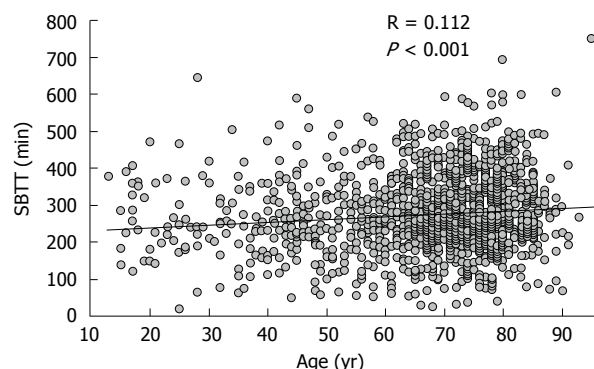
Variables	Center's volume	Age	SBTT	P0-1/P2 lesion
Center's volume	1.000	0.006	0.009	0.112
Age	0.006	1.000	0.112	0.160
SBTT	0.009	0.112	1.000	0.064
P0-1/P2 lesion	0.112	0.160	0.064	1.000

P0-1/P2 lesion is the dependent variable. SBTT: Small-bowel transit time.

package was used for statistical computations.

## RESULTS

We retrieved and scrutinized 1433 out of 2295 SBCE records (62.4%) fulfilling the inclusion criteria (Figure 1). Patients were  $67 \pm 15$  years of age (range: 13-95) and 815 (57%) were males. Seventy-six, 431 and 926 patients were examined in low-, mid- and high-volume centers, respectively. Small-bowel cleanliness was deemed adequate in 1376 patients (96%), and P2 lesions were encountered in 760 (54%) patients. Findings of the P2 group were artero-venous malformations (AVM) (54.5%), inflammatory lesions (23.6%), mass/tumor (10.4%), and luminal blood (11.5%). The main features of the two groups are summarized in Table 2. In comparison with patients of P0-1 Group ( $n = 657$ ; 46%), those of P2 Group ( $n = 776$ , 54%) were older ( $69 \pm 13$  years vs  $64 \pm 16$  years of age,  $P < 0.0001$ ), with a longer SBTT ( $283 \pm 105$  min vs  $269 \pm 98$  min,  $P = 0.0015$ ). Furthermore, more P2 patients were examined in low-volume centers (low-volume 7% vs 3%; mid-volume 33% vs 27%; high-volume 60% vs 70%,  $P < 0.001$ ), and adequate bowel cleanliness was more frequent in patients of the P2 group, with a borderline statistical significance (97% vs 95%,  $P = 0.06$ ). Among variables, we found a significant correlation between age and SBTT (Pearson's  $R = 0.112$ ,  $P < 0.001$ ; Figure 2). In the final model of multivariable analysis-including age, SBTT, and center's volume-age was the independent predictor for the detection of P2 lesions ( $\beta = 0.16$ ;  $P < 0.01$ ; Table 3).

**Figure 2** Pearson's correlation between age (years) and small-bowel transit time (min). SBTT: Small-bowel transit time.

## DISCUSSION

Several factors are known to influence the DY of SBCE in patients with OGB and other indications, but the role of SBTT has been only recently highlighted. In a retrospective series of 212 patients with OGB and complete small-bowel visualization, Buscaglia *et al.*<sup>[6]</sup> showed a two-fold increase in the number of DY in patients with an SBTT longer than six hours. Westerhof and coworkers in a retrospective series of 690 consecutive patients found a correlation between SBTT and DY for all indications, but suspected Crohn's disease<sup>[7]</sup>. One explanation of this finding may be that a slower passage of the capsule in the small-bowel may allow a better DR of significant lesions. Accordingly, the colonoscopic concept that a longer time for withdrawal of the scope corresponds to greater adenoma detection rate<sup>[10]</sup> could be directly translated to the domain of capsule endoscopy. This finding is not futile, having weight in the controversy regarding the administration of prokinetics prior to SBCE. Although prokinetics are advocated to overcome a slow gastric emptying, they, in turn can jeopardize the visualization of the entire small-bowel<sup>[11]</sup>, their indiscriminate use may actually lower the DY. Metoclopramide improves the CR by reducing the gastric transit time, whereas its effect on SBTT is less clear<sup>[12]</sup>. Nevertheless, a reduction of SBTT by mosapride (a drug pharmacologically related to metoclopramide for antagonism of 5-HT<sub>3</sub> receptors) has been reported<sup>[13]</sup>. Interestingly, Koulaouzidis and coworkers found that orally administered domperidone prior to SBCE, performed for various indications with the Pillcam system, increased the CR at expenses of a reduced DY<sup>[14]</sup>. Erythromycin, which increases the CR of SBCE without reducing the SBTT in healthy volunteers<sup>[15,16]</sup>, may be the prokinetic of choice, but a controlled retrospective study was disappointing<sup>[17]</sup>. To achieve a complete examination, at least for the Pillcam SB system, the real-time display of the recorder may be helpful in selecting patients who benefit from intravenous prokinetic, by administration of i.v. prokinetic if the gastric folds are still visible after 45 min from capsule ingestion.

Our study shows that a longer SBTT increases the DR of P2 lesions in patients submitted to SBCE for OGB with complete small-bowel visualization. However, contrary to a study by Buscaglia, but in accordance with others<sup>[18]</sup>, we found a correlation between age and SBTT, with age, in our investigation, being the stronger predictor of P2 findings in a multivariable analysis. One possible explanation of this discrepancy may be due to a skewness toward an aged population of our cohort and - as expected - a prevalence of AVMs in the P2 group.

Not surprisingly, we found that adequate small-bowel cleanliness was associated with an improved DR, albeit with borderline statistical significance. Conversely, the better DR of SBCE performed in low-volume centers is far from obvious. One may speculate that the limited resources constrain low-volume centers to more rigorous patient selection, or, alternatively, the reduced workload of low-volume centers allows the referring physician a longer time to review videos.

Two major limitations of this study restrict the generalizability of our findings and comparability with those of others: our inability to stratify patients into obscure-overt and obscure-occult bleeding, and our lack of data on blood loss severity. In fact, there is compelling evidence that the DY of SBCE in patients with the overt type of OGB critically depend on the time elapsed from bleeding to SBCE evaluation and by the severity of anemia<sup>[19,20]</sup>. Of course, it would be of interest examine different weights of the included variables between the two types of gastrointestinal bleeding. Furthermore, the study lacks a centralized blinded review of SBCE studies, which is a weakness for a diagnostic tool such as SBCE, which is affected by a sub-optimal inter-observer agreement<sup>[21]</sup>. Finally, we were unable to adjust our data for drugs consumption, because many drugs, especially opioids, can slow SBTT. However, this study has several strengths: its large sample size, prospective design, the participation of secondary care referral centers, and the multicenter evaluation of consecutive patients referred to SBCE, well representing real life.

In conclusion, this large multicenter prospective study of patients with OGB and complete SBCE shows that a longer SBTT increases the DR of SBCE and correlates with age, with older age serving as an independent predictor for P2 lesions. Our data argue against the customary use of prokinetics for SBCE. However, further comparative studies are needed to determine the advantage of increasing the completion rate by prokinetics at the expense of a faster SBTT.

## COMMENTS

### Background

Small-bowel capsule endoscopy (SBCE) is the first-line investigation in patients with obscure gastrointestinal bleeding (OGB). The diagnostic yield of SBCE is related to its completion rate (*i.e.*, visualization of the entire small-bowel

mucosa); for this reason, many clinicians use prokinetics before or during the procedure. However, small retrospective series suggested that a longer small-bowel transit time (SBTT) increases the diagnostic yield of SBCE.

### Innovations and breakthroughs

The present study - an analysis of a large database of consecutive, prospectively collected, complete SBCE performed for obscure gastrointestinal bleeding - confirms that a longer SBTT increases the detection rate of significant findings. However, the authors found a correlation between SBTT and age, with age serving as an independent predictor on multivariable analysis.

### Applications

Unselective use of prokinetics may hamper the diagnostic yield of SBCE.

### Research frontiers

Future studies are needed to show if prokinetics are useful for patients in which the gastric folds are still visible after 45 min after capsule ingestion.

### Terminology

Obscure gastrointestinal bleeding: a gastrointestinal bleeding in which the source is not detected by esophagogastroduodenoscopy and colonoscopy; it is of two types, namely: (1) obscure-overt, presenting with melena; and (2) obscure-occult, presenting with iron-deficiency anemia and positive fecal occult blood test. Prokinetic: a drug which enhances the motility of the gastrointestinal tract. Small-bowel transit time: the time elapsed from the first frame of the duodenal cap to the first frame of the cecum.

### Peer-review

This is a nice study, from a database that most will envy. Well done for this work, can be accepted following some corrections.

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**L- Editor:** A **E- Editor:** Liu WX



## Observational Study

# Role of capsule endoscopy in suspected celiac disease: A European multi-centre study

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## Abstract

### AIM

To analyze the diagnostic yield (DY), therapeutic impact (TI) and safety of capsule endoscopy (CE).

### METHODS

This is a multi-centre, observational, analytical, retrospective study. A total of 163 patients with suspicion of celiac disease (CD) (mean age =  $46.4 \pm 17.3$  years, 68.1% women) who underwent CE from 2003 to 2015 were included. Patients were divided into four groups: seronegative CD with atrophy (Group-I,  $n = 19$ ), seropositive CD without atrophy (Group-II,  $n = 39$ ), contraindication to gastroscopy (Group-III,  $n = 6$ ), seronegative CD without atrophy, but with a compatible context (Group-IV,  $n = 99$ ). DY, TI and the safety of CE were analysed.

### RESULTS

The overall DY was 54% and the final diagnosis was villous atrophy ( $n = 65$ , 39.9%), complicated CD ( $n = 12$ , 7.4%) and other enteropathies ( $n = 11$ , 6.8%; 8 Crohn's). DY for groups I to IV was 73.7%, 69.2%, 50% and 44.4%, respectively. Atrophy was located in duodenum in 24 cases (36.9%), diffuse in 19 (29.2%), jejunal in 11 (16.9%), and patchy in 10 cases (15.4%). Factors associated with a greater DY were positive serology (68.3% *vs* 49.2%,  $P = 0.034$ ) and older age ( $P = 0.008$ ). On the other hand, neither sex nor clinical presentation, family background, positive histology or HLA status were associated with DY. CE results

changed the therapeutic approach in 71.8% of the cases. Atrophy was associated with a greater TI (92.3% *vs* 45.3%,  $P < 0.001$ ) and 81.9% of the patients responded to diet. There was one case of capsule retention (0.6%). Agreement between CE findings and subsequent histology was 100% for diagnosing normal/other conditions, 70% for suspected CD and 50% for complicated CD.

### CONCLUSION

CE has a high DY in cases of suspicion of CD and it leads to changes in the clinical course of the disease. CE is safe procedure with a high degree of concordance with histology and it helps in the differential diagnosis of CD.

**Key words:** Capsule endoscopy; Celiac disease; Anti-transglutaminase antibodies; Gluten-free diet; Non-celiac gluten sensitivity

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**Core tip:** We present the experience of 14 European centers in the indication of impact of capsule endoscopy for suspected celiac disease. It is the study with more patients published to date. We describe the diagnostic and therapeutic impact of capsule in celiac disease, as well as the safety of the technique for this indication.

Luján-Sanchis M, Pérez-Cuadrado-Robles E, García-Lledó J, Juanmartínez Fernández JF, Elli L, Jiménez-García VA, Egea-Valenzuela J, Valle-Muñoz J, Carretero-Ribón C, Fernández-Urién-Sainz I, López-Higueras A, Alonso-Lázaro N, Sanjuan-Acosta M, Sánchez-Ceballos F, Rosa B, González-Vázquez S, Branchi F, Ruano-Díaz L, Prieto-de-Frías C, Pons-Beltrán V, Borque-Barrera P, González-Suárez B, Xavier S, Argüelles-Arias F, Herreras-Gutiérrez JM, Pérez-Cuadrado-Martínez E, Sempere-García-Argüelles J. Role of capsule endoscopy in suspected celiac disease: A European multi-centre study. *World J Gastroenterol* 2017; 23(4): 703-711 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/703.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.703>

## INTRODUCTION

Celiac disease (CD) is the most common autoimmune enteropathy<sup>[1]</sup>, and it is characterized by gluten-induced chronic inflammation of the small bowel (SB). The diagnosis of CD requires the analysis of clinical, histopathological, and serological factors. Genetic factors are not performed routinely, they only help in dubious cases. These has a role primarily exclusion of this diagnosis by its high negative predictive value. Currently, serology anti-transglutaminase antibodies (ATG) is the test of choice for the initial diagnosis and monitoring<sup>[2,3]</sup>, although gaved by false positives

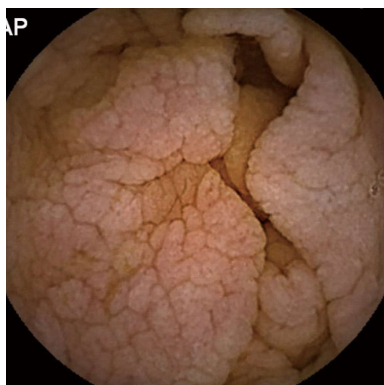


Figure 1 Image suggestive of celiac disease by capsule endoscopy.

and negatives<sup>[4]</sup>. The presence of villous atrophy on duodenal biopsy (DB) through upper digestive endoscopy remains the gold standard for diagnosis in adults, although patchy intestinal impairment can yield to false negatives<sup>[5]</sup>. Thus, there are a significant proportion of patients without classic CD diagnostic criteria who present with discordant data and pose a diagnostic challenge. Today allowed 4 of the 5 criteria Salerno<sup>[6]</sup>: clinical, high titer serology, HLA feature biopsy and/or clinical and histological response to gluten-free diet (GFD). Seronegative cases have led to the recent description of a spectrum of diseases related to gluten, such as seronegative CD and non-celiac gluten sensitivity (NCGS)<sup>[7]</sup>. The prevalence of NCGS is high, as shown in a multi-centre randomized and controlled study, which found NCGS in 14% of 140 patients with functional gastrointestinal symptoms. The absence of SB damage is necessary to suspect a NCGS, thus, in presence of atrophy it is impossible to diagnose of NCGS<sup>[8]</sup>. These new nosological concepts help to classify patients who do not meet the classic criteria and could benefit from a GFD<sup>[9]</sup>.

Capsule endoscopy (CE) is an endoscopy technique, which visualizes the entire SB and has proven useful in patients with negative serology and intestinal atrophy or Marsh- I / II<sup>[10]</sup>. The most common signs compatible with CD are the reduction or absence of Kerckring folds (65%), followed by scalloping (55%) and a mosaic pattern with nodularity (32%)<sup>[10]</sup> (Figure 1). This technique can detect villous atrophy with greater sensitivity than conventional endoscopy (92% vs 55%)<sup>[11]</sup> and it has demonstrated high cost effectiveness and diagnostic accuracy for CD<sup>[12-14]</sup>. However, recent European guidelines<sup>[15]</sup> relegate the role of CE to an alternative for patients who do not want or cannot undergo conventional endoscopy<sup>[16]</sup>, possibly due the shortage of relevant publications, which also comprise low numbers of cases. Therefore, our objective was to analyse the impact of CE in a European multi-centre study of patients with suspected CD who do not meet the classic CD criteria or who have discordant results in common diagnostic tests.

## MATERIALS AND METHODS

### Patients and definitions

An analytical retrospective observational study involving 14 hospitals in Europe. This article was coordinated by the Valencia University General Hospital Consortium. It has been approved by the local institutional review board and approved by its Ethics Committee.

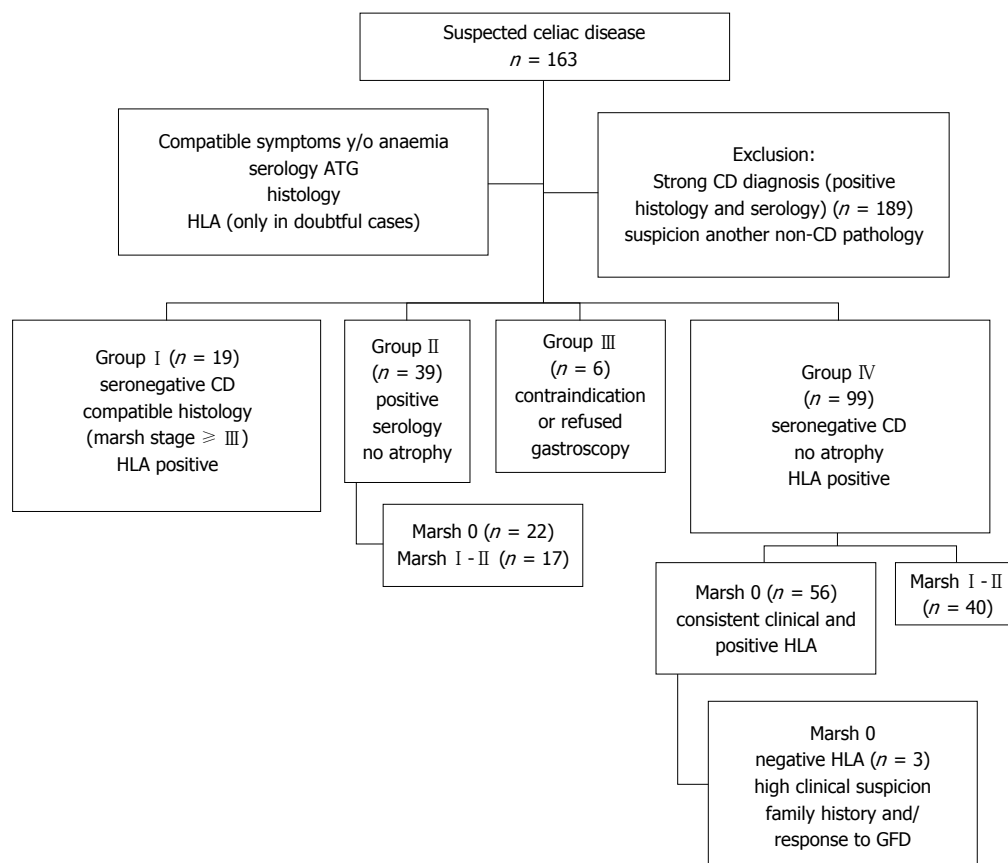
**Inclusion criteria:** An analysis of the clinical histories of 163 patients (mean age:  $46.4 \pm 17.3$  years, range: 11-85; 68.1% women) was conducted; the patients underwent CE for suspected CD during the years 2003-2015. The suspicion of CD was based on a clinical assessment indicating symptoms that were compatible with CD, serology (ATG and to rule IgA deficit) and histology. In selected cases HLA status DQ2/DQ8 (exclusively in doubtful cases). Family history of CD were collected. All patients underwent gastroscopy to determine the basal histology except when endoscopy was contraindicated. Depending on the protocol for each centre, there were 2-6 DB (of the bulb and/or second duodenal portion). Intestinal damage was calculated using the Marsh classification<sup>[17]</sup>, with stages III and higher considered positive. Based on the various findings, patients were classified into 4 suspect groups, as follows (Figure 2): (1) Group- I ( $n = 19$ ): Seronegative CD. Patients with negative serology, histology compatible with CD and positive HLA; (2) Group- II ( $n = 39$ ): Patients with positive serology, no atrophy and Marsh stages 0 ( $n = 22$ ) or I - II ( $n = 17$ ); (3) Group- III ( $n = 6$ ): Patients with contraindications or refusal to undergo gastroscopy; and (4) Group- IV ( $n = 99$ ): Seronegative patients without atrophy, with clinical digestive symptoms and/or anaemia. Studied according to their histology, which indicated Marsh stages 0 ( $n = 59$ ) or I- II ( $n = 40$ ). Patients with Marsh 0, showed consistent clinical and positive HLA. Patients with Marsh 0 and negative HLA ( $n = 3$ ) were included by high clinical suspicion, other family members and/or to advise those who need for accurate diagnosis for response to GFD and need to maintain or withdraw it (Figure 2).

**Exclusion criteria:** Patients with a strong CD diagnosis (positive histology and serology) were excluded ( $n = 189$ ), as were those who underwent CE due to suspicion of another non-celiac pathology, or requests for obscure gastrointestinal bleeding without other data suspicion of CD. Those who presented with negative serology and histology without compatible clinical/analytical characteristics (regardless of the HLA), and those who were HLA negative when the only suspect data point was clinical presentation.

### Procedure

Multiple CE systems were used, including *Pillcam SB*,





**Figure 2** Groups with suspected celiac disease included and excluded in the study. CE: Capsule endoscopy; CD: Celiac disease; ATG: Anti-transglutaminase antibodies; GFD: Gluten-free diet.



**Figure 3** Image suggestive of celiac disease complicated by ulcerative jejunitis.

SB2, SB3, COLON 1 and COLON 2 (Medtronic Inc, Dublin, Ireland) and Mirocam (Intromedic, Seoul, Korea). Prior capsule patency was indicated in 6 patients (3.7%) and was normal in all cases. The indication of the type of CE, patency and the prior preparation followed the protocol of each centre. The location of the lesions and their extent in the various SB segments were recorded.

The diagnostic yield (DY) of CE was considered positive when CE found pathological findings (nodular mucosa, mosaic pattern, villous atrophy, scalloping folds) (Figure 1), either intestinal atrophy,

complications from CD (ulcerative lesions mainly in jejunum-Figure 3, neoformation), or diagnoses than CD. The distinction between CD and other enteropathies was made by each center based on clinical, analytical, radiological, endoscopic and response criteria to specific treatments.

The therapeutic impact (TI) was considered positive when the CE changed the therapeutic approach or the patient's evolutionary course, including the modifications of a GFD or a specific treatment for CD or other enteropathies or subsequent digestive endoscopies. Both impacts were analysed based on the different groups previously described. When the CE indicated the implementation of new endoscopic procedures with biopsy, the agreement with histology was analysed.

### Statistical analysis

Categorical variables were compared using a  $\chi^2$ -test or Fisher's test. Normally distributed continuous variables were presented as the mean, standard deviation and analysed by a Student *t*-test. *P*-values < 0.05 were considered statistically significant. SPSS version 23 was used (IBM, SPSS Inc., IL, United States).

## RESULTS

### Patients

The overall prevalence of positive serology by ATG

**Table 1** Diagnostic performance of capsule endoscopy for the subgroups

Subgroup ( <i>n</i> , %yield) <sup>1</sup>	Normal	Intestinal atrophy	Complicated CD	Other enteropathies
I ( <i>n</i> = 14/19, 73.7%)	5 (26.3)	9 (47.4)	5 (26.3)	0
II ( <i>n</i> = 27/39, 69.2%)	12 (30.8)	25 (64.1)	1 (2.6)	1 (2.6)
III ( <i>n</i> = 3/6, 50%)	3 (50.0)	3 (50.0)	0	0
IV ( <i>n</i> = 44/99, 44.4%)	55 (55.6)	28 (28.3)	6 (6.1)	10 (10.1)

<sup>1</sup>Group-I: Seronegative celiac disease; Group-II: Positive serology with no atrophy; Group-III: Contraindications or refusal to undergo gastroscopy; Group-IV: Seronegative patients without atrophy, with clinical digestive symptoms and/or anaemia. CD: Celiac disease.

(*n* = 38) or anti-gliadin/endomysium antibodies (*n* = 3) was 25.15%. The HLA was positive (*n* = 68, 41.7%), negative (*n* = 8, 4.9%) and in most it was not performed (*n* = 86, 52.8%). All cases of negative HLA (three Marsh 0, four Marsh- I and one Marsh- II) corresponded to the patients in Group-IV.

The presentation forms were clinical digestive symptoms (*n* = 95, 58.3%), iron-deficiency anaemia or iron deficiency (*n* = 22, 13.5%), or both (*n* = 42, 25.8%). In addition, the associated dermatitis herpetiformis (*n* = 4, 2.5%), neurological syndromes (*n* = 3, 1.8%, one in the form of ataxia with suspected Gobbi syndrome) and stunted growth (*n* = 2, 1.2%) were found. The family history of CD (*n* = 11, 6.8%) was also collected.

### Diagnostic yield of capsule endoscopy

The average SB transit time was 232.1 ± 89.9 min, with full visualization in 92.6% cases. There were 6 incomplete procedures, and 50% of them reached a diagnosis. There was only one complication (0.6%) due to retention of the CE secondary to ulcerative jejunitis (UJ), and the CE was extracted by balloon-assisted enteroscopy (BAE), which confirmed the diagnosis.

Overall, the DY of the CE diagnosis was 54% (*n* = 88). The DY obtained by the subgroups is shown in Table 1. The CE results were suggestive of intestinal atrophy (*n* = 65, 39.9%), UJ (Figure 3) (*n* = 11, 6.8%), intestinal lymphoma in the jejunum (*n* = 1, 0.6%) and other enteropathies (*n* = 11, 6.8%). Positive serology (68.3% vs 49.2%, *P* = 0.034) and age (50 ± 17 vs 43 ± 17, *P* = 0.008) were associated with a larger impact on diagnosis, but positive histology at baseline (73.7% vs 51.5%, *P* = 0.068), HLA (60.3% vs 55.6%, *P* = 0.785, sex (*P* = 0.717), clinical presentation (*P* = 0.993) and family background (*P* = 0.745) were not. In seropositive patients (Group- II), there were no differences between those with DY statuses for the Marsh-0 and Marsh- I / II stages (59.1% vs 82.4%, *P* = 0.119).

The atrophy was exclusively duodenal (*n* = 24, 36.9%), jejunal (*n* = 11, 16.9%), or ileal (*n* = 1, 1.5%), was diffuse in at least 2 areas (*n* = 19, 29.2%) and was patchy (*n* = 10, 15.4%). The diagnosis of atrophy was associated with a greater TI than when the CE result was normal (92.3% vs 45.3%, *P* < 0.001). Three patients with UJ also presented duodenal involvement, and 3 were exclusively ileal. In a case when the UJ affected the entire SB, a sprue-like enteropathy associated with olmesartan was eventually confirmed. Of these patients, at least 2 initially presented with digestive symptoms and had negative serology. Patient who had positive serology and a biopsy indicating Marsh- I, was diagnosed with suspected of jejunal lymphoma by CE; however, subsequent biopsies using BAE did not confirm this finding and showed intestinal atrophy corresponding to Marsh-III. The CE results indicated diagnoses of non-CD enteropathies, mostly in Group-IV (*n* = 11, 6.7%) and Group- II (*n* = 1, 2.6%). The most frequent was Crohn's disease (*n* = 8, 72.7%), and the location was exclusively jejunal (*n* = 3), duodenojejunal (*n* = 1), jejunoileal (*n* = 2) and exclusively ileal (*n* = 1). A stenosis was detected by CE in a patient with jejunal Crohn's, but the capsule could still pass through the stenosis. Only one patient was finally confirmed as having Crohn's disease and associated CD; this patient responded positively to corticosteroids and GFD. In addition, one patient was diagnosed with proctosigmoiditis (ulcerative colitis) through colon CE and a SB intussusception associated with non-specific enteritis and an enteropathy treated with nonsteroidal anti-inflammatory drugs.

### Therapeutic impact of capsule endoscopy

The global TI was 71.8% (*n* = 117), with the suggested changes including that a GFD should be used (*n* = 85, 72.7%) or should be stopped (*n* = 4, 3.4%) and that specific drugs should be used (*n* = 24, 20.5%). In addition, further endoscopy was suggested by the CE results in 36 cases (30.8%), including BAE (*n* = 18), new gastroscopy (*n* = 15) and ileocolonoscopy for Crohn (*n* = 3). One patient was diagnosed with T-cell intestinal lymphoma of the jejunum, and BAE with biopsy was indicated after the discovery of diffuse intestinal atrophy by CE. There were 18 BAE following CE results. However, half of the cases (*n* = 9) were carried out in patients presenting with complicated CD by CE or suspected Crohn disease and only 9 presented with atrophy by CE. Thus, the impact of BAE vs conventional endoscopy in this setting can not be concluded because of the low number of cases.

The CE results agreed with the endoscopy results when endoscopy was suggested (Table 2); villous atrophy suggestive of CD agreed in 70% of the cases. The impact for the subgroups and the therapeutic response to the indicated GFD are shown in Table 3. Overall, 81.2% of the patients responded to the

**Table 2 Comparison of capsule endoscopy results with a subsequent histology evaluation of the same patient**

CE diagnostic yield	Biopsied cases	Biopsy result	Agreement
Normal	<i>n</i> = 2	Normal ( <i>n</i> = 2)	100%
Atrophy	<i>n</i> = 10	Normal ( <i>n</i> = 2), atrophy ( <i>n</i> = 7) <sup>2</sup> , lymphoma ( <i>n</i> = 1)	70%
Complicated CD <sup>1</sup>	<i>n</i> = 4	Atrophy ( <i>n</i> = 2), UJ ( <i>n</i> = 2)	50%
Other diagnoses	<i>n</i> = 3	Crohn's disease ( <i>n</i> = 2), Ulcerative colitis ( <i>n</i> = 1)	100%

<sup>1</sup>Three ulcerative jejunoileitis (UJ) and one lymphoma; <sup>2</sup>One of the biopsies reported Marsh III and eosinophilic gastroenteropathy. CE: Capsule endoscopy; CD: Celiac disease.

**Table 3 Modifications and response to the gluten-free diet in the different subgroups of patients *n* (%)**

Subgroup <sup>1</sup>	Therapeutic yield	GFD Indicated	GFD removed	Responded to GFD	Responded to the withdrawal
I ( <i>n</i> = 19)	16 (84.2)	12 (63.2)	1 (5.3)	8 (66.7)	0
II ( <i>n</i> = 39)	31 (79.5)	27 (69.2)	0	18 (66.7)	-
III ( <i>n</i> = 6)	6 (100)	5 (83.3)	0	4 (80)	-
IV ( <i>n</i> = 99)	64 (64.7)	41 (41.4)	3 (3.1)	39 (95.1)	3 (100)

<sup>1</sup>Group- I : seronegative celiac disease; Group- II : Positive serology with no atrophy; Group- III: Contraindications or refusal to undergo gastroscopy; Group- IV: Seronegative patients without atrophy, with clinical digestive symptoms and/or anaemia. GFD: Gluten-free diet.

GDF. In the two cases that did not show a response, autoimmune enteropathy was diagnosed after evidence of villous atrophy was found by the CE. Only one patient worsened after the withdrawal of the GFD. In the other three cases, the non-responders (Group-IV) showed no atrophy, as observed by CE. The response to specific drugs was 58.3%. Complications from the CD and other enteropathies, such as Crohn's disease, were treated according to the usual protocol of each centre. Of the patients with normal CE results, 29.3% (*n* = 22/75) responded to the GFD, whereas 63.1% (*n* = 41/65) of the patients for whom atrophy was observed by CE responded to the GFD; this difference was significant (*P* < 0.001). Of the 41 patients in Group-IV, 95.1% responded favourably to the GFD, without a significant difference in the response between Marsh- I / II (*n* = 21) and Marsh-0 (*n* = 20) (90.5% vs 100%, *P* = 0.488). NCGS was diagnosed in symptomatic patients of Group-IV (seronegative CD without atrophy) when they clinically responded to the GFD (*n* = 15/39, 38.5%), which was started after normal CE results without any further confirmation of classic CD.

## DISCUSSION

The present multi-centre study describes a series of 163 patients with suspected CD in whom CE was

performed in the absence of traditional diagnostic evidence. In these cases, the CE is the first non-invasive alternative diagnostic test in SB when suspicion is high<sup>[18]</sup>. The global DY of CE was beneficial for more than half of the cases. The most frequent finding was intestinal atrophy, followed by complicated CD and other enteropathies. Most of the patients included presented with clinical symptoms, with positive serologic markers and negative atrophy. In nearly 40% of them, when the CE result was normal, NCGS could be diagnosed. Likewise, more than half of seropositive patients without atrophy were diagnosed as having CD. The CE results influenced the therapeutic approach or evolutionary course in approximately 70% of the cases, and in most of them, the patients responded to the GFD.

The sensitivity and specificity of CE in CD is 83%-89% and 95%-98%, respectively<sup>[13,14]</sup>, and CE is a cost-effective technique for the diagnosis of intestinal atrophy. In addition, CE allows a differential diagnosis to be performed based on observations of the entire SB. In patients with suspected CD, CE is indicated when conventional endoscopy is contraindicated or refused; this technique was initially used in cases of refractory CD and in cases of suspected complications<sup>[15,19,20]</sup>. Another potential indication includes the ambiguous cases of CD that show disagreement between serology and histology.

In our study, the diagnostic performance was greater in patients with positive serology and in the seronegative patients with positive histology. HLA, sex, clinical presentation and family background were not associated with CD and cannot serve as a guide for indicating CE. In patients with seronegative CD (Group- I ), the absence of antibodies could be associated with fluctuating antibodies, advanced age or a GFD<sup>[4]</sup>. The negative serology is inversely related to the degree of atrophy because this entity includes initial states (latent and potential atypical CD)<sup>[21]</sup>, reducing the benefits of CE<sup>[22]</sup>. However, in our study, CE is very beneficial in these cases because CE has a high global diagnosis effect, providing a diagnosis of villous atrophy in almost half of the patients and in a significant proportion of the patients who had complicated CD. Other authors have demonstrated that the benefits of CE will be greater for patients corresponding to Marsh- III vs Marsh- I - II (28% vs 7%)<sup>[10]</sup>. However, in our study, the presence of villous atrophy at baseline was not significantly associated with a higher DY, but it was associated with more severe patterns of the disease.

On the other hand, the majority of patients with positive serology who are not treated have the typical histological changes of CD<sup>[23,24]</sup>; however, those without villous atrophy (Group- II ) account for one-third of CD cases<sup>[25,26]</sup>. In these cases, the false negative histology can be caused by the patchy distribution, which most often affects the distal or latent forms of the disease<sup>[4,27,28]</sup>. In our study, the benefit of CE in this

context was very high given that CE allows observation of injuries consistent with villous atrophy in areas that are not accessible to the biopsy<sup>[29]</sup>. For some authors, the confirmation of CD in these groups of seropositive patients with a normal DB is the clinical and serological response to the GFD<sup>[20]</sup>. Patients who do not want to undergo endoscopy or for whom endoscopy is contraindicated (Group-III) constituted a minority. In these patients, CE is an alternative accepted method of diagnosis<sup>[30]</sup> and, in our experience indicates, is beneficial to half of the patients.

Finally, the majority of patients in which CE was requested in our series (Group-IV) were seronegative, lacked duodenal atrophy and had a positive HLA and a clinical presentation compatible with CD. The sensitivity of CE is lower in the absence of atrophy when endoscopic signs may remain unnoticed. However, the diagnostic impact in these cases was approximately 40%, and the main observation was intestinal atrophy. All new cases of NCGS and most other enteropathies belonged to this group. The distribution pattern observed using CE is frequently extensive enteropathy (duodenal continuing into patchy jejunal)<sup>[11]</sup>. This pattern occurred in 66.6% of the patients who exhibited symptoms in the proximal ID, and 11.1% had panenteric symptoms<sup>[31]</sup>. Similarly, in our study, the most frequent location of the atrophy was duodenal, followed by a widespread distribution, jejunal distribution, patchy distribution and occasionally an isolated ileal distribution. We found that almost 20% of the patients had atrophy unreachable by conventional endoscopy. The relevance of the spread of the enteropathy characteristics found using CE correlates to the ATG results but not to the clinical symptoms<sup>[10]</sup>. However, as in other studies<sup>[11]</sup>, the percentage of the response to the GFD was higher in our patients who had villous atrophy.

As for the finding of ulcers in the UJ, they are distinguished from those found in other enteropathies or in patients without pathology because they are more numerous ( $\geq 5$ ) and larger and distal<sup>[32]</sup>. Likewise, the distinction with other ulcerative diseases should be made in a context of adequate suspicion and after response to specific treatment.

Regarding the TI, the CE findings influenced the therapeutic approach in more than 70% of the patients, with the majority responding to the GFD. Similarly, a previous study reported that CE findings were consistent with histology findings in 78% of cases<sup>[33]</sup>. In our experience, there was a total agreement between the CE findings and the histology findings when the CE indicated normal results and 70% agreement for the diagnosis of atrophy.

### Weaknesses of the study

Our study has several limitations in addition to its retrospective design, which include the following: the use of different systems for CE, subjective CE

criteria for CD diagnosis, with successive new criteria in histological classifications and clinical practice guidelines, additional endoscopic instrumentation (BAE), the lack of HLA assays for all cases, the broad time interval of the data collection, and the use of different investigators. Nevertheless, this study represents one of the largest series published to date for this type of patient, who are frequently encountered in normal clinical practice.

In conclusion, CE has a fundamental role with a high diagnosis impact in cases of misleading diagnosis for CD, with the CE modifying the clinical course, especially in cases with positive serology at baseline. In addition, the atrophy observed by CE has a high concordance with the results of subsequent histology and relates to the response to the GFD. This procedure is safe and useful, even when it indicates a normal result. The diagnostic performance along with the response to the GFD allowed a differentiation between CD, NCGS and other enteropathies. Therefore, our data suggest that in cases of misleading CD, CE can complement serology and biopsy in the early and differential diagnosis of this disease.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Celiac disease (CD) is an autoimmune disease characterized by an increased immune response to gluten. Prevalence rates in populations in the America and Europe are estimated at 0.2%-1.0%. The diagnostic test is the histology of the small intestine through superior endoscopy demonstrating the presence of atrophy of the villi. The diagnosis of CD becomes a real challenge when all the factors of suspicion are not fulfilled. For this reason there is a growing interest in the role of the capsule endoscopy (CE) in this disease. Due to its ability to increase the intestinal image, it can detect villous atrophy compatible with celiac disease and other enteropathies or complications associated to this disease.

### Research frontiers

Studies have shown a utility of the endoscopic capsule for the diagnosis of celiac disease atrophy with sensitivity, specificity and positive and negative predictive values of CE of 70%-100%, 64%-100%, 96%-100% and 71%-93%, respectively. There is currently no clinical practice guide that accurately defines the role of the CE in this context as the published series show a small number of cases. For this reason, the authors conducted this European multicenter study that allowed the inclusion of a greater number of cases, in order to define the appropriate use of CE in the suspicion of CD.

### Innovations and breakthroughs

CE has an important role with a high diagnostic impact in cases of misleading diagnosis for CD, with the CE modifying the clinical course, especially in cases with positive serology at baseline. In addition, the atrophy observed by CE has a high concordance with the results of subsequent histology and relates to the response to the gluten-free diet (GFD). This procedure is safe and useful, even when it indicates a normal result. The diagnostic performance along with the response to the GFD allowed a differentiation between CD, non-celiac gluten sensitivity and other enteropathies.



## Applications

Therefore, these data suggest that in cases of misleading CD, CE can complement serology and biopsy in the early and differential diagnosis of this disease.

## Terminology

CE is a non-invasive tool that displays the entire SB and is an alternative to duodenal biopsy in doubtful cases of celiac disease.

## Peer-review

This study shows the impact of CE on diagnosis and therapies for patients suspected of celiac disease.

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## Observational Study

# Association between endotoxemia and histological features of nonalcoholic fatty liver disease

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## Abstract

### AIM

To assess whether surrogate biomarkers of endotoxemia were correlated with the histological features of

nonalcoholic fatty liver disease (NAFLD).

## METHODS

One hundred twenty-six NAFLD patients who had undergone percutaneous liver biopsy were enrolled. Serum lipopolysaccharide (LPS)-binding protein (LBP) and anti-endotoxin core immunoglobulin G (EndoCab IgG) antibody concentrations at the time of liver biopsy were measured using the enzyme-linked immunosorbent assays to examine for relationships between biomarker levels and histological scores.

## RESULTS

Serum LBP concentration was significantly increased in nonalcoholic steatohepatitis (NASH) patients as compared with nonalcoholic fatty liver (NAFL) subjects and was correlated with steatosis ( $r = 0.38$ ,  $P < 0.0001$ ) and ballooning scores ( $r = 0.23$ ,  $P = 0.01$ ), but not with the severity of lobular inflammation or fibrosis. Multivariate linear regression analysis revealed that LBP was associated with steatosis score and circulating C-reactive protein, aspartate aminotransferase, and fibrinogen levels. Serum EndoCab IgG concentration was comparable between NASH and NAFL patients. No meaningful correlations were detected between EndoCab IgG and histological findings.

## CONCLUSION

LBP/EndoCab IgG were not correlated with lobular inflammation or fibrosis. More accurate LPS biomarkers are required to stringently assess the contribution of endotoxemia to conventional NASH.

**Key words:** Nonalcoholic steatohepatitis; Endotoxemia; Lipopolysaccharide-binding protein; EndoCab IgG; Fibrosis; Steatosis

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**Core tip:** This is the first study simultaneously measuring two surrogate endotoxemia markers, lipopolysaccharide-binding protein (LBP) and EndoCab IgG, in biopsy-proven nonalcoholic fatty liver disease (NAFLD) patients in order to assess for relationships with the histological features of NAFLD. Serum LBP/EndoCab IgG were not correlated with lobular inflammation or fibrosis. It remains elusive whether portal endotoxemia promotes hepatitis/fibrosis in human conventional NAFLD/nonalcoholic steatohepatitis.

Kitabatake H, Tanaka N, Fujimori N, Komatsu M, Okubo A, Kakegawa K, Kimura T, Sugiura A, Yamazaki T, Shibata S, Ichikawa Y, Joshita S, Umemura T, Matsumoto A, Koinuma M, Sano K, Aoyama T, Tanaka E. Association between endotoxemia and histological features of nonalcoholic fatty liver disease. *World J Gastroenterol* 2017; 23(4): 712-722 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/712.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.712>

## INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide. NAFLD includes a wide spectrum of disorders, ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) and resultant liver cirrhosis and hepatocellular carcinoma<sup>[1-4]</sup>. NASH is characterized by the presence of hepatocyte ballooning, lobular inflammation and/or various degree of fibrosis in addition to macrovesicular steatosis<sup>[1-3]</sup>. Although several pathogenic factors, such as lipotoxicity, endoplasmic reticulum stress, iron accumulation, and inflammatory signaling, reportedly contribute to the progression from steatosis to steatohepatitis/steatofibrosis, the mechanism of NASH development has not been fully clarified.

Recent murine studies have demonstrated a key role of endotoxin/lipopolysaccharide (LPS) in the onset of NASH<sup>[5,6]</sup>. For example, repeated LPS injection into *ob/ob* mice led to steatohepatitis<sup>[7]</sup>, while mice lacking the gene encoding Toll-like receptor (TLR) 4, a central molecule in LPS-mediated signaling, were resistant to NASH development<sup>[8]</sup>. It is generally accepted that Kupffer cells are activated when the gut mucosa becomes inflamed and fragile or when gut bacteria overgrow and LPS subsequently flows into the portal vein. However, it remains unclear whether the gut barrier is disrupted and portal LPS levels are elevated in typical obesity-, metabolic syndrome-related NAFLD patients without accompanying active inflammatory bowel disease<sup>[9,10]</sup> or a history of gastrointestinal surgery, such as pancreaticoduodenectomy and blind loop construction<sup>[11,12]</sup>.

Another issue requiring attention when considering the contribution of endotoxin/LPS to human NASH is the lack of appropriate systems to determine portal LPS concentration. Since portal LPS is rapidly eliminated in the liver, systemic LPS levels often do not mirror those in the portal vein, and the half-life of circulating LPS is as short as 2 h<sup>[13]</sup>. These shortcomings obscure the evaluation of endotoxin/LPS contribution to NASH.

Circulating LPS-binding protein (LBP) and anti-endotoxin core immunoglobulin G (EndoCab IgG) antibody are commercially available surrogate markers of endotoxemia<sup>[10,14]</sup>. LBP is a soluble acute-phase protein that binds to bacterial endotoxin. When the liver senses bacterial endotoxin, LBP is rapidly synthesized by hepatocytes to neutralize the toxin and then secreted into the circulation. The EndoCab IgG assay measures endotoxin core antibodies to reflect the immune response against persistent endotoxin exposure. Since both of these biomarkers are more stable than LPS, they represent possible indicators of endotoxemia.

In the current study, serum levels of LBP and EndoCab IgG were measured in biopsy-proven NAFLD patients and their correlation with the histological



severity of NAFLD was assessed to clarify associations between endotoxemia and NASH development.

## MATERIALS AND METHODS

### Patients

This study was approved by the Committee for Medical Ethics of Shinshu University School of Medicine (Approval number: 2802) and conducted in accordance with the 1983 revision of the Helsinki declaration of 1975. Informed written consent was obtained from all patients. One hundred twenty-six NAFLD patients who were admitted to Shinshu University Hospital between February 2009 and April 2015 for percutaneous liver biopsy were enrolled. NAFLD had been suspected based on the following criteria: (1) the presence of hepatorenal contrast and increased hepatic echogenicity on abdominal ultrasonography; (2) ethanol consumption of < 20 g/d; and (3) the absence of other causes of liver dysfunction, such as viral hepatitis, drug-induced liver injury, autoimmune liver diseases, primary sclerosing cholangitis, Wilson's disease, hereditary hemochromatosis, and citrin deficiency<sup>[15-18]</sup>. The diagnosis of NAFLD/NASH was confirmed based on the histological findings of biopsied specimens.

Body weight and height were measured before liver biopsy performed in a fasting state. The presence of obesity was defined as a body mass index of  $\geq 25$  kg/m<sup>2</sup> according to criteria released by the Japan Society for the Study of Obesity<sup>[19]</sup>. Medical information was also recorded, and the presence of hypertension, hyperlipidemia, and diabetes was evaluated as described previously<sup>[20,21]</sup>. Blood samples were obtained on the day of the liver biopsy in a fasting state and routine examinations, such as complete blood counts, coagulation, and blood chemistry that included serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were carried out using standard laboratory methods. The remaining sera samples were immediately frozen and kept at -80 °C until further use.

### Histopathological analysis

Biopsy specimens were obtained from liver segment 5 or 8 using a 14-gauge needle as described previously and immediately fixed in 10% neutral formalin. Sections were cut at a 4- $\mu$ m thickness and stained by means of the hematoxylin and eosin and Azan-Mallory methods. The histological activity of NAFLD was assessed by an independent expert pathologist (KS) in a blinded manner for the degrees of steatosis, lobular inflammation, ballooning, and fibrosis according to the system proposed by Kleiner *et al.*<sup>[22]</sup>, as steatosis grade 1: 5%-33% of hepatocytes affected, grade 2: 33%-66% of hepatocytes affected, and grade 3: > 66% of hepatocytes affected; lobular inflammation grade 0: no inflammatory foci, grade 1: < 2 foci per

200  $\times$  field, grade 2: 2-4 foci per 200  $\times$  field, and grade 3: > 4 foci per 200  $\times$  field; ballooning grade 0: no ballooned hepatocytes, grade 1: a few ballooned hepatocytes, and grade 2: many/prominent ballooned hepatocytes; and fibrosis stage (F) 0: no fibrosis, F1: perisinusoidal, perivenular, or portal/periportal fibrosis, F2: perisinusoidal and portal/periportal fibrosis, F3: bridging fibrosis, and F4: cirrhosis. NASH was defined as the presence of macrovesicular steatosis ( $\geq 5\%$  of hepatocytes affected) and hepatocyte ballooning with or without lobular inflammation and fibrosis. NAFLD patients having macrovesicular steatosis without ballooning were diagnosed as having NAFL. The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (1-3), lobular inflammation (0-3), and ballooning (0-2), ranging from 1 to 8.

### Measurement of serum LBP and EndoCab IgG levels

Frozen serum samples obtained at the time of liver biopsy were diluted in 1000- and 200-fold with dilution buffer, and serum LBP and EndoCab IgG concentrations were measured in duplicates using the LBP ELISA kit (HK315-01, Hycult Biotech, Uden, the Netherlands) and the ENDOCAB ELISA kit (HK504, Hycult Biotech), respectively, according to the manufacturer's instructions.

### Measurement of serum cytokeratin 18 fragment levels

Serum concentrations of caspase-cleaved cytokeratin 18 (CK18) fragments were measured using the M30 Apoptosense® ELISA kit (VLVbio AB, Nacka, Sweden) as described previously<sup>[15]</sup>.

### Statistical analysis

Data are expressed as number (percentage) or median (25<sup>th</sup>, 75<sup>th</sup> percentiles). Comparisons between the groups were carried out using the Mann-Whitney *U* test,  $\chi^2$  test, or one-way ANOVA with Bonferroni's correction. Spearman's test was adopted to examine for correlations among LBP, EndoCab IgG, and biochemical/histological data. Multivariate linear regression analysis was conducted to search for independent predictors of LBP. Statistical analyses were performed using StatFlex Ver6.0 software (Artech Co., Ltd., Osaka, Japan). A *P* value of < 0.05 was considered to be statistically significant.

## RESULTS

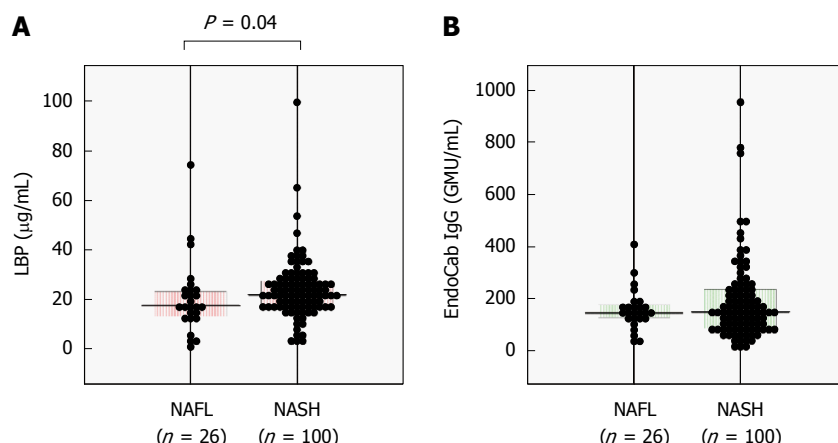
### Serum LBP/EndoCab IgG levels in NAFLD patients

The clinicopathological features of the 126 NAFLD patients are summarized in Table 1. One-hundred patients (79%) were diagnosed as having NASH and 29 (23%) had advanced fibrosis of stage 3 or 4. Serum LBP concentration was significantly increased in NASH patients as compared with NAFL patients (Figure 1A), whereas serum EndoCab IgG was comparable

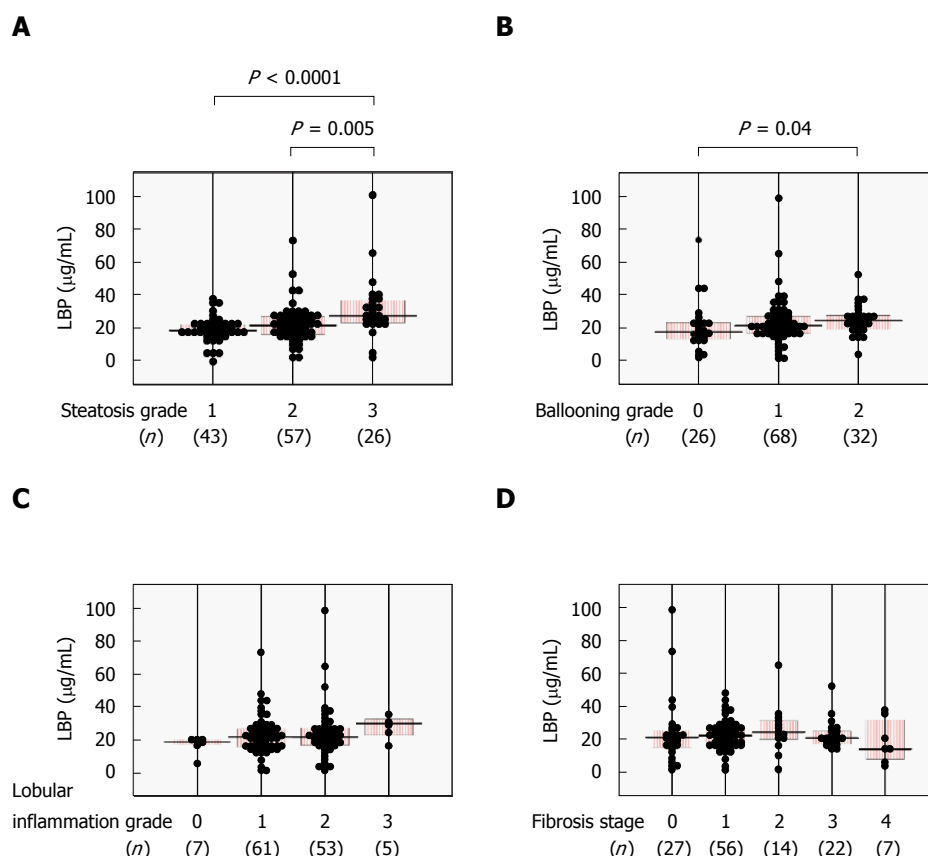
**Table 1** Clinicopathological features of nonalcoholic fatty liver disease patients *n* (%)

Parameter	All ( <i>n</i> = 126)	NAFL ( <i>n</i> = 26)	NASH ( <i>n</i> = 100)	<i>P</i> value
Clinical findings				
Age (yr)	56 (44-65)	52 (42-58)	58 (45-65)	0.17
Male	54 (43)	14 (54)	40 (40)	0.20
BMI $\geq$ 25 kg/m <sup>2</sup>	76 (60)	12 (46)	64 (64)	0.10
Diabetes	43 (34)	5 (19)	38 (38)	0.07
Hypertension	49 (39)	8 (31)	41 (41)	0.34
Hyperlipidemia	82 (65)	19 (73)	63 (63)	0.34
BMI (kg/m <sup>2</sup> )	25.8 (23.6-29.4)	24.6 (22.6-28.5)	26.4 (23.9-29.5)	< 0.05
Ethanol (g/d)	0 (0-5)	0 (0-2.8)	0 (0-5)	0.26
Leukocytes ( $\times 10^3/\mu\text{L}$ )	5.57 (4.73-6.82)	5.07 (4.76-5.75)	5.72 (4.72-6.86)	0.24
Erythrocytes ( $\times 10^4/\mu\text{L}$ )	490 (453-515)	490 (474-512)	489 (452-515)	0.36
Hemoglobin (g/dL)	14.9 (14.0-15.8)	15.1 (14.6-15.9)	14.8 (13.9-15.8)	0.14
Hematocrit (%)	44.2 (41.6-46.6)	45.3 (43.3-46.9)	43.9 (41.5-46.6)	0.31
MCV (fL)	91.2 (88.5-93.7)	91.5 (89.1-93.0)	91.1 (88.4-94.1)	0.81
MCH (pg)	30.7 (29.6-31.7)	31 (30.2-31.6)	30.5 (29.6-31.7)	0.42
MCHC (%)	33.6 (33.0-34.3)	33.9 (33.5-34.4)	33.4 (32.9-34.2)	0.08
Platelets ( $\times 10^4/\mu\text{L}$ )	23.8 (17.9-26.8)	23.3 (19.1-26.7)	23.8 (17.8-26.9)	0.88
PT-INR	1.00 (0.98-1.05)	1.00 (0.98-1.03)	1.01 (0.97-1.05)	0.40
APTT (s)	28.6 (26.6-31.2)	28.6 (27.5-30.1)	28.6 (26.3-31.3)	0.84
FIBG (mg/dL)	287 (243-314)	284 (247-321)	287 (242-313)	0.73
Total protein (g/dL)	7.6 (7.3-7.9)	7.6 (7.3-7.9)	7.6 (7.3-7.8)	0.97
Albumin (g/dL)	4.5 (4.3-4.7)	4.6 (4.5-4.7)	4.5 (4.3-4.7)	0.14
Bilirubin (mg/dL)	0.9 (0.7-1.1)	0.8 (0.7-1.1)	0.9 (0.7-1.1)	0.30
AST (U/L)	40 (29-63)	27 (23-33)	47 (33-70)	< 0.00001
ALT (U/L)	61 (36-92)	39 (30-56)	69 (42-104)	0.0002
LDH (U/L)	214 (186-240)	189 (165-231)	219 (191-249)	0.02
ALP (U/L)	254 (214-323)	246 (218-312)	258 (214-323)	0.86
$\gamma$ GT (U/L)	53 (35-82)	44 (26-70)	54 (37-87)	0.21
ChE (U/L)	384 (342-429)	399 (369-445)	376 (340-427)	0.12
Urea nitrogen (mg/dL)	13 (11-15)	13.2 (12-14.5)	13 (11-15)	0.65
Creatinine (mg/dL)	0.70 (0.58-0.82)	0.79 (0.62-0.84)	0.66 (0.57-0.82)	0.17
Uric acid (mg/dL)	5.6 (4.8-6.8)	6.0 (5.2-7.5)	5.6 (4.8-6.6)	0.26
eGFR (mL/min/1.73 m <sup>2</sup> )	79 (69-89)	77 (59-88)	79 (70-89)	0.53
Total cholesterol (mg/dL)	209 (181-233)	218 (175-247)	206 (182-230)	0.41
Triglycerides (mg/dL)	119 (91-156)	122 (88-165)	119 (93-156)	0.95
LDL-cholesterol (mg/dL)	134 (109-152)	136 (103-155)	131 (113-147)	0.80
HDL-cholesterol (mg/dL)	52 (45-59)	53 (45-61)	52 (45-57)	0.59
Glucose (mg/dL)	106 (97-120)	99 (92-112)	107 (98-122)	0.02
Insulin ( $\mu\text{U/mL}$ )	12 (7.3-17.6)	7.5 (5.1-11.5)	13.1 (8.2-17.9)	0.002
HbA1c (%)	5.8 (5.6-6.4)	5.8 (5.4-6.0)	5.9 (5.6-6.6)	0.02
HOMA-IR	3.2 (1.9-4.8)	2.0 (1.2-3.2)	3.5 (2.2-4.9)	0.001
IgG (mg/dL)	1261 (1089-1535)	1264 (1118-1524)	1261 (1086-1541)	0.77
IgM (mg/dL)	94 (65-126)	83 (54-113)	97 (72-129)	0.18
IgA (mg/dL)	269 (180-333)	252 (185-293)	272 (180-349)	0.56
CRP (mg/dL)	0.10 (0.04-0.17)	0.05 (0.03-0.16)	0.08 (0.05-0.17)	0.13
Hyaluronic acid (mg/dL)	40 (24-74)	29 (23-52)	47 (25-88)	0.08
Type 4 collagen 7S (mg/dL)	4.5 (3.7-5.5)	3.7 (3.6-4.5)	4.5 (3.8-6.3)	0.003
CK18 fragment (U/L)	298 (155-561)	164 (121-293)	351 (178-645) <sup>1</sup>	0.0003
Histological findings				
Steatosis 1/2/3	43/57/26	15/9/2	28/48/24	0.004
Ballooning 0/1/2	26/68/32	26/0/0	0/68/32	< 0.00001
Lobular inflammation 0/1/2/3	7/61/53/5	3/19/4/0	4/42/49/5	0.0003
Fibrosis 0/1/2/3/4	27/56/14/22/7	16/10/0/0/0	11/46/14/22/7	< 0.00001

<sup>1</sup>*n* = 96. Data are expressed as number (percentage) or median (25<sup>th</sup>, 75<sup>th</sup> percentiles). Histological findings were scored according to the criteria proposed by Kleiner *et al.*<sup>[22]</sup> Comparisons between NAFL and NASH groups were carried out using the  $\chi^2$  test or Mann-Whitney *U* test. BMI: Body mass index; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PT-INR: International ratio of prothrombin time; APTT: Activated partial thromboplastin test; FIBG: Fibrinogen; AST: Aspartate aminotransferase; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; eGFR: Estimated glomerular filtration rate; ALP: Alkaline phosphatase;  $\gamma$ GT:  $\gamma$ -glutamyltransferase; ChE: Cholinesterase; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; HbA1c: Hemoglobin A1c; HOMA-IR: Homeostasis model assessment for insulin resistance; CRP: C-reactive protein; CK18: Cytokeratin 18.



**Figure 1** Comparisons of serum lipopolysaccharide-binding protein (A) and EndoCab IgG (B) between 26 nonalcoholic fatty liver and 100 nonalcoholic steatohepatitis patients. The clinicopathological features of the patients are shown in Table 1. Bars represent median values.  $P$  values were calculated using the Mann-Whitney  $U$  test. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis.



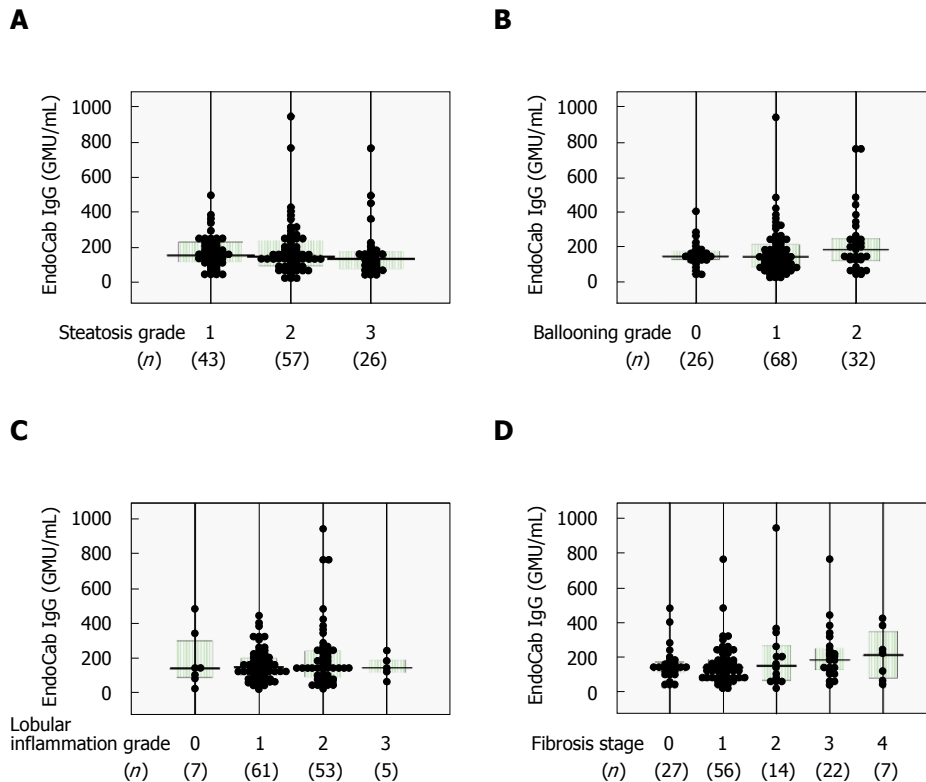
**Figure 2** Relationships between serum lipopolysaccharide-binding protein and histological findings in 126 biopsy-proven nonalcoholic fatty liver disease patients. A: Steatosis (1-3); B: Ballooning (0-2); C: Lobular inflammation (0-3); and D: Fibrosis (0-4) scores. Comparisons among subgroups were conducted using one-way ANOVA with Bonferroni's correction. LBP: Lipopolysaccharide-binding protein.

between the groups (Figure 1B).

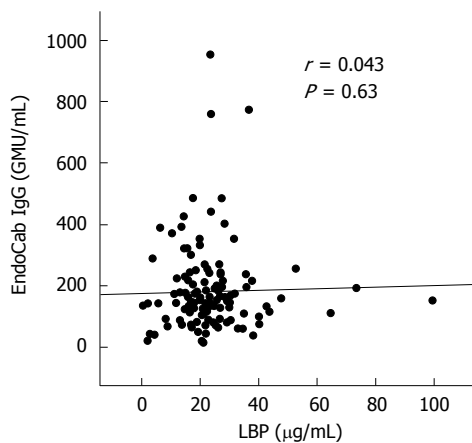
### Relationships between LBP/EndoCab IgG levels and histological findings

We next examined whether serum LBP levels correlated with histological severity in NAFLD patients. Serum LBP was significantly higher in the steatosis

grade 3 group as compared with the steatosis grade 1 or 2 groups (Figure 2A) and was positively correlated with steatosis score (Table 2). Significantly increased serum LBP was seen in the ballooning grade 2 group over the ballooning grade 0 group (Figure 2B), with a positive correlation between LBP and ballooning score (Table 2). There were no significant relationships



**Figure 3 Relationships between serum EndoCab IgG and histological findings in 126 biopsy-proven nonalcoholic fatty liver disease patients.** A: Steatosis (1-3); B: Ballooning (0-2); C: Lobular inflammation (0-3); and D: Fibrosis (0-4) scores. Comparisons among subgroups were conducted using one-way ANOVA with Bonferroni's correction.



**Figure 4 Correlation between serum lipopolysaccharide-binding protein and EndoCab IgG in 126 biopsy-proven nonalcoholic fatty liver disease patients.** A correlation coefficient ( $r$ ) and  $P$  value were calculated using Spearman's test. LBP: Lipopolysaccharide-binding protein.

between serum LBP and lobular inflammation grade (Figure 2C and Table 2) or fibrosis stage (Figure 2D and Table 2).

Similar analyses were carried out for serum EndoCab IgG, which yielded comparable findings among all subgroups (Figure 3) and no correlations with histological scores (Table 2). The lack of agreement between LBP and EndoCab IgG (Figure 4) may explain the discrepant results between LBP and

**Table 2 Correlations between lipopolysaccharide-binding protein/EndoCab IgG and individual pathological features**

	LBP		EndoCab IgG	
	$r$	$P$ value	$r$	$P$ value
Steatosis	0.38	< 0.0001	-0.10	0.25
Ballooning	0.23	0.01	0.11	0.22
Lobular inflammation	0.13	0.14	0.05	0.94
Fibrosis	0.03	0.74	0.13	0.13

Histological findings were scored according to the criteria proposed by Kleiner *et al.*<sup>[22]</sup> Correlation coefficients ( $r$ ) and  $P$  values were calculated by Spearman's test. LBP: Lipopolysaccharide-binding protein.

EndoCab IgG for histological scores.

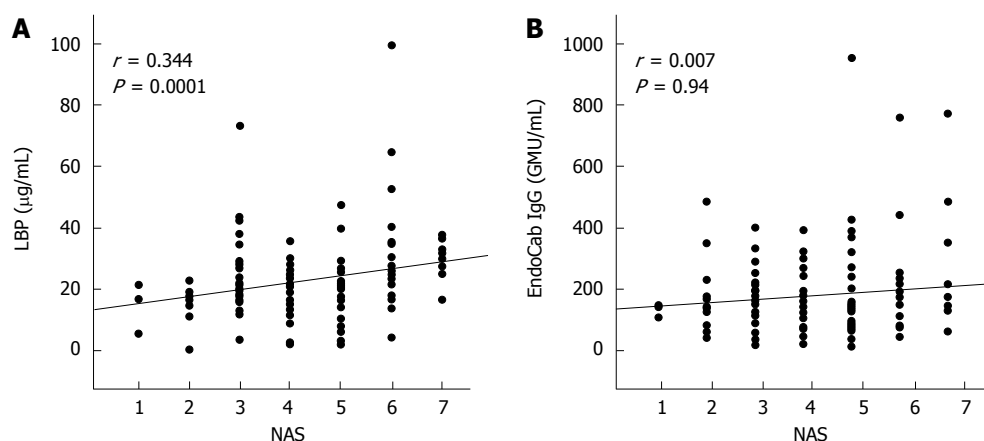
#### Correlations between LBP/EndoCab levels and NAS

There was a significant positive correlation for serum LBP and NAS, an indicator of histological NAFLD activity, but none for EndoCab IgG (Figure 5).

#### Correlations between LBP/EndoCab levels and laboratory data

Serum LBP was significantly positively correlated with AST, ALT, leukocyte count, fibrinogen (FIBG), C-reactive protein (CRP), and CK18 fragment (Table 3), while EndoCab IgG was correlated positively with age, HbA1c, IgG, IgA, and hyaluronic acid and negatively





**Figure 5** Correlation of nonalcoholic fatty liver disease activity score with serum lipopolysaccharide-binding protein (A) and EndoCab IgG (B) in 126 biopsy-proven nonalcoholic fatty liver disease patients. Correlation coefficients ( $r$ ) and  $P$  values were calculated using Spearman's test. LBP: Lipopolysaccharide-binding protein; NAS: Nonalcoholic fatty liver disease activity score.

with platelet count (Table 3). Factor analysis using several laboratory data parameters distinguished a clear separation between the LBP and EndoCab IgG groups; the former group included inflammation-related parameters, such as CRP, FIBG, and leukocyte count, whereas the latter group contained HbA1c, age, and hyaluronic acid. Multivariate linear regression analysis revealed that steatosis score ( $P = 0.0033$ ), circulating CRP ( $P = 0.0032$ ), AST ( $P = 0.0151$ ), and FIBG ( $P = 0.0181$ ) were independent predictors of LBP.

## DISCUSSION

To our knowledge, this is the first study examining the relationship between two surrogate LPS markers and histological severity in NAFLD. Unexpectedly, serum EndoCab IgG did not correlate with any histological findings, nor was serum LBP associated with lobular inflammation grade or fibrosis stage. Based on these results, we could not conclude whether LPS played a crucial role in hepatitis development or fibrosis progression in NAFLD patients, which deviated from findings obtained in murine models.

Although serum LPS is reportedly elevated in NASH patients<sup>[23]</sup>, the direct measurement of LPS has several flaws. First, LPS has a very short half-life and is rapidly eliminated in the liver, and thus serum LPS values rarely reflect actual endotoxemia. Second, the limulus amoebocyte lysate assay is widely used for LPS determination but may be influenced by exogenous LPS contamination due to its high sensitivity. The assay is also disrupted by detergents, urea, and pH<sup>[24]</sup>. We therefore adopted the more stable biomarkers LBP and EndoCab IgG as indicators of endotoxemia.

Since LBP is rapidly induced by LPS and EndoCab IgG reflects the immune response to endotoxin core, these biomarkers measure acute/intermittent and chronic/persistent endotoxemia, respectively. Previous studies have examined either serum LBP or

EndoCab IgG in NAFLD patients. LBP was similar<sup>[25]</sup> between control and NAFLD subjects and increased in NAFLD/NASH patients with severe fibrosis<sup>[26,27]</sup>. EndoCab IgG was comparable<sup>[28]</sup> between control and NAFLD/NASH groups and higher in NASH<sup>[29]</sup>. However, the number of NAFLD patients was relatively small (less than 40) in these investigations. Moreover, there have been no reports simultaneously measuring LBP and EndoCab IgG in histologically-proven NAFLD patients or examining these factors for associations with histological findings. The absence of a correlation between LBP and EndoCab IgG has been supported by a large recent study of 920 participants<sup>[14]</sup>. Although LBP and EndoCab IgG were simultaneously measured in the previous study<sup>[14]</sup>, histological evaluation of the liver was not performed.

In the present series, the severity of lobular inflammation and fibrosis did not correlate with either serum LBP or EndoCab IgG. EndoCab IgG tended to associate with age, hyaluronic acid, and IgG, which was indicative of the possibility of nonspecific IgG elevation due to chronic liver disease and/or aging. Thus, evidence that LPS promoted fibrosis and hepatitis in the context of conventional human NAFLD/NASH could not be demonstrated.

The degree of steatosis correlated with serum LBP level. An earlier study also demonstrated a significant correlation for serum LBP and intrahepatic triglyceride content as determined by proton-magnetic resonance spectroscopy ( $r = 0.366$ ,  $P < 0.001$ )<sup>[14]</sup>, but not for EndoCab IgG. These similar findings prompted us to consider that LBP might be induced independently of LPS in NAFLD patients. Indeed, the fact that disruption of the LBP-encoding gene in mice decreased basal expression levels of fatty acid-synthesizing enzymes and suppressed steatogenesis<sup>[30]</sup> implied a direct link between increased LBP and hepatosteatosis. LBP is also up-regulated in hypertrophied adipocytes and acts as an adipokine<sup>[31]</sup>. Along with CRP and

**Table 3** Correlations between lipopolysaccharide-binding protein/EndoCab IgG and routine clinical data

	LBP		EndoCab IgG	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Age	-0.10	0.25	0.34 <sup>1</sup>	0.0001 <sup>1</sup>
BMI	0.11	0.23	-0.02	0.86
Leukocyte	0.31 <sup>1</sup>	0.0005 <sup>1</sup>	-0.07	0.44
Erythrocyte	0.11	0.23	-0.23	0.01
Hemoglobin	0.02	0.87	-0.20	0.024
Hematocrit	0.05	0.55	-0.22	0.013
MCV	-0.15	0.10	0.08	0.37
MCH	-0.15	0.92	0.10	0.25
MCHC	-0.15	0.11	0.02	0.81
Platelet	0.22	0.015	-0.29	0.001
PT-INR	-0.13	0.15	0.12	0.18
APTT	0.06	0.51	0.08	0.37
FIBG	0.30 <sup>1</sup>	0.0009 <sup>1</sup>	-0.05	0.59
Total protein	-0.01	0.91	0.12	0.18
Albumin	-0.02	0.84	-0.17	0.06
Bilirubin	-0.23	0.01	-0.03	0.73
AST	0.38 <sup>1</sup>	< 0.0001 <sup>1</sup>	0.07	0.44
ALT	0.35 <sup>1</sup>	< 0.0001 <sup>1</sup>	-0.09	0.31
LDH	0.24	0.006	0.13	0.16
ALP	0.16	0.07	0.08	0.39
γGT	0.21	0.02	-0.06	0.53
ChE	-0.04	0.69	-0.21	0.02
Urea nitrogen	-0.24	0.007	-0.08	0.35
Creatinine	-0.05	0.60	-0.26	0.004
Uric acid	0.07	0.47	-0.21	0.02
eGFR	0.15	0.12	-0.02	0.85
Total cholesterol	0.09	0.31	0.02	0.86
Triglycerides	0.07	0.46	0.11	0.22
LDL-cholesterol	0.08	0.37	0.001	0.99
HDL-cholesterol	-0.06	0.54	-0.001	0.99
Glucose	-0.08	0.35	0.19	0.04
Insulin	0.17	0.07	0.01	0.96
HbA1c	0.05	0.59	0.27	0.002
HOMA-IR	0.14	0.14	0.04	0.65
IgG	-0.09	0.33	0.30 <sup>1</sup>	0.0009 <sup>1</sup>
IgM	0.12	0.19	-0.02	0.80
IgA	-0.10	0.28	0.25	0.006
CRP	0.47 <sup>1</sup>	< 0.0001 <sup>1</sup>	0.05	0.61
Hyaluronic acid	-0.08	0.42	0.36 <sup>1</sup>	0.0002 <sup>1</sup>
Type 4 collagen 7S	-0.04	0.65	0.17	0.08
CK18 fragment	0.28	0.002	0.002	0.98

<sup>1</sup>Items with  $r \geq 0.3$  and  $P < 0.05$ . Correlation coefficients ( $r$ ) and  $P$  values were calculated by Spearman's test. BMI: Body mass index; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PT-INR: International ratio of prothrombin time; APTT: Activated partial thromboplastin test; FIBG: Fibrinogen; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; eGFR: Estimated glomerular filtration rate; ALP: Alkaline phosphatase; γGT: γ-glutamyltransferase; ChE: Cholinesterase; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; HbA1c: Hemoglobin A1c; HOMA-IR: Homeostasis model assessment for insulin resistance; CRP: C-reactive protein; CK18: Cytokeratin 18.

FIBG, LBP is an acute phase reactant as well. It is noteworthy that LBP can be induced by interleukin-6 (IL-6)<sup>[32]</sup>, a pro-inflammatory cytokine, in hepatocytes. IL-6 is markedly up-regulated in steatotic livers<sup>[33]</sup>, indicating a possible link among steatosis, IL-6, and LBP. Although the possibility that LPS directly induced steatosis cannot be ruled out completely, the above

findings corroborate a possible association between serum LBP and steatosis independently of LPS/endotoxemia.

This study uncovered a weak, but statistically significant, correlation between serum LBP level and the incidence of ballooned hepatocytes. In hepatocytes with ballooning degeneration, activated c-Jun N-terminal kinase (JNK) and ensuing lipoapoptosis have been documented<sup>[34]</sup>. *Escherichia coli* LPS induces LBP in human oral keratinocytes through the activation of JNK in addition to nuclear factor kappa B and p38 mitogen-activated protein kinase<sup>[35]</sup>. Therefore, a positive relationship between LBP expression and ballooning score might reflect activated JNK-mediated signaling in degenerated hepatocytes.

Yuan *et al.*<sup>[36]</sup> demonstrated that circulating LPS levels in pediatric NASH patients were distributed dichotomously at either high or normal levels, suggesting that endotoxemia was present in specific NASH patients only. Serum LPS levels exhibited no impact on NAS or fibrosis stage and no meaningful relationship was detected between LPS and the proportion of intestinal Gram-negative bacteria. Therefore, they concluded that gut microbiome composition did not contribute to endotoxemia in NASH, nor was endotoxemia always required in the pathogenesis of NASH, which were partially consistent with our results. The observation that antibiotics/probiotics can attenuate NAFLD may not directly support a key role of LPS in NASH development since intestine-derived metabolites/toxicants other than LPS, such as deoxycholic acid and ceramides, promote NAFLD/NASH development<sup>[37,38]</sup>. The therapeutic use of probiotics/prebiotics has not been supported by high-quality clinical studies<sup>[39]</sup>. Additionally, the notion that increased hepatic TLR4 expression in NASH indicates an important role of portal endotoxemia may be inappropriate since TLRs are activated by several molecules other than LPS, including palmitic acid<sup>[40]</sup>.

A key limitation of this study was that it did not directly measure LPS concentrations. Since the present investigation used cryogenically stored samples, we were concerned about the accuracy of LPS value measurement due to the abovementioned flaws in the LPS assay system. Future studies will benefit from assessment of LPS using freshly prepared serum samples along with improvements in LPS assay systems. Another limitation was that we could not examine the association between LBP and *PNPLA3* polymorphisms, which might impact the degree of hepatic steatosis. However, a recent study showed no relationship between *PNPLA3* variants and circulating LBP levels in chronic hepatitis C patients<sup>[41]</sup>.

It is reasonable to consider that chronic LPS challenge to steatotic livers, such as NAFLD accompanied with severe gingivitis or inflammatory bowel disease, may be detrimental to liver condition. However, establishing a key contribution of LPS to the histological

severity of human primary NASH was not possible in the current study. The lack of appropriate endotoxin markers is a major limitation at present, as is the low sensitivity/specificity of LBP/EndoCab IgG assays. Further improvements in LPS detection systems may provide novel information on the role of LPS/endotoxemia in the pathogenesis of conventional NASH.

## ACKNOWLEDGMENTS

The authors thank Mr. Trevor Ralph for his editorial assistance.

## COMMENTS

### Background

Recent murine studies have demonstrated a key role of endotoxin/lipopolysaccharide (LPS) in the onset of nonalcoholic steatohepatitis (NASH). The contribution of intestinal bacterial overgrowth, increased intestinal permeability, and portal endotoxemia to the progression from nonalcoholic fatty liver (NAFL) to NASH has attracted considerable recent attention. However, evaluating the LPS contribution to human NASH is challenging since LPS is rapidly eliminated in the liver, and therefore venous LPS concentrations often do not reflect portal ones.

### Research frontiers

Circulating LPS-binding protein (LBP) and the anti-endotoxin core immunoglobulin G (EndoCab IgG) antibody are commercially available surrogate markers of endotoxemia that are more stable than LPS. The research hotspot is to examine whether these endotoxemia markers correlate with histological severity in nonalcoholic fatty liver disease (NAFLD).

### Innovations and breakthroughs

This is the first study simultaneously measuring two surrogate endotoxemia markers, LBP and EndoCab IgG, in biopsy-proven NAFLD patients in order to assess for relationships with the histological scores of NAFLD. Serum LBP concentration was significantly increased in NASH patients and was correlated with steatosis and ballooning scores, but not with the severity of lobular inflammation or fibrosis. Serum EndoCab IgG concentration was comparable between NASH and NAFL patients.

### Applications

It is reasonable to consider that chronic LPS challenge to steatotic livers, such as NAFLD accompanied with severe gingivitis or inflammatory bowel disease, may be detrimental to liver condition. However, the contribution of LPS to the histological severity of human primary NASH could not be confirmed in the current study. The lack of appropriate endotoxin markers is a major limitation at present, as is the low sensitivity/specificity of LBP/EndoCab IgG assays. Further improvements in LPS detection systems may provide novel information on the role of LPS/endotoxemia in the pathogenesis of conventional NASH.

### Terminology

NAFLD is a chronic liver disease increasing worldwide that includes a wide spectrum of disorders, ranging from NAFL to NASH and resultant liver cirrhosis and hepatocellular carcinoma. NASH is characterized by the presence of hepatocyte ballooning, lobular inflammation and/or various degrees of fibrosis in addition to macrovesicular steatosis. Increased intestinal permeability and ensuing portal endotoxemia is presumed to be one of the contributors to the progression of NASH.

### Peer-review

This report, written by Kitabatake *et al.*, is of an important retrospective study that has been carried out to disclose pathological mechanisms of NAFLD/NASH

as well as its diagnosis. Especially, the close relationship between LBP and NASH is very interesting.

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## Observational Study

# Long-term prognosis in patients continuing taking antithrombotics after peptic ulcer bleeding

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## Abstract

### AIM

To investigate the long-term prognosis in peptic ulcer patients continuing taking antithrombotics after ulcer bleeding, and to determine the risk factors that influence the prognosis.

### METHODS

All clinical data of peptic ulcer patients treated from January 1, 2009 to January 1, 2014 were retrospectively collected and analyzed. Patients were divided into either a continuing group to continue taking antithrombotic drugs after ulcer bleeding or a discontinuing group to discontinue antithrombotic drugs. The primary outcome of follow-up in peptic ulcer bleeding patients was recurrent bleeding, and secondary outcome was death or acute cardiovascular disease occurrence. The final date of follow-up was December 31, 2014. Basic demographic data, complications, and disease classifications were analyzed and compared by *t*- or  $\chi^2$ -test. The number of patients that achieved various outcomes was counted and analyzed statistically. A survival curve was drawn using the Kaplan-Meier method, and the difference

was compared using the log-rank test. COX regression multivariate analysis was applied to analyze risk factors for the prognosis of peptic ulcer patients.

## RESULTS

A total of 167 patients were enrolled into this study. As for the baseline information, differences in age, smoking, alcohol abuse, and acute cardiovascular diseases were statistically significant between the continuing and discontinuing groups ( $70.8 \pm 11.4$  vs  $62.4 \pm 12.0$ ,  $P < 0.001$ ; 8 (8.2%) vs 15 (21.7%),  $P < 0.05$ ; 65 (66.3%) vs 13 (18.8%),  $P < 0.001$ ). At the end of the study, 18 patients had recurrent bleeding and three patients died or had acute cardiovascular disease in the continuing group, while four patients had recurrent bleeding and 15 patients died or had acute cardiovascular disease in the discontinuing group. The differences in these results were statistically significant ( $P = 0.022$ ,  $P = 0.000$ ). The Kaplan-Meier survival curve indicated that the incidence of recurrent bleeding was higher in patients in the continuing group, and the risk of death and developing acute cardiovascular disease was higher in patients in the discontinuing group (log-rank test,  $P = 0.000$  for both). Furthermore, COX regression multivariate analysis revealed that the hazard ratio (HR) for recurrent bleeding was 2.986 (95%CI: 0.67-8.356,  $P = 0.015$ ) in the continuing group, while HR for death or acute cardiovascular disease was 5.216 (95%CI: 1.035-26.278,  $P = 0.028$ ).

## CONCLUSION

After the occurrence of peptic ulcer bleeding, continuing antithrombotics increases the risk of recurrent bleeding events, while discontinuing antithrombotics would increase the risk of death and developing cardiovascular disease. This suggests that clinicians should comprehensively consider the use of antithrombotics after peptic ulcer bleeding.

**Key words:** Peptic ulcer bleeding; Antithrombotics; Cardiovascular disease; Risk factor; Survival curve

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**Core tip:** Patients with peptic ulcer bleeding were enrolled into our study, and clinical information was analyzed by statistical method. We found that continuing antithrombotic drugs for peptic ulcer patients increased the risk of recurrent bleeding events, and discontinuing antithrombotic drugs increased the risk of death or cardiovascular events. Our results indicate that clinicians should balance the usage of antithrombotics to reduce the risk of peptic ulcer bleeding.

Wang XX, Dong B, Hong B, Gong YQ, Wang W, Wang J, Zhou ZZ, Jiang WJ. Long-term prognosis in patients continuing taking antithrombotics after peptic ulcer bleeding. *World J Gastroenterol* 2017; 23(4): 723-729 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/723.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.723>

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## INTRODUCTION

Peptic ulcer is a highly prevalent illness<sup>[1]</sup>, and it tremendously threatens the health of humans due to high morbidity and severe complications<sup>[2-5]</sup>. Among all complications, peptic ulcer bleeding is one of the common clinical diseases<sup>[6]</sup>. In recent years, despite the application of proton pump inhibitors (PPIs)<sup>[7-9]</sup> and *Helicobacter pylori* eradication<sup>[10-12]</sup>, the morbidity of peptic ulcer bleeding has not decreased<sup>[13]</sup> at least partially due to the use of antiplatelet agents, anticoagulants, and thrombin inhibitors. These drugs have recently been used extensively for the treatment of thromboembolic disease<sup>[14-16]</sup>. It has also been estimated that the usage of these drugs has been increasing worldwide as cardiovascular morbidity increases in the aged population<sup>[17,18]</sup>. This would induce a high incidence of peptic ulcer bleeding<sup>[19,20]</sup>. Aspirin is an antithrombotic drug that has been widely applied in view of the benefit in preventing cardiovascular disease<sup>[21,22]</sup>. However, patients with cardiovascular disease are recommended to immediately discontinue the usage of aspirin after successful endoscopic treatment of peptic ulcer bleeding, in order to prevent death or acute disease occurrence, according to the Medication Guide<sup>[23]</sup>. In a randomized double-blind study, Sung *et al*<sup>[24]</sup> found that recurrent bleeding events due to the continued usage of aspirin severely influences the prognosis of patients. Therefore, there is a dilemma to the clinical usage of antithrombotic drugs. Furthermore, there are few studies on antithrombotics usage for treating peptic ulcer bleeding patients, and there is increasing concern on these patients. Hence, we collected the clinical data of patients with peptic ulcer bleeding treated at our hospital in recent five years, aiming to investigate the effect of continued administration of antithrombotic drugs and identify the risk factors for prognosis.

## MATERIALS AND METHODS

### Study objects

Patients with peptic ulcer treated at Tongren Hospital affiliated to Shanghai Jiao Tong University from January 1, 2009 to January 1, 2014 were included in this study. The study ended on December 31, 2014. Upper gastrointestinal hemorrhage was defined as hemoptysis, hematochezia, melena, fainting, or dizziness with anemia. The following patients were excluded: patients with non-peptic ulcer bleeding, esophageal varices, vascular dysplasia, esophageal or gastric cancer, and ulcer perforation; patients with peptic ulcer bleeding who had an unsuccessful

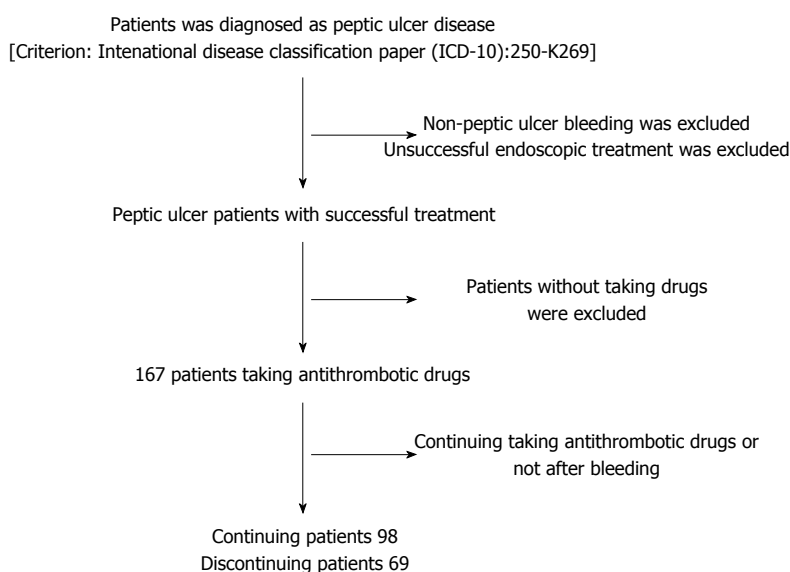


Figure 1 Flow of enrollment of patients with peptic ulcer.

endoscopic treatment; patients who did not receive antithrombotic drugs after a successful therapy; patients who received PIPs to prevent damage from antithrombotic drugs. The enrollment process is shown in Figure 1.

Finally, a total of 167 patients were enrolled in this study. Based on whether drug administration was continued or discontinued after peptic ulcer bleeding healed following endoscopic treatment, the patients were divided into either a continuing group ( $n = 98$ ) or a discontinuing group ( $n = 69$ ). The continuing group included the patients who continued taking the drugs after the bleeding healed, while the discontinuing group included the patients who discontinued taking the drugs after the bleeding healed. All patients in this study provided a signed informed consent form, and this study was approved by the hospital ethics committee.

### Study methods

The clinical data of the patients, including demographic data and complications, were analyzed statistically. The time to end point was strictly calculated. Primary end point was recurrent bleeding events within 30 d, including hemoptysis, melaena,  $> 2$  g/dL of hemoglobin within 24 h, and unstable blood flow (systolic blood pressure  $\leq 90$  mmHg or heart rate  $\geq 110$  times/min). Patients with one of the aforementioned or combined characteristics were defined to achieve the primary end point. Secondary outcomes were death, acute cardiovascular disease, acute myocardial infarction, and ischemia or transient ischemia. The number of patients with different outcomes was counted, and the difference was compared statistically. Survival time was collected to draw the survival rate.

### Statistical analysis

All data were analyzed using SPSS 19.0 software. Age, hemoglobin levels, and body mass index (BMI) measurements are expressed as mean  $\pm$  SD. Categorical data are expressed as percentages. Measurement data following a normal distribution were compared by the  $t$ -test. Frequency data were compared by the  $\chi^2$ -test. Kaplan-Meier method was applied to calculate the survival rate and draw the survival curve. Differences were compared using the log-rank test. The multivariable COX proportional regression model was applied to analyze the risk factors for prognosis in patients with peptic ulcer bleeding.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Baseline data of patients with peptic ulcer bleeding

After screening, a total of 167 patients with peptic ulcer bleeding were enrolled into this study. Among these patients, 98 continued receiving antithrombotic drugs, and 69 discontinued. The average age of the patients who continued and discontinued receiving antithrombotic drugs was  $70.8 \pm 11.4$  years and  $62.4 \pm 12.0$  years, respectively ( $P = 0.000$ ). The percentage of patients with a history of smoking or alcohol abuse was significantly higher in the continuing group than in the discontinuing group ( $P = 0.012$ ). Furthermore, the rate of cardiovascular complications was significantly higher in the continuing group ( $P = 0.000$ ; Table 1).

### Comparison of various outcomes between the two groups

Recurrent bleeding occurred in 18 patients in the



**Table 1** Baseline information between the continuing and discontinuing groups *n* (%)

Characteristic	Continuing group	Discontinuing group	<i>T</i> / $\chi^2$	<i>P</i> value
No. of patients	98	69		
Demographic data				
Gender (male/%)	68 (69.39)	42 (60.87)	1.307	0.253
Age (mean $\pm$ SD)	70.8 $\pm$ 11.4	62.4 $\pm$ 12.0	4.588	0.000
BMI (kg/m <sup>2</sup> , mean $\pm$ SD)	21.4 $\pm$ 4.5	22.0 $\pm$ 4.2	0.872	0.385
Complications				
Smoking and alcohol abuse	8 (8.2)	15 (21.7)	6.284	0.012
Diabetes	32 (32.7)	15 (21.7)	2.385	0.123
Hypertension	71 (72.4)	56 (81.2)	1.338	0.247
Chronic kidney disease	23 (23.4)	8 (11.6)	3.777	0.052
Chronic obstructive pulmonary disease	10 (10.2)	8 (11.6)	0.081	0.775
Acute cardiovascular disease	65 (66.3)	13 (18.8)	36.681	0.000
Non-antithrombotic drug usage				
Aspirin	84 (85.7)	59 (85.5)	0.001	0.970
Forrest classification				
I - II	31 (31.6)	28 (40.6)	1.419	0.234
III	67 (68.4)	41 (59.4)	1.419	0.234
Hemoglobin (g/dL)	9.0 $\pm$ 2.4	8.6 $\pm$ 2.1	1.116	0.266

**Table 2** Comparison of various outcomes achieved between the two groups *n* (%)

	Continuing group	Discontinuing group	$\chi^2$	<i>P</i> value
Recurrent ulcer bleeding events	18 (18.4)	4 (5.8)	5.594	0.018
Death or cardiovascular disease	3 (3.1)	15 (21.7)	14.689	0.000

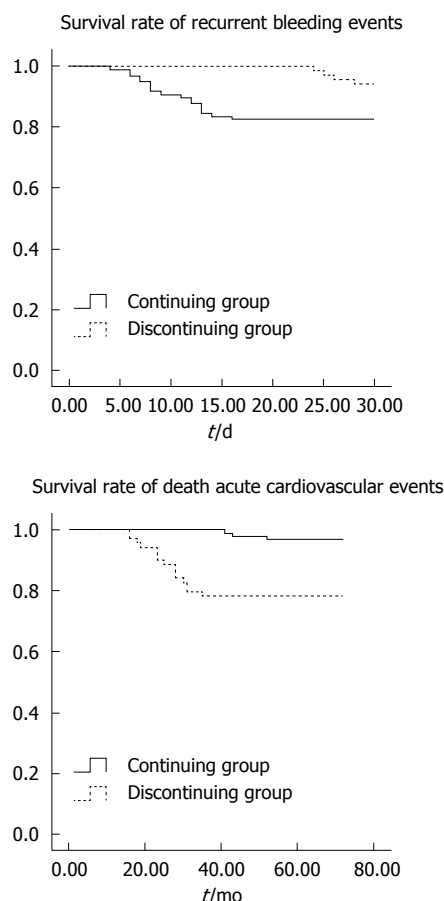
continuing group and in four patients in the discontinuing group. Death or acute cardiovascular disease occurred in three patients in the continuing group and in 15 patients in the discontinuing group. The differences in the rates of primary and secondary outcomes between the two groups were statistically significant ( $P = 0.018$ ,  $P = 0.000$ ; Table 2).

### Survival curve in the two groups

Kaplan-Meier results indicated that bleeding occurred more frequently in patients in the continuing group, while survival rate was significantly higher in patients in the discontinuing group (Log-rank test,  $P = 0.022$ ,  $P = 0.000$ ; Figure 2).

### Risk factors for prognosis of patients

The multivariable COX proportional regression model indicated that continuing intake of antithrombotic drugs increased the risk of recurrent bleeding events (95%CI: 1.067-8.356, OR = 2.986,  $P = 0.015$ ), while discontinuing intake of antithrombotic drugs increased the risk of death or acute cardiovascular

**Figure 2** Kaplan-Meier survival curves for various outcomes.

disease (95%CI: 1.035-26.278, OR = 5.216,  $P = 0.028$ ; Table 3).

## DISCUSSION

Peptic ulcer is one of the most common clinical gastrointestinal tract diseases at present<sup>[6,25]</sup>, and it is generally induced by damage of the gastric or duodenal mucosa. Gastric acid and protease play a crucial role in disease progression<sup>[26,27]</sup>. The aged population accounts for most of the cases, and ulcer bleeding, perforation, and pyloric obstruction are the most common complications<sup>[28-31]</sup>. *Helicobacter pylori* infection, excessive secretion of gastric acid, and excessive antithrombotic drug intake often trigger the occurrence of ulcer bleeding<sup>[32-34]</sup>. In recent years, peptic ulcer morbidity increased slowly due to medical technology progression<sup>[35]</sup>; however, the incidence of ulcer bleeding has been continuously increasing<sup>[36,37]</sup>.

Cardiovascular disease is defined as ischemic or hemorrhagic disease occurring in all tissues due to atherosclerosis and blood viscosity<sup>[38]</sup>. In view of its high morbidity and mortality, more and more people, even healthy people, tend to take antithrombotics to prevent and reduce its risk<sup>[39]</sup>. However, excessive drug usage will increase the risk of bleeding in patients with ulcer bleeding.

**Table 3** COX regression multivariate analysis of risk factors for prognosis

	Recurrent ulcer bleeding events				Death or cardiovascular occurrence			
	$\beta$	OR	95%CI	P value	$\beta$	OR	95%CI	P value
Usage of antithrombotics	1.094	2.986	1.067-8.356	0.015	1.652	5.216	1.035-26.278	0.028

As the aged population has an increased necessity for preventing acute cardiovascular disease, aspirin and other antithrombotics continue to be broadly used<sup>[40-42]</sup>. It has been reported that most patients with established cardiovascular disease ignore the risk of aspirin, and continue to take aspirin or other antithrombotics for secondary prevention<sup>[43,44]</sup>. Even more, the literature has shown that cardiovascular disease complications occur more frequently in patients who have discontinued antiplatelet drug therapy, compared to patients who continue this therapy<sup>[45]</sup>. Nevertheless, continuing intake of aspirin or antithrombotic drugs will increase the risk of hemorrhage complications in surgery instead<sup>[46,47]</sup>. Accordingly, clinicians could not balance the risk of cardiovascular disease and hemorrhage complications. In addition, there are few studies on the prognosis of patients with peptic ulcer bleeding. Hence, the clinical data of patients with peptic ulcer bleeding treated at our hospital were collected and analyzed to discuss the prognosis of patients, hoping to provide guidance for clinical applications.

#### **Age, smoking, and alcohol abuse influence the usage of antithrombotics in patients with peptic ulcer bleeding**

Our study revealed that patients were older in the continuing group than in the discontinuing group, indicating that aged patients need more antithrombotics. This is consistent to the current social situation. The number of patients with cardiovascular complications was higher in the continuing group than in the discontinuing group, which is in agreement with a previous report that patients with an established disease tend to continue taking drugs. In addition, there was a difference in smoking and alcohol abuse between the two groups; there were more of these patients in the discontinuing group. It is plausible that patients taking drugs tended to reduce smoking or alcohol consumption.

#### **Taking antithrombotics affects survival rate in patients with peptic ulcer bleeding**

Our study indicated that patients who continued to take the drugs had a higher risk of recurrent bleeding events. In contrast, patients in the discontinuing group had a higher risk of death or acute cardiovascular disease. This result is inconsistent with recent studies reporting that there was no difference between the two groups. On one hand, our follow-up time did not have a limit. However, patients with less than two months of follow-up were excluded in the study conducted

by Kim *et al.*<sup>[48]</sup>. Furthermore, it has been widely accepted that recurrent bleeding time was shorter than normal bleeding time. On the other hand, the number of patients in these two studies is different. Consistent with our results, Sung *et al.*<sup>[24]</sup> estimated a higher incidence of recurrent bleeding events in patients continuing taking aspirin in a randomized double-blind study (a likelihood ratio of nearly 2). Through retrospective research, we also obtained similar results (a likelihood ratio of nearly 3)<sup>[49]</sup>, which further supports this view. This implies that more attention should be given when continuing taking antithrombotics.

#### **Limitations and prospects**

This study has some limitations. First, a limited number of patients could not sufficiently support our conclusion. Second, we did not distinguish different antithrombotic drugs. However, many studies have shown that the single or combined application of drugs would result in an obvious difference. Finally, the definite time of bleeding was lacking. Hence, we were not able to obtain the precise time when to discontinue or continue drugs.

In conclusion, our results demonstrate that after the occurrence of peptic ulcer bleeding, continuing the intake of drugs would increase the risk of recurrent bleeding events, while discontinuing the intake of drugs will increase the risk of death and acute cardiovascular occurrence. These indicate that clinicians must extensively weigh the benefits and risks when using antithrombotics for treating patients with peptic ulcer bleeding.

## **COMMENTS**

### **Background**

Peptic ulcer is one of the most common gastrointestinal diseases which is generally induced by damage of the gastric or duodenal mucosa. Gastric acid and protease play a crucial role in disease progression. The aged population accounts for most of the cases, and ulcer bleeding, perforation, and pyloric obstruction are the most common complications. *Helicobacter pylori* infection, excessive secretion of gastric acid, and excessive antithrombotic drug taking would trigger the occurrence of ulcer bleeding. In recent years, peptic ulcer morbidity has increased slowly due to medical technology progression, but ulcer bleeding incidence rate has been increasing all the time. Cardiovascular disease is defined as ischemic or hemorrhagic disease occurring in all tissues due to atherosclerosis and blood viscosity. In view of its high morbidity and mortality, more and more people, even healthy people, tend to take antithrombotics to prevent and reduce its risk. However, excessive drug usage will increase bleeding risk instead in ulcer bleeding patients.

### **Research frontiers**

As the aged population has an increased necessity for preventing acute

cardiovascular disease, aspirin and other antithrombotics are broadly used. It is reported that most patients with established cardiovascular disease ignore the risk of aspirin and still insist in taking aspirin or other antithrombotics for secondary prevention. Even more, the literature shows that patients have cardiovascular disease complication more easily in those discontinuing antiplatelet drug therapy compared to those continuing antiplatelet drug therapy. Nevertheless, continuing aspirin or antithrombotic drugs will increase hemorrhage complication risk in surgery instead. Accordingly, clinicians could not balance risk of cardiovascular disease and hemorrhage complication.

### Innovations and breakthroughs

The authors investigated the prognosis and risk factors in peptic ulcer bleeding patients. Even more, they showed two survival curves with disparate outcomes to demonstrate survival difference in patients continuing or discontinuing taking antithrombotics. These results suggest that clinicians must take more attention in the usage of antithrombotic drugs.

### Applications

This study demonstrates that after occurrence of peptic ulcer bleeding, continuing taking drugs will increase the risk of recurrent bleeding events, and discontinuing drugs will increase risk of death and acute cardiovascular occurrence, which indicates that clinicians must weigh the risks and benefits when using antithrombotics to treat ulcer bleeding patients.

### Peer-review

Patients with ulcer peptic bleeding were enrolled in this study and clinical information was analyzed by statistical method. Authors found that continuing antithrombotic drugs in ulcer peptic patients increased the risk of recurrent bleeding events while discontinuing drugs increased risk of death or cardiovascular events. The results indicated that clinicians should balance the usage of antithrombotics to reduce risk in peptic ulcer bleeding patients

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## Real case of primitive embryonal duodenal carcinoma in a young man

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### Abstract

We report here the case of a young man suffering from a rare germ cell tumour. The patient was a 25-year-old man who was referred to our centre for asthenia, stinging epigastric pain, and an iron deficiency anaemia. Gastroscopy revealed a circumferential vegetating lesion on the second portion of the duodenum. The lesion was indurated at the third portion of the duodenum, responsible for a tight stenosis. A computerized tomography-scan of the chest, abdomen and pelvis, and a pancreatic MRI showed a circumferential lesion with a bi-ductal dilatation (*i.e.*, of the common bile duct and Wirsung's duct) without metastatic localisation. The patient underwent a pancreaticoduodenectomy with lymph node dissection including all cellular adipose tissues of the hepatic pedicle from the hepatic common artery and of the retroportal lamina. Histological findings were suggestive of a duodenal embryonal carcinoma with pancreatic infiltration. This is the second published case highlighting the duodenal primitive localisation of an embryonal carcinoma with pancreatic infiltration.

**Key words:** Embryonal carcinoma; Germ cell tumour; Duodenum; Young male; Pancreaticoduodenectomy

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**Core tip:** Duodenal embryonal carcinoma is a rare germ cell localisation. This lesion may be revealed by a chronic or acute haemorrhage. Our patient presented with an iron deficiency anaemia associated with asthenia and epigastric pain. Imaging studies and endoscopy showed a tight stenosis of the third portion of the duodenum with a circumferential lesion responsible for a common bile duct and Wirsung's duct dilatation without any metastatic localisation. The patient underwent a pancreaticoduodenectomy and histological findings helped to identify a duodenal embryonal carcinoma with pancreatic infiltration.

Barbieux J, Memeo R, De Blasi V, Suciu S, Faucher V, Averous G, Roy C, Marescaux J, Mutter D, Pessaux P. Real case of primitive embryonal duodenal carcinoma in a young man. *World J Gastroenterol* 2017; 23(4): 730-734 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/730.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.730>

## INTRODUCTION

In young men, aged between 15 and 35, testicular cancer is the leading cause of neoplasia, with an incidence rate of 2.1 for 100000<sup>[1]</sup>. It should be noted that about 5% of these patients may present with a metastatic localisation on the digestive tract<sup>[2]</sup>. The most frequent origin for embryonal carcinoma is testicular (33% of cases), as confirmed by the literature<sup>[1]</sup>. The pineal gland, the mediastinal region, the digestive tract, the lungs and the retroperitoneum could well be the primitive origin of an embryonal carcinoma<sup>[3]</sup>.

Here, we reported the case of an embryonal duodenal carcinoma with pancreatic infiltration.

## CASE REPORT

A 25-year-old Bulgarian man was referred to our centre by his regular medical doctor for a strong asthenia, which lasted for the past 3 wk, and stinging epigastric pain, which was paroxysmal with dorsal irradiation responsible for nocturnal awakening getting worse since 1 mo.

The patient had neither lost weight recently nor did he present with anorexia.

Apart from a moderate active smoking, he did not have any significant surgical and medical history.

Clinical examination showed that the patient has a body mass index of 19. Examination of the abdomen revealed an epigastric sensitivity without any abdominal mass. Bowel movements were regular; however, dark stools were noted for the last week. A digital rectal exam revealed neither mass nor blood.



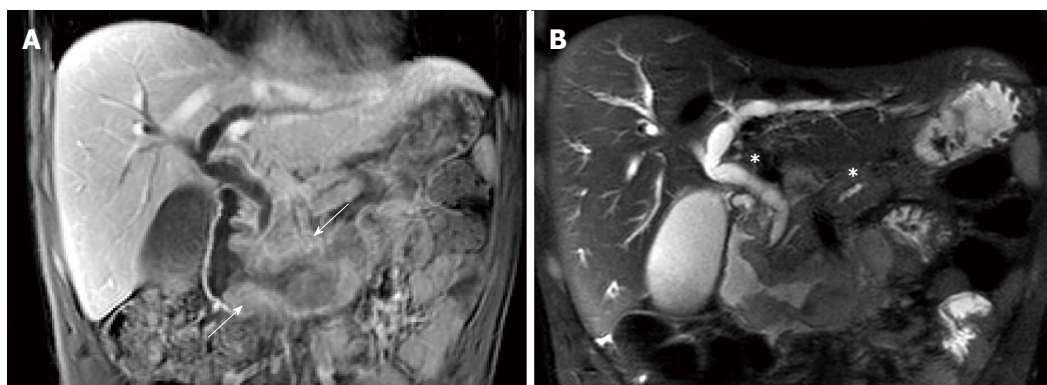
Figure 1 Gastroscopy shows a circumferential vegetating mass with a villous appearance on the second portion of the duodenum.

The patient was afebrile and presented with a marked skin pallor.

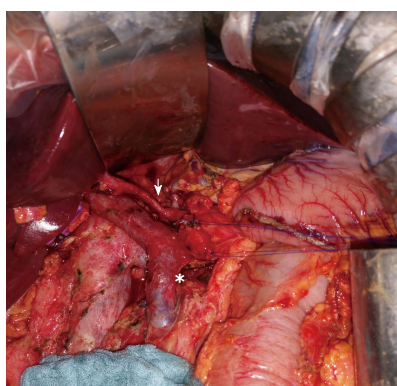
Biologically, the patient presented with haemoglobin at 6.8 g/dL in relation with an iron deficiency anaemia (serum iron: 2 µmol/L, mean corpuscular volume: 76.5 fL and ferritin: 4 µmol/L). For this reason he was transfused 4 units of packed red blood cells as soon as he was admitted to our department. There was no inflammatory syndrome (leukocytes: 8.88 Giga/L and C-reactive protein: 22.3 mg/L). Liver function test results showed a cytolysis (glutamic pyruvic transaminase: 218 U/L and glutamic oxaloacetic transaminase: 76 U/L) as well as an anicteric cholestasis (gamma-glutamyl transferase: 644 U/L, alkaline phosphatase: 507 U/L and total bilirubin: 2.7 µmol/L). Lipase was at 2591 U/L and quickly decreased. Tumoural markers were measured: carcinoembryonic antigen: < 1 µg/L; carbohydrate antigen 19-9: 17.2 kU/L; alpha foetoprotein: 2.1 µg/L; β human chorionic gonadotropin < 3 UI/L; lactate dehydrogenase: 117 U/L.

Rectoscopy performed until 40 cm from the anal margin did not show anything specific. Gastroscopy has revealed a circumferential vegetating lesion with a villous appearance on the second portion of the duodenum (Figure 1). This lesion became indurated and ulcerated at the third portion of the duodenum and was responsible for a tight stenosis. Biopsy findings were evocative of a slightly differentiated adenocarcinoma of biliopancreatic origin (cytokeratin 7+ and cytokeratin 20-).

A computerized tomography (CT) of the chest, abdomen and pelvis showed a circumferential lesion thickening of up to 2 cm at the level of the second and third portions of the duodenum with a bi-ductal dilatation (of the common bile duct and main pancreatic duct (Figure 2). An 8 mm adenomegaly could be noted in a retropancreatic position. No secondary lesion was observed. Magnetic resonance imaging (MRI) of the pancreas and magnetic resonance cholangiopancreatography (MRCP) confirmed this duodenal tissue thickening spreading from the proximal part of the second portion of the duodenum



**Figure 2 Magnetic resonance imaging.** A: MRI, coronal T1-weighted MR image with contrast showing the duodenal tissue thickening spreading from the second duodenum proximal part up to the duodenojejunal flexure (white arrow); B: MRI, coronal T2-weighted MR image showing the pancreatic duct and bile duct upstream swelling (white asterisks) without any secondary hepatic lesion. MRI: Magnetic resonance imaging.



**Figure 3 Intraoperative view of the pancreaticoduodenectomy showing lymph node dissection with excision of all cellular adipose tissues of the hepatic pedicle from the common hepatic artery (white arrow) and of the retroportal lamina (white asterisk).**

up to the duodenojejunal flexure, which was accountable for pancreatic duct and bile duct upstream swelling without any secondary hepatic lesion (Figure 2). Some very short contact adenomegalies were also observed there.

After a multidisciplinary meeting, pancreaticoduodenectomy was decided. Surgery was performed by RM. Intraoperatively, examination of the abdominal cavity did not find any peritoneal carcinomatosis or any other tumoural lesions. Picking analysis of para-aortic lymph nodes and two frozen section examination at the level of the upper part of the mesenteric pedicle did not help to identify any sign of malignancy. A pancreaticoduodenectomy was performed with a pancreaticogastric anastomosis and a hepatico-jejunal anastomosis. Lymph node dissection included all cellular adipose tissues of the hepatic hilum from the common hepatic artery and the retroportal lamina (Figure 3). Histological findings were suggestive of a duodenal embryonal carcinoma with pancreatic infiltration rated T4N0 (0 out of 48 lymph node) M0 R0 (Figure 4A). Specimen cells expressed cytokeratin 7 (Figure 4B), CD30 (Figure 4C) and SALL4 (Figure 4D) in

immunohistochemical staining.

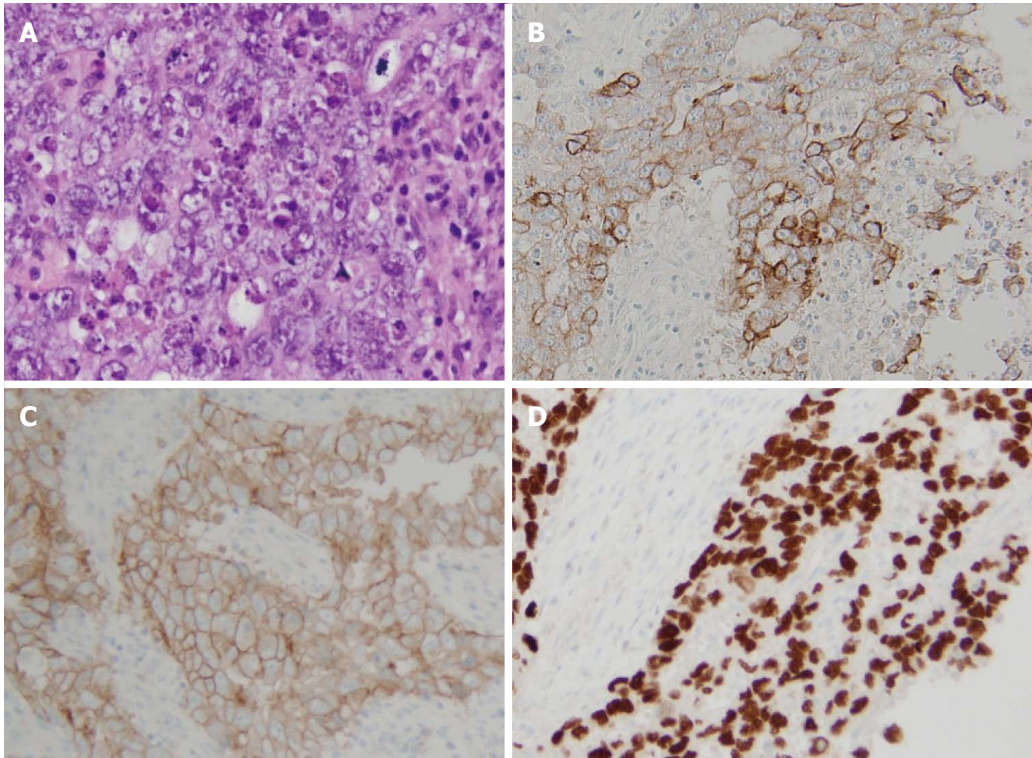
In order not to undermine a primitive cancer, a testicular ultrasound and CT-scan of the head and neck were performed without any repeat finding of the tumoural lesion. The case was presented to the oncology staff in order to put forward an adjuvant chemotherapy after cryogenic sperm preservation.

## DISCUSSION

Melanoma, renal, mammary, bronchopulmonary, gastric or testicular neoplasia<sup>[4]</sup> represent the most frequent metastatic tumours at the level of the small bowel, and particularly at the level of the duodenum. However, in young men, a testicular origin is to be favoured due to the preferential epidemiology of these cancers in this population group<sup>[1]</sup>. It should be noted that tumours with testicular germ cells are dichotomized between seminomas and non-seminomatous germ cell tumours, such as embryonal carcinomas and teratomas<sup>[5]</sup>. The sites of dissemination of testicular embryonal carcinomas which are most frequently mentioned are the lungs, the liver and the retroperitoneal space. The digestive tract provides a rare but known dissemination area (5%)<sup>[1]</sup>. Fu *et al*<sup>[6]</sup> reported the case of a young patient with a symptomatic duodenal metastasis which induced low digestive bleeding. On the other hand, we did not highlight any acute haemorrhagic episode but rather an iron deficiency with chronic haemorrhagic suffusion. The literature shows that patients presenting with bowel metastasis of an embryonic cancer develop, in most cases, a haemorrhagic symptom (14 out of 15 patients in the study by Fu *et al*<sup>[6]</sup>) or a digestive occlusion<sup>[6,7]</sup>. Tumoural dissemination by contiguity is the most frequently identified tumoural expansion mode at the expense of haematogenic or lymphatic pathways<sup>[6]</sup>. This accounts for the pancreatic lesions found in our case.

A primitive duodenal tumour is a hypothesis which must be discussed because clinical and imaging examinations have not led to the identification





**Figure 4** Histological findings of the operative specimen showing an embryonal carcinoma: pleomorphic cell proliferation with marked cytonuclear atypia, granular or clear cytoplasm, arranged in nests or solid pattern, associated with a fibrous stroma and an abundant lymphocytic inflammatory infiltrate (A). Specimen cells expressing cytokeratin 7 (B), CD30 (C) and SALL4 (D) are evidenced in immunohistochemical staining. A: HE  $\times 400$ ; B: Cytokeratin 7  $\times 200$ ; C: CD30  $\times 200$ ; D: SALL4  $\times 200$ .

of another primitive lesion. A duodenal primitive embryonal carcinoma was already described in a young man<sup>[8]</sup>. The hypothesis of a retro-differentiation of adenocarcinoma cells at the level of embryonal ectodermal cells of a metaplasia or differentiation in trophoblastic precursor seems to explain the histology of this primitive embryonal duodenal tumour<sup>[9]</sup>. As well as metastasis with choriocarcinoma of testicular origin, the tumoural cellular profile is not homogeneous<sup>[11]</sup>. This accounts for the biopsy initial result matching with a limited sample of the tumoural lesion. This last one was evocative of an adenocarcinoma with a biliopancreatic profile of an embryonal carcinoma at the final review.

Regarding tumour markers, high rates of  $\beta$  human chorionic gonadotropin are frequently described with non-seminomatous tumours, particularly choriocarcinomas. The alpha foetoprotein seems rather ascending in case of a non-seminomatous lesion, particularly embryonal carcinomas with a yolk sac differentiation<sup>[5]</sup>. We did not notice any modification of tumoural markers in our case as opposed to the case described by Küçüköner *et al.*<sup>[8]</sup>.

In conclusion, the onset of a strong anaemia in a young man requires endoscopic and imaging explorations in order to search for an embryonal tumoural lesion on the digestive tract since they are very frequently revealed by a chronic or acute haemorrhage.

## COMMENTS

### Case characteristics

A 25-year-old Bulgarian man was referred to our centre for a strong asthenia, and stinging epigastric pain getting worse since 1 mo.

### Clinical diagnosis

Clinical examination showed that the patient has a marked skin pallor with epigastric sensitivity without any abdominal mass nor recent weight loss or anorexia.

### Differential diagnosis

A slightly differentiated adenocarcinoma of biliopancreatic origin or another germ cell tumour.

### Laboratory diagnosis

The patient presented with haemoglobin of 6.8 g/dL in relation with an iron deficiency anaemia, associated with a cytolysis as well as an anicteric cholestasis.

### Imaging diagnosis

Radiological exams showed a circumferential lesion thickening of up to 2 cm at the level of the second and third portions of the duodenum with a bi-ductal dilatation without any secondary lesion.

### Pathological diagnosis

Histological findings were suggestive of a duodenal embryonal carcinoma with pancreatic infiltration associated with, in immunohistochemical staining, a specimen cells' expression of cytokeratin 7, CD30 and SALL4.

### Treatment

After a multidisciplinary meeting, pancreaticoduodenectomy was decided and



performed.

### Related reports

This is the second published case highlighting the duodenal primitive localisation of an embryonal carcinoma with pancreatic infiltration.

### Experiences and lessons

A pancreatic tumour in a young man may not be of biliopancreatic origin and requires extensive investigations to not ignore the uncommon entities as a germ cell tumour.

### Peer-review

This is an important case report of a most unusual embryonal tumour involving the duodenum and adjacent pancreas and needs publication.

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## Late-onset severe biliary bleeding after endoscopic pigtail plastic stent insertion

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### Abstract

Here, we report our experience with a case of severe biliary bleeding due to a hepatic arterial pseudoaneurysm that had developed 1 year after endoscopic biliary plastic stent insertion. The patient, a 78-year-old woman, presented with hematemesis and obstructive jaundice. Ruptured hepatic arterial pseudoaneurysm was diagnosed, which was suspected to have been caused by long-term placement of an endoscopic retrograde biliary drainage (ERBD) stent. This episode of biliary bleeding was successfully treated by transarterial embolization (TAE). Pseudoaneurysm leading to hemobilia is a rare but potentially fatal complication in patients with long-term placement of ERBD. TAE is a minimally invasive procedure that offers effective treatment for biliary bleeding.

**Key words:** Biliary stent; Plastic stent; Biliary bleeding; Pseudoaneurysm; Pigtail stent

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**Core tip:** Biliary bleeding after endoscopic pigtail plastic stent insertion is a rare but potentially fatal complication. Transarterial embolization (TAE) is a minimally invasive procedure that offers effective treatment for pseudoaneurysm. We report here a case of biliary bleeding caused by long-term placement of a pigtail plastic stent, which was inserted without removal of common bile duct stones due to the patient's age; the TAE treatment was successful. This case report will help similar patients yet to be encountered but likely to increase in number due to ageing of the world's population.

Yasuda M, Sato H, Koyama Y, Sakakida T, Kawakami T, Nishimura T, Fujii H, Nakatsugawa Y, Yamada S, Tomatsuri N, Okuyama Y, Kimura H, Ito T, Morishita H, Yoshida N. Late-onset severe biliary bleeding after endoscopic pigtail plastic stent insertion. *World J Gastroenterol* 2017; 23(4): 735-739 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/735.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.735>

## INTRODUCTION

Biliary bleeding is an uncommon cause of upper gastrointestinal bleeding and biliary tract obstruction. Biliary stent-related biliary bleeding that occurs after an endoscopic procedure has been reported less often than biliary bleeding related to the percutaneous transhepatic procedure; however, its incidence has been increasing due to the general increase in endoscopic interventions. Transarterial embolization (TAE) is an effective treatment for traumatic pseudoaneurysm of visceral arteries. We describe, herein, a patient with gastrointestinal bleeding and biliary tract obstruction due to intrabiliary rupture of hepatic arterial pseudoaneurysm, which was successfully treated by TAE and percutaneous transhepatic biliary drainage (PTBD).

## CASE REPORT

### Patient

A 78-year-old woman.

### Chief complaint

Hematemesis.

### Past related medical history

Thirty years before the current admission, the patient had undergone left hepatectomy, cholecystectomy and choledochectomy for intrahepatic and common bile duct (CBD) stones, in conjunction with Roux-en-Y and biliary reconstruction. At 1 year prior to the current admission, the patient had developed acute obstructive cholangitis due to CBD stones and had subsequently undergone single-balloon endoscopy. During that procedure, a 7 Fr pigtail type plastic biliary stent had been

inserted into the CBD, which was already dilated due to the previous biliary reconstruction. The proximal edge of the plastic stent had been placed, coiled around, in the CBD improperly (Figure 1). After the cholangitis had been resolved, the patient refused removal of her CBD stones, citing age as her reasoning.

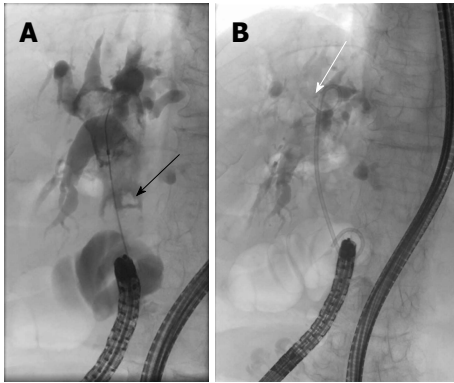
### Family history

We found nothing of significance in the patient's family history.

### Case presentation

The patient presented to our emergency department with repeated episodes of hematemesis, which had occurred for 6 h. She was not on any medications. On examination, she had pallor of the palpebral conjunctiva, subfebrile temperature (37.3 °C) and tachycardia (heart rate of 99 bpm); her blood pressure was 14.4/5.9 kPa and her respiration rate was 20/min. She had no abdominal pain. Laboratory findings were as follows; hemoglobin of 9.6 g/dL (normal: 11.3-15.2 mg/dL); aspartate aminotransferase/alanine aminotransferase of 155/58 IU/L (normal: 13-33/2-27 IU/L); alkaline phosphatase of 1208 IU/L (normal: 115-359 IU/L); total bilirubin of 1.8 mg/dL (normal: 0.3-1.2 mg/dL); C-reactive protein of 2.6 mg/dL (normal: < 0.3 mg/dL); white blood cell count of 10180/μL (normal: 4000-8000/μL). Non-enhanced computed tomography (CT) revealed dilation of the CBD, as well as high-density masses in the bile duct and in the afferent intestinal loop. Single-balloon endoscopy of the small intestine revealed copious fresh coagulum in the afferent loop, but the site of bleeding was not detected. Contrast-enhanced CT showed 13 mm × 10 mm pseudoaneurysm of the hepatic artery, proximal to the edge of the plastic biliary stent (Figure 2). Abdominal angiography was performed and the pseudoaneurysm was detected close to the stent in the anterior segment artery of the right hepatic artery. N-butyl-2-cyanoacrylate was applied to embolize the pseudoaneurysm (Figure 3), after which a PTBD tube was inserted in the direction from B6 (the inferior branch of right posterior bile duct, corresponding to S6 of the Couinaud classification adopted by Bismuth) to the afferent loop. As a result, the patient's jaundice improved and she was discharged on the 34<sup>th</sup> hospital day.

On the 3<sup>rd</sup> day after discharge, the patient returned to the emergency department with hematemesis, jaundice and shock. Enhanced CT showed extravasation of contrast medium at the site of the pseudoaneurysm, suggesting that the embolic material may have migrated to the jejunum. Emergent abdominal angiography was performed and the digital subtraction angiogram (DSA) revealed an aneurysm at A8 (the anterior arterial branch supplying the superior subsegment) with an arterio-biliary fistula (Figure 4A). Collateral arteries to the anterior segment of the right lobe were also seen. Embolization was performed for



**Figure 1** Endoscopic retrograde cholangiopancreatography image. A: Stones in common bile duct (black arrow); B: The plastic stent had been placed improperly, and the edge was coiled around in the common bile duct (white arrow). There was no sign of collapse or leakage in the biliary tract.



**Figure 2** Abdominal computed tomography angiography. A 13 mm × 10 mm pseudoaneurysm was found at the proximal anterior segment of the right hepatic artery; The hepatic side edge of the plastic stent (black arrow) was close to the pseudoaneurysm.

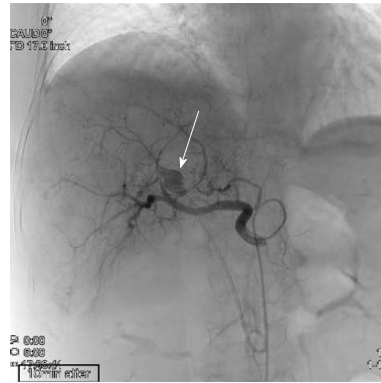
A8, proximal to the pseudoaneurysm (Figure 4B). Subsequently, the patient developed an S8 (Couinaud classification) liver abscess, which was treated successfully by percutaneous transhepatic drainage.

The patient has survived for 1 year since final discharge, without any new health issues.

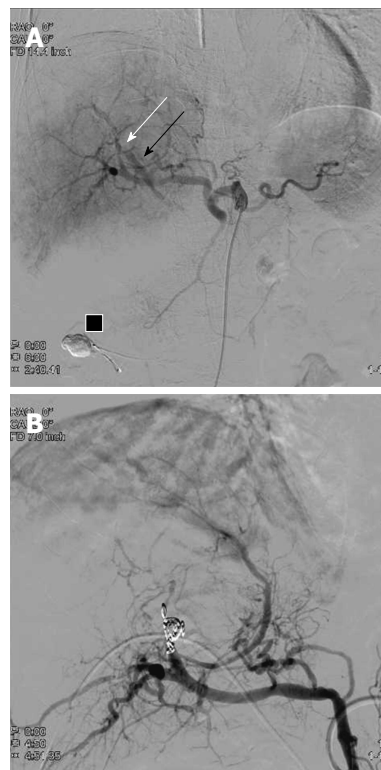
## DISCUSSION

Various reports about biliary bleeding have been published. Although biliary hemorrhage is relatively common after surgery, percutaneous transhepatic procedures and placement of the self-expanding metallic biliary stents<sup>[1-5]</sup>, it is rare after insertion of a plastic stent. Likewise, only a few articles have reported on biliary bleeding associated with plastic stents<sup>[6-8]</sup>.

We managed events of both life-threatening bleeding and acute obstructive suppurative cholangitis in a patient with a history of intestinal tract reconstruction. Due to this patient's previous surgery and severe bleeding, endoscopic biliary drainage was relatively difficult, so we chose to perform PTBD for obstructive



**Figure 3** The pseudoaneurysm was detected upon celiac axis injection, with the aneurysmal sac slightly lateral to the edge of the plastic stent (arrow). The sac was no longer seen on a selective digital subtraction angiography image after successful embolization. Blood flow was maintained in the anterior segment of the right hepatic artery.



**Figure 4** The 2<sup>nd</sup> digital subtraction angiography image. A: Selective digital subtraction angiography image of the common hepatic artery showing an arterio-biliary fistula (white arrow) between A8 and the common bile duct (black arrow). Embolic material that migrated into the afferent loop is present (black box); B: Proximal A8 embolization obliterated the pseudoaneurysm. Leakage of contrast medium was completely controlled after embolization.

jaundice. We presume that this hepatic pseudoaneurysm might have formed as a result of traumatic stimulation related to the plastic stent placement because it had been placed improperly, with its tip located at the site of the aneurysm. Yet, this may not be the only cause. The patient's history of cholangitis and CBD stones could have also contributed to the formation of the pseudoaneurysm. In daily clinical



practice, an ERBD stent is usually placed for temporary drainage. However, we sometimes leave biliary stones and the ERBD stent in elderly patients due to their comorbidities, so it is not uncommon for us to see such patients<sup>[9]</sup>.

In the study conducted by Green *et al.*<sup>[10]</sup>, cases of biliary bleeding, including those due to pseudoaneurysm, were mostly treated conservatively (43%) or by TAE (36%), with 20% undergoing surgery. In general, the preferred method to stop life-threatening biliary tract bleeding is TAE because it is less invasive and shows higher efficacy than the surgical approach<sup>[10]</sup>; moreover, TAE has lower reported rates of post-treatment mortality and morbidity than surgery<sup>[11,12]</sup>. However, cases of post-TAE hepatic artery occlusion and other complications, including fatal hepatic necrosis and intrahepatic abscess formation, have been reported<sup>[12-16]</sup>. Selective embolization of the vessel, as close as possible to the pseudoaneurysm, is desirable, both to reduce the risk of recurrent biliary bleeding and decrease the likelihood of hepatic necrosis<sup>[13]</sup>.

For the patient described herein, we initially performed selective embolization and maintained peripheral flow because the pseudoaneurysm sac was detected by the first angiogram. Upon repeat angiography, an arterio-biliary fistula was found, instead of a sac, so we were forced to perform non-selective embolization of the anterior segment of the right hepatic artery. After the second TAE, the patient developed local liver necrosis and an intrahepatic abscess of S8, but she was successfully treated by percutaneous drainage and antibiotics. She has remained well for more than 1 year since final discharge.

In conclusion, hepatic artery pseudoaneurysm is a rare but potentially fatal complication in patients with a long-term placement of ERBD. TAE is a minimally invasive procedure that offers effective treatment for pseudoaneurysm.

## COMMENTS

### Case characteristics

A post-choledochectomy 78-year-old woman, with a pigtail biliary stent and CBD stones left in after previous treatment for cholangitis 1 year prior, presented with hematemesis.

### Clinical diagnosis

Biliary bleeding from a hepatic pseudoaneurysm and obstructive jaundice were detected by imaging examination.

### Differential diagnosis

Combination of peptic ulcer and biliary stent obstruction.

### Laboratory diagnosis

Elevated hepatobiliary system enzyme and C-reactive protein levels and leukocytosis suggestive of obstructive cholangitis. Anemia suggestive of bleeding.

### Imaging diagnosis

Contrast-enhanced computed tomography scan showed a 13 mm × 10 mm

pseudoaneurysm of the hepatic artery, proximal to the edge of the plastic biliary stent.

### Treatment

Transarterial embolization (TAE) and percutaneous transhepatic biliary drainage.

### Related reports

Although biliary hemorrhage is relatively common after surgery, percutaneous transhepatic procedures and placement of self-expanding metallic biliary stents, it is rare after insertion of a plastic stent.

### Term explanation

The Couinaud classification is used to describe functional liver anatomy. It has emerged as the preferred anatomy classification system since it divides the liver into eight independent functional units.

### Experiences and lessons

The pigtail plastic stent is often used for temporary biliary drainage. However, it is important to remember that this stent type can cause fatal bleeding. TAE is a more effective and less invasive treatment for biliary bleeding than surgery.

### Peer-review

Although trauma caused by the proximal tip of the pigtail stent is highly suspicious, the authors explain that there might be other additional factors for the formation of the hepatic pseudoaneurysm.

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**L- Editor:** A **E- Editor:** Liu WX



## Correcting for non-compliance when determining colonic transit time with radio-opaque markers

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**Author contributions:** Ibarra A, Olli K and Ouwehand AC wrote this letter.

**Conflict-of-interest statement:** Alvin Ibarra, Kaisa Olli and Arthur C Ouwehand were employees of DuPont during the preparation of the Letter to the Editor and declare no other conflicts of interest.

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### Abstract

The use of radio-opaque markers and abdominal X-ray is the standard method for determining colonic transit time (CTT). However, when there are deviations in the intake of these markers by participants in clinical trials it is desirable to improve observations by introducing

corrections, where possible. To date, there is no standard procedure to adjust for such deviations. This report proposes a series of alternatives based on possible scenarios for deviations from the intended intake of radio-opaque markers. The proposed method to correct for missed or delayed consumption of radio-opaque markers can help to increase the accuracy of the CTT measurements in clinical trials.

**Key words:** Colonic transit time; Gastroenterology; Gut transit time; Radio-opaque marker; X-ray

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**Core tip:** Ibarra A *et al* correcting for non-compliance when determining colonic transit time with radio-opaque markers.

Ibarra A, Olli K, Ouwehand AC. Correcting for non-compliance when determining colonic transit time with radio-opaque markers. *World J Gastroenterol* 2017; 23(4): 740-742 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i4/740.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i4.740>

### TO THE EDITOR

The use of radio-opaque markers and abdominal X-ray is the standard approach for determining colonic transit time (CTT)<sup>[1,2]</sup>. This technique is simple, inexpensive, reliable and reproducible<sup>[1,2]</sup>. The principle of the method is based on the consumption of radio-opaque markers for six consecutive days, creating an equilibrium between incoming and outgoing markers, followed by an abdominal X-ray on day 7. The number of markers that can be identified on the X-ray is an expression of CTT per the following equation:

$$CTT = n_i \times (t/N)$$

**Table 1** Examples of scenarios of compliance and non-compliance with radio-opaque marker consumption and their potential correction

Days	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5	Scenario 6	Scenario 7
1	●	●	●	●	●	●	●
2	●	●	○	●	●	●	●
3	●	● (± $\alpha$ h)	○	●	● <sup>1</sup>	●	●
4	● <sup>1</sup>	● <sup>1</sup>	●	● <sup>1</sup>	● <sup>1</sup>	○	● (+ 4 h) <sup>1</sup>
5	● <sup>1</sup>	● <sup>1</sup>	●	● (± $\beta$ h) <sup>1</sup>	● <sup>1</sup>	● <sup>1</sup>	● <sup>1</sup>
6	● <sup>1</sup>	● <sup>1</sup>	● <sup>1</sup>	● <sup>1</sup>	○ <sup>1</sup>	● <sup>1</sup>	● <sup>1</sup>
<i>n</i>	24	24	24	24	24	24	72
<i>t</i> (h)	24	24	24	24	24	24	72 (-4)
<i>ni</i> (X-ray)	70	52	24	60	69 (+24)	48	65
CTT (h)	70	52	24	60	93	48	61.4

<sup>1</sup>The period indicated by the estimated colonic transit time (CTT)-*i.e.*, the markers on the days that are visualized on the X-ray. Scenarios 1 to 6: *n* is the total number of markers ingested each day, *t* is the time between marker ingestion in hours, *ni* is the number of markers observed on X-ray, and CTT is the estimated colonic transit time. Scenario 7: *n* is the total number of markers ingested during the estimated CTT period, *t* is the number of days in hours on which the markers are detected minus the number of hours by which consumption is delayed, *ni* is the number of markers observed on X-ray, and CTT is the estimated colonic transit time. Missed markers are indicated as open circles, consumed markers are indicated as closed circles.  $\alpha$  and  $\beta$  are hypothetical deviation times of radio-opaque marker consumption. Values between parenthesis indicate corrections.

where *n<sub>i</sub>* is the number of markers that is observed on X-ray, *t* is the time between the ingestion of markers in hours and *N* is the total number of markers that is ingested each day. Thus, if markers are consumed at 24-h intervals and the number of markers per day is 24, CTT equals the total marker count on the X-ray<sup>[1,2]</sup>.

This method was developed to diagnose constipation subtypes: those with "normal" transit times and those with "slow transit", the latter of which can be subdivided into "colonic inertia", "hindgut dysfunction" and "outlet obstruction"<sup>[1,2]</sup>. In addition, this protocol is used for research purposes to study the effects of certain dietary interventions on CTT.

This procedure requires compliance with marker consumption and abdominal X-ray. However, patients and volunteers in nutritional studies might fail to adhere to the protocol. Non-compliance can lead to substantial underestimation of CTT. Bouchoucha and colleagues<sup>[3]</sup> determined the influence of non-compliance on the diagnosis (delayed transit and site of the delay) and concluded that skipping the ingestion of markers for one or two days still allows for an acceptable clinical diagnosis.

However, it is unknown whether non-compliance is also tolerable for dietary intervention studies. Dietary intervention studies that involve, for example, fiber, prebiotics and probiotics will often aim to detect modest changes in CTT<sup>[4]</sup>. Non-compliance with radio-opaque marker consumption is likely to increase the variations between baseline and treatment values and between those of the treatments and placebo, impeding the ability to detect subtle changes due to a nutritional intervention. Thus, what may be acceptable for a diagnosis might be unsuitable for dietary research. In this report, we propose a method for correcting some of the errors that are introduced by non-compliance with radio-opaque marker consumption and X-ray.

Table 1 lists various scenarios for radio-opaque

marker consumption. In these examples, it is assumed that 24 markers are to be consumed per day. Scenario 1 represents full compliance with marker consumption; thus, as discussed above, the number of markers that are observed indicates the CTT. Deviations from the marker consumption protocol can be divided in two types: those that have occurred "outside" of versus "within" the calculated CTT. To examine the influence of non-compliance and determine whether a correction can be performed to mitigate it, the day and time at which the non-compliance occurred must be known. Instances of non-compliance that took place "outside" of the CTT can be assumed to have had no influence on the transit time as calculated (Scenario 2). Had the markers that were omitted or consumed at the wrong time been ingested as the protocol requires, they could reasonably be assumed to have been excreted (Scenario 3). Thus, Scenarios 2 and 3 do not require any correction and are assumed to have accurately estimated the CTT, consistent with Bouchoucha and colleagues<sup>[3]</sup>.

When a day on which the markers were consumed too early or too late falls "within" the calculated CTT (Scenario 4), no correction is needed, because all markers will be observed, regardless of whether they are consumed at the scheduled time. If the markers are missed (*i.e.*, not taken) on a day "within" the calculated CTT, we can assume that had those markers been consumed, they would have been detected by X-ray (Scenario 5). Thus, a correction can be made, adding the missed markers. A similar correction can be made if the X-ray is delayed by a day (*i.e.*, without providing extra radio-opaque markers), because such a case can be treated as if the markers that were scheduled for the last day were not consumed (Scenario 5). When a marker has been missed just outside of the calculated CTT (Scenario 6), no correction can be made, because it is uncertain whether the markers, if consumed, would have been detected. When radio-



opaque markers have been consumed too late and when these markers are the “oldest” that are still observed, according to the calculated CTT (Scenario 7), a correction can be applied, assuming that instead of  $t = 24$  (in the example),  $t$  is the number of days (in hours) on which the markers are detected minus the number of hours by which consumption was delayed, and  $N$  is the number of markers that was consumed during this period.

To correct the various scenarios accurately, the exact day and time of non-compliance of radio-opaque marker intake during the intervention must be known. These values can be tracked with a diary that is completed by the subject throughout the administration of radio-opaque markers according to the protocol, helping to identify the precise time of non-compliance.

In conclusion, the proposed method to correct for missed or delayed consumption of radio-opaque

markers can help to increase the accuracy of the CTT measurements in clinical trials.

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