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Gastric acid level of humans must decrease in the future

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Abstract

Proton pump inhibitors strongly inhibit gastric acid production, but digestion problems do not generally arise. We can intake almost ordinary food even after total gastrectomy. Small intestine itself can digest and absorb food using various digestive enzymes without digestion in the stomach. The pH level of gastric acid in humans is much lower than that of most animals, and very close to that of carrion-eating animals called scavengers. It is assumed that ancient humans became bipedal approximately 4 million years ago. It was difficult for humans, who just started unstable bipedal locomotion, to catch quadrupedal-walking animals that can move faster, without special hunting tools. They may have eaten remaining carcasses, which is mainly the leftovers of carnivora species, as animal-derived food. The benefit to produce a volume of gastric acid for humans is carrion eating, in which disinfection by gastric acid is important. Humans produce a high concentration of gastric acid to enable consumption of a diet containing some bacteria and support this lifestyle by consuming significant energy to protect themselves from gastric acid. Now, the opportunity for strong deleterious bacteria to enter the gastrointestinal tract has decreased because of the organized clean environment. If this hygienic environment is maintained for a long time, our gastric acid level must be decreased gradually.

Key Words: Gastric acid; Proton pump inhibitor; Digestion; Scavenger; Carrion eating; Ancient humans

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Core Tip: We can intake almost ordinary food even after total gastrectomy. Small intestine itself can digest and absorb food without digestion in the stomach. The pH level of gastric acid in humans is much lower than that of most animals, and very close to that of carrion-eating animals. The benefit to produce a volume of gastric acid for humans is carrion eating, in which disinfection by gastric acid is important. Now, we

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have decreased risk of food poisoning because of clean environment. If this hygienic environment is maintained for a long time, our gastric acid level must be decreased gradually.

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INTRODUCTION

In recent years, the number of patients with reflux esophagitis has increased due to life-span extension and food satiation, and proton pump inhibitors (PPIs), strong acid reducers, are increasingly prescribed. People are concerned about side effects of PPIs associated with the increase in the number of prescription^[1]. However, it is generally understood that PPIs are relatively safe; PPIs strongly inhibit gastric acid production, but digestion problems do not generally arise, with some impaired absorption of vitamin B12. We discuss gastric acid in humans by focusing on why strong inhibition of gastric acid does not significantly affect digestion.

GASTRIC ACID IN HUMANS

Digestion by gastric acid and pepsin is potent and can completely decompose animal-derived food. However, the decomposition takes time. For example, a snake swallows a whole animal and digests it at a gastric acid level (pH 1.5-2.0), the same as that in humans, but it takes approximately one week for the digestion process, depending on the size of the swallowed animal^[2,3]. Modern humans maintain food in the stomach for approximately four hours, and there is a limitation to the digestive ability of the stomach^[4]. The less cooked food that might have been served by ancient people would need more than four hours to digest in the stomach. Of course, the digestion time in the stomach in the ancient period might have been longer than that in present time, but strong digestive ability of the small intestine in modern humans is not necessary if digestion in the stomach is adequate.

We can intake almost ordinary food even after total gastrectomy, although we need to take food in small increments since the stomach cannot hold food. We are able to do so because the small intestine can digest and absorb food using various digestive enzymes without digestion in the stomach. Although impaired absorption of vitamins and minerals occurs due to the evolved absorption system of humans, the small intestine can still digest food. We captured how solid food is digested in the small intestine using capsule endoscopy and found that not only carbohydrates and meat but also the cytoplasm of plants are absorbed in the small intestine^[5]. In other words, we endoscopically confirmed that the passage of current formed food through the stomach does not influence digestion in the small intestine. Because a patient who takes PPIs does not have any major digestive/absorption problems, the importance of gastric acid in the digestive system of humans is thought to be low. Nevertheless, the gastric acid level of humans is considerably high compared to that of many other animals^[6].

The pH of gastric acid in humans is 1.5-2.0. According to a report summarized by Beasley *et al*^[6], the pH level is much lower than that of most animals, including anthropoids (≥ 3.0), and very close to that of carrion-eating animals called scavengers, such as falconine birds and vultures^[6]. This report shows a trend that pH in the stomach is the highest in herbivores and decreases in order of carnivores, omnivores, and scavengers (Figure 1). The pH of humans is lower among omnivores and equal to scavengers. Herbivores eat raw plants that are protected by sunlight and antibacterial agents produced from the plant, therefore have less-toxic bacteria. Also, normal carnivores eat non-festering meat that is freshly killed. Carrion that is left over of such carnivores has no small highly virulent bacteria and carrion-eating needs a system to disinfect the bacteria. It has been thought that one of the disinfection system is the strong acid in the stomach. Living organisms use great energy to produce gastric acid.

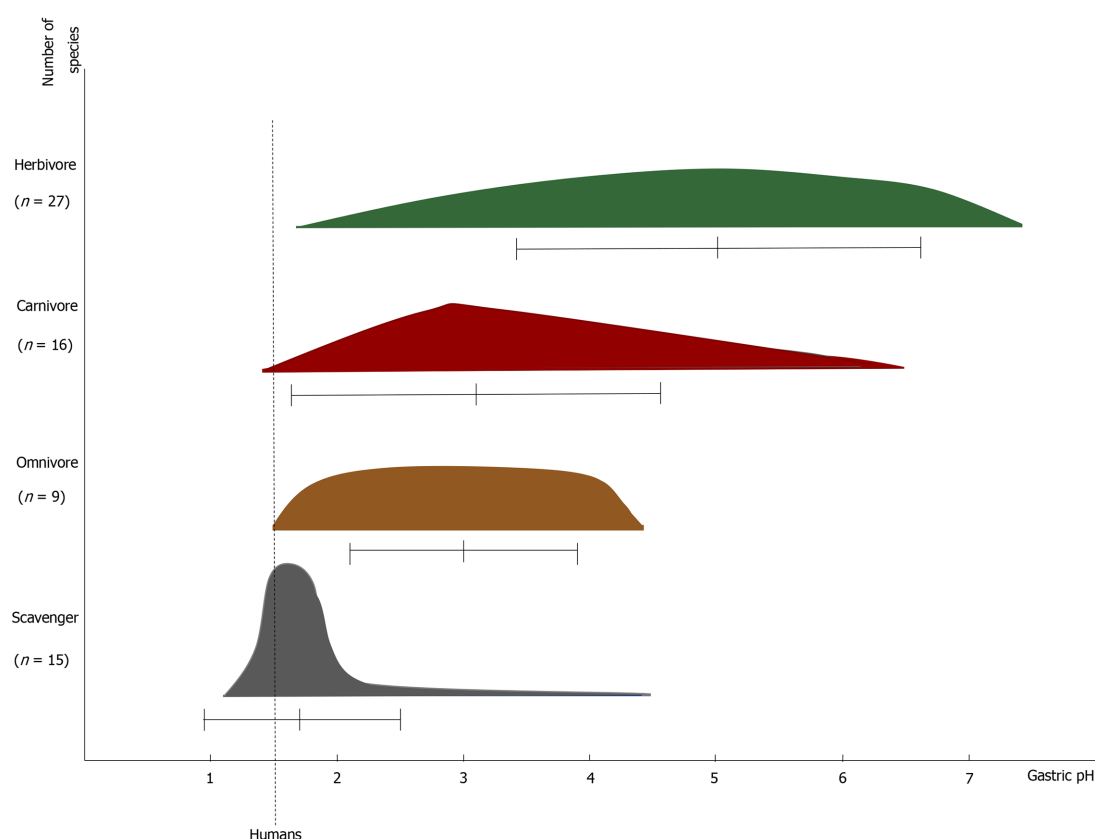


Figure 1 pH of mammals and avian species according to feeding habit. Forty-two mammals and 25 avian species except human summarized by Beasley *et al.*^[6] were categorized in herbivore, carnivore, omnivore, and scavenger and the distribution of species against pH in the stomach was expressed in a scheme. The scheme was deformed due to its large variance. mean \pm SD of the pH is inserted below.

First, they need energy to produce gastric acid itself^[7]. In addition, they need to protect the gastric mucosa from gastric acid, protect against back-flow of gastric fluid in the esophagogastric junction, and neutralize gastric acid immediately in the duodenum for protection^[8,9]. The benefit of the effort to produce gastric acid for humans is carrion eating, in which disinfection by gastric acid is important.

It is assumed that ancient humans became bipedal approximately 4 million years ago^[10]. It was difficult for humans, who just started unstable bipedal locomotion without special hunting tools, to catch quadrupedal walking animals that can move faster. They may have eaten remaining carcasses, which is mainly the leftovers of carnivora species (bone marrow), as animal-derived food^[11]. This hypothesis has been proven from bone-destroying stone artifacts. In other words, humans may have survived and developed as a carrion-eating animal. To enable this method of subsistence, humans needed enhanced bactericidal power, and individuals adopted increased gastric acid levels, which is preserved in modern humans. This high gastric acid level enables relatively long-term use of animal-derived food, which is difficult to preserve, and may support a wide variety of dietary habits of humans as omnivores.

CONCLUSION

As stated above, humans have adjusted to the environment as omnivorous mammals that can consume carrion. Humans produce a high concentration of gastric acid to enable consumption of a diet containing some bacteria and support this lifestyle by spending significant energy to protect themselves from gastric acid. We have decreased the opportunity for strong deleterious bacteria to enter the gastrointestinal tract because of the organized clean environment, especially in developed countries. If this hygienic environment is maintained for a long time, our gastric acid level must be decreased gradually, with some modification in the absorption system.

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First United Arab Emirates consensus on diagnosis and management of inflammatory bowel diseases: A 2020 Delphi consensus

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Abstract

Ulcerative colitis and Crohn's disease are the main entities of inflammatory bowel disease characterized by chronic remittent inflammation of the gastrointestinal tract. The incidence and prevalence are on the rise worldwide, and the heterogeneity between patients and within individuals over time is striking. The progressive advance in our understanding of the etiopathogenesis coupled with an unprecedented increase in therapeutic options have changed the management towards evidence-based interventions by clinicians with patients. This guideline was stimulated and supported by the Emirates Gastroenterology and Hepatology Society following a systematic review and a Delphi consensus process that provided evidence- and expert opinion-based recommendations. Comprehensive up-to-date guidance is provided regarding diagnosis, evaluation of disease severity, appropriate and timely use of different investigations, choice of appropriate therapy for induction and remission phase according to disease severity, and management of main complications.

Key Words: Ulcerative colitis; Crohn's disease; Infliximab; Adalimumab; Vedolizumab; Ustekinumab; Tofacitinib

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Core Tip: The prevalence of ulcerative colitis and Crohn's disease is on the rise. The advance in our understanding of the etiopathogenesis with an unprecedented increase in therapeutic options have changed the management towards tailoring evidence-based interventions. In this consensus, the diagnosis of inflammatory bowel diseases are based on the clinical evaluation and a combination of endoscopic, histological, radiological, and/or biochemical findings. The therapeutic options have been revised in view of the treatment goals, which now aim to treat beyond "symptoms" to achieve mucosal healing when possible and to minimize intestinal injury and bowel damage with resultant disability.

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INTRODUCTION

Inflammatory bowel diseases (IBD) comprising Crohn's disease (CD) and ulcerative colitis (UC), are chronic, relapsing, progressive, and potentially disabling conditions affecting the gastrointestinal (GI) tract^[1-6]. The incidence and prevalence of IBD is increasing worldwide at an alarming rate including in African countries, the Middle East, and the Asia Pacific^[4,7-12]. Meanwhile, the aetiology remains unknown, although IBD is believed to be triggered by aberrant immune responses often in a genetically predisposed individual to certain environmental triggers^[13-15]. Rapid strides in our understanding of the etiopathogenesis of IBD coupled with an unprecedented increase in therapeutic options including biological and "small molecule" therapies have redefined our treatment goals, which now aim to treat beyond "symptoms" to achieve mucosal healing when possible and limit intestinal injury and bowel damage with resultant disability^[16-21].

Due to the progressive change of IBD management, a two-round Delphi technique method was used to reach a consensus among nine expert gastroenterologists working in the United Arab Emirates.

METHODOLOGY OF THE CONSENSUS

The consensus was promoted and supported by the Emirates Society of Gastroenterology and Hepatology; the president (MAK) selected the members of the committee among the public and private sectors across different Emirates of United Arab Emirates based on their competence on IBD. An extensive research of the relevant scientific literature was made by a medical writer company on Medline and EMBASE databases from the first published until December 2018 and then updated to December 2019 and made available to the committee members. Clinical priorities covered by the consensus were: adult cases, definition, clinical characteristics and diagnosis, investigations including imaging, monitoring, treatment of active phases and maintaining the remission, managing of perianal disease, and prevention of postsurgical recurrence in CD. Published guidelines and consensus from the European Crohn's Colitis Organization, British Society of Gastroenterology, American College of Gastroenterology, and American Gastroenterological Association were also taken into consideration.

Draft of statements/recommendations was compiled by a medical writer and distributed to the entire panel for the first assessment of the agreement. The statements were finalized by the panel in two face-to-face meetings. A Likert-type scale (1, strongly disagree; 2, disagree; 3, neutral; 4, agree; and 5, strongly agree) was used to measure the agreement. In cases of disagreement, uncertainty, or agreement less than 75% of participants, the panelists were required to submit comments and propose changes. In case of debate or conflict, revoting was recommended. The updated statements were then re-evaluated by the entire panel in the second round.

An agreement of 75% or more represented a strong recommendation; 50%-74.9% represented a recommendation, and less than 50% was represented as a suggestion. Percentage of the final agreement is given between brackets ([Table 1](#)) ([Supplementary Table 1](#)).

CLINICAL FEATURES AND DIFFERENTIAL DIAGNOSIS

UC

Statement 1: UC is suspected when a patient, especially in late adolescence or early adulthood, reports having bloody diarrhoea for more than two weeks, rectal bleeding, rectal urgency, tenesmus, mucopurulent exudate, faecal incontinence, nocturnal defecation, and crampy abdominal pain. [100]

Statement 2: Examination of suspected patients with mild or moderate UC is usually unremarkable; digital rectal examination can be done to confirm fresh blood in the rectum. [100]

Statement 3: IBD is suspected when the patient has a family history of UC or CD. [88.8]

Statement 4: Severe colitis is suspected when a patient presents with increased bowel frequency (six or more per day), abdominal pain/tenderness, anorexia, weight loss, tachycardia, reduced bowel sounds, and fever. [100]

UC is characterized by mucosal inflammation affecting the large intestine, typically starting in the rectum but often extending proximally to involve the colon to a variable distance^[4,22,23]. Rectal sparing may occur in up to 3% of patients, and patchy inflammation may be present in those treated with topical therapy^[24-27]. Likewise, localized inflammation at the cecum or around the appendix as well as so-called backwash ileitis may also be found^[24-26]. UC may present at any age but typically presents between ages 15-30 and follows a relapsing-remitting course^[4]. Up to 90% of patients may experience one or more episodes of relapse after the first attack, and active disease with frequent relapses in the first two years after diagnosis is linked with a worse prognosis^[22,28]. UC typically presents with rectal bleeding, diarrhoea, tenesmus (a sense of pressure), and urgency often associated with crampy abdominal pain, faecal incontinence, and nocturnal diarrhoea^[4,29].

CD is characterized by transmural inflammation affecting any part of the digestive tract but typically involves the ileum and colon in a typically discontinuous manner^[1,30,31]. Hallmark clinical symptoms are abdominal pain, diarrhoea (with or without bleeding), weight loss, fatigue, anaemia, recurrent fistulae, and growth failure (in children)^[30]. A family history may raise the index of suspicion, but disease activity or severity are unaffected by family history^[32,33]. Although more susceptibility loci are

Table 1 Breakdown of the agreement on different statements (*n* = 117)

Agreement (%)	Number of statements	Strength of recommendation
100	35	STRONG recommendation
88.8	48	STRONG recommendation
77.7	26	STRONG recommendation
66.6	3	Recommendation
50.0	4	Recommendation
37.5	1	Suggestion

known for both CD and UC, only a few have been associated with disease outcomes, such as the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) polymorphism, which is associated with a more progressive disease course in CD with a higher risk of intestinal obstruction and need for surgery^[34,35]. Genetic testing cannot be used to diagnose IBD^[36,37].

Statement 5: The diagnosis of IBD is established by clinical evaluation and a combination of endoscopic, histological, radiological, and/or biochemical investigations. [100]

The diagnosis of IBD is therefore not based on a single diagnostic criterion but in fact a combination of clinical features, biochemical (including stool) examination, endoscopic assessment, histopathology, and in the case of CD radiological investigations to visualize the small intestine^[38].

At diagnosis, blood counts, electrolytes, liver enzymes, and inflammatory markers [C-reactive protein (CRP) and stool calprotectin] should be checked^[39–41]. Physical examination may be unremarkable in mild to moderate disease, although digital rectal examination may show evidence of fresh blood in the rectum. Anaemia (iron and/or vitamin B₁₂ deficiency) and an elevated platelet count are common at diagnosis^[39–41]. Serum CRP is an acute-phase reactant produced by the liver that rises in any case of inflammation including active IBD^[42]. Its half-life is of 19 h, which makes it a useful marker to detect and monitor inflammation. Erythrocyte sedimentation rate (ESR) is a nonspecific marker of inflammation that may be increased in patients with IBD. Although sometimes helpful individually, it is not specific for active IBD^[41]. Because up to half of patients with IBD can have normal CRP and ESR values when the activity is mild, their use in monitoring is limited^[41]. An elevated CRP may correlate with the clinical activity in CD but less well with UC except in acute severe UC^[29,30,43,44]. Faecal calprotectin (FC) is a calcium binding protein, derived from neutrophils with a specific release in the presence of intestinal inflammation, correlating well with endoscopic findings, and is a good marker of relapse and response to treatment and in differentiating patients with IBD from those with irritable bowel syndrome^[45,46]. It lacks specificity to distinguish IBD from other causes of bowel inflammation^[47].

Stool cultures and *Clostridium difficile* toxin assay are recommended to rule out infective aetiology^[48,49]. Loose stools for over 6 wk help to distinguish infective causes from inflammatory bowel disease^[50,51].

The diagnosis of IBD needs an endoscopic assessment, typically an ileo-colonoscopy for CD and sigmoidoscopy in the case of UC followed by an ileo-colonoscopy within 12 mo of diagnosis to establish disease phenotype, determine full disease extent, and risk stratify for dysplasia^[52–54]. Accordingly, UC is characterized according to the Montreal classification as proctitis (E1), left-sided colitis (E2) (up to splenic flexure), and extensive colitis proximal to the splenic flexure or pancolitis up to the cecum (E3)^[55,56]. As per the Truelove and Witts criteria, mild colitis is defined as fewer than four bowel movements daily with no fever, normal heart rate, haemoglobin > 11g/dL, and ESR < 20 mm/hr. The disease is defined as severe by bowel frequency greater than six times a day along with fever, tachycardia, anaemia, or an elevation in ESR^[57].

At histology, a combination of features including basal plasmacytosis, diffuse crypt atrophy and distortion, villous surface irregularity, and mucous depletion support a diagnosis of UC when in agreement with clinical symptoms^[31]. Although no single feature is diagnostic^[31,58].

CD

Statement 6: CD is suspected when a patient, especially young, presents with abdominal pain, weight loss, constipation, or chronic diarrhoea. [88.8]

Statement 7: A patient with CD commonly presents with systemic symptoms of malaise, anorexia, or fever. [77,7]

Statement 8: Symptoms of CD are nonspecific and mimics that of irritable bowel syndrome. Unexplained anaemia and growth failure should be considered to avoid delayed diagnosis. [100]

Statement 9: The symptoms of acute terminal ileal CD may be mistaken for acute appendicitis. [88,8]

Statement 10: Physical examination of patients with CD may reveal tenderness or a palpable mass. Some may present with perianal disease (abscess, fistula, fissure) [88,8]

Statement 11: Infections or drug-induced colitis must always be excluded [88,8]

CD is a chronic, progressive inflammatory disorder of the GI tract^[1]. Classical symptoms include abdominal pain, chronic diarrhoea and fatigue, although patients may also present with weight loss, fever, anaemia, growth failure, recurrent fistulae, and extraintestinal manifestations^[59]. Physical examination may reveal these findings. Additionally, abdominal examination may reveal tenderness particularly in the right iliac fossa or a palpable mass in the affected area. Some patients may also present with perianal disease in the form of an abscess, fistula, or fissure.

The most common symptom of CD is chronic diarrhoea, although some patients may have a normal bowel habit^[60]. Terminal ileal disease may be mistaken for acute appendicitis. Fatigue is a very common and less well understood symptom of CD, which may be multifactorial and stem from inflammation, anaemia, and micronutrient, vitamin, and mineral deficiency. Fever, weight loss, or even growth failure (in younger individuals) may be presenting features^[1,59]. This myriad of symptoms can often be mistaken for irritable bowel syndrome, which may delay the diagnosis of CD^[1,2,16].

In a population-based study from Manitoba, Canada, 41 % of patients had a three year or greater delay in diagnosis from the onset of symptoms^[61]. In another study, the time to diagnose was on average seven years in patients with CD, even when those individuals meeting Rome criteria for irritable bowel syndrome were excluded from the analysis as compared with less than one year to diagnosis for patients with UC^[62]. Stool cultures and *Clostridium difficile* toxin assay are recommended to rule out infective aetiologies^[48,49]. Loose stools for over 6 wk help to distinguish infective causes from IBD^[50,51]. Drug-induced colitis must also be excluded^[59,63].

Statement 12: Genetic or serological testing is currently not recommended yet for routine diagnosis of IBD. [100]

Statement 13: If diagnosis of IBD is in doubt despite an interval of appropriate treatment, a repeated endoscopy is required. [88,8]

The diagnosis of CD is based on a combination of investigative modalities. A clinical history and examination as appropriate, ileo-colonoscopy, small intestinal radiological assessment, laboratory tests, and histological examination of mucosal biopsies from endoscopy^[64]. Mucosal biopsies from endoscopy or surgical resection specimens may show inflammation and/or crypt distortion. Endoscopic features more suggestive of CD are discontinuous segments of disease ("skip lesions"), ileal involvement, and granulomatous inflammation. Approximately 3% of UC patients may be reclassified as Crohn's colitis^[65]. Conversely, 0.6%-3% will be reclassified to alternative colitis after initial diagnosis of CD^[66].

CD may be diagnosed on surgical samples when at least three histological features suggestive of CD [segmental crypt architectural abnormalities and mucin depletion, mucin preservation at the active sites, and focal chronic inflammation (without crypt atrophy)] are found in the absence of granulomas, or when an epithelioid granuloma is detected in combination with an additional feature^[31,58]. Some patients cannot be assigned to either CD or answer to colitis and are labelled as IBD unclassified^[65,66]. Thus, if the diagnosis of IBD is in doubt despite an interval of appropriate treatment, repeat endoscopy is required to help establish the diagnosis. Despite a growing number of identified susceptibility loci in both CD and UC, few have been associated with disease outcomes, such as the nucleotide-binding oligomerization domain-containing protein 2 polymorphism, which is associated with a more aggressive disease course in CD with a higher risk of intestinal stenosis and need for surgery^[34,35]. Genetic testing cannot be used to diagnose IBD^[36,37].

CLASSIFICATION AND SEVERITY

UC

Statement 14: UC disease extent is defined by the maximal macroscopic extent at colonoscopy and classified as proctitis, left-sided colitis, and extensive colitis (according to the Montreal classification). [100]

The Montreal classification in adults^[55] and Paris classification in children^[67] are useful for the phenotypic classification of patients.

Knowledge of disease extent is a key determinant of prognosis as the risk of colectomy hinges on disease extension^[68]. A systematic review reported a ten-year colectomy rate of 19% for extensive colitis, 8% for left-sided colitis, and 5% for proctitis^[4]. Further risk factors for colectomy are male gender, young age, and elevated inflammatory biomarkers at diagnosis^[4]. Backwash ileitis was associated with more aggressive disease and with primary sclerosing cholangitis^[4]. Those with extensive colitis also have the highest risk of developing colorectal cancer^[69,70]. Notably, disease extent can change after diagnosis^[56,71]. Up to half with proctitis or proctosigmoiditis will develop more extensive disease. Of patients with proctitis initially, 10% will ultimately have extensive colitis^[4,22,71]. Disease extent should be reported as the maximum extent of inflammation, remembering that information may regress over time. Furthermore, and especially in quiescent UC, endoscopic appearances may be underestimated. Biopsies should be taken to determine the full extent of involvement.

Statement 15: According to severity, UC is either in remission characterized by the absence of symptoms and the absence of an endoscopic acute inflammatory changes, or an active disease characterized by the presence of symptoms and endoscopically active mucosal findings. [77.7]

Statement 16: The severity of UC can be classified into mild, moderate, and severe based on clinical symptoms and signs, blood tests, and endoscopy. [100]

Although the Truelove and Witts criteria (discussed under statement 5) are relatively easy to use and useful in determining the opportunity for hospital admission, the index is not designed to provide a measure of severity and does not include nocturnal symptoms. Furthermore, it does not consider endoscopic severity.

Several scoring systems are available to classify disease severity in UC^[72]. They aid objective assessment of disease and guide therapeutic and monitoring strategies. Their use allows clinicians to monitor patient progress during follow-up^[17,54].

The simple colitis clinical activity index is a reliable and responsive score with clear definitions for clinical response and remission^[73]. Simple colitis clinical activity index scores range between 0 and 19 points and include nocturnal bowel movements and faecal urgency, which affect patient quality of life^[73]. A SCCAI score < 2 indicates clinical remission, and a decrease of > 1.5 points from baseline correlates with patient-defined significant improvement^[74].

The Mayo Clinic Score (MCS) or index (partial Mayo Clinic index and endoscopic subscore) and ulcerative colitis disease activity index (UCDAI) represent a composite assessment of clinical symptoms (stool frequency and rectal bleeding) and endoscopic severity^[75,76].

Although not validated, the MCS is easy to calculate and has been applied for evaluating therapeutic outcomes in clinical trials^[77]. The score evaluates stool frequency, rectal bleeding, a physician's global assessment, and mucosal inflammation at endoscopy with a value ranging from 0 to 3 and a maximum score of 12 points. Clinical improvement is generally defined as the drop of baseline scores by ≥ 3 points whereas clinical remission as an overall score ≤ 2 (and no individual sub-score > 1) or UCDAI ≤ 1 ^[75,76]. A partial Mayo score (PMS) < 1 indicates remission^[17]. The PMS uses the clinical elements of the MCS and is well correlated with perceptions of treatment efficacy by the patients^[78,79].

Recently, the patient reported outcome (PRO), derived from components of the Mayo score has been put forward as an interim outcome measure when in combination with endoscopic findings. PROs appear to be well correlated with disease activity and may predict patient-defined remission^[80].

Several endoscopic scoring systems for UC are available. They include numerous descriptors namely, vascular pattern, mucosal erythema, mucosal granularity, mucosal oedema, mucopurulent exudate, bleeding, friability, erosions, and ulcers^[54,81,82]. An extensive review of scoring systems is beyond the scope of this manuscript, but the reader is referred to an exhaustive review with images and videos for reference^[54].

The endoscopic component [Mayo endoscopic sub-score (MES)] of the MCS is the most widely used, and it assesses inflammation based on a 4-point scale (0-3): (0)

normal; (1) erythema, decreased vascular pattern, mild friability; (2) marked erythema, absent vascular pattern, friability, erosions; and (3) ulceration, spontaneous bleeding^[75]. Mucosal healing is defined as a sub-score of 0-1, although this has not been formally validated^[75,83].

A post-hoc analysis of the active ulcerative colitis trial (ACT)-1 trial demonstrated that patients achieving a post-treatment MES of 0 or 1 had similar colectomy rates and were significantly less likely to undergo colectomy in the subsequent year than those with higher MES^[84]. Higher steroid-free remission rates were noted in patients with MES of 0 at 1 year than those with an MES of 1^[84]. Furthermore, MES of 0 was associated with a lower risk of clinical relapse than MES of 1 (5.0% *vs* 9.4%, respectively)^[26,85,86]. The MES is relatively easier to use and has been used extensively in clinical trials. The lack of validation, inability to distinguish superficial from deep ulcers, that it reflects appearances of the most severely affected visualized segment and has no minimal insertion length are significant limitations^[87].

Variability in interobserver agreement can result from variability in the assessment of friability. This has led to the development of the Modified Mayo Score (MMES)^[88]. The MMES classifies any degree of friability with a sub-score of 2. It divides the colon into five segments, and the score for each segment is added to give a Modified Score, which is multiplied by the maximal extent of inflammation and divided by the number of segments with active inflammation to give the final MMES^[88]. Although MMES correlates with clinical, biological, and histological activity, this has not been validated. The Mayo score is most widely used in clinical practice.

Two scoring systems were developed in an attempt to develop a prospective validated tool: The ulcerative colitis endoscopic index of severity (UCEIS) and the ulcerative colitis colonoscopic index of severity (UCCIS)^[89-91]. The UCEIS considers three endoscopic findings; vascular pattern, bleeding, and erosions/ulcers scored in the most severely affected part of the colon. It was initially developed as an 11-point score and then simplified to an 8-point tool scoring erosions/ulcers (0-2), vascular pattern (0-2), and bleeding (1-4) with a satisfactory interobserver agreement (kappa 0.5)^[90]. Friability was excluded from this index. Although easy to use in clinical practice, thresholds for mild, moderate, and severe disease have not been defined. UCEIS does not define the extent of disease as it scores the most affected segment. It does demonstrate more responsiveness to ulcer size, depth, and change following treatment than the MCS^[92-94]. Furthermore, a UCEIS of zero was associated with a lower risk of relapse than UCEIS of 1 (5% *vs* 22.4%)^[94]. Both scores demonstrated a high degree of correlation for disease assessment by sigmoidoscopy and colonoscopy^[95].

The UCCIS has been prospectively validated^[91]. It includes six variables: (1) Vascular pattern; (2) Granularity; (3) Ulceration; (4) Bleeding/friability; (5) Grading of segmental and global assessment of endoscopic severity with a predefined 4-point scale; and (6) Global assessment of endoscopic severity on a 10-cm visual analogue scale with good to excellent interobserver agreement^[91]. Although correlation with clinical activity and CRP was demonstrated, a cut off level for endoscopic response and remission is not currently known^[91].

CD

Statement 17: CD is classified according to disease location: terminal ileum, colon, ileocolonic, and upper GI (Montreal classification). [77.7]

Statement 18: CD is divided according to disease behaviour (stricturing, penetrating, nonstricturing/nonpenetrating, with or without perianal involvement) (according to Montreal classification). [100]

CD is widely classified using the Montreal classification in adults and the Paris classification in the paediatric IBD population^[55,67]. Thus, L1 relates to terminal ileal CD, L2 to ileo-colonic CD, and L3 to colonic CD. Isolated upper GI disease (L4) is further subdivided into L4a (upper disease proximal to the ligament of Treitz) and L4b (upper disease distal to the ligament of Treitz and proximal to distal 1/3 of the ileum)^[55]. Perianal disease is denoted by "P"^[72]. Disease behaviour is designated as B1 (nonstricturing-nonpenetrating), B2 (stricturing), and B3 (penetrating) disease. Furthermore, age at diagnosis is denoted as A1 (< 17 years), A2 (17-40 years), and A3 (> 40 years).

The majority of patients (56%-81%) have an inflammatory phenotype at diagnosis and between 5% and 25 have stricturing or penetrating disease^[1]. Indeed, a large population-based study reported a cumulative risk of 51% to develop an intestinal complication after 20 years of follow-up in patients presenting with inflammatory behaviour at diagnosis^[96]. In addition, at multivariate analysis, ileal, ileocolonic, or upper GI involvement compared to colonic involvement alone were significantly

associated with onset of intestinal complications^[96]. Risk factors for severe disease include young age at diagnosis^[97], initial extensive GI involvement, ileal/ileocolonic involvement, perianal/severe rectal disease, and patients presenting with a penetrating or stenotic disease phenotype^[98]. Visceral adiposity is also associated with an increased risk of penetrating disease^[99].

INVESTIGATIONS FOR IBD

Statement 19: Ileocolonoscopy with multiple biopsy specimens is the first-line procedure for diagnosing and determining the severity of IBD. [100]

Statement 20: For a reliable diagnosis of CD or UC, a minimum of two biopsies from five sites around the colon (including the rectum) as well as from the ileum should be obtained. [100]

The diagnosis of IBD needs an endoscopic assessment, typically an ileo-colonoscopy for CD and sigmoidoscopy in the case of UC followed by an ileo-colonoscopy within 12 mo of diagnosis to establish disease phenotype, determine full disease extent, and risk stratify for dysplasia^[52-54]. A minimum of two biopsy samples must be obtained from five sites including the ileum and rectum at the first endoscopic assessment.

Endoscopic appearances of UC have been described above. The rectum is always involved but rectal sparing may be seen in up to 3% of patients^[24]. Patchy inflammation of the rectum is seen in patients who have received topical therapy^[26,27,100]. An isolated involvement of cecum or around the appendix as well as backwash ileitis may be seen in UC, but in the absence of supportive clinical presentation and histology, small bowel assessment should be arranged to consider CD^[25]. Backwash ileitis may occur in up to 20% of patients with extensive UC^[100].

At histology, a combination of features including basal plasmacytosis, diffuse crypt atrophy and distortion, villous surface irregularity, and mucous depletion suggest a diagnosis of UC^[31]. Although granulomas and focal crypt architectural abnormalities with focal or patchy chronic inflammation or mucin preservation may suggest CD, no single feature is diagnostic^[31,58]. Endoscopic features suggestive of CD are discontinuous segments of disease (“skip lesions”), ileal involvement, and granulomatous inflammation. Approximately 3% of UC patients may be reclassified as Crohn’s colitis^[65]. Conversely, 0.6%-3% will be reclassified to alternative colitis after initial diagnosis of CD^[66]. Although a diagnosis of CD may be made on surgical samples^[31,58], some patients cannot be assigned to either CD or UC and are labelled as IBD unclassified^[65,66]. Thus, if the diagnosis of IBD is in doubt despite an interval of appropriate treatment, repeat endoscopy is required to help establish the diagnosis^[63].

Statement 21: Stool specimens should be obtained to exclude common pathogens and specifically assayed for *Clostridium difficile* toxin. [88.8]

Statement 22: Laboratory markers of chronic inflammation may correlate with the severity of IBD. [50]

Statement 23: CRP broadly correlates with clinical severity. [77.7]

Statement 24: Elevated ESR, CRP, anaemia, number of bowel movements, and hypoalbuminemia are signs of severe clinical activity that predict the need for colectomy in severe acute colitis. [88.8]

Statement 25: Microbial testing should be done in patients with colitis with every disease flare. [100]

Stool cultures and *Clostridium difficile* toxin assay are recommended to exclude infective aetiologies^[48,49]. Loose stools for over 6 wk help to distinguish infective causes from IBD^[50,51]. In a recent UK study, 10% of IBD relapses were associated with enteric infections, 50% of which were *Clostridium difficile* related^[101]. An American study reported that 18.1% and 16.1% of samples from CD and UC patients, respectively, were positive for GI pathogens using a multiplex PCR^[102]. Norovirus and *Campylobacter* were more likely in CD patients, while UC patients were more likely to have *Campylobacter*, *Plesiomonas*, and *Escherichia coli* (compared with non-IBD samples)^[102]. Thus, a comprehensive infection screen considering relevant clues from the clinical history should be considered for every flare of IBD. *Clostridium difficile* infection has been associated with worse outcomes in hospitalized IBD patients^[103,104].

An elevated ESR, CRP, anaemia, number of bowel movements, and hypoalbuminemia are signs of severe clinical activity that can predict the need for

colectomy in acute severe UC. A stool frequency $> 8/d$ or stool frequency $> 3/d$ with CRP > 45 mg/L on day 3 of intravenous corticosteroid predicts the need for rescue therapy to prevent colectomy^[44]. Several other mathematical models comprising stool frequency, CRP, and albumin on day 3 may also guide clinicians to use “rescue therapy” with infliximab (IFX) or cyclosporine to prevent the need for colectomy^[105-107].

FC is a calcium binding protein derived from neutrophils with a role in intestinal inflammation regulation. It is a useful inflammatory marker, correlating well with endoscopic findings. It is also a good marker of relapse and response to treatment and in differentiating patients with IBD from those with irritable bowel syndrome^[45,46]. It lacks specificity to distinguish IBD from other causes of bowel inflammation^[47]. Calprotectin values correlate well with endoscopic indices of disease activity and is therefore very helpful in different clinical settings, including diagnosis, relapse, and response to therapy^[46,108-110]. A clear-cut threshold value to distinguish between IBD and functional bowel diseases has not been set yet^[41,111]. However, an acceptable diagnostic accuracy can potentially be obtained at a cut-off value of $150 \mu\text{g/g}$ ^[41]. It also appears to be strongly correlated with endoscopic inflammation in UC^[112]. An elevated faecal calprotectin $> 1000 \mu\text{g/g}$ stool on day 3 of intravenous corticosteroid along with a UCEIS > 6 on admission is also a predictor of the need for colectomy^[113].

Statement 26: Serological testing currently available is not recommended for differentiating colonic CD from UC. [77.7]

Serological markers may have a role in supporting a diagnosis, though the accuracy of the best available tests (perinuclear anti-neutrophil cytoplasmic antibodies and anti-*Saccharomyces cerevisiae* antibodies) is rather weak and therefore not effective at discriminating colonic CD from UC^[114]. The incremental diagnostic value of antiglycan and antimicrobial antibodies, such as anti-OmpC (*Escherichia coli* outer membrane porin C) and CBIR1 (CBIR1 flagellin), is minimal and not clinically relevant^[115]. Despite a growing number of identified susceptibility loci in both CD and UC, only a few have been associated with disease outcomes, such as the *NOD2* polymorphism that is associated with a more aggressive disease course in CD with a higher risk of intestinal stenosis and need for first surgery^[34,35]. Genetic testing cannot be used to diagnose IBD^[36,37].

Statement 27: Diagnostic criteria for iron deficiency depend on the level of inflammation. In patients without clinical, endoscopic, or biochemical evidence of active disease, serum ferritin $< 30 \mu\text{g/L}$ is an appropriate criterion. In the presence of inflammation, serum ferritin up to $100 \mu\text{g/L}$ may still be consistent with iron deficiency. [88.8]

Up to 1/3 of patients with active IBD will have iron deficiency anaemia^[116] leading to fatigue and negative impact on quality of life. It should be remembered that systemic inflammation may inhibit iron absorption^[116]. In the presence of active disease, oral iron supplementation should be avoided, and in patients with inactive IBD, no more than $100 \mu\text{g/L}$ of elemental iron should be administered daily^[117,118]. Ferritin is an acute phase reactant, and it should be remembered that iron deficiency may be present with a level up to $100 \mu\text{g/L}$ in case of active inflammation. The evaluation of transferrin saturation is therefore essential to distinguish true iron deficiency from a falsely elevated ferritin^[63,117,118]. Intravenous iron may be administered to patients who are intolerant to oral iron or have active IBD with moderate to severe iron deficiency anaemia^[116].

Statement 28: Cross-sectional imaging magnetic resonance imaging (MRI) and computed tomography (CT) enterography and transabdominal ultrasonography (US) are used to complement endoscopy. [100]

Statement 29: Complementary radiological techniques using MRI, CT, and US should be used to rule-out stenotic lesions and are necessary when the lesion is impassable with the endoscope. [88.8]

MRI and CT enterography and US are used to complement endoscopy and have largely replaced fluoroscopic techniques over the years^[63,72]. These techniques have the advantage of better classifying disease phenotype and behaviour by providing information on the bowel wall and extra enteric soft tissue pathology^[119]. Small bowel US may be performed with or without bowel distension and the use of oral contrast^[119]. Meta-analyses have shown no significant difference in accuracy for CD diagnosis with CT enterography, small bowel US, and MRI enterography^[120-123]. Sensitivity and specificity ranges between 85% and 95%^[120-123]. CT is extremely accurate for evaluation and monitoring of mural and extramural complications in CD patients, although it is not recommended to monitor disease activity due to significant radiation

exposure^[124,125]. State-of-the-art low radiation dose CT scanners may significantly reduce the dose, but the use of nonionizing imaging is preferable, considering the young age of these patients and the need of repetition during the lifespan^[121,124,125]. Thus, CT should be ideally used only in the emergency setting, and if small bowel US and MRI are not available^[72,121].

Magnetic resonance enterography and CT have shown high accuracy for detecting active CD^[120,121]. Magnetic resonance enterography features of active disease include an increase in intestinal wall thickness and vascularity, contrast enhancement on T2 and diffusion weighted imaging signals, presence of ulcers, and extraluminal complications^[120,121]. These findings are usually reported with specific activity scores^[126,127]. Data supporting small bowel US are less consistent currently^[128].

They may also be useful when assessing areas impassable with an endoscope, particularly stenotic lesions^[63]. Patients with CD have a 2-3 fold increased risk of colorectal cancer compared to an age-matched population and the risk of small bowel malignancy between 18 and 27 times. Stricturing in the context of CD may be complicated by dysplasia or cancer. Therefore, endoscopy and biopsies should be performed. Fibrotic structures may be assessed using modern imaging such as MRI and techniques in development such as magnetization transfer sequences, delayed contrast enhancement, contrast enhanced US, and elastography^[127,129,130]. US detection of strictures may be improved by the use of oral contrast^[131].

Statement 30: CD patients with symptoms of the upper GI tract should receive esophagogastroduodenoscopy to rule out proximal involvement. [88.8]

CD affecting the upper GI tract may be present in 13%-16% of patients. It is usually accompanied by ileal and/or colonic disease^[132,133]. Upper GI endoscopy should be performed in patients with dyspepsia, vomiting, or other upper GI symptoms but is not routinely indicated in adults with proven or suspected CD^[51,72]. Focal gastritis may be a feature of CD^[134]. In patients with IBD-unspecified, upper GI endoscopy may help to differentiate between UC and CD^[63]. Upper GI endoscopy should be performed in patients with suspected CD^[135].

Statement 31: Small bowel capsule endoscopy (SBCE) should only be used when the clinical suspicion for CD remains high despite negative evaluations with ileocolonoscopy and radiological examinations (MRI/CT). This could be useful to assess the disease extent. [66.6]

Statement 32: SBCE is contraindicated in GI obstruction, strictures, and swallowing disorders. [100]

SBCE enables the entire small bowel to be assessed by a wireless capsule^[136]. Specificity for the diagnosis of IBD was reported as 53% in one study using a consensus standard^[137,138]. Minor mucosal abnormalities may obscure the diagnosis such as in those using nonsteroidal anti-inflammatory drugs^[139,140].

Several scoring systems are available to grade lesions^[54,136,141], but they are not used widely. At the present time, the use of capsule endoscopy is currently restricted to patients with a high index of suspicion of CD when cross-sectional imaging has been normal or nondiagnostic^[63,140].

SBCE is contraindicated in the presence of GI obstruction, strictures, and swallowing disorders. Given the size and rigidity of capsules, they may be retained within the small bowel in the context of stricturing disease. In a recent meta-analysis, the risk of capsule retention was 3.6% [95% confidence interval (CI): 1.7%-8.6%] despite considerable heterogeneity in studies^[142]. Retention rates were much lower after use of a patency capsule or stricture exclusion with cross-sectional imaging at 2.7% (95%CI: 1.1%-6.4%)^[142]. The use of a patency capsule is therefore recommended in patients with stricturing CD, suspected strictures, abdominal pain, distension, nausea, vomiting, history of small intestinal resection, abdominal or pelvic radiation, and chronic nonsteroidal anti-inflammatory drugs use^[140,143].

Statement 33: Device-assisted enteroscopy is an invasive procedure that may only be performed by an expert if the histological diagnosis of CD is needed or when endoscopic therapy is indicated, including dilatation of strictures, retrieval of impacted capsules, and treatment of bleeding. [88.8]

Balloon assisted enteroscopy may help visualize areas outside the scope of a standard endoscope and in that respect may have a similar diagnostic yield as capsule endoscopy. It offers the added advantage in expert hands of tissue biopsy or endoscopic therapy^[144]. The examination is invasive, requires sedation or general anaesthesia, is expensive, and has risks^[145]. A perforation rate of 0.15% (95%CI: 0.05%-0.45%) and complication rate (including perforation and bleeding) of 0.72% (95%CI:

0.56%-0.90%) have been reported^[145,146].

Statement 34: In acute severe UC, a plain abdominal radiograph should be performed to exclude colonic dilatation. [37.5]

Abdominal radiographs may provide vital information in patients with acute severe ulcerative colitis (ASUC). Mucosal islands (or 'thumb printing') are predictors of failure of medical therapy. The presence of faecal residue suggests uninflamed or normal colonic mucosa. Proximal constipation may be associated with left-sided or distal colonic inflammation. Proximal constipation may worsen distal disease and require laxatives in addition to treatment of UC^[43,63]. Transverse colonic and caecal diameter (diameter greater than 5.5 cm is consistent with dilatation of the colon and impending toxic dilatation)^[43,58]. Cross-sectional imaging may be required in patients suspected of having extraluminal complications and perforation^[147].

Statement 35: Flexible sigmoidoscopy should be used to confirm the diagnosis of severe colitis and help exclude infection, particularly cytomegalovirus (CMV). [88.8]

Statement 36: Enema preparation before flexible sigmoidoscopy is considered safe in patients with severe UC. [77.7]

Endoscopic assessment with a sigmoidoscopy helps to confirm the diagnosis of ASUC and also to obtain biopsies to diagnose or exclude CMV colitis^[147]. Colonoscopy in ASUC is associated with a significant risk of colonic dilation and perforation. A sigmoidoscopy is preferred with minimal air insufflation and by an experienced endoscopist^[43,147]. Although an enema if required may be administered, it is often unnecessary. Deep ulceration at sigmoidoscopy correlates with failure of corticosteroid therapy and need for rescue therapy or colectomy^[148]. Corte *et al*^[149] reported that a UCEIS score ≥ 5 was associated with a 50% likelihood of need for medical rescue treatment and 33% risk of colectomy compared with 27% and 9%, respectively, for those who scored ≤ 4 . In a retrospective review of 92 patients with ASUC, the UCEIS score correlated with MES (Spearman's rho, 0.762; $P < 0.001$) and requirement for colectomy [adjusted odds ratio (OR) 3.25; 95%CI: 1.77- 5.97; $P < 0.001$]^[150].

CMV colitis can affect 30% of patients with ASUC refractory to corticosteroid therapy^[151,152]. Medically refractory disease treatment with corticosteroids and the presence of endoscopic ulceration are risk factors for CMV colitis. Biopsy should be taken from the base of the ulcer as CMV infection has a predilection for actively inflamed tissue. Histology for a viral cytopathic effect on haematoxylin eosin staining has poor sensitivity, but immunohistochemistry with culture methods and PCR based assays are favoured to confirm CMV colitis^[151-153]. The presence of CMV infection increases the risk of medical refractoriness and colectomy. As such, if CMV infection is diagnosed in steroid refractory cases, then it should be treated with antiviral therapy^[151,152]. The most commonly used agent is ganciclovir administered intravenously and then for 14 d with response rates approaching 70%. Oral therapy with valganciclovir may also be used in selected patients after discussion with infectious disease physicians^[151,152].

Statement 37: If colonic stenosis occurs in UC, then multiple endoscopic biopsies should be taken. CT should be performed to exclude carcinoma. [77.7]

Detection of a new colonic stricture should prompt multiple biopsies to rule out malignancy. Colonic strictures at diagnosis or during follow-up were associated in one study, with a 3.6% and 4.9% probability of colorectal cancer at five and ten years, respectively^[154]. In the Groupe d'Etudes Thérapeutiques des Affections Inflammatoires du tube Digestif study (GETAID), dysplasia or cancer was detected in 3.5% of patients with IBD with colonic strictures^[155]. Biopsies before endoscopic balloon dilatation are recommended^[155,156].

Statement 38: Endoscopic reassessment is appropriate whenever it seems necessary to change management. [88.8]

Statement 39: Colonoscopy is also recommended to determine response to treatment and for surveillance of cancer development. [100]

Statement 40: In IBD, therapy and follow-up can be guided by CRP levels and faecal markers, which are able to predict clinical relapse. [77.7]

Endoscopy is currently the standard for the diagnosis and assessment of mucosal activity, dysplasia surveillance, and the assessment of response to treatment^[17,54]. The lack of correlation between symptoms and objective measures of disease activity and knowledge that chronic inflammation may lead to progressive gut damage with complications has brought the concept of "treating to target," which aims to treat gut

damage or disability^[1,5,16].

Endoscopic remission (mucosal healing) has emerged as a key goal of therapy. The “treat to target” concept was developed by the Selecting Therapeutic Targets in Inflammatory Bowel Disease committee (STRIDE), consisting of experts from the International Organization for the Study of IBD^[17]. Its premise is the identification of a target for which treatment is commenced and optimized with periodic monitoring until the goal is achieved, taking patient considerations into account. Specifically, they refer to resolution of abdominal pain and normalization of bowel habits and endoscopic remission (or cross-sectional imaging when endoscopy is not feasible in CD)^[17]. Mucosal healing is linked to a lasting clinical response and reduction in undesirable outcomes such as corticosteroid use, hospitalization, surgery, and colorectal cancer complicating IBD^[157-162]. Meanwhile, endoscopy is the standard for mucosal assessment and allows adjustments in treatment to “target”^[17,54,163]. Mucosal healing, or endoscopic remission, refers to the absence of ulceration in CD and resolution in mucosal friability or ulceration at colonoscopy or sigmoidoscopy in UC^[17,52]. Several endoscopic scoring systems are available for UC and CD, which provide objectivity^[17,52,54].

Although biochemical targets (CRP or faecal calprotectin) were considered adjunctive targets in the STRIDE consensus in the absence of sufficient evidence at the time, recent evidence supports use of biochemical markers such as CRP and faecal calprotectin in monitoring response^[100,164]. In a meta-analysis of 2822 IBD patients and 298 controls, a calprotectin level of 50 µg/g showed the best sensitivity (90.6%) to detect endoscopic disease activity with specificity (78.2%) at calprotectin > 100 µg/g^[165]. In a separate meta-analysis calprotectin of 250 µg/g provided specificity of 82% compared to thresholds of 100 µg/g and 50 µg/g (specificity of 66% and 60%, respectively) to differentiate active IBD from remission^[111]. FC of 250 µg/g had sensitivity of 80% compared to a sensitivity of 84% and 92% at cut-offs of 100 µg/g and 50 µg/g, respectively^[111]. Calprotectin may also be used to guide therapy changes. FC is able to distinguish between active and inactive IBD with greater accuracy for UC than CD^[111,166]. Calprotectin correlates with endoscopic and histologic inflammation in UC^[167,168].

The CALM (open-label randomized effect of tight control management on Crohn’s disease) study showed that a calprotectin level less than 250 µg/g stool, CD activity index (CDAI) < 150, CRP < 5 mg/L, and corticosteroid-free remission can be used as a target with dose increase of adalimumab and azathioprine (AZA) until these objectives were achieved^[164]. At 12 mo, the ‘treat to target’ group achieved the primary end-point (CDEIS score < 4, without deep ulcers) in 45.9%, whilst 30.3% of the control group had a CDEIS score < 4, without deep ulcers ($P = 0.010$)^[164]. Calprotectin may also predict future relapse to enable considerations with treatment de-escalation^[63,164]. In the STORI (Stop Infliximab in Patients With Crohn’s Disease) trial, patients stopping anti-tumour necrosis factor (TNF) who achieved mucosal healing and a calprotectin ≥ 300 µg/g had a 30% relapse rate whereas those with both mucosal healing and a lower calprotectin had relapse rates of between 10% and 20%^[169,170]. Serial measurements are more predictive of the likelihood of relapse^[63].

MANAGEMENT–ACTIVE UC

Statement 41: The treatment strategy for UC is mainly based on the severity, distribution, and pattern of disease. Disease extent influences treatment modality and choice of (oral, topical) therapy. [77.7]

The therapeutic strategy for UC should be based on the specific diagnosis, an assessment of disease activity, distribution, and disease prognosis. Thus, patients with clinically mild active UC but with a history of steroid dependence or previous hospitalization should be considered for treatment appropriate for moderate to severe active UC given the significant impact these factors may have on disease prognosis^[171]. Also, deep ulceration at sigmoidoscopy predicts the failure of corticosteroid therapy and need for rescue therapy or colectomy^[148].

Proctitis

Statement 42: Suppository is the preferred initial treatment for mild or moderately active proctitis. Aminosalicylate (5-ASA) foam or enemas can be used as an alternative though less tolerated. [88.8]

Statement 43: Combined (oral and topical) therapy is more effective than topical alone

for the treatment of proctitis. [77.7]

5-ASA suppositories are the preferred initial treatment for mild or moderately active proctitis^[172]. 5-ASA suppositories achieve higher mucosal concentrations through a topical effect than oral 5-ASA alone^[173]. Suppositories are preferable over enema preparations as they may be better tolerated, and enemas may pool higher up in the sigmoid^[172]. When oral 5-ASA is combined with topical therapies, response rates are higher^[173,174]. Furthermore, topical 5-ASA is more effective than topical hydrocortisone enemas and corticosteroids^[175,176].

Although a dose response relationship has been observed for oral 5-ASA, this has not been the case with rectal 5-ASA. In a study comparing 1 g 5-ASA suppositories daily with 500 mg three times a day, the once daily dose was found to be more convenient and had similar efficacy^[177]. No significant differences were observed with doses (1 g or 4 g daily) or formulation (liquid, gel, foam, or suppository) for topical therapy. 5-ASA was noted superior to rectal corticosteroids to induce symptomatic remission (OR 1.65; 95%CI: 1.1-2.5)^[172]. 5-ASA suppositories are also effective for the maintenance of remission in ulcerative proctitis and it appears that alternate day or every third day therapy does not reduce the rate of remission^[172,176].

Statement 44: Refractory proctitis may require treatment with systemic steroids, immunosuppressants, and/or biologics. [100]

Before labelling proctitis as “refractory,” it is important to ensure that the patient has been adherent to the treatment and that a differential diagnosis is considered. Proximal constipation maybe an exacerbating factor, which should be addressed as also coexisting irritable bowel symptoms. Infections (lymphogranuloma venereum, herpes simplex virus, syphilis, *Neisseria gonorrhoeae*, Giardia, and amoebiasis) and other pathologies such as solitary rectal ulcer, psoriatic colitis, rectal prolapse, and chemical colitis should also be considered^[178].

If compliance is established for those not responding to optimized 5-ASA, then a 5 mg prednisolone suppository may be added while continuing 5-ASA suppositories^[176,179]. If the patient does not respond to this strategy, then a course of corticosteroid may be needed. Escalation to a thiopurine or biologic may be necessary^[180,181].

Left sided UC

Statement 45: 5-ASA enema combined with oral 5-ASA is more effective than oral or topical 5-ASA or topical steroids alone in the treatment of mild to moderate active left-sided UC. [88.8]

Statement 46: Topical steroids alone are not more effective than topical 5-ASA. [50]

Statement 47: Once-daily dosing with 5-ASA is as effective as divided doses in mild to moderate active left- sided UC. [77.7]

Statement 48: Systemic corticosteroids are appropriate in patients with moderate to severe activity and in those with mild activity who do not respond to 5-ASA. [88.8]

Statement 49: Budesonide multimatrix (MMX) can be considered in patients with mild to moderate disease who are intolerant or refractory to 5-ASA. [77.7]

In a meta-analysis of 4 randomized controlled trials (RCT), combination therapy with rectal 5-ASA enemas (1 g/d) and oral 5-ASA (at least 2 g/d) was more effective than using oral 5-ASA on its own to induce remission in left sided UC [relative risk (RR) induction failure 0.65; 95%CI: 0.47-0.91]^[174]. These findings were supported by another study that showed when comparing the two regimens an RR of 0.86 for failure with combination treatment (95%CI: 0.81-0.91)^[182]. An oral 5-ASA dose of at least 2 g/d is recommended to induce remission with mildly active UC.^[182,183]

Topical steroid preparations are not more effective than 5-ASA, and they should be used when patients either do not tolerate or are unresponsive to 5-ASA^[172,179]. A 5 mg prednisolone suppository may be added for patients not responding to 5-ASA topical therapy while continuing 5-ASA suppositories at bedtime^[63]. A double-blind double-dummy four-group prospective randomized trial compared budesonide 2 mg, budesonide 4 mg, 5-ASA 1 g, or budesonide 2 mg and 5-ASA 1 g in an 8-wk study. The primary endpoint was resolution of clinical symptoms for three consecutive days. Budesonide 4 mg was more effective than 2 mg but no different to 1 g 5-ASA or the combination of budesonide 2 mg and 5-ASA at a dose of 1 g^[184].

Budesonide MMX is a topically acting corticosteroid with high first pass metabolism and few systemic side-effects. In a recent study in UC with failure of 5-ASA,

budesonide MMX 9 mg for 8 wk was superior at achieving clinical and endoscopic remission as compared to ongoing 5-ASA and placebo ($P = 0.049$)^[185]. Oral budesonide is also safe and more effective than placebo for the induction of remission in patients with mild active UC. In a RCT, clinical remission was achieved by 17.9% of patients given 9 mg budesonide MMX, 13.2% given 6 mg budesonide MMX, and 12.1% of those receiving 5-ASA as compared to 7.4% in the placebo group ($P = 0.0143$, $P = 0.139$, $P = 0.22$)^[183]. A Cochrane systematic review rated the quality of evidence as moderate. No clear benefit for extensive UC was demonstrated, but the efficacy was significant for left sided disease with endoscopic healing rates at 27.6% *vs* 17.1% for budesonide MMX and placebo, respectively^[186,187]. Budesonide MMX is associated with fewer systemic side effects than classical corticosteroids (33% *vs* 55%) but not associated with either adrenal suppression or significant reduction in bone mineral density. Although there are no adequately powered comparative studies between budesonide MMX and conventional corticosteroids, budesonide MMX could be positioned as an alternative to conventional corticosteroids in mild to moderate UC unresponsive to 5-ASA^[188-191].

Moderate-severe and extensive UC

Statement 50: Mild to moderate active extensive UC should initially be treated with a 5-ASA enema combined with oral 5-ASA. [88.8]

Statement 51: Corticosteroids have potent anti-inflammatory properties and are effective for induction of remission in UC but have no efficacy for maintenance of remission, and their long-term use can lead to adverse events. [88.8]

The dose response effect of 5-ASA for induction of response in UC was investigated in the ASCEND (Delayed-release oral mesalamine for the treatment of mildly to moderately active ulcerative colitis) trials^[171,192,193]. In ASCEND I, patients with mild to moderate active UC were randomized to 2.4 g or 4.8 g of mesalazine^[192]. At week 6, the proportion of patients experiencing improvement in either group was similar (51% *vs* 56%, $P =$ not significant). Patients with moderate active UC responded better to 4.8 g daily, but those with mildly active disease did not^[192]. The ASCEND II study showed that patients with moderately active UC had a better response to 4.8 g daily than 2.4 g daily (72% *vs* 59%, $P = 0.036$)^[193]. Post hoc analysis of ASCEND I and II showed greater mucosal healing in the 4.8 g/d group as compared with 2.4 g/d^[194]. The ASCEND III trial randomized patients with moderate active UC to receive 2.4 g daily or 4.8 g daily mesalazine^[171]. The primary endpoint of treatment success was defined as complete clinical remission or partial response showed no differences between the groups. A small but significant difference in remission with 43% of patients on 4.8 g/d *vs* 35% on 2.4 g daily was observed at 6 wk^[171]. In a subgroup analysis, patients receiving oral 5-ASA and rectal therapies had a greater likelihood of response to 4.8 g/d^[171].

In a recent meta-analysis, the low dose of 2-2.4 g of 5-ASA was equally effective as 4.8 g/d (RR 0.91; 95%CI: 0.85-0.98)^[182], but the subgroup patients with moderate active UC might benefit from a higher dose of 4.8 g per day^[195]. Symptomatic remission following the initiation of 5-ASA was achieved at week 2 in 10%-30% of patients, by week 4 in 30%-45%, and by week 8 in 35%-50%^[196-199]. Once daily dosing of 5-ASA has been shown to be as effective as divided doses and could improve compliance^[195].

For patients with mild active UC who do not respond to 5-ASA and those with moderate to severe UC activity, oral corticosteroids can induce remission. Corticosteroids were more effective than placebo for the induction of remission (RR 0.65; 95%CI: 0.45-0.93) in a meta-analysis^[200]. Prednisolone is usually started at a dose of 40-60 mg daily with a clinical response typically within 5-7 d of treatment and no benefit with doses over 60 mg daily^[201]. Tapering should be tailored by clinical symptoms and response. However, steroid sparing agents should also be considered appropriately as corticosteroids have no role in maintenance of remission^[202,203].

Statement 52: In moderate disease refractory to oral steroids, anti-TNF, vedolizumab (VDZ), ustekinumab, and tofacitinib may be valid options. [88.8]

Anti-TNF, anti-integrin, Tofacitinib (Janus kinase inhibitor), and ustekinumab (anti-p40 subunit inhibitor of IL-12/23) are currently licensed for patients with moderately active UC refractory to oral corticosteroids.

Anti-TNF agents (IFX, adalimumab, and golimumab) have all demonstrated superiority over placebo in the induction of clinical response and remission in UC^[204-206]. In the ACT-1 and ACT-2 studies, patients with moderate to severe UC, failing corticosteroids and/or thiopurines (and/or 5-ASA for ACT-2) received 5 mg/kg or 10 mg/kg IFX or placebo at 0, 2, and 6 wk and were followed through week 54 (ACT-1) or week 30 (ACT-2)^[83]. Patients in both 5 mg/kg and 10 mg/kg had a similar clinical response at week 8 with pooled data showing 67% for 5 mg/kg *vs* 33%

for placebo. Clinical remission rates at week 30 were 30% for 5 mg/kg (placebo 13%) with remission sustained through week 54. Corticosteroid free remission rates were 22% for 5 mg/kg by week 30 and sustained through week 54^[83]. The SUCCESS (Efficacy and Safety of Infliximab Monotherapy *vs* Combination Therapy *vs* AZA Monotherapy in Ulcerative Colitis) study showed that in patients in whom corticosteroid therapy had failed, the combination of IFX and AZA was more effective with higher clinical remission rates at week 16 (40%) compared to IFX alone (22%)^[207].

In the ULTRA (Ulcerative Colitis Long-Term Remission and Maintenance with Adalimumab) 1 and 2 trials, adalimumab showed efficacy for induction and maintenance of remission in UC^[208,209]. Adalimumab 160 mg at week 0, followed by 80 mg at week 2, and then 40 mg every other week achieved remission in 19% of patients in ULTRA 1 (9% placebo) and 21% of patients in ULTRA 2 (11% placebo). In the ULTRA 2 maintenance study, clinical remission at week 52 was 22% (12% for placebo) in the anti-TNF-naïve group^[208,209]. Corticosteroid free remission was achieved by week 52 in 14% patients (placebo 6%). In the open label ULTRA 3 extension study, 25% of patients remained in clinical remission at four years^[210].

Golimumab is the third anti-TNF to have regulatory approval for the treatment of moderate to severe active UC^[211,212]. In the PURSUIT (An Efficacy and Safety Study of Golimumab in Participants With Moderately to Severely Active Ulcerative Colitis) trial, patients with UC with failure to respond to 5-ASA, oral corticosteroids, AZA, mercaptopurine, or those steroid-dependent were enrolled. These patients were all anti-TNF naïve. At week 6, 51% of patients achieved clinical response on 200 mg/100 mg and 54.9% on 400 mg/200 mg, both proving significantly superior to placebo (30.3%, $P < 0.0001$). Clinical remission was achieved at week 6 in 17.8% in both 200 mg/100 mg and 17.9% (400 mg/200 mg) *vs* placebo (6.4%, $P < 0.0001$)^[211,212]. Real world studies also demonstrated similar effectiveness data^[213,214].

VDZ was approved for the management of moderate to severe active UC in 2015. In the GEMINI (Vedolizumab as Induction and Maintenance Therapy for Ulcerative Colitis) I study, the primary endpoint was clinical response at week 6 (defined by a reduction in the Mayo score of ≥ 3 points and a decrease of at least 30% from baseline, with a decrease of ≥ 1 point on the rectal bleeding subscore, absolute score 0–1)^[215]. For maintenance, the primary endpoint was clinical remission at week 52. Of 374 patients randomized to VDZ or placebo, clinical response at week 6 was achieved in 47.1% in the VDZ group as compared with 25.5% in the placebo group (95%CI: 11.6–31.7; $P < 0.001$). At 52 wk, 41.8% of patients on VDZ 8-weekly, 44.8% on VDZ 4-weekly, and 15.9% of patients receiving placebo achieved clinical remission. VDZ was noted to be superior to placebo for clinical response (RR = 0.82, 95%CI: 0.75–0.91), induction of remission (RR = 0.86, 95%CI: 0.80–0.91), endoscopic remission (RR = 0.82, 95%CI: 0.75–0.91), and remission at 52 wk in week 6 responders (RR = 2.73, 95%CI: 1.78–4.18) in a Cochrane review^[216].

In the GEMINI open label extension, patients with ≥ 248 wk of cumulative VDZ treatment were included ($n = 154$). Among patients responding to induction therapy who completed the maintenance study, 40.9% of patients had 248 wk of treatment; 98% achieved clinical response, and 90% had clinical remission^[217]. Post hoc analysis noted improvements in PROs of reduction in rectal bleeding and stool frequency by 2 wk^[218]. Real-world data provided further evidence for effectiveness and safety of VDZ^[217,219–221].

Recent systematic reviews with network meta-analysis ranked VDZ and IFX highest for induction of clinical remission in biologic-naïve UC patients^[222], and VDZ was associated with the lowest risk of serious adverse events and infections^[223]. The VARSITY (Vedolizumab *vs* Adalimumab for Moderate-to-Severe Ulcerative Colitis) trial was the first head-to-head comparison that compared intravenous infusions of VDZ with subcutaneous adalimumab in a double-blind, double-dummy RCT^[224]. Clinical remission at week 52 was achieved in significantly more patients who received VDZ than in those receiving adalimumab (31.3% *vs* 22.5%) as did endoscopic improvement (39.7% *vs* 27.7%). The percentage of patients who had corticosteroid free remission at week 52 (a key secondary endpoint) was higher in the adalimumab group (21.8% *vs* 12.6%). Adverse events were higher in the adalimumab group. Previous anti-TNF exposure was allowed (but capped at 25%), and no dose escalation was allowed^[225].

Tofacitinib, a nonselective inhibitor of the Janus kinase enzyme was approved by the United States Food and Drug Administration for treatment of moderate to severe UC in 2018. In the OCTAVE (Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis) 1 and 2 studies, patients with moderate to severe active UC who had failed conventional treatment with 5-ASA, corticosteroids, and/or immunomodulators and approximately 50% of whom had also failed anti-TNF

therapies were treated with 10 mg bd tofacitinib in the induction trials^[226]. Clinical remission at week 8 was achieved in more patients receiving tofacitinib 10 mg twice a day (bd) (18.5% and 16.6%, respectively) than those receiving placebo (8.2% and 3.6%, respectively)^[226]. In the maintenance trial at 52 wk, 46% of patients receiving tofacitinib 10 mg bd and 34.3% of patients receiving 5 mg bd achieved remission compared with 11.1% who received placebo^[226].

Clinical responders to induction therapy entered the maintenance trial SUSTAIN (Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis) and demonstrated clinical remission in 34.3% of patients treated with tofacitinib 5 mg bd and 40.6% in the 10 mg bd group as opposed to 11.1% in the placebo group ($P < 0.001$ in both treatment arms *vs* placebo)^[226].

Herpes zoster was seen in 5.1% of tofacitinib treated patients at 10 mg bd as compared to 0.5% patients on placebo^[226]. Herpes zoster vaccination should be considered before starting treatment in people above 50 years, who are considered at high risk for recurrent shingles. No live vaccination should be administered for three months after stopping a biologic, and tofacitinib should not be commenced for 4 wk after live vaccine administration^[227]. An open label study in patients with rheumatoid arthritis comparing tofacitinib 5 mg or 10 mg bd with anti-TNF noted a 5-fold increased risk of pulmonary embolism in individuals over 50 years and with at least one cardiovascular risk factor in patients on tofacitinib 10 mg bd compared to those on anti-TNF^[227,228]. The European Medical Agency advises against using the higher (10 mg bd) dose of tofacitinib in patients with an increased risk of pulmonary embolus including recent surgery, coagulation disorders, previous thromboembolism, heart failure, malignancy, combined contraception, or hormone replace therapy^[228].

Ustekinumab is the most recently approved biologic for moderate to severe active UC patients who have failed to respond or have intolerance to corticosteroids, immunomodulators, anti-TNF therapy, or VDZ^[229]. The UNIFI trial randomized patients 1:1:1 to receive a single intravenous dose of placebo, 130 mg ustekinumab, or approximately 6 mg/kg ustekinumab (patients weighing ≤ 55 kg received 260 mg; patients weighing > 55 kg and ≤ 85 kg received 390 mg; and patients weighing > 85 kg received 520 mg). Clinical remission at week 8 (Mayo score ≤ 2 points with no individual subscore > 1) was the primary endpoint and achieved by 15.6% on 130 mg ustekinumab, 15.5% on the approximately 6 mg/kg dose, and 5.3% on placebo ($P < 0.001$)^[229]. Endoscopic healing (Mayo endoscopy subscore of 0 or 1) was 26.3%, 27%, and 13.8% in the three groups, respectively ($P < 0.001$). Clinical response (decrease from baseline Mayo score of $\geq 30\%$ and ≥ 3 points with either a decrease from baseline in the rectal bleeding subscore of ≥ 1 or a rectal bleeding subscore of 0 or 1) was 51.3%, 61.8%, and 31.3% in the three respective groups ($P < 0.001$). Improvement in the IBD questionnaire, mucosal healing, and histological healing (defined as $0 \leq 5\%$ neutrophils in epithelium, no crypt destruction, and no erosions, ulcerations, or granulations) was noted in active treatment arms compared to placebo at 20.3%, 18.4%, and 8.9%, respectively, at week 8. There were no differences in adverse events compared to placebo. In particular, there were no malignancies, opportunistic infections, or tuberculosis. Five-hundred and twenty three patients in clinical response at week 8 were rerandomized in the maintenance study (lasting 44 wk) to placebo, 8-weekly, or 12-weekly dosing with remission rates of 24%, 38.4%, and 43.8%, respectively ($P = 0.002$ for 8-weekly and $P < 0.001$ for 12-weekly *vs* placebo^[229]). There were no statistically significant benefits of 8-weekly dosing (compared to 12-weekly, although numerically higher) and were restricted to the anti-TNF refractory population. The safety profile was similar to that in CD UNITI studies^[229].

Acute Severe UC

Statement 53: Acute severe UC is defined as having bloody diarrhoea ≥ 6 /d. Any signs of systemic toxicity are an indication for hospital admission. [88.8]

Statement 54: Patients with severe UC should be assessed on the third day of intravenous (IV) steroid therapy; nonresponders are shifted to IFX or cyclosporine. Colectomy is recommended if there is no improvement following 4–7 d of salvage therapy. [77.7]

Statement 55: In severe active UC, in case of serious contraindication to steroids, IFX or cyclosporine are an alternative to the recommended IV steroids. [88.8]

Although the majority of patients tend to have a mild to moderate disease course, between 20%-25% of patients may experience a severe flare needing hospitalization for medical treatment and consideration for colectomy if medical therapy fails^[230]. ASUC is associated with a 30%-40% risk of colectomy after one or more severe exacerbations,

and 10%-20% may need colectomy at their first admission^[231-233]. ASUC is defined by the Truelove and Witts criteria^[57] with ≥ 6 bloody stools per day and pulse rate > 90 per minute, temperature > 37.8 °C, or haemoglobin < 10.5 g/L or ESR > 30 mm/h and European Crohn's and Colitis Organization criteria^[49], which includes CRP > 30 mg/L^[49]. Patients with ASUC should be hospitalized in a specialist gastroenterology facility for multidisciplinary care. The aim is clinical remission as defined by ≤ 3 stools per day without rectal bleeding^[49].

A full blood count, urea, electrolytes (including serum magnesium), creatinine, ESR, CRP, liver chemistry, lipid profile, abdominal radiograph, and stool tests for culture, microscopy, and sensitivity along with *Clostridium difficile* testing should be arranged^[43,49,63]. Nearly 50% of patients may prove refractory to IV hydrocortisone therapy^[234]. In anticipation for rescue therapy, additional tests including tuberculosis screening (interferon gamma release assay and chest radiograph), hepatitis B serology (hepatitis B surface antigen and hepatitis B core antibody; commonly known as HbsAg and HbcAb respectively), thiopurine methyltransferase activity (if not known previously), CMV IgG and IgM, human immunodeficiency virus, and varicella zoster serology should be arranged for patients who do not show a response by day 3 of IV steroid therapy^[43,49,63].

Intravenous corticosteroid therapy, namely hydrocortisone (100 mg three to four times a day) or methylprednisolone (60 mg/d), is the cornerstone of treatment. Higher doses offer no additional benefit. In the study by Truelove *et al.*^[235], 49 patients with ASUC were treated with intravenous prednisone, and a clinical remission rate of 73 percent was noted 5 d after treatment. In a subsequent systematic review of 32 trials of corticosteroid therapy for ASUC involving 1991 patients, 67% of patients responded to steroids with 29% having a colectomy^[201]. Mortality was 1%, and outcomes had not changed between 1974 and 2006^[201]. Rescue therapy (or surgery) should be considered for patients unresponsive to steroid therapy between days 3 and 5. The more recently reported CONSTRUCT (Infliximab *vs* ciclosporin for steroid-resistant acute severe ulcerative colitis) study also demonstrated a response rate of intravenous steroids in 49% of patients^[234]. Prolonged corticosteroid therapy beyond 7-10 d provided no additional benefit and increased the risk of toxicity^[43,201].

There are many stratification tools using clinical criteria to predict the need for timely rescue therapy or colectomy^[43,49,63]. Of these, the Travis criterion is widely used and suggests that at day 3 of corticosteroid therapy, patients with a stool frequency greater than 8 per day or a stool frequency of 3 per day and CRP > 45 mg/dL have an 85% likelihood of requiring colectomy in the current hospitalization^[44]. Rescue therapy in the form of either IFX or cyclosporine may be effective. Patients not responding to rescue therapy with either IFX or cyclosporine should be offered prompt colectomy.

The efficacy of cyclosporine in acute steroid-refractory UC was studied by Lichtiger *et al.*^[236]. Of 11 patients with steroid-refractory UC, 9 who received cyclosporine (4 mg/kg) as a continuous intravenous infusion improved, whereas the 9 patients who received placebo had no improvement. Colectomy was needed in 3 of 11 and 4 of 9 patients in the cyclosporine and placebo groups, respectively^[236]. In an RCT^[237] comparing 4 mg/kg with 2 mg/kg intravenous cyclosporine, both groups showed equal efficacy for severe steroid-refractory UC^[237]. At day 8, response was 82% and 83%, respectively, in both groups with no difference in short-term colectomy rates.

Long-term success of cyclosporine rescue requires concomitant immunosuppression with a thiopurine^[238]. A study of ASUC patients treated with intravenous cyclosporine with a median follow-up of 1.5 yrs showed that concomitant thiopurine therapy was the only factor associated with reduction in the risk of colectomy (OR 0.01; 95% CI: 0.00-0.09; $P < 0.0001$)^[238].

If oral cyclosporine is used as bridging therapy, then a thiopurine should be added, aiming to taper the dose of cyclosporine in a few months^[239-241]. Patients with previous inadequate response to thiopurine are unsuitable for cyclosporine therapy^[49]. A number of serious infections have been associated with cyclosporine in 5% of patients and mortality in 1%-3%^[239,242,243]. A number of adverse events are linked to cyclosporine therapy and include nephrotoxicity (6.3%), seizures (3.6%), anaphylaxis (0.9%), and death (1.8%). Additionally, paraesthesia, hypertension, hypertrichosis, headache, minor infections, hyperkalaemia, hypomagnesaemia, and gingival swelling are also known to occur^[239,242,243]. Cyclosporine is administered intravenously at a dose of 2 mg/kg/d aiming for levels between 150 ng/mL and 250 ng/mL using a monoclonal assay. Responders may be switched to an oral dose, twice the intravenous dose, administered in two doses and aiming for a trough concentration of 100-200 ng/mL^[239-241]. A thiopurine should be initiated during hospitalization. Prophylaxis for *Pneumocystis jirovecii* (sulfamethoxazole and trimethoprim) must also be initiated for the duration of triple therapy for 3 mo. At the end, cyclosporine can be stopped, and

AZA continued^[244-246].

IFX is widely used as rescue therapy in ASUC^[247]. In an early study, Jarnerot *et al*^[249] randomized 45 patients with acute severe steroid-refractory UC (4 d after initiating steroids) to either a single infusion of IFX (5 mg/kg) or placebo. In the IFX group, 7 of 24 patients (29%) had a colectomy within 3 mo as compared with 14 of 21 patients in the placebo group^[248]. Long-term (3 yrs) follow-up data of this cohort revealed a lower colectomy rate in IFX compared to controls (50% *vs* 76%) ($P = 0.01$)^[249]. IFX carries risks such as reactivation of latent tuberculosis, opportunistic infections, and sepsis that require screening for tuberculosis and hepatitis and should be avoided in the presence of infection or sepsis^[244-246]. Contraindications for IFX include congestive cardiac failure (New York Heart Association Class III/IV), demyelinating disease, sepsis, active tuberculosis, and active infection^[244-246]. Mortality risk between IFX and cyclosporine are comparable^[247].

Statement 56: The choice between options for salvage/rescue, either IFX or cyclosporine, should be individualized. [88.8]

Statement 57: Prolonged use of corticosteroids should be avoided as being ineffective as maintenance therapy and may lead to increased risk of postoperative complications. [88.8]

Statement 58: Only a single attempt at rescue therapy with IFX or cyclosporine should be considered before referral for colectomy. [100]

Cyclosporine and IFX have demonstrated equal efficacy in head-to-head comparison studies^[234,250,251]. The open-label CySIF (Cyclosporine *vs* IFX in patients with severe ulcerative colitis) trial included 115 patients previously naïve to IFX and cyclosporine with a Lichtiger score > 10 points (range 0-21) with ASUC refractory to at 5 d of intravenous steroids^[251]. The patients were 1:1 randomized to receive intravenous cyclosporine (2 mg/kg per day for 1 wk, followed by oral cyclosporine until day 98) or IFX (5 mg/kg on days 0, 14 and 42). AZA was commenced in both groups at day 7 in patients with a clinical response. Treatment failure defined by absence of a clinical response at day 7 was the primary endpoint as was relapse between day 7 and day 98, absence of steroid-free remission at day 98, any severe adverse event leading to treatment discontinuation, colectomy, or death^[251]. There were no statistically significant differences in treatment failure in patients given cyclosporine (60%) and those on IFX (54%) ($P = 0.52$). Nine (16%) patients in the cyclosporine group and 14 (25%) in the IFX group had severe adverse events but not statistically different^[251]. Mucosal healing was similar in both groups (47% in the cyclosporine group and 45% in IFX-treated patients) and colectomy rates (17% in the cyclosporine group and 21% in IFX-treated patients) were also comparable^[251]. Long-term follow-up of patients treated in the CySIF trial showed no difference in colectomy-free survival at 1 year and 5 years in patients treated with either cyclosporine or IFX^[252].

The CONSTRUCT (Infliximab *vs* ciclosporin for steroid-resistant acute severe ulcerative colitis) trial was a mixed-methods, open-label, pragmatic randomized trial including 270 patients^[234]. Patients were randomly allocated (1:1) to receive either IFX (5 mg/kg intravenous at baseline and again at 2 wk and 6 wk after the first infusion) or cyclosporine (2 mg/kg per day by continuous infusion for up to 7 d, followed by twice-daily tablets delivering 5.5 mg/kg per day for 12 wk). The primary outcome was quality-adjusted survival. There was no statistically significant difference between groups for the primary endpoint or for the secondary endpoints of colectomy rates, time to colectomy, serious adverse events, or death. IFX, however, was associated with a greater treatment cost^[234]. The availability of biologics similar to IFX will now positively impact the cost of IFX treatment. A meta-analysis did not show any difference in short-term response at 3 mo and 12 mo in RCTs^[253].

In a study of 740 patients with steroid-refractory ASUC with median follow-up of 71 mo, there was no difference in colectomy rates between patients treated with IFX or cyclosporine (26.2% *vs* 25.4%) at 5 yrs, but there was a significantly lower rate of serious adverse events in the cyclosporine group *vs* IFX (15.4% *vs* 26.5%) ($P = 0.001$)^[254].

The choice of either IFX or cyclosporine as rescue therapy must be individualized considering nuances of each therapy. The statistical power (80% power to detect a 30% difference between groups) in the CysIF study was low, making a type II error likely, which is not detecting a difference between drugs if there is a difference. IFX was administered as per the standard induction regimen whilst cyclosporine levels were monitored. Increasingly, our knowledge of IFX dosing is being informed by the understanding of the pharmacokinetics of IFX^[43,247,255]. A high TNF burden, proteolytic degradation of drug^[256], and increased intestinal loss of IFX influences the success of

IFX therapy^[257,258]. Elevated CRP (a surrogate for inflammatory activity), low albumin (active UC with increase in intestinal permeability and faecal drug loss), and severe endoscopic lesions can predict poor outcomes^[257,258]. IFX therapy was associated with a reduction in hospital stay (median 4 d) (interquartile range 4.0-5.75) with IFX compared with 11 d (interquartile range 7.75-13.25) with cyclosporine^[259]. In the CONSTRUCT trial, patients and physicians noted greater treatment satisfaction with IFX^[234]. Improving knowledge of pharmacokinetics of IFX in ASUC will enable more efficient use of IFX as a rescue treatment.

Sequential rescue therapy is not currently recommended before referral for a colectomy. Studies of cyclosporine and IFX as sequential rescue therapy show limited efficacy, and the risk of adverse effects is concerning. A German study found a high risk of serious side effects (16% patients) including one death^[260]. Another study of 86 patients receiving sequential therapy (cyclosporine followed by IFX) reported colectomy-free rates of 61% and 41% at 3 and 12 mo, respectively; the risk of infectious complications was 10%^[261]. Chaparro *et al.*^[262] reported a 30% colectomy-free survival for sequential therapy (cyclosporine followed by IFX), 23% rate of adverse effects, and a death from nosocomial pneumonia^[262]. A systematic review of ten studies with 314 participants receiving sequential therapy noted short term response rates of 62.4% and remission rate of 38.9% with colectomy rates of 28.3% at 3 mo and 42.3% at 12 mo^[263]. There is insufficient evidence to support sequential therapy currently. With risks of severe immunosuppression, this approach is not supported by current guidelines^[49,63,147,264]. Prolonged corticosteroid therapy beyond 7-10 d provides no additional benefit and increases the risk of toxicity and postoperative complications^[43,201,265].

Colectomy involves excision of the colon, proximal rectum, and distal rectal mucosa and may be considered “curative” for acute colitis. Colectomy needs to be urgently considered in emergencies including refractory haemorrhage, toxic dilatation, intestinal perforation, or inadequate response to medical therapy. Inappropriate delay in considering colectomy can lead to risk of adverse outcomes and/or death^[266,267]. This emphasizes the need for an “exit strategy” in steroid-refractory ASUC patients by day 3-5 of hospitalization by the gastroenterologist involving the patient, surgeon, and a stoma therapist. Timely consideration (within 7 d) of colectomy in patients with ASUC or steroid-refractory disease is associated with improved perioperative outcomes and reduced in-hospital mortality and morbidity^[268].

Steroid-dependent UC

Statement 59: Azathioprine can be used as a steroid-sparing agent in steroid-dependent UC. [100]

Statement 60: Anti-TNF agents, VDZ, tofacitinib, and ustekinumab are effective for induction of remission in steroid-refractory or steroid-dependent moderate to severe UC. They are also effective in moderate colitis refractory to thiopurines. [88.8]

Thiopurines (AZA and mercaptopurine) are effective steroid sparing agents for the maintenance of remission in moderate to severe active UC^[269-271]. They are not effective for induction of remission. In a meta-analysis of three randomized studies, thiopurine maintenance favoured placebo (RR 0.6, 95%CI: 0.37-0.95)^[270]. In a subsequent meta-analysis, treatment with thiopurine was associated with 23% absolute risk reduction (number needed to treat = 5) to prevent one recurrence (OR 2.59, 95%CI: 1.26-5.3). The most recently published Cochrane review of four studies of AZA *vs* placebo reported a benefit of AZA over placebo (44% *vs* 65% failure, respectively, RR 0.68; 95%CI: 0.54-0.86)^[272].

Anti-TNF and anti-integrin agents are effective for the induction of remission in steroid refractory and steroid dependent moderate to severe active UC. Janus kinase inhibitor (tofacitinib) and ustekinumab (anti-p40 subunit inhibitor of IL12/23) are also currently licensed for patients with moderate active UC refractory to oral corticosteroids. They are also effective in moderate to severe active UC refractory to thiopurines. A full discussion of this can be found under Statement 52 and 55 above.

MANAGEMENT-MAINTENANCE OF REMISSION IN UC

Statement 61: The goal of maintenance therapy in UC is to maintain steroid-free remission defined clinically and endoscopically. [100]

Statement 62: Choice of maintenance treatment is determined by disease extent, disease course, response to previous maintenance treatment, severity, and treatment of

the most recent flare as well as the safety of maintenance treatment. [88.8]

Statement 63: For patients achieving remission with 5-ASA, the use of 5-ASA oral and/or topical should be used as maintenance therapy depending on disease extent. [88.8]

Statement 64: Once daily oral 5-ASA preparations are preferred over sulphasalazine for maintaining remission due to reducing toxicity. [88.8]

The aim of maintenance treatment in UC is steroid-free remission defined both clinically and endoscopically^[273]. Several scoring systems are available to classify disease severity in UC^[72]. They aid objective assessment of disease and guide therapeutic and monitoring strategies, and their strength lies in the potential to monitor patient progress during follow-up^[17,54]. Several clinical scoring systems are available and are discussed in detail under statements 16 and 17.

Of these, the MCS is easier to use and has been used widely in adult clinical trials^[77]. The MCS (0-12) includes stool frequency, rectal bleeding, a physician's global assessment, and endoscopic assessment. Clinical improvement is defined as the reduction of baseline scores by ≥ 3 points and clinical remission as an overall score ≤ 2 (and no individual sub-score > 1) or UCDAI ≤ 1 ^[75,76]. A PMS < 1 indicates remission^[17]. The PMS uses the nonendoscopic components of the total score and correlates well with PROs with treatment^[78,79]. Recently, the PRO2, derived from components of the Mayo score, has been proposed as an interim outcome measure when combined with endoscopic data. PROs appear to correlate well with established activity and may improve the ability to predict patient defined remission^[80].

Endoscopic remission (mucosal healing) has emerged as a key goal of therapy. "Treat to target" has emerged as our new standard in well-selected patients and is discussed under statement 40.

The therapeutic strategy for maintenance of remission in UC is ascertained by disease extent, disease course response to previous maintenance treatment severity, and treatment of the recent flare as well as safety of maintenance treatment. These are discussed in detail under statements 41 through 55.

Patients achieving remission on 5-ASA therapy should have maintenance treatment with 5-ASA long term to maintain remission. 5-ASA has shown efficacy at doses of 2 g daily or higher in a Cochrane meta-analysis as discussed under 5-ASA above^[195]. Topical therapy is also effective and should be continued where possible to maintain remission^[172,174]. 5-ASA adherence can be a challenge and particularly so with topical therapy and once daily dosing. Alternate day or every third day therapy for topical treatment does not reduce the rate of remission and may be associated with better adherence^[172,176,274].

Oral 5-ASA was associated with a higher rate of failure to maintain clinical or endoscopic remission (RR 1.14; 95%CI: 1.03-1.27) than sulfasalazine and a higher rate of failure to maintain remission in general (RR 1.08; 95%CI: 0.92-1.26) in a Cochrane review^[275]. Sulfasalazine may often cause side effects such as headache and nausea. Additionally, allergy to the sulpha moiety and need for multiple dosing can limit its use^[147]. Furthermore, it has a nonuniversal, reversible, nondose dependent effect on male infertility^[276,277]. Resolution of sperm abnormalities occurs approximately three months after sulfasalazine discontinuation. Prospective fathers should discontinue sulfasalazine 3-4 mo prior to attempting conception given the negative impact on semen quality. They can be switched to a 5-ASA compound that is compatible with use throughout conception^[278].

Statement 65: 5-ASA maintenance treatment should be continued long-term; this may reduce the risk of colon cancer. [77.7]

5-ASA has been shown to have a protective effect against colorectal neoplasia risk in IBD^[279]. A systematic review concluded that 5-ASA therapy was associated with an OR of 0.6 (95%CI: 0.42-0.9; $P = 0.04$) for the development of colorectal cancer^[280]. Two recent meta-analyses confirmed these findings^[281,282]. It is unclear if this is a true biological effect or hinges on control of inflammation, which is known to be a driver of colorectal neoplasia risk in IBD^[283-285]. Indeed, in patients on immunosuppressive therapy, data have shown no additional benefits of 5-ASA and stopping 5-ASA in patients on anti-TNF therapy did not worsen disease course^[286]. Given the weight of the evidence currently, 5-ASA maintenance treatment should be continued long term for the chemoprophylactic benefit in addition to maintenance of remission achieved.

Statement 66: Thiopurines are effective in maintaining remission in patients with early or frequent relapse while taking 5-ASA, patients who are intolerant to it, patients who

are steroid-dependent, and patients responding to cyclosporine. [88.8]

Thiopurines (AZA and mercaptopurine) are effective steroid sparing agents for the maintenance of remission in moderate to severe active UC^[269-271]. They are not effective for induction of remission. In a meta-analysis of three randomized studies, thiopurine maintenance favoured placebo (RR 0.6; 95%CI: 0.37-0.95)^[270]. In a subsequent meta-analysis, treatment with thiopurine was associated with an absolute risk reduction of 23% (number needed to treat = 5) to prevent one recurrence (OR 2.59; 95%CI: 1.26-5.3). A Cochrane review of four thiopurine maintenance studies *vs* placebo showed a benefit of AZA (44% *vs* 65% failure, respectively, RR 0.68; 95%CI: 0.54-0.86)^[272].

Statement 67: In patients responding to anti-TNF, maintaining remission by continuing anti-TNF therapy with or without thiopurines is appropriate. [100]

Patients who have responded to anti-TNF therapy should be maintained on anti-TNF therapy with or without thiopurines. A systematic review and meta-analysis of six placebo-controlled, double-blind studies showed that IFX, adalimumab, and golimumab demonstrated more efficacy than placebo for clinical remission maintenance in UC^[205]. The UC-SUCCESS study showed that in patients in whom corticosteroid therapy had failed, the combination of IFX and AZA was more effective with higher clinical remission rates at week 16 (40%) compared to IFX alone (22%)^[207].

Statement 68: VDZ is efficient in inducing and maintaining remission in patients who failed anti-TNF. [88.8]

In the GEMINI trials, the primary endpoint was clinical remission at week 52. Of 374 patients randomized to VDZ or placebo, 47.1% achieved clinical response at week 6 in the VDZ group when compared with 25.5% in the placebo group (95%CI: 11.6–31.7; $P < 0.001$). At week 52, 41.8% of patients treated with VDZ 8 weekly, 44.8% treated with VDZ 4 weekly, and 15.9% of patients receiving placebo were in clinical remission. VDZ was superior to placebo for clinical response (RR = 0.82; 95%CI: 0.75-0.91), induction of remission (RR = 0.86; 95%CI: 0.80-0.91), endoscopic remission (RR = 0.82; 95%CI: 0.75-0.91), and remission at 52 wk in week 6 responders (RR = 2.73; 95%CI: 1.78-4.18) in a Cochrane systematic review^[216]. More patients naïve to TNF antagonists achieved endoscopic remission than patients with TNF antagonist failure at weeks 26 and 52. The GEMINI open label extension included patients with a minimum 248 wk of cumulative VDZ treatment ($n = 154$). Among induction responders and those who completed the maintenance study, 40.9% of patients had 248 wk of treatment; 98% achieved clinical response, and 90% had clinical remission^[217]. Significant improvements in PROs of reduction in rectal bleeding and stool frequency as early as 2 wk were reported in post hoc analysis of GEMINI trials^[218].

The US-VICTORY (Vedolizumab for Health OuTcomes in InflammatORY Bowel Diseases) study reported on 321 VDZ-treated UC patients, 71% of whom had failed anti-TNF treatment^[217]. Clinical and endoscopic remission was achieved at 12 mo by 51% and 41% of patients, respectively. Lower rates of clinical [hazard ratio (HR): 0.53; 95%CI: 0.38–0.75] and endoscopic remission (HR: 0.51; 95%CI: 0.29–0.88) were noted in those with previous anti-TNF exposure^[217]. The EVOLVE (Retrospective Real-World Comparative Analysis Highlights Safety of Vedolizumab and Anti-TNF α Therapies in Biologic-Naïve Patients study for UC) was a retrospective study of the safety and effectiveness of VDZ compared with anti-TNF agents in a real-world cohort of biologic naïve patients^[221]. At 24 mo, clinical response (91% *vs* 86%), clinical remission (79% *vs* 66%), and mucosal healing (92% *vs* 84%) were high in VDZ and anti-TNF patients, respectively, with no real differences between groups. Treatment persistence (75% *vs* 54%; $P < 0.01$) was greater with VDZ than anti-TNF, whilst more anti-TNF treated patients required dose escalation than the VDZ group (25% *vs* 31%; $P < 0.05$)^[221].

Statement 69: Thiopurines are appropriate to maintain remission in thiopurine-naïve patients. [100]

The role of thiopurines in the maintenance of remission in steroid dependent UC has been discussed above under statement 66.

MANAGEMENT–ACTIVE CD

Statement 70: The management plan for a patient with CD should take into account the activity, site, and behaviour of disease, and should always be discussed with the patient. [100]

Statement 71: Determining the activity of disease may be more difficult in CD than UC and should rely on objective evidence, *e.g.*, inflammatory markers or colonoscopy. [77.7]

Statement 72: It is important to confirm disease activity as a cause of recurrent symptoms, although unnecessary to re-evaluate the distribution of disease unless this will alter management [77.7]

Management decisions for CD must take into account disease location, behaviour, and activity. Accordingly the Montreal classification is used to classify CD according to age (A1 < 17 years at diagnosis, A2 17-40, A3 > 40), location (L1-ileal, L2-colonic, L3- ileocolonic, L4 isolated upper GI), and disease behaviour (B1-nonstricturing nonpenetrating, B2-structuring, B3-fistulizing, and “p” for perianal disease modifier)^[55,56]. These classification systems aid clinical decision making regarding medical or surgical treatment. A third of patients will have ileal, ileocolonic, or colonic disease, and in 6%–14% the disease location may change over time^[287,288]. Inflammatory disease is noted in 56% to 81% at diagnosis, whilst 5% to 25% will have stricturing or penetrating disease behaviour at diagnosis^[287].

The likelihood of developing an intestinal complication in patients with inflammatory behaviour was 51% at 20 years after diagnosis. Ileal, ileocolonic, or upper GI involvement compared to colonic involvement were significantly associated with shorter time to the development of intestinal complications^[96].

In a population-based study, perianal fistulae occurred in 10%-26%, and the cumulative risk was 26% at 20 years after diagnosis^[96].

Determining clinical disease activity should be based on objective and validated scoring systems^[72]. The CDAI is time-consuming, requires data input from patients, and focuses on diarrhoea (which may have other pathophysiological reasons and not only inflammation), cannot be used in patients with stomas, and is not validated for use after surgery. The Harvey Bradshaw score is more practical for clinical application^[72].

Biochemical markers can be a useful surrogate of inflammation, but 40% of patients with IBD and mild inflammation can have normal CRP and ESR values limiting their use in monitoring^[289].

Ileocolonoscopy with biopsy is the gold standard first line investigation for suspected CD. Ileoscopy with biopsy histology is superior in establishing the diagnosis of mild ileal CD, although terminal ileal intubation may not always be possible^[72].

Patients may have isolated proximal small bowel disease beyond the reach of even complete ileocolonoscopy. Ileoscopy and radiological imaging are complementary in diagnosis of ileal CD^[290,291]. Dedicated small bowel should be conducted in addition to ileocolonoscopy in patients with suspected CD and those with unspecified colitis at ileocolonoscopy^[72]. Mucosal biopsy is necessary for a comprehensive assessment of the colon and distal ileum^[52,290].

Endoscopic assessment is not always necessary for the monitoring of disease activity or detection of recrudescence of inflammation. Faecal markers may have a role in the noninvasive monitoring of CD activity. FC is a sensitive marker of disease activity and correlates with several endoscopic activity indices^[292]. A calprotectin > 160 µg/g has a sensitivity of 91.7% and a specificity of 82.9% to predict relapse in patients with IFX-induced remission^[293-295].

Mild to moderate active luminal CD

Statement 73: Oral budesonide is the preferred treatment for the mildly active localized ileocecal CD. [88.8]

Controlled ileal release (CIR) budesonide is effective for control of symptoms of mild to moderate active localized ileocecal CD. CIR budesonide is a pH-dependent ileal release oral corticosteroid with high topical activity and low systemic bioavailability (about 10%-20%)^[296,297]. A randomized double blind trial reported that CIR budesonide 9 mg daily for 8 wk was as effective as prednisolone 40 mg daily (tapering to 5 mg at 8 wk) for the induction of remission in patients with mild to moderate active ileo-caecal CD with efficacy (CDAI < 150) at 51% for budesonide at 8 wk compared with 52.5% for prednisolone and significantly fewer side effects^[298]. Once daily 9 mg is as effective as 3 mg three times daily^[299] and was demonstrated effective over placebo at achieving remission^[300-302]. Somewhat lower efficacy of CIR budesonide should be considered in context of its pharmacokinetic profile, releasing drug in the ileum and right colon, topical effect with extensive first-pass metabolism, and consequent lower systemic corticosteroid exposure^[300-302]. Budesonide is inferior to prednisolone (RR 0.52, 95% CI 0.28-0.95) in the context of more severe disease (CDAI >

300). When remission is achieved budesonide may be tapered over 1-2 wk^[296,297].

Statement 74: 5-ASA should not be used for induction of remission and achieving mucosal healing in patients with active CD. [88.8]

Oral and topical mesalamine is no more effective than placebo for the induction of remission and achieving mucosal healing in patients with active CD^[303-305]. Although sulfasalazine (3-6 g daily) may be an effective treatment of mild to moderate active colonic CD and/or ileocolonic CD (but not small bowel disease), it is not more effective than placebo for achieving mucosal healing in CD^[303,306,307].

Statement 75: Metronidazole should not be used as primary therapy for luminal inflammatory CD. [100]

A wide range of antibiotics have been studied in the induction of remission for CD^[308]. The precise mechanisms whereby broad-spectrum antibiotics work is uncertain but might include immunosuppressive activity (*e.g.*, metronidazole), treatment of bacterial overgrowth, and suppression of a bacteria-induced antigenic stimulus. However, metronidazole has been demonstrated as not more effective than placebo at inducing remission in patients with CD^[309].

In a paediatric randomized trial of 73 patients with CD, azithromycin 75 mg/kg for 5 d a week for 4 wk followed by metronidazole 20 mg/kg for 4 wk was compared with metronidazole only for 8 wk. A remission rate of 66% for azithromycin/metronidazole was noted as compared to 29% for metronidazole ($P = 0.025$)^[310].

Statement 76: Antimycobacterial therapy has not been shown to be effective and should not be used as primary therapy. [100]

In a randomized placebo-controlled trial of 2 yrs of treatment with clarithromycin, rifabutin, and clofazimine in 213 patients with active CD, early benefit of antibiotics was noted. However, there were no significant differences in relapse rates during follow-up^[311]. In another phase 3 trial using higher doses, 331 patients with moderate to severe active CD were randomized to RHB-104 (clarithromycin 95 mg, rifabutin 45 mg, and clofazimine 10 mg) five capsules twice daily or placebo for 52 wk in addition to the pre-study therapy. Remission was achieved at 26 wk in 37% *vs* 23% on placebo ($P = 0.007$), and durable remission (from week 16 to 52) was achieved in 18% *vs* 9% on placebo ($P = 0.019$)^[312]. Data on mucosal healing or duration of benefit at the end of therapy are not available. In the absence of credible supportive evidence, antimycobacterial therapy should not be used as primary therapy for CD.

Statement 77: Biologic therapy should be considered in CD patients with high disease activity and features indicating a poor prognosis. [88.8]

Anti-TNF therapies (IFX, adalimumab, and certolizumab pegol) have been shown to be effective for treatment of patients with CD with an inadequate response to treatment with corticosteroids, thiopurines, and methotrexate^[83,84,313-316]. Notably, combo therapy using IFX with immunomodulators is more effective than monotherapy with either agent and are discussed under statement 89^[317-320]. Anti-integrin (VDZ) and anti-interleukin ustekinumab (blocking p40 subunit of IL12/23) have been licensed for the management of steroid and/or immunomodulator refractory CD^[215,220,321-328]. Details on trials and supportive evidence are discussed under individual statements below.

Statement 78: Mild oesophageal or gastroduodenal CD may be treated with a proton pump inhibitor with close monitoring, while more severe or refractory disease requires additional systemic corticosteroids or a biologic-based strategy. [88.8]

The prevalence of upper GI tract CD in adults is 0.3%-5%^[134]. Routine endoscopic evaluation in asymptomatic patients (predominantly children) may disclose mild endoscopic changes in nearly 64% of patients and histological abnormalities in up to 70% of patients^[329]. Endoscopic lesions may be mucosal nodularity, ulcers (aphthous and linear), antral thickening, and duodenal strictures^[330]. Histologic features may include granuloma, focal cryptitis of the duodenum, and focally enhanced gastritis^[331].

The European Panel on the Appropriateness of Crohn's Disease Therapy (EPACT II) was a multidisciplinary international panel that published guidance on the management of special situations including upper GI CD^[332]. The EPACT II panel recommends proton pump inhibitors as first line therapy in the absence of stenosis and steroids as second line and IFX being recommended as third line treatment, respectively^[332]. The Panel deemed adalimumab of uncertain benefit, and 5-ASA certolizumab and natalizumab were deemed inappropriate. Balloon dilatation was recommended as first line therapy in the presence of stenosis followed by proton pump inhibitors and then steroids, thiopurines, or surgery as subsequent options. The benefit of IFX was uncertain. Enteral nutrition may be required and is best delivered

by a gastrostomy tube in the context of severe or stricturing disease^[332]. In isolated lesions of the oesophagus, other diagnoses such as reflux disease, tuberculosis, fungal disease, sarcoidosis, Behcet's disease, and malignancy should be considered^[333].

Statement 79: Any patient who has an early relapse after a course of steroids should started immunomodulator or biologic therapy to reduce the risk of a further relapse and/or prolonged steroid therapy. [88.8]

In patients with moderate to severe CD who have a relapse after corticosteroid therapy, thiopurine analogues (6-mercaptopurine and AZA) may be used. Mercaptopurine and its prodrug, AZA, have steroid-sparing effects for the maintenance of remission in CD^[334,335]. Their slow onset (8-12 wk) makes them ineffective for short-term induction in active, symptomatic disease^[334,335]. Thiopurines were more effective than placebo for maintenance of remission in CD, although quality of evidence for this has been reported as low [number needed to treat (NNT) = 9]^[335]. A systematic review with network meta-analysis showed a benefit of thiopurines compared with placebo for the maintenance of remission of CD, although anti-TNF therapy was more effective than thiopurines^[334]. Intramuscular methotrexate 25 mg weekly given to patients with chronic active CD despite 3 mo of prednisolone therapy showed improved clinical remission rates compared with placebo at 16 wk with reduction in corticosteroid requirements^[336]. Methotrexate was found efficacious as maintenance therapy^[337]. These findings were confirmed by a Cochrane review^[338] and another network meta-analysis showing benefit of methotrexate (OR 0.24; 95% CI: 1.1-4.8)^[334]. Dosing and monitoring of thiopurines is discussed under statement 97 below. The side effect profile of AZA and 6-mercaptopurine includes allergic reactions, pancreatitis, myelosuppression, nausea, infections, hepatotoxicity, and malignancy, especially nonmelanoma skin cancer and lymphoma^[339,340].

If methotrexate is considered in women with child-bearing capability, highly effective contraception must be in place^[341]. There is some inconsistency in the evidence regarding whether men should be advised not to conceive whilst using methotrexate or within three months of stopping it in view of concerns regarding its effects on spermatogenesis and teratogenicity^[278,342]. Adverse effects of methotrexate include nausea and vomiting, liver and pulmonary toxicity, bone marrow suppression, and skin cancer; risk of lymphoma has not been convincingly demonstrated in patients with CD^[63,336,343,344]. The white cell count and liver biochemistry should be routinely monitored during methotrexate therapy^[63,336,343,344].

Anti-TNF antibodies (IFX, adalimumab, and certolizumab pegol) are effective for treatment of patients with CD who respond inadequately to standard^[83,84,313-316]. Combination therapy of IFX with immunomodulators is more effective than either agent given alone is discussed under statement 89^[317-320]. Anti-integrin (VDZ) and anti-interleukin ustekinumab (blocking p40 subunit of IL12/23) have been licensed for the management of steroid and/or immunomodulator refractory CD^[215,220,321-328]. Details on trials and supportive evidence are discussed under individual statements below.

Statement 80: Particular care should be taken to consider serious infections as a complication of immunosuppressive therapy, including biologics and steroids. [88.8]

Infection is the most frequently encountered consequence of biological therapy in patients with IBD^[245,246]. A systematic review and meta-analysis of 49 randomized placebo controlled studies reported that biological agents were associated with a moderate increase in the risk of any infection (OR 1.19; 95% CI: 1.10-1.29) and significantly increased risk for opportunistic infections (OR 1.90; 95% CI: 1.21-3.01) but do not influence the risk of serious infections in patients with IBD^[345]. Serious infection risk was significantly lower with biologic studies with a low risk of bias, perhaps reflecting control of active inflammation (OR 0.56; 95% CI: 0.35-0.90)^[345].

A pooled analysis of primary safety data across ten IBD clinical trials in adults receiving IFX and immunomodulatory therapy did not find an increase in the risk of infections or serious infections with long term IFX treatment compared to placebo^[346]. Patients with UC but not CD who received immunomodulator treatment (versus treatment without immunomodulator) demonstrated an increased risk of infection^[346]. Patients should be screened and vaccinated for vaccine preventable illnesses in line with international guidelines^[63,244-246].

A recent systematic review and meta-analysis evaluated the comparative risk of serious infections from immunosuppressive therapy^[344]. As compared to anti-TNF monotherapy, the risk of serious infection was higher with combination of anti-TNF and an immunosuppressive agent (RR 1.19; 95% CI: 1.03-1.37), with anti-TNF and a corticosteroid (RR 1.64; 95% CI: 1.33-2.03), or with all three drugs (RR 1.35; 95% CI: 1.04-1.77). Monotherapy with an immunosuppressive agent was associated with a lower

risk of serious infections than monotherapy with an anti-TNF agent (RR 0.61; 95%CI: 0.44-0.84) or a TNF antagonist with an immunosuppressive agent (RR 0.56; 95%CI: 0.39-0.81). IFX was associated with a lower risk of serious infections compared with adalimumab therapy in patients with UC (RR 0.57; 95%CI: 0.33-0.97) but not CD (RR 0.91; 95%CI: 0.49-1.70). Combination therapies for IBD including TNF antagonists, especially with corticosteroids, are associated with a higher risk of serious infection, whereas monotherapy with an immunosuppressive agent is associated with a lower risk compared with monotherapy with a TNF antagonist^[344].

Statement 81: The choice of biologic therapy depends on availability, route of delivery, patient preference and cost because they all have similar efficacy in luminal CD and similar adverse-event profiles. [88.8]

Biological therapies appear to have similar efficacy in luminal CD. In the absence of head to head studies, network meta-analyses suggested that in biologic naïve patients IFX and adalimumab ranked highest for the induction of clinical remission, whilst in anti-TNF experienced patients, ustekinumab and VDZ were ranked highest for the induction of clinical remission^[222]. For maintenance therapy, adalimumab and IFX were most likely to maintain remission in responders, whilst ustekinumab had the lowest risk of serious infections^[222].

There is no head-to-head comparison of ustekinumab *vs* VDZ in patients with IBD who have failed anti-TNF therapy, but indirect comparisons do not show differences in efficacy^[321]. Response is generally worse in patients with a longer disease duration or those refractory to other therapies^[323,347,348]. The VICTORY study compared outcomes among VDZ-treated and anti-TNF-treated patients with CD. After propensity score matching, with more than 500 patients included in the analysis, there was no significant difference in clinical and steroid free remission at 1-yr^[349]. Shorter disease duration was associated with higher response rates to VDZ in CD but not in UC^[349].

Advancing age and increasing comorbidity or possibly with a history of malignancy may favour nonsystemic therapy. Route of administration and cost of therapy may also drive treatment choice, with significant reductions in cost through biosimilar anti-TNF drugs^[350]. Drug factors, route of administration, disease severity and activity, and informed patient choice will govern the decision on which biologic is chosen^[350].

Moderate-to-severe CD

Statement 82: The severely active localized ileocecal CD should initially be treated with systemic corticosteroids. [100]

Statement 83: Conventional corticosteroids do not consistently achieve mucosal healing and should be used sparingly as a bridge to more tailored therapy. [88.8]

Statement 84: Intravenous corticosteroids and or biologic therapy can be used to treat severe CD in the absence of any contraindications. [100]

Patients with moderate to severe active localized ileocecal CD should be offered conventional corticosteroids orally, or intravenous for more severe disease, to alleviate symptoms of a flare^[306,307]. Every effort should be made to limit exposure^[202,203] as even short-term use of corticosteroids are associated with adverse events, such as bone loss, mood disorder, insomnia, hypertension, elevated blood glucose, glaucoma, acne, weight gain, and hypoadrenalism. Prednisolone is typically commenced at doses ranging from 40 to 60 mg/d^[351], continued for 1-2 wk and tapered by 5 mg weekly until 20 mg and then by 2.5-5.0 mg weekly^[351]. Corticosteroid tapers should generally not be carried out for more than 3 mo^[306,307]. Oral prednisone doses or equivalent corticosteroids exceeding 60 mg a day offer no additional advantage. Notably 1 in 5 patients will be refractory to steroids, and an additional third may become steroid dependent with symptoms of relapse at the attempt to taper^[351,352]. Corticosteroids are unable to maintain remission or achieve mucosal healing^[84,200,317]. In addition, corticosteroids may favour perforating complications (abscess and fistula) and are therefore relatively contraindicated in such cases. As such, steroid-sparing agents should be used in patients with severe disease phenotypes and to achieve and maintain meaningful remission^[1,16,17,20,158].

Statement 85: For patients with moderate to severe active CD, biologic therapy without an immunomodulator should be used for induction of symptomatic remission. [77.7]

Anti-TNF therapies are effective for patients with CD with an inadequate response to treatment with corticosteroids, thiopurines, and methotrexate^[83,84,313-316]. Combination therapy using IFX and immunomodulators is more effective than either drug as monotherapy^[317-320]. The effect of combining an immunomodulator with adalimumab

or certolizumab pegol is less well studied^[353,354]. Evidence to support continuation of an immunomodulator when an anti-TNF antibody is commenced after failure of the immunomodulator is lacking. The rationale in that situation would be to prevent antidrug antibody formation in the light of significant disease^[353,354]. Anti-TNF treatments are more effective than placebo for induction of response, remission, and complete and partial mucosal healing in patients with CD^[204,355]. Anti-TNF treatments have a rapid onset of action, as early as 2 wk of initiating therapy. Anti-TNF treatments are more effective when given earlier in the course of disease, especially when given within 2 yrs of disease-onset^[161,164,210,317].

Statement 86: Combination therapy of IFX with immunomodulators (thiopurines) is more effective than treatment with either immunomodulators alone or IFX alone in patients who are naïve to those agents. [100]

The SONIC (Infliximab, Azathioprine, or Combination Therapy for Crohn's Disease) study demonstrated that combination therapy with AZA and IFX was more effective than immunomodulators or IFX monotherapy in patients who were naïve to these drugs^[317]. This is likely to be a synergistic effect with the additional benefit of preventing immunogenicity from IFX. Adding an immunosuppressant may reduce requirement for dose escalation with IFX and also need for drug switching through reduction of immunogenicity and boosting trough levels^[318]. A network meta-analysis also demonstrated superiority of combination therapy using IFX with AZA over monotherapy^[334]. The recently reported PANTS (Personalized anti-TNF therapy in Crohn's disease) study (a 3 year observational cohort) also demonstrated immunogenicity rates of 26% and 28% with originator and biosimilar IFX, respectively, and that immunomodulator use reduced the risk of immunogenicity (HR = 0.37; $P < 0.0001$)^[356].

Combination therapy using IFX and methotrexate was not noted to be more effective than IFX monotherapy in maintaining remission up to 50 wk, although it was deemed as safe^[338,343]. A recent Cochrane review also reached a similar conclusion. Methotrexate does reduce immunogenicity to IFX^[338,343]. Taken together, the evidence suggests that when using IFX combined with AZA where possible (or methotrexate if AZA cannot be used) should always be preferred. If there are contraindications to both AZA and methotrexate, an alternative to IFX should be considered unless there is a strong clinical reason to use IFX, such as perianal CD^[357].

Statement 87: Cyclosporine, mycophenolate mofetil, and tacrolimus should not be used for CD. [50]

Cyclosporine, tacrolimus, and mycophenolate mofetil therapy are not recommended for use in CD^[358-361].

Statement 88: Immunosuppressive naïve patients who are dependent on corticosteroids should be treated with immunosuppressants and/or biologic therapy. [77.7]

A full discussion can be found under statements 79, 80, and 86.

Steroid-dependent CD

Statement 89: Methotrexate (up to 25 mg once weekly intramuscularly or subcutaneously) may be effective and should be considered in patients with steroid-dependent CD. [66.6]

Intramuscular methotrexate 25 mg weekly given to patients with chronic active CD despite 3 mo of prednisolone therapy showed improved clinical remission rates compared with placebo at 16 wk with reduction in corticosteroid requirements^[336]. Methotrexate was found efficacious as maintenance therapy^[337]. These findings were confirmed by a Cochrane review^[338] and another network meta-analysis showing benefit of methotrexate (OR 0.24; 95%CI: 1.1-4.8)^[334]. Oral methotrexate has reduced bioavailability compared with parenteral administration, and 15 milligrams of subcutaneous methotrexate may be preferred to intramuscular under the circumstances as it may be easier to administer and less painful^[362,363]. It is recommended that induction therapy is administered *via* the subcutaneous route, and patients may be switched to oral methotrexate during the maintenance phase. Folic acid should be administered at a dose of 5 mg weekly (typically one to two days after administration of methotrexate) or at a dose of 1 milligram daily to reduce GI and liver toxicity^[63].

Statement 90: Biologics should be used to treat CD that is resistant/dependent to treatment with corticosteroids. [100]

Anti-TNF therapies (IFX, adalimumab, and certolizumab pegol) are effective for

patients with CD with an inadequate response to treatment with corticosteroids, thiopurines, and methotrexate^[83,84,313-316]. Combination therapy using IFX and immunomodulators is more effective than either drug^[317-320]. The effect of combining an immunomodulator with adalimumab or certolizumab pegol is less well studied but is likely to be superior to therapy with anti-TNF agent monotherapy because all anti-TNF treatments are eventually immunogenic, and immunomodulators may reduce the rate of antidrug antibody formation^[204,335]. Anti-TNF treatments have rapid onset of effect with benefit often noted within 2 wk of initiating therapy. Anti-TNF treatments are more effective when given earlier in the course of disease, especially within 2 yrs of disease onset^[161,164,210,317]. Anti-integrin (VDZ) and anti-interleukin ustekinumab (blocking p40 subunit of IL12/23) have been licensed for the management of steroid and/or immunomodulator refractory CD^[215,220,321-328]. Details on trials and supportive evidence are discussed under individual statements below.

Statement 91: In view of the adverse effects of cigarette smoking on the course of CD, smoking should be discouraged in all patients. [100]

Cigarette smoking has an adverse effect on disease activity in CD^[364]. All IBD patients should be asked about cigarette smoking. Smokers with CD have an increased rate of surgery, IBD-related hospital admissions, and peripheral arthritis compared to nonsmokers^[365,366]. Active smoking is associated with penetrating disease and increases relapse risk even after discontinuation of biologic therapy^[367,368]. Conversely, in those who stop smoking, there are fewer flares and a reduced need for steroids and immunomodulatory therapy^[369]. Smokers with CD should be offered smoking cessation advice^[370,371] by suggesting behavioural therapy in combination with pharmacotherapy (nicotine replacement, bupropion, or varenicline)^[372]. Pregnant women should be counselled regarding risks and benefits of nicotine replacement therapy^[372].

Statement 92: In localized disease, thiopurines or methotrexate should be considered for maintaining remission achieved by systemic steroids. [77.7]

Thiopurines and methotrexate are not effective for induction of remission. Thiopurines have been demonstrated to be more effective than placebo for maintenance of remission in CD, although quality of evidence for this has been reported as low (NNT = 9)^[335]. A systematic review with network meta-analysis showed a benefit of thiopurines compared with placebo for the maintenance of remission of CD, although anti-TNF therapy was more effective than thiopurines^[334]. The role of methotrexate in maintenance of remission is discussed under statement 79.

Statement 93: Thiopurine S-methyltransferase (TPMT) testing should be performed before initial use of thiopurines. [77.7]

Statement 94: Upon relapse, escalation of the maintenance treatment can be considered to prevent disease progression. Steroids should not be used to maintain remission. [100]

TPMT is involved in the metabolism of AZA. A subset of patients with low TPMT activity may be at increased risk of adverse events from AZA and consequent discontinuation of therapy^[373,374]. In a prospective study patients dosed according to TPMT status performed better than those in the standard therapy group (2.6% risk of haematological side effects with TPMT directed dosing *vs* 22.9% in standard therapy group, RR 0.11, 95% CI: 0.01-0.85)^[375]. TPMT-directed dosing appears to be cost-effective^[373,374].

The starting dose of AZA is 2-2.5 mg/kg body weight and for mercaptopurine 1-1.5mg/kg in those with normal TPMT activity^[63]. Patients homozygous or compound heterozygous for TPMT (absent TPMT activity) have a very high risk of thiopurine-induced myelosuppression, and azathioprine should be avoided. In individuals who have heterozygous TPMT type, 50% of the standard dose may improve tolerance^[375]. Genetic variation in the *NUDT15* (*Nudix hydrolase 15*) enzyme has also been associated with myelosuppression^[376,377]. Although initially described in East Asians, this is also seen in those of European ancestry^[378]. The Clinical Pharmacogenetics Implementation Consortium recommends *NUDT15* testing in Asians with dose reduction or thiopurine avoidance in deficiency states^[376].

Thiopurines may be started at the full dose after determining TPMT status. There is no clear evidence in favour of gradually increasing the dose from low to the maximum weight-based dose. Such a strategy may in fact cause a significant delay in achieving a target dose and consequent clinical response^[379]. Measurement of thiopurine metabolites, thioguanine, and methyl-mercaptopurine may enable monitoring by detection of nonadherence to treatment, suboptimal dosing, or administration of a

high dose^[380].

Thioguanine levels of 230–400 pmol/8Å~108 erythrocytes are associated with better clinical response, and methyl-mercaptopurine levels over 5000 pmol/8Å~108 erythrocytes are associated with more liver toxicity^[381]. Small prospective studies have not shown clinical benefit of a metabolite driven strategy^[382,383]. In patients on combination therapy with IFX and thiopurines, a lower thioguanine of 125 pmol/8Å~108 erythrocytes appears adequate to achieve therapeutic levels of IFX^[384]. Another recent study suggested a target level of > 105 pmol/8Å~108 erythrocytes^[385]. Optimizing thiopurine doses in nonresponders can be considered before further escalation or change in treatment where possible.

MANAGEMENT–MAINTENANCE OF REMISSION IN CD

Statement 95: For CD patients with extensive disease, thiopurines and/or biologics are recommended for maintenance of remission. [77.7]

The role of thiopurines in the maintenance of remission in CD is discussed above under statement 94 The role of biologics for the maintenance of remission is discussed below.

Statement 96: In CD patients with aggressive/severe disease course or poor prognostic factors, biologics approved for the disease should be considered. [88.8]

Statement 97: Biologics should be given for CD refractory to thiopurine or methotrexate [88.8]

Biologics should be considered for the treatment of aggressive CD or associated with poor prognostic factors and for CD refractory to thiopurines or methotrexate.

Features linked with a severe disease course include young age at diagnosis^[97], extensive GI involvement, ileal/ileocolonic involvement, perianal/severe rectal disease, and patients presenting with a penetrating or stricturing disease phenotype^[98]. Visceral adiposity is also a marker for greater risk of penetrating disease^[99]. A detailed discussion can be found under statements 88, 89, 93, and specific biological agents discussed below.

Statement 98: If remission has been achieved with the combination of anti-TNF therapy and thiopurines in treatment naïve CD patients, maintenance with the same regimen is recommended. [88.8]

The data and merits of combination therapy are discussed under statement 86.

Statement 99: If remission has been achieved in CD patients with biologic monotherapy, maintenance with biologic monotherapy is appropriate. [88.8]

IFX was the first anti-TNF (and biologic) used in IBD demonstrating benefit in luminal CD. In the ACCENT 1 (Maintenance infliximab for Crohn's disease) study, 573 patients with active luminal disease were administered a single 5 mg/kg intravenous dose. Response was assessed at week 2, and patients were then randomized to placebo injections at weeks 2, 6, and then 8 weekly (group 1), or IFX 5 mg/kg at the same schedule, or IFX 5 mg/kg at weeks 2 and 6, then 10 mg/kg 8 weekly^[314]. By week 2, clinical response was noted in 58%. Among responders at week 30, 39% treated with 5 mg/kg maintenance and 45% on 10 mg/kg were in clinical remission with similar remission rates observed at week 54^[314].

In the CLASSIC (Adalimumab induced clinical remission in Crohn's disease) I study of adalimumab in moderate to severe CD naïve to anti-TNF therapy, adalimumab was administered 160 mg subcutaneously followed by 80 mg at week 2. Clinical remission (CDAI < 150) was achieved in 36% ($P = 0.001$ vs placebo) compared to 24% (80 mg/40 mg), 18% (40 mg/20 mg), and 12% on placebo^[386]. In CHARM (The Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance) study of maintenance therapy, induction responders (to 80 mg subcutaneous and 40 mg at two weeks) were given placebo, 40 mg every two weeks, or 40 mg weekly, with 12%, 36% and 41%, respectively, in clinical remission at week 56^[315]. The GAIN (Gauging Adalimumab Efficacy in Infliximab Nonresponders) trial showed efficacy of adalimumab in patients with active CD and loss of response or intolerance to IFX^[316]. In the EXTEND (extend the safety and efficacy of adalimumab through endoscopic healing) trial, adalimumab demonstrated efficacy in inducing and maintaining endoscopic mucosal healing^[313] with improved outcomes in those who achieved deep remission^[387]. These data demonstrate that anti-TNF treatments may maintain remission when it has been induced as monotherapy. The benefits of combination

therapy (at least with IFX) are discussed under statement 103.

Statement 100: IFX monotherapy is effective at maintaining biologics-induced remission, but because of the potential for immunogenicity and loss of response, the combination with thiopurines or methotrexate should be considered. [88.8]

A discussion of the data and merits of combination therapy with IFX can be found under statement 86.

The data for combination therapy with adalimumab and an immunomodulator is not as strong as for IFX. A meta-analysis demonstrated that combination therapy of adalimumab with an immunomodulator was slightly better than adalimumab monotherapy for induction of remission, but 1 year remission rates were not different, and dose escalation was not reduced compared to monotherapy^[353]. The DIAMOND (Adalimumab Monotherapy and a Combination with Azathioprine for Crohn's Disease) trial compared adalimumab monotherapy to combination therapy with AZA in 176 Japanese CD patients and showed similar rates of remission at week 26 and 52^[354].

The weight of the evidence suggests that although both IFX and adalimumab are immunogenic, at least for IFX, the combination with thiopurines or methotrexate should be considered.

Statement 101: Combination therapies are associated with increased risk of malignancies, and their use should always be balanced carefully against the substantial benefits associated with these treatments and discussed with the patient. [88.8]

The benefits and risks of combination therapy must be individualized in addition to infection risk. The potential risks of malignancies need to be carefully considered and discussed with the patient. Patients treated with thiopurines may have a higher risk, especially among males and those patients diagnosed at younger ages and in those over 65 years of age^[335,340,388]. The rare but increased risk of hepatosplenic T-cell lymphoma in young patients when a thiopurine is combined with an anti-TNF should be considered and discussed^[389].

Adverse effects of thiopurines include nausea, infections, allergic reactions, pancreatitis, myelosuppression, hepatotoxicity, and malignancy, particularly lymphoma and nonmelanoma skin cancer^[339,340].

A recent nationwide study from France reported that the risk of lymphoma was higher among those exposed to thiopurine monotherapy [adjusted hazard ratio (aHR), 2.60; 95% CI: 1.96-3.44; $P < 0.001$], anti-TNF monotherapy (aHR 2.41; 95% CI: 1.60-3.64; $P < 0.001$), or combination therapy (aHR 6.11; 95% CI: 3.46-10.8; $P < 0.001$)^[390]. The risk was higher in patients exposed to combination therapy *vs* those exposed to thiopurine monotherapy (aHR 2.35; 95% CI: 1.31-4.22; $P < 0.001$) or anti-TNF monotherapy (aHR 2.53; 95% CI: 1.35-4.77; $P < .001$). The use of thiopurine monotherapy or anti-TNF monotherapy was associated with a small but statistically significant increased risk of lymphoma compared with exposure to neither medication, and this risk was higher with combination therapy than with each of these treatments used alone^[390]. In the context of prior malignancy, biological treatment must be carefully considered. A delay of at least two years after successful cancer eradication seems appropriate and increased up to five years for malignancies with a high risk of late metastatic spread (including breast, melanoma, and renal cell carcinoma)^[391]. The New York Crohn's and Colitis Organization followed 333 patients with IBD and a history of cancer for up to five years and found no differences in the rate of cancer free survival between patients treated with anti-TNF alone or in combination or no immunosuppression^[392]. Recent European Crohn's Colitis Organization guidelines provide a comprehensive discussion on malignancy in relation to IBD and its treatment^[393].

Statement 102: VDZ should be used for maintenance of remission of VDZ-induced remission of CD. [100]

VDZ was shown to be effective at inducing and maintaining remission in moderately active CD in the GEMINI 2 trial^[394]. It included patients with moderate to severe active CD and evidence of inflammation (CRP > 2.87 mg/L, faecal calprotectin > 250 μ g/g stool and evidence of ulceration at colonoscopy and imaging). Two coprimary endpoints were considered at week 6: clinical remission (CDAI ≤ 150 points) and a CDAI-100 response (≥ 100 -point decrease in CDAI). The primary endpoint for maintenance treatment was clinical remission at week 52. Of 368 randomized patients, 14.5% achieved remission on VDZ as opposed to 6.8% on placebo ($P = 0.02$); a CDAI-100 response was achieved by 31.3% of VDZ-treated patients as opposed to 25.7% on placebo ($P = 0.23$). Clinical remission was achieved at week 52 in 39% of those treated with VDZ 8 weekly, 36.4% receiving VDZ 4 weekly,

and 21.6% receiving placebo^[394].

In the GEMINI 3 trial during a 10 wk induction period, 416 patients with moderate to severe active CD were included, 76% of whom anti-TNF therapy had previously failed^[395]. The primary endpoint was clinical remission at week 6. Secondary endpoints included clinical remission at week 10 and a CDAI-100 response at week 6 and week 10. Of 315 CD patients with anti-TNF intolerance or failure, 15.2% treated with VDZ and 12.1% on placebo achieved clinical remission at week 6 ($P = 0.433$). Clinical remission was achieved at week 10 by 26.6% patients treated with VDZ as opposed to 12.1% on placebo (95%CI: 1.3–3.6, $P < 0.001$). In a subgroup analysis, VDZ proved more effective than placebo for induction of remission in anti-TNF naïve patients (35.3 vs 16.0%, $P = 0.025$)^[395].

A subsequent analysis of patients in GEMINI 2 and GEMINI 3 with 516 anti-TNF naïve and 960 anti-TNF exposed patients reported clinical remission in anti-TNF naïve patients at week 6 (22.7% vs 10.6%, 95%CI: 3.7–21.4) and week 10 (26.6 vs 15.4%, 95%CI: 1.5–21.1)^[396]. Anti-TNF naïve patients achieved higher rates of clinical remission at week 52 compared with placebo (48.9% vs 26.8%, 95%CI: 8.9–35.4). Although clinical remission with VDZ and placebo at week 6 were comparable (13.3% vs 9.7%, 95%CI: 1.6–9.8) in patients previously unresponsive to anti-TNF agents, clinical remission at week 10 was higher in VDZ-treated patients (21.8% vs 11.0%, 95%CI: 4.5–18.6)^[396].

In the maintenance study, higher clinical remission rates were noted for VDZ-treated patients with prior anti-TNF failure against placebo at week 52 (27.7% vs 12.8%, 95%CI: 4.7–25.0)^[396]. Prior anti-TNF antagonist failure is associated with more refractoriness to induction therapy, but responders to VDZ have a durable treatment benefit irrespective of prior TNF antagonist exposure.

In the GEMINI open label extension study, clinical responders from the randomized trials who completed at least 52 wk of treatment were followed^[397,398]. There were 61 of 146 patients who had 248 wk of therapy. Clinical response and remission were maintained in 95% and 89%, respectively, of these patients with treatment benefits through weeks 52 and 248^[397,398]. A systematic review including 994 participants reported clinical response and remission rates at week 6 of 54% (95%CI: 41%–66%) and 22% (95%CI: 13%–35%), respectively, with similar rates at week 14. Clinical remission was noted in 32% (95%CI: 12%–62%) of patients at week 52^[219]. A recent systematic review with meta-analysis of real-world studies reported that 30% of CD patients were in clinical remission by week 14 (95%CI: 25%–34%) and at 12 mo (95%CI: 20%–42%) with higher rates in bio-naïve patients [48% of patients at week 14 (95%CI: 28%–68%) and 44% of patients at 12 mo (95%CI: 18%–75%)]^[399].

The VICTORY (Vedolizumab for Health OuTcomes in InflammatORY Bowel Diseases) study of 212 patients with moderate to severe CD reported 12 mo clinical remission, mucosal healing, and deep remission (clinical remission and mucosal healing) rates of 35%, 63%, and 26%, respectively^[400]. Clinical remission was less likely in individuals with prior TNF-antagonist exposure (HR 0.40; 95%CI: 0.20–0.81), smoking history (HR 0.47; 95%CI: 0.25–0.89), active perianal disease (HR 0.49; 95%CI: 0.27–0.88), and severe active disease activity (HR 0.54; 95%CI: 0.31–0.95)^[400]. After adjusting for disease-related factors including previous exposure to TNF antagonists, patients with early CD (< 2 yrs) were significantly more likely to achieve clinical remission than patients with later-stage CD (aHR 1.59; 95%CI: 1.02–2.49), corticosteroid-free clinical remission (aHR, 3.39; 95%CI: 1.66–6.92), and endoscopic remission (aHR 1.90; 95%CI: 1.06–3.39)^[349]. The VERSIFY (efficacy of vedolizumab on endoscopic healing in moderately to severely active Crohn's disease) study reported endoscopic, radiologic, and histologic healing in 101 patients who received VDZ therapy with moderate to severe CD (CDAI 220–450) and a simple endoscopic score for CD (SES-CD) of 7 or more, and failure of conventional therapy^[401]. The primary endpoint was endoscopic remission (SES-CD score ≤ 4) at week 26 and was achieved by 11.9% of patients (95%CI: 6.3–9.8). At week 52, 17.9% of patients were in endoscopic remission (95%CI: 8.9–30.4). Anti-TNF naïve patients achieved higher rates of endoscopic remission than patients with TNF-antagonist failure at weeks 26 and 52. A higher proportion of patients with moderate CD (SES-CD 7–15) achieved endoscopic remission at weeks 26 and 52 than patients with severe CD (SES-CD scores above 15). Magnetic resonance enterography evidence of remission was noted in 21.9% of patients at week 26 (95%CI: 9.3–40.0) and in 38.1% at week 52 (95%CI: 18.1–61.6). By week 52, 20.5% of patients had a histologic response in the colon (95%CI: 9.8–35.3) and 34.3% of patients had a histologic response in the ileum (95%CI: 19.1–52.2)^[401].

Statement 103: Ustekinumab should be used for the maintenance of remission of the ustekinumab-induced response of CD. [100]

Ustekinumab inhibits the p-40 subunit of IL-12 and IL-23 and is efficacious in CD

patients exposed to corticosteroids and/or immunomodulators or anti-TNF agents. Ustekinumab was evaluated in the Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease (UNITI and IM-UNITI) trials in patients with CD. UNITI-1 enrolled patients who had prior anti-TNF failure or intolerance. Clinical response was achieved at week 8 in 37.8% of ustekinumab 6 mg/kg treated patients ($P < 0.001$ vs placebo), 33.5% with 130 mg, ($P = 0.001$ vs placebo), and 20.2% with placebo^[323].

In the UNITI-2 study, patients without previous failure of treatment (mostly anti-TNF naïve, and some with previous successful use of anti-TNF therapy) were enrolled. By 8 wk, 57.9% in the 6 mg/kg, 47.4% receiving 130 mg, and 32.1% on placebo ($P < 0.001$ vs both doses) had a clinical response^[402]. Patients who responded at week 8 in both studies were randomized to the IM-UNITI maintenance study. Among these, 53.1% on 90 mg subcutaneously every 8 wk, ($P = 0.005$ vs placebo), 48.8% on 90 mg subcutaneously every 12 wk ($P = 0.04$ vs placebo), and 35.9% on placebo were in remission at 44 wk. IM-UNITI had 45% anti-TNF refractory patients. By week 44, 41.1% were in remission on ustekinumab 90 mg subcutaneously every 8 wk compared to 26.2% on placebo ($P = 0.10$). An increasing body of real-world experience supports the benefit of ustekinumab^[324-326,328].

Although there are no published head-to-head studies comparing ustekinumab and other biologics, indirect comparisons suggest no difference in efficacy^[321]. Experience from multiple clinical trials in CD has shown that patients with longer disease duration have lower response rates as do those who have proven refractory to other therapies^[347]. Data for ustekinumab use in pregnancy are limited to case studies and registry data. In the rheumatological literature, ustekinumab was not associated with increased risk of miscarriage or congenital malformation, although controlled data are not available^[403,404]. Ustekinumab is contraindicated in patients with active tuberculosis, sepsis, or opportunistic infections, including gut infections such as *Clostridium difficile*. The safety profile appears reassuring. Data from psoriasis studies have shown that anti-TNF therapies are associated with a greater risk of serious infection (1.9-2.9/100 patient years) compared to ustekinumab (0.93/100 patient years), although the dose of ustekinumab used in CD was lower^[405].

In a study of 167 Crohn's patients failing anti-TNF therapy and treated with ustekinumab, no malignancy, tuberculosis, or deaths were attributed to ustekinumab. Although 11.4% developed arthralgia^[328], this was not noted on active treatment compared to the placebo in the IM-UNITI maintenance trial^[323].

Statement 104: Loss of response to a biologic agent should be first managed by dose optimization guided by measurement of serum levels, if available, and antidrug antibodies followed by switching to a different drug within class or a different mechanism of action. [77.7]

Loss of response to an anti-TNF may be a primary (no response to induction therapy) or a secondary loss of response. In patients who have primary nonresponse to an anti-TNF, the probability of response to a second agent is low. Switching mechanism may be more successful. Therapeutic drug monitoring has a role, with emerging evidence suggesting that drug levels in primary nonresponders may be lower than in responders, and antidrug antibodies may occur within a few weeks of treatment initiation^[406,407].

Secondary loss of response to anti-TNF therapy may be due to immune-mediated neutralizing antibodies to the drug, non-neutralizing, drug-clearing antibodies, or nonimmune-mediated mechanisms. Therapeutic drug monitoring may help in decision-making and prove cost-effective^[408,409].

In a prospective study of IBD patients with secondary loss of response to IFX, mucosal healing occurred in half of patients who had their dose increased and was associated with a rise in trough levels^[410]. Increasing trough levels of IFX by shortening the infusion interval to six weeks may be as effective as shortening to 4 wk or administering 10 mg/kg dose^[411]. A proportion of patients may have detectable drug levels and a low titre of antidrug antibodies^[412]. Starting immunomodulator therapy (unless already started) may negate the antibodies and recapture response, particularly with an increase in anti-TNF dose^[413]. If the antibody titre is high with a low drug level, a switch possibly in the same class may be appropriate if the patient had responded to this class of therapy. There is a risk of forming antibodies to the next drug in class and switching to another mechanism of action may also be appropriate^[414]. Another important scenario to consider with loss of response and active disease is the presence of "adequate" drug levels. This implies mechanistic failure relating to the drug and switch to an alternative mechanism of action ("out of class").

Statement 105: 5-ASA is not recommended for maintenance of medically induced

remission in CD. [77.7]

A Cochrane review found that oral 5-ASA has no efficacy in maintaining clinical remission in CD^[415]. Several meta-analyses have reported similar negative findings for both induction and maintenance of remission with 5-ASA^[301,302,416,417]. Although some studies have shown possible benefit for sulphasalazine in the induction of clinical remission^[306,307], there was no benefit in maintenance and no benefit for 5-ASA for the induction of remission in colonic CD^[418]. As such, 5-ASA cannot be recommended for the maintenance of medically induced remission in CD.

Statement 106: Antibiotics are not effective for induction of remission in CD. [88.8]

A wide range of antibiotics have been studied in the induction of remission for CD^[308]. The precise mechanisms whereby broad-spectrum antibiotics work is uncertain but might include direct immunosuppression (*e.g.*, metronidazole), reduction of bacterial overgrowth, and suppression of a bacteria-induced antigenic stimulus. Metronidazole has not been shown to be effective as primary therapy for luminal inflammatory CD. Ciprofloxacin appears to have similar efficacy to mesalamine in active CD but is not more effective than placebo to induce remission^[308,419,420]. Neither of these agents influences mucosal healing in CD^[308,419,420].

Statement 107: Budesonide should not be used to maintain remission of CD beyond 6 mo. [77.7]

Six randomized placebo-controlled studies have evaluated the effect of budesonide on maintenance of remission in CD^[421-426]. Relapse rates at 12 mo for 3 to 6 mg budesonide ranged from 40% to 74% and were not significantly different from placebo. Subsequent meta-analyses have shown lack of efficacy in maintenance of remission with only slight improvements in mean time to symptom relapse^[427-430]. These meta-analyses included budesonide doses of 3 mg and 6 mg wherein more adverse events, such as abnormal adrenocorticoid stimulation tests and alteration in bone mineral density, were reported compared with placebo; however changes were lower than those with conventional corticosteroids^[427-430]. It is not recommended that budesonide be used for the maintenance of remission of CD beyond 6 mo.

FISTULIZING CD

Statement 108: Drainage of abscesses should be undertaken before treatment of fistulizing CD with biologics. [100]

Statement 109: Placement of setons increases the efficacy of anti-TNF and should be considered in treating complex perianal fistula. [88.8]

Statement 110: Anti-TNF with and without thiopurines is effective and should be considered in treating complex perianal fistulae in CD. [100]

Statement 111: The addition of antibiotics to anti-TNF is more effective than anti-TNF alone and should be considered in treating complex perianal fistulae. [50]

Statement 112: Antibiotics may be effective and should be considered in treating simple perianal fistulae. [88.8]

Upon suspicion of an intra-abdominal abscess, abdominopelvic cross-sectional imaging of the abdomen is recommended. Both CT enterography and MR enterography have a diagnostic accuracy greater than 90% for the preoperative diagnosis of abscesses^[431]. Preoperative CT guided abscess drainage may lead to a lower rate of postsurgical complications^[432]. For smaller abscesses (< 5 mm), surgical drainage is not required.

Asymptomatic simple perianal fistulae do not need specific treatment. Symptomatic simple fistulae are addressed with noncutting setons or fistulotomy. Complex fistulae with or without abscess require seton placement together with medical treatment^[433]. The decision on when to remove setons depends on treatment and drainage of any abscess. Surgical advancement flaps may be employed to close simple or complex fistulae in the absence of active infection or inflammation^[434,435].

Major fistulae (such as gastric-small bowel, proximal small bowel) may be associated with diarrhoea or small intestinal bacterial overgrowth for which surgery or medical therapy may be required. Prior to the initiation of immunosuppressive therapy with either biologics or antimetabolite therapy, complications such as abscesses should be considered using cross-sectional imaging and treated with

drainage before initiation of biologic therapy or immunosuppression^[436]. High-output fistulae need surgical intervention (proximal bowel diversion, bowel segment resection, or fistula closure). Low-output fistulae may respond to immunomodulator or biologic therapy as monotherapy or as combination treatment; evidence supporting the use of immunomodulation is weak^[357].

Proximal diversion (“defunctioning”) to enable rectal and/or perianal healing is required in conjunction with initiation of anti-TNF therapy to promote healing of the perianal disease. A systematic review reported that the long-term success of diverting ostomy is rather low^[437].

Patients with simple fistulae with no active mucosal involvement in the rectum have good response to fistulotomy or mucosal advancement flap surgery, whilst patients with mucosal involvement are more likely to benefit from seton placement than fistulotomy^[433-435]. Setons enable drainage of sepsis and inflammatory fistula tracts; this should be conducted before initiation of biologic therapy and has been associated with a better overall fistula healing and recurrence rate, longer duration of fistula closure, and prevention of repeated abscesses^[438-441]. Although other agents such as immunomodulators, VDZ, or anti-TNF- α agents may also be considered in individual circumstances, the weight of the evidence supports IFX^[357]. Thiopurines may alleviate symptoms of perianal fistulae but have a slow onset of action and have been shown to be effective for treating fistulizing CD^[357].

In the ACCENT II (A Crohn’s Disease Clinical Trial Evaluating Infliximab In a New Long-term Treatment Regimen in Patients with Fistulizing Crohn’s Disease) trial, induction response with fistula closure occurred in 69% of patients at 14 wk. Patient randomization to IFX subsequently had a significantly longer median time to loss of response compared to those receiving placebo (> 40 wk *vs* 14 wk), with 36% of patients with absence of fistula drainage after 54 wk of IFX treatment compared to 19% of placebo patients ($P = 0.009$)^[357]. Higher IFX trough levels may be needed for perianal fistulizing disease, with target levels > 10 $\mu\text{g/mL}$ associated with improved outcomes^[442].

The efficacy of adalimumab has not been tested in a trial with the primary endpoint of fistula healing. In the CHARM (Crohn’s Trial of the Fully Human Antibody Adalimumab for Remission Maintenance) trial, adalimumab had increased efficacy compared to placebo for closure of abdominal or perianal fistulae as a secondary endpoint^[315].

Of 117 patients with draining fistulae at baseline, complete fistula closure at week 56 was seen in 33% of patients on adalimumab as opposed to 13% on placebo ($P = 0.016$)^[315].

Furthermore, 90% (28/31) of patients with healed fistulae by week 56 (including those on placebo) maintained healing after a year on open label treatment^[443].

In another RCT of 76 patients with active perianal fistulizing disease receiving open label therapy with 24 wk adalimumab combined with either ciprofloxacin 500 mg BD for 12 wk or placebo, after 12 wk the primary endpoint of 50% reduction in draining fistulae was seen in 71% in the combination adalimumab/ciprofloxacin group compared to 47% in the adalimumab/placebo group ($P = 0.047$)^[444]. Notably, at week 24 (12 wk after stopping antibiotic therapy) there was no significant difference between the two groups, suggesting that antibiotics may assist healing but not affect long-term outcome^[444].

Antibiotics may heal simple, superficial perianal fistulae and have an adjunctive role in treating perianal sepsis associated with more complex fistulae as discussed above^[445-447]. Treatment usually involves metronidazole (10-20 mg/kg/d orally for 4-8 wk) and/or ciprofloxacin (500 mg orally twice daily for 4-8 wk), or levofloxacin (500-750 mg once daily for 4-8 wk) for both fistula and treatment of concurrent mucosal disease. Metronidazole and ciprofloxacin conversely have not demonstrated effective healing for complex perianal fistulae but may improve fistula-related symptoms^[448].

Statement 113: Anti-TNF with or without thiopurines may be effective and should be considered in treating enterocutaneous and rectovaginal fistulae in CD. [88.8]

Internal fistulae more challenging to treat and data on the management of enterocutaneous, enterovesical, and rectovaginal fistulae are limited.

Response to medical therapy (fistulae closure) as reported in a systematic review was complete in 38.3% of rectovaginal fistulae and 65.9% of enterovesical fistulae^[449]. In the case of enterovesical fistulae, risk factors for the need for surgery include sigmoid origin, small bowel obstruction, abscess formation, ureteric obstruction, or urinary tract infection^[450].

In a study of 47 patients with genital fistulae, antibiotics did not demonstrate

efficacy, thiopurines showed 13% complete and 24% partial response, and anti TNF- α therapy showed 17% complete and 30% partial response. A third of patients had surgery with 22% showing complete response after first surgery and 39% after reintervention. Overall, fistula closure was achieved in 30% of patients^[451].

Current optimal management of rectovaginal fistulae involves use of medical therapy (thiopurine monotherapy or anti-TNF or combination therapy) as first-line treatment. The aim of medical therapy is to heal mucosal inflammation and facilitate surgical intervention. Surgical options for the treatment of rectovaginal fistulae includes fistula excision with interposition of healthy tissue between the rectum and vagina. Active infection must be treated and resolved before attempting repair. Subsequently, a mucosal advancement flap can then be performed.

Likewise, enterocutaneous or enterovesical fistulae may be treated with immunomodulator therapy or anti-TNF antibodies or both. Prospective trial data are lacking. The fistulae and any associated complications need to be addressed collectively^[452,453].

Medical therapy has a role in the treatment of a fistula associated with active inflammation but not to treat a postoperative fistula^[454]. In the GETAID study of 48 patients with enterocutaneous fistula and 21 postoperative fistulae (within 30 days of surgery, mainly intestinal resections), approximately 33% had multiple tracts, and 25% had high output^[455].

Of patients receiving anti-TNF therapy, fistula healing occurred in a third, 50% of whom relapsed over a median 3 year follow-up. A third developed an intra-abdominal abscess whilst receiving anti-TNF therapy. Fifty-four percent of patients required surgery. Predictors of poor healing and surgery included complexity (multiple tracts) and associated stenosis^[455]. Complex fistulae are associated with adverse outcomes including mortality^[455]. Patients with enterocutaneous fistulae need multidisciplinary management decisions.

Among anti-TNF agents, higher quality evidence is available for IFX relative to adalimumab in the setting of nonperianal fistulizing CD. In the ACCENT II trial that had 25 women with 27 draining rectovaginal fistulae at baseline, fistula closure at week 14 was achieved for 45% of fistulae with IFX induction therapy^[357]. IFX maintenance was associated with a longer duration of fistula closure compared to placebo therapy (median 46 wk *vs* 33 wk). Enterocutaneous fistulae were present in less than 10% of the total number of patients. There has been no RCT of efficacy in fistulizing CD with adalimumab^[303].

PREVENTION OF POST-SURGICAL RECURRENCE

Statement 114: Thiopurines may be used to prevent clinical and endoscopic recurrence and are more effective than 5-ASA or placebo. However, they are not effective at preventing severe endoscopic recurrence. [88.8]

Statement 115: In high-risk patients, biologics should be started within 4 wk of surgery in order to prevent postoperative CD. [88.8]

Statement 116: In postoperative CD, IFX can be combined with an immunomodulator to decrease immunogenicity and decrease the loss of response. [66.6]

Up to 50% of patients with CD may require intestinal resection within ten years of diagnosis. The cumulative risk of surgery in patients with CD at one, five and ten years is estimated at 16.3%, 30.3%, and 46.6%, respectively^[456]. Surgery is not curative, and a quarter of these patients will need further resection within five years of index surgery^[456]. Furthermore, endoscopic recurrence defined by using the Rutgeert's score occurs in 30%-90% of patients at the neoterminal ileum within 12 mo of surgery and in most patients by five years^[457-462]. Several risk factors, such as penetrating disease phenotype, ≥ 2 previous CD-related surgeries and cigarette smoking, may adversely affect postoperative disease course^[463-469]. Additional risk factors include perianal disease, extensive small bowel resection a short interval between diagnosis and first surgery (< 10 yrs), and young age of onset (< 30 yrs)^[462,470].

Thiopurines have been shown to be effective at maintenance of remission after surgery in the TOPPIC (Trial of Mercaptopurine *vs* Placebo to Prevent Recurrence of Crohn's Disease) trial. Clinical or endoscopic recurrence at three years were similar, although a post hoc analysis did find that the mercaptopurine treated group was more likely to achieve endoscopic healing (Rutgeert's score i0)^[471]. In the POCER trial (Postoperative Crohn's Endoscopic Recurrence Study), thiopurine use was associated with lower endoscopic and clinical recurrence^[472]. Thiopurines are slower to act and as

such may be more suited to less severe endoscopic recurrence (Rutgeert's i2)^[470,472].

In another meta-analysis, thiopurines showed more efficacy than placebo for the prevention of clinical recurrence at one year (mean difference, 8%; 95% CI: 1%–15%; $P = 0.021$; NNT = 13) and two years (mean difference, 13%; 95% CI: 2%–24%; $P = 0.018$; NNT = 8) after surgery, and endoscopic recurrence (i2-4) (mean difference, 23%; 95% CI: 9–37%; $P = 0.0016$; NNT = 4) at one year after surgery^[473]. A meta-analysis of five controlled trials showed that they were not more effective than placebo or mesalamine (controls) for preventing clinical recurrence (year 1 RR 0.88; 95% CI: 0.60–1.30; $P = 0.53$ and year 2 RR 0.76; 95% CI: 0.55–1.05; $P = 0.10$) but did show more efficacy for the prevention of endoscopic recurrence (year 1 RR 0.71; 95% CI: 0.53–0.94; $P = 0.02$)^[474]. Notably, patients receiving thiopurines had more adverse events leading to drug withdrawal as compared with controls (RR 2.57; 95% CI: 1.47–4.51; $P = 0.001$)^[474].

There is little evidence to support the use of mesalazine in the prevention of postoperative recurrence of CD^[470]. A systematic review with meta-analysis showed modest benefit with reduction in clinical recurrence compared to placebo (RR 0.59; 95% CI: 0.43–0.82), but the evidence supporting its use to prevent endoscopic recurrence was subject to imprecision, inconsistency, and publication bias^[475].

Antibiotics have the potential to avoid relapse for at least three months after surgery^[476]. Metronidazole (20 mg/kg daily) was not well tolerated (23% drop out) during the three month trial and was associated with neuropathic complications, which are dose-related and raise concerns with longer term use^[477]. Ornidazole may also be effective but is also limited by significant side-effects (32% drop out during the one year trial treatment)^[478]. In a small pilot study, ciprofloxacin did not show much benefit over placebo^[479]. Trials with rifaximin are awaited in the postoperative setting, and its gut specificity may carry appeal^[480].

Anti-TNF therapy has been shown in several studies to reduce clinical and endoscopic recurrence^[470,475,481–483]. A randomized three arm study compared postoperative adalimumab against AZA and 5-ASA with a two year follow-up. Endoscopic recurrence was significantly lower in the adalimumab group (adalimumab 6.3%, AZA 64.7%, 5-ASA 83.3%) and significantly reduced clinical recurrence (12.5%, 64.7%, and 50%, respectively)^[484]. The POCER (Postoperative Crohn's Endoscopic Recurrence Study) trial was a randomized trial that compared an active care model using endoscopic assessment at six months postoperatively with standard care (no colonoscopy at six months)^[472]. Patients received metronidazole for three months postoperatively, and those at high risk of recurrence received thiopurines (or adalimumab if intolerant). Treatment was escalated in the active care group if endoscopic recurrence was found at the 6 mo colonoscopy to thiopurine, fortnightly adalimumab with thiopurine, or weekly adalimumab. Endoscopic recurrence at 18 mo was 49% in the active group *vs* 67% in the standard care group ($P = 0.03$), and clinical recurrence was 27% and 40%, respectively ($P = 0.08$)^[472].

Two meta-analyses have studied the efficacy of anti-TNF treatments for preventing postoperative CD^[476,485]. Van Loo *et al*^[485] demonstrated IFX superiority to placebo in preventing clinical and endoscopic recurrence. A network meta-analysis of 21 controlled trials across 5-ASA, antibiotic, and immunomodulator treatments showed that anti-TNF monotherapy was associated with a reduced risk of clinical relapse (RR 0.04; 95% CI: 0.00–0.14) and endoscopic relapse (RR 0.01; 95% CI: 0.00–0.05) compared with placebo^[476]. Anti-TNF monotherapy was the most effective intervention for prevention of postoperative recurrence (clinical relapse RR 0.02–0.20; endoscopic relapse RR 0.005–0.04). On this basis, anti-TNF therapy can be considered first line for patients at high risk for postoperative recurrence and for patients who have tried and failed or are intolerant to thiopurines. It is not known if combination thiopurine with an anti-TNF therapy is more effective than monotherapy, although the combination of IFX and AZA achieves higher response and remission rates and may also prevent immunogenicity to IFX^[317].

MANAGEMENT–INTRA-ABDOMINAL ABSCESS

Statement 117: An intra-abdominal abscess should be treated with antibiotics and a drainage procedure, either radiographically or surgical. [77.7]

When an intra-abdominal abscess is suspected, abdominopelvic cross-sectional imaging should be arranged. Both CT enterography and MR enterography have a diagnostic accuracy of greater than 90% for the preoperative detection of abscesses^[431]. Preoperative CT guided abscess drainage may lead to a lower rate of postsurgical

complications^[432]. For smaller abscesses (< 5 mm), surgical drainage is not required.

CONCLUSION

The incidence and prevalence of UC and CD are on the rise worldwide, and their heterogeneity between patients and within individuals over time is striking. The progressive advance in our understanding of the etiopathogenesis coupled with an unprecedented increase in therapeutic options have progressively changed the management towards tailoring evidence-based interventions by clinicians in partnership with patients.

This guideline was stimulated and supported by the Emirates Gastroenterology and Hepatology Society following an extensive systematic review and a Delphi consensus process provides evidence- and expert opinion-based recommendations.

Comprehensive up-to-date guidance is provided regarding evaluation of disease severity, appropriate and timely use of different investigations, and choice of appropriate therapy for induction and remission phase in view of the treatment goals, which now aim to treat beyond symptoms to achieve mucosal healing when possible and to minimize intestinal injury and bowel damage with resultant disability.

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Hepatocellular carcinoma after direct-acting antiviral hepatitis C virus therapy: A debate near the end

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Abstract

Direct acting antivirals (DAAs) have revolutionized the treatment of hepatitis C virus (HCV) infection, achieving high rates ($\geq 95\%$) of sustained virological response, with a good safety profile and high compliance rates. Consequently, it had been expected that viral clearance will reduce morbidity and mortality rates, as well as the risk of hepatocellular carcinoma (HCC). However, since 2016, concerns have been raised over an unexpected high rate of HCC occurrence and recurrence after DAA therapy, which led to an avalanche of studies with contradictory results. We aimed to review the most recent and relevant articles regarding the risk of HCC after DAA treatment and identify the associated risk factors.

Key Words: Hepatocellular carcinoma; Direct acting antivirals therapy; Hepatitis C virus infection; Sustained virological response; Risk factors of hepatocellular carcinoma; Review

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Core Tip: The risk of hepatocellular carcinoma (HCC) occurrence or recurrence in patients with chronic hepatitis C virus (HCV) infection receiving direct acting antivirals (DAAs) has been debated through the last 4 years. Data provided by current literature indicate a decreasing incidence rate of HCC (both *de novo* and recurrent) in patients with chronic hepatitis C, HCV-related cirrhosis, and HCV-related HCC after achieving sustained virological response with DAAs.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents the most frequent histologic type among primary liver neoplasia, and is the fifth most common cancer globally comprising 5.6% of all cancers and the second most common cause of cancer death^[1]. The leading risk factor for HCC is chronic hepatitis C virus (HCV) infection with a 3% annual risk in patients with HCV liver cirrhosis^[2]. Data from WHO's global hepatitis report shows that 1% of the world population is infected with HCV^[3]. Beside inducing liver injury and fibrosis which subsequently will lead to liver cirrhosis, HCV has a direct carcinogenic potential with pro-oncogenic effects upon the infected cell through oxidative stress, DNA damage and deregulation of host cell checkpoints^[4].

Most studies regarding the risk of HCC in patients with HCV chronic infection treated with interferon (IFN)-based therapy reported that achieving sustained viral response (SVR) reduced the risk to 0.5%-1% per year^[5,6]. However, IFN-based therapy was limited by a low SVR rate (approximately 40%-50%) and a poor tolerance among patients with cirrhosis due to multiple adverse events^[7].

The therapy of HCV infection was revolutionized by the introduction of the currently approved IFN-free regimens containing all-oral direct-acting antivirals (DAAs) which target viral proteins such as NS3/4A protease, NS5B polymerase and the NS5A replication complex, achieving SVR rates in over 95% of patients, with good safety profile and excellent tolerance^[8-10]. Thus, it was expected that viral clearance will reduce morbidity and mortality rates implying a decreased risk of HCC. Several studies which assessed the risk of HCC occurrence and recurrence in patients treated with IFN-based therapy, have shown that the risk is significantly lower in those who achieved a SVR than in those who did not^[11]. In addition, SVR-achieving patients benefit from long-term preserved liver function and consequently a longer survival^[12]. However, data provided by 2 studies published in 2016 were a matter of concern regarding the high risk of HCC occurrence and recurrence after DAA therapies^[13,14]. The debate was continued by several studies reporting conflicting data and thus casting a shadow over the relation between DAA therapy and HCC. Although it is commonly known that the risk of HCC remains even after HCV clearance, it is important to clarify whether DAAs have a role in suppressing the development of HCC.

We carried out a review of the most recent and relevant articles regarding the risk of HCC after DAA therapy and identify the associated risk factors.

THE RELATION BETWEEN HCC AND CHRONIC HCV INFECTION

Commonly, HCC develops in a liver with histologic abnormalities, the presence of chronic liver disease representing a potential risk for tumour initiation and progression. In about 90% of the cases, HCC is associated with a known risk factor^[2]. The most important risk factor for the development of HCC is liver cirrhosis of whatever etiology, which is considered a premalignant lesion and is present in over 70% of cases^[15]. Liver cirrhosis marks the final stage of all chronic hepatopathies and the most common causes are chronic infection with HBV or HCV, alcohol consumption, hereditary metabolic diseases such as hemochromatosis or alpha-1-antitrypsin deficiency and non-alcoholic steatohepatitis^[16]. All etiologic forms of cirrhosis may be complicated by the development of HCC, but patients with chronic HBV and HCV infection are under a higher risk.

HCV is an RNA virus that belongs to the *Flaviviridae* family, consisting of single-stranded RNA whose genome encodes a protein comprising 3000 amino acids from which, *via* proteolysis, result structural and nonstructural proteins. Structural proteins (core, envelope E1 and E2) play an important role in determining the morphological viral characteristics and in the invasion process of host-cells^[15]. Nonstructural proteins (P1, NS2, NS3, NS4A, NS4B, NS5A and NS5B) are involved in viral replication and the pathogenesis of secondary liver injury^[17]. The HCV genome is very heterogeneous

with at least seven genotypes and several subtypes reported so far. HCV genotype 1, 3 and 6 have been incriminated in a poor clinical outcome as compared to the other genotypes, with a higher prevalence of cirrhosis or HCC^[18-20]. The hepatocarcinogenesis induced by chronic HCV infection is a multistage and multifactorial process, in which direct and indirect mechanisms interact leading to the creation of a pro-carcinogenic microenvironment represented by liver cirrhosis, in which viral protein structures act as promoters of malignant degeneration^[21]. HCC development due to HCV is a gradual process spanning two to four decades^[22]. HCV carcinogenesis is mediated by viral-induced factors and host immunologic response which is mediated by tumoral necrosis factor, IFNs and chronic inflammation secondary to HCV^[23]. Cell cycles are associated with mutations that can transform hepatocytes to malignant cells. Telomerase reverse transcriptase, tumour protein 53, β catenin are the most frequent genes mutated in HCC^[24].

HCC OCCURRENCE AFTER DAA THERAPY

IFN-based therapy provided undisputed clinical benefit in pre-cirrhotic and cirrhotic SVR-achieving patients, with a significant reduction in disease progression and complications, including HCC when compared to those without SVR or untreated^[11,25-27]. The risk factors associated with HCC development in IFN-treated patients achieving SVR are older age, male gender, advanced liver fibrosis, fatty liver, and a high posttreatment serum alpha-fetoprotein (AFP) level^[28-30]. However, due to restrictive inclusion criteria, low SVR rates and high treatment-related toxicity, IFN-based therapy was not an ideal treatment in patients with chronic HCV infection.

The IFN-free regimens using new DAAs represent a turning point in the treatment of patients with chronic HCV infection, providing high SVR rates and fair tolerance, which raised the expectations of preventing complications of advanced liver disease in HCV patients, including HCC. These prospects are based on data provided by previous studies carried out in the IFN era, demonstrating a decline in HCC incidence in SVR-achieving patients^[31,32]. Nonetheless, despite evidences that achieving SVR provide protection against HCC development, several articles published in 2016 and 2017 reported an unexpected increased occurrence, recurrence and a more aggressive pattern of HCC after DAA therapy in cirrhotic HCV patients^[13,14,33]. The results of these studies were countered by many because of the small cohort size, the absence of control groups, and short follow-up periods (Table 1).

Retrospective studies comparing outcomes after DAAs vs IFN-based therapy

A large cohort study which compared 30183 DAA-treated patients to 137502 patients without evidence of HCV treatment and 12948 IFN-treated patients, identified a more advanced age, predominance of male sex and cirrhosis at baseline in DAA-treated patients compared to those untreated. After adjustments for variables, the authors reported a significantly reduced risk of HCC relative to no treatment (adjusted HR = 0.84, 95% CI: 0.73-0.96), and relative to IFN-based treatment (HR = 0.69, 95% CI: 0.59-0.81)^[34]. Similarly, Ioannou *et al*^[35] conducted a study in which 60000 patients with antiviral treatment were enrolled between 1999 and 2015. The antiviral regimens were divided into 3 groups: 35871 IFN-only, 4535 DAA and IFN, and 21948 DAA-only with a mean follow-up time of 6.1 years for all patients and 1.53 years for the DAA-only group. The study found a significant reduction in HCC occurrence risk of 71% (adjusted HR = 0.29; 95% CI: 0.23-0.37) in patients with DAA-induced SVR. Janjua *et al*^[36] recently published a study which evaluated a large cohort treated with DAAs compared with a retrospective cohort treated with IFN. The authors found a similar rate reduction in HCC risk in patients who achieved SVR obtained either with DAAs or with IFN-based regimens (70% reduction for DAAs and 79% for IFN-based therapy).

Retrospective studies assessing outcomes after DAAs

Kanwal *et al*^[37] conducted a cohort study which evaluated the risk of HCC in 22500 DAA-treated patients with a mean follow-up period of 1.02 years. The study demonstrated that the risk of HCC in patients with SVR is significantly reduced as compared with non-SVR patients [0.90 vs 3.45 HCC/100 person year (PY); adjusted HR = 0.28, 95% CI: 0.22-0.36]. Similarly, in a study including almost 4000 DAA-treated HCV patients from several centers across Spain, Calleja *et al*^[38] aimed to evaluate the effectiveness, safety and clinical outcomes of DAA-based therapy in HCV genotype 1 infection, reported a HCC incidence of 0.93% within 18 mo of starting DAAs treatment

Table 1 *De novo* hepatocellular carcinoma incidence after direct-acting antiviral treatment

Ref.	Type of study	Patient (n) and characteristics	Follow-up time	<i>De novo</i> HCC incidence
Conti <i>et al</i> ^[14]	Retrospective study	Cirrhotic patients treated with DAAs (n = 285)	Mean FU: 5.6 mo	3.16%
Ravi <i>et al</i> ^[33]	Retrospective study	Cirrhotic patients treated with DAAs (n = 66)	From SOT to 6 mo after EOT	9.1%
Singer <i>et al</i> ^[34]	Retrospective study	DAA-treated (n = 30183), IFN-treated (n = 12948), untreated (n = 137502)	Mean FU: 1.05 yr	1.18 per 100 PY
Nahon <i>et al</i> ^[43]	Retrospective study	All compensated cirrhotic patients DAA-treated (n = 336), IFN-treated with SVR (n = 495), IFN-treated without SVR (n = 439)	Median FU: 21.2 mo (IQR: 13.5-26.9)	2.6 per 100 PY
Ioannou <i>et al</i> ^[35]	Retrospective study	DAA-treated (n = 21948), IFN-treated (n = 35871), DAA + IFN treated (n = 4535)	Mean FU: 6.1 yr	1.32 per 100 PY
Kanwal <i>et al</i> ^[37]	Retrospective study	DAA-treated (n = 22500)	Mean FU: 1.02 yr	1.18 per 100 PY
Cheung <i>et al</i> ^[42]	Prospectivestudy	DAA-treated (n = 406), untreated (n = 261)	Median FU: 18 mo	4%
Calleja <i>et al</i> ^[38]	Retrospectivestudy	DAA-treated (n = 3325)	Mean FU: 18 mo	11.3%
Mettke <i>et al</i> ^[44]	Prospective study	DAA-treated (n = 158), untreated (n = 184)	Median FU: 440 d	2.90 per 100 PY
Carrat <i>et al</i> ^[45]	Prospectivestudy	DAA-treated (n = 7344), untreated (n = 2551)	Median FU: 33.4 mo (IQR: 24.0-40.7)	1.40 per 100 PY
Janjua <i>et al</i> ^[36]	Retrospective study	IFN-treated (n = 8871), DAA-treated (n = 3905)	Median FU: 1.0 yr	6.9 per 1000 PY
Poordad <i>et al</i> ^[46]	Prospective study	DAA-treated (n = 2211)	156 wk from EOT	1.4%
Sangiovanni <i>et al</i> ^[47]	Prospective study	DAA-treated (n = 1285)	Mean FU: 17 mo	3.1 per 100 PY
Kanwal <i>et al</i> ^[39]	Retrospective study	DAA-treated (n = 18076)	Mean FU: 2.9 yr	3%
Romano <i>et al</i> ^[48]	Prospective study	DAA-treated (n = 3917)	Median FU: 523 d, (IQR: 381-699 d)	0.97 per 100 PY
Tani <i>et al</i> ^[40]	Retrospective study	DAA-treated (n = 1088)	Median FU: 13.8 mo	2.38%
Watanabe <i>et al</i> ^[41]	Retrospective study	DAA-treated (n = 1438)	Median FU: 803 d	3.82%

DAA: Direct-acting antivirals; SOT: Start of treatment; FU: Follow-up; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; IFN: Interferon; IQR: Interquartile range; PY: Person-year; EOT: End of treatment; SVR: Sustained viral response.

with ombitasvir/paritaprevir/ritonavir plus dasabuvir (OMV/PTV/r+DSV) and ledipasvir/sofosbuvir (LDV/SOF). It should be mentioned that measuring the incidence of HCC was not an objective of this study. In contrast to these results, the same team that had previously reported a reduced risk of HCC in patients with chronic HCV infection treated with DAAs^[37], recently published another retrospective cohort study in which evaluated the long-term risk of HCC in patients with SVR to DAAs, followed up for over 3.5 years after SVR^[39]. From 18076 patients who achieved SVR with DAAs, they found 544 patients with *de novo* HCC, with 1-, 2-, and 3-year cumulative risks of HCC of 1.1%, 1.9% and 2.8%, respectively. Results from another two recently published studies are consistent with those reported by this second study by Kanwal *et al*^[39]. In a retrospective study, Tani *et al*^[40] demonstrated that the 12- and 36-mo cumulative incidences of HCC were 1.88 and 6.00%, respectively. Similarly, Watanabe *et al*^[41] reported 1- and 2-year cumulative incidences of HCC of 1.9 and 4.1%, respectively.

Prospective studies

A prospective study by Cheung *et al*^[42] which included 406 patients with decompensated cirrhosis found no evidence of an increased risk for HCC during DAA therapy or during the 12-mo follow-up. The authors found a 4.2% HCC incidence in the first six months from the start of DAA treatment, the equivalent to the occurrence seen in a matched control group containing untreated patients. Furthermore, the authors suggested the possibility of pre-existing undiagnosed cancer in patients which developed HCC during DAA treatment. Another large prospective study from ANRS CO12 CirVir cohort including 1270 HCV patients with compensated biopsy-proven

cirrhosis reported that after Cox analysis there was no statistically significant increase in the risk of HCC development associated with DAAs use^[43]. A large prospective study by Mettke *et al*^[44] containing 158 HCV-related cirrhotic patients treated with DAAs and 184 HCV-related cirrhotic patients without treatment, demonstrated a similar HCC incidence over a short period of time in the two groups (HCC developed in 6 DAA-treated patients and 14 untreated patients, yielding HCC incidence rates of 2.90 and 4.48 per 100 person-years, respectively). A multi-center prospective cohort study published by Carrat *et al*^[45] in France also found that treatment with DAAs was associated with a reduced risk for mortality and HCC. The study included 7344 patients with DAA treatment and 2551 patients without treatment, with a mean follow-up period of 33.4 mo. After adjustment for variables, DAA treatment was associated with a decrease in HCC (adjusted HR = 0.66, 95%CI: 0.46-0.91) and all-cause mortality (adjusted HR = 0.48, 95%CI: 0.33-0.70). Also, recent results from the ongoing phase 3b trials TOPAZ-I and TOPAZ-II demonstrated a low incidence rate of HCC in DAA-treated HCV patients. The combined interim results from both the TOPAZ-I and TOPAZ-II studies performed up to 156 wk posttreatment showed that the rates of liver transplantation, liver decompensation, HCC and liver-related death were 0.6%, 2.0%, 1.4%, and 0.3%, respectively^[46]. Another recent prospective study by Sangiovanni *et al*^[47], including 1285 consecutive patients with HCV-related cirrhosis without any history of HCC (group 1), and 124 cirrhotics with previous HCC and complete response to treatment (group 2) found an yearly incidence of 3.1/100 PY-recent data from a prospective study by Romano *et al*^[48] showed a HCC incidence rate of 0.97 per 100 PY (95%CI: 0.73-1.26) and a sharp decline in HCC risk in the 2nd year of follow-up in patients with HCV-related cirrhosis treated with DAAs.

Ma *et al*^[49] recently published a large meta-analysis in which 276848 HCV-infected patients treated either with IFN-based therapy or with DAAs were included. The authors reported that DAA treatment is not better than IFN-based therapies in preventing the development of HCC, indicating that IFN-based therapy might currently be irreplaceable in the prophylaxis of HCC in patients with chronic hepatitis C.

HCC RECURRENCE AFTER DAA THERAPY

In patients with early disease stage HCC - Barcelona Clinic Liver Cancer Stage 0/A - BCLC 0/A, there are available potentially curative treatments such as surgical resection and local ablation, with high 5-year overall survival rate. However, tumour recurrence and decompensation of underlying cirrhosis contribute to long-term mortality even after curative treatment. As stated by previous research, HCC recurrence after an initial complete response may develop through either the dissemination of cells from the original tumor prior to curative therapy, or through de-novo cancers arising in the cirrhotic genetically altered microenvironment^[50]. Thus, it seems appropriate to stratify the recurrence of HCC into: (1) Intra-hepatic metastasis of the original tumor; and (2) Multicentric carcinogenesis. The distinction between these models is mandatory and it could be made using the amount of time between curative therapy and recurrence. Thereby, an early recurrence within 1-2 years may be attributed to intrahepatic metastasis, whereas a recurrence > 2 years is supposedly due to metachronous HCC^[51]. Microscopic vascular invasion and/or satellites are high risk hallmarks for dissemination whilst sustained inflammation with persistent liver damage is predictive for multicentric carcinogenesis/metachronous tumors^[52]. The most important factor in predicting the growth of nested malignant cell from the primary tumor is the immune cancer surveillance which in a normal setting triggers the activation of stromal cells and lymphocyte recruitment, secondary leading to the suppression of cell clones^[53]. Several studies from the IFN era highlighted the beneficial effect on HCC recurrence exerted by IFN-based therapy^[54-57]. One of the major differences between IFN and DAA obtained SVR is the kinetics of viral suppression which may be the key in explaining the high recurrence rate of HCC. The mechanism proposed for HCC recurrence in patients with SVR obtained with DAAs in prior HCV-related HCC patients, consists of a disruption of the immune cancer surveillance due to an abrupt resolution of a chronic inflammatory state as the suppression of HCV replication occurs in the first days after therapy^[58].

However, available data regarding HCC recurrence in patients with initial complete response to hepatic resection or local ablation following DAA-induced SVR are scarce and conflicting (Table 2).

Table 2 Recurrent hepatocellular carcinoma incidence after direct-acting antiviral treatment

Ref.	Type of study	Patient (n) and characteristics	Follow-up time	Recurrent HCC incidence
Reig <i>et al</i> ^[13]	Retrospective study	DAA-treated (n = 103)	Mean FU: 5.7 mo	27.6%
Conti <i>et al</i> ^[14]	Retrospective study	DAA-treated (n = 59)	Mean FU: 5.6 mo	28.8%
ANRS CO22 HEPATHER ^[64]	Prospective study	DAA-treated (n = 189), untreated (n = 78)	Mean FU: 20.2 mo	0.73 per 100 person-months
ANRS CO12 CirVir ^[64]	Prospective study	All biopsy proven cirrhotic patients, DAA-treated (n = 13), untreated (n = 66)	Median FU: 21.3 mo (IQR: 13.0-33.5)	1.11 per 100 person-months
ANRS CO23 CUPILT ^[64]	Prospective study	LT recipients for HCC, treated with DAA (n = 314)	Mean FU: 70 ± 64 mo after LT	2.2%
Cabibbo <i>et al</i> ^[65]	Prospective study	DAA-treated (n = 143)	Mean FU: 8.7 mo	20.3%
Lin <i>et al</i> ^[59]	Retrospective study	DAA-treated (n = 60), untreated (n = 47)	Median FU: 20 mo	37.1%
Singal <i>et al</i> ^[60]	Retrospective study	DAA-treated (n = 304), IFN-treated (n = 489)	Median FU: 10.4 mo (IQR: 5.3-20.8) since complete remission	DAA treated 42.1%, untreated 52.9%
Nagata <i>et al</i> ^[62]	Retrospective study	DAA-treated (n = 83), IFN-treated (n = 60)	Mean FU: IFN 81.6 mo, DAA 21.6 mo	IFN-treated 54.2%, DAA-treated 45.1%
Imai <i>et al</i> ^[63]	Retrospective study	DAA-treated (n = 13), IFN-treated (n = 34), untreated (n = 70)	N/A	

DAA: Direct-acting antivirals; FU: Follow-up; HCC: Hepatocellular carcinoma; IFN: Interferon; IQR: Interquartile range; LT: Liver transplant; N/A: Not available.

Retrospective studies

In 2016, Reig *et al*^[13] reported an unexpected high rate of 27.6% of early tumor recurrence in patients with HCV-related HCC undergoing DAA treatment. Similar results were found by Conti *et al*^[14] in a single-center retrospective cohort study, with a recurrence rate of HCC after DAAs of 28.81%. Contrasting results were reported by Lin *et al*^[59] in a recently published retrospective study which included 107 patients with HCV-related HCC, of whom 60 received DAA therapy after treatment for HCC. After a median follow-up of 20 mo, 37.1% patients had HCC recurrence after DAAs. The authors concluded that, compared to untreated patients, DAA therapy did not increase recurrent HCC after curative treatment and also improved the survival outcome of HCC patients. In line with these results, the largest retrospective cohort study ever reported was recently published, including untreated control arm, based on 31 health systems throughout the United States and Canada. The study included 793 HCV-related HCC patients of which 304 (38.3%) received DAA therapy and 489 (61.7%) were untreated. The rate of tumor recurrence was 42.1% in DAA-treated patients and 58.9% in the untreated group. After variable adjustments, the study reported that DAA exposure is not associated with an increased risk of HCC recurrence (HR = 0.90; 95%CI: 0.70-1.16)^[60]. A meta-analysis published by Lui *et al*^[61] also showed that the use of DAA therapy is associated with a significantly lower risk of HCC development compared to patients without DAA treatment. The authors found a > 60% lower risk of HCC recurrence in patients exposed to DAA compared to controls (OR = 0.36, 95%CI: 0.27-0.47; *P* < 0.001; *I*² = 88%).

A retrospective cohort study which compared outcomes of patients with prior HCV-related HCC treated with DAAs *vs* IFN-based therapy found no significant difference between IFN-based and IFN-free therapy groups by propensity score-matched analysis (5-year incidence; 54.2% in IFN-based, 45.1% in IFN-free therapy; *P* = 0.54)^[62]. Consistent with these findings, results from a recent retrospective cohort from Japan showed that SVR by therapy with DAAs exhibited an anti-liver tumorigenesis effect equal to that of IFN-based therapy and reduced the risk of HCC recurrence (*P* = 0.564)^[63].

Prospective studies

A prospective multicenter French study that included ANRS cohorts concluded that the rate of HCC recurrence was not different between DAA-treated and untreated

groups^[64]. Cabibbo *et al*^[65] conducted a prospective multicenter study in Italy in which were included 143 patients with successfully treated BCLC 0/A HCC, and subsequently treated with DAAs. They found an HCC-recurrence incidence of 12%, 26.6%, and 29.1%, respectively in 6-, 12-, and 18-mo of follow-up, and concluded that although the risk of HCC recurrence remains high, it is comparable between the DAA group and the untreated group.

RISK FACTORS FOR *DE NOVO* AND RECURRENT HCC

The increasing number of patients who will obtain HCV clearance with DAAs and the continued risk of hepatocarcinogenesis even after SVR require the identification of patients at highest risk of developing HCC. Regarding the host risk factors such as older age, male gender and family history, HBV or HIV co-infection, alcohol consumption, steatohepatitis and advanced liver disease are well known as associated risk factors^[66]. Also, tobacco smoking and exposure to aflatoxin are the most studied environmental risk factors involved in the development of HCC^[67]. The risk factors incriminated in HCC occurrence and recurrence, in patients treated with DAAs, are mainly older age, non-SVR and advanced liver disease (Table 3).

Retrospective studies

A study conducted by Kanwal *et al*^[37] which included DAA treated patients, reported a 4.7-fold higher HCC risk in cirrhotic patients than in those without cirrhosis (adjusted HR = 4.73; 95%CI: 3.34-6.68). Similar findings were disclosed by Ioannou *et al*^[35] in a large cohort retrospective study, concluding that the incidence of HCC was highest in patients with cirrhosis and treatment failure. Singer *et al*^[34] demonstrated that older age, male gender, liver cirrhosis, thrombocytopenia, portal hypertension, diabetes, tobacco use, alcoholic liver disease, and use of betablockers and anti-hypertensives were associated with an increased risk of HCC in multivariable adjusted models. Despite using different inclusion criteria and study methods, these three cohort studies demonstrated that the presence of cirrhosis and the absence of SVR were the major risk factors of HCC occurrence in HCV patients^[34,35,37]. A high FIB-4 index and posttreatment AFP were identified as independent factors that contributed to HCC occurrence in two recent studies^[39,41].

In the 2016 paper, Conti *et al*^[14] reported that decompensated cirrhosis characterized by a high Child-Pugh-Turcotte score (OR = 4.18, 95%CI: 1.17-14.8, $P = 0.03$) and a history of HCC (OR = 12.0, 95%CI: 4.02-35.74, $P < 0.0001$) were associated with HCC. In addition, a large comparative study from Japan revealed that posttreatment Wisteria floribunda agglutinin positive Mac-2 binding protein (WFA⁺M2BP) was significantly associated with HCC recurrence in patients with HCV without advanced liver fibrosis. In addition, the comparative study for occurrence and recurrence of HCC in IFN-based *vs* DAAs, showed that AFP (> 5.4 ng/mL) and WFA⁺M2BP levels (> 1.8 COI) were strongly associated with *de novo* HCC in those with DAA therapy^[61]. In a recently published retrospective study, Sangiovanni *et al*^[47] found at multivariable Cox regression models that ascites and AFP log-value were independently associated with HCC occurrence, while a history of alcohol abuse and HCC recurrence was associated with HCC recurrence.

Prospective studies

In addition to these findings from retrospective studies, Ide *et al*^[68] found that besides male gender and an older age, higher FIB-4 index and GGTP levels, were independently associated with HCC occurrence. Also, Calvaruso *et al*^[69] found in their prospective study that albumin level (< 3.5 g/dL), platelets < 120 × 10⁹/L and failure to achieve SVR were associated with an increased risk of HCC development. The failure in achieving SVR was also incriminated as a risk factor for HCC occurrence along with HBV coinfection and APRI > 2.5, in another study from Italy^[48]. Another study which enrolled patients with a history of successful radiofrequency ablation treatment for HCV-related HCC who had received antiviral therapy with DAAs (147 patients) or IFN (156 patients) reported that a higher AFP-L3 level, larger number of HCC treatments, and a shorter interval between the last HCC treatment and the initiation of antiviral therapy were associated with the risk of HCC recurrence^[70]. A recent study from Egypt, which enrolled 160 DAA-treated and 80 untreated HCV patients, showed that an ultrasound measured adequate liver volume (at a cutoff of 495 mL) predicted HCC occurrence after DAAs^[71].

Most of the studies we reviewed indicated that the major risk factors for HCC

Table 3 Risk factors for de novo and recurrent hepatocellular carcinoma after direct-acting antiviral therapy

Ref.	Type of study	Patient (n) and characteristics	Risk factors
Conti <i>et al</i> ^[14]	Retrospective study	Cirrhotic patients treated with DAAs (n = 285)	No associated factor for <i>de novo</i> HCC, older age, liver stiffness for HCC recurrence
Singer <i>et al</i> ^[34]	Retrospective study	DAA-treated (n = 30183), IFN-treated (n = 12948), untreated (n = 137502)	Older age, male gender, cirrhosis, thrombocytopenia, portal hypertension, diabetes, tobacco use, alcoholic liver disease
Ioannou <i>et al</i> ^[35]	Retrospective study	DAA-treated (n = 21948), IFN-treated (n = 35871), DAA + IFN treated (n = 4535)	Non-SVR, cirrhosis
Kanwal <i>et al</i> ^[37]	Retrospective study	DAA-treated (n = 22500)	Non-SVR, alcohol use, non-African Americans, cirrhosis
Hanafy <i>et al</i> ^[71]	Prospective study	All decompensated cirrhotic patients, DAA-treated (n = 160), untreated (n = 80)	An adequate baseline liver volume measured by ultrasound was associated with less HCC occurrence and better short-term survival
Kanwal <i>et al</i> ^[39]	Retrospective study	DAA-treated (n = 18076)	High FIB-4/ APRI, alcohol use, older age, genotype 3
Watanabe <i>et al</i> ^[41]	Retrospective study	DAA-treated (n = 1438)	High FIB-4 index, AFP
Nagata <i>et al</i> ^[62]	Retrospective study	DAA-treated (n = 83), IFN-treated (n = 60)	IL-28 genetic polymorphism, post-treatment WFA ⁺ M2BP, AFP (> 5.4 ng/mL)
Ide <i>et al</i> ^[68]	Prospective study	CHC DAA-treated (n = 2552)	Age ≥ 62 yr, male gender, FIB-4 index ≥ 4.6, GGTP level ≥ 44 IU/L
Calvaruso <i>et al</i> ^[69]	Prospective study	HCV cirrhosis DAA-treated (n = 2249)	Albumin < 3.5 g/dL, platelets < 120 × 10 ⁹ /L, absence of SVR
Romano <i>et al</i> ^[48]	Prospective study	CHC > F3 DAA-treated (n = 3917)	HBsAg+, APRI ≥ 2.5, CPT B, treatment failure
Sangiovanni <i>et al</i> ^[47]	Retrospective study	1161 HCC-free HCV cirrhotics, DAA treated, 124 HCV cirrhotics who had received a curative treatment for an HCC DAA treated	<i>De novo</i> HCC: Ascites, AFP, recurrent HCC; History of alcohol abuse, history of HCC recurrence

DAA: Direct-acting antivirals; FU: Follow-up; HCC: Hepatocellular carcinoma; SVR: Sustained virological response; IFN: Interferon; IQR: Interquartile range; LT: Liver transplant; CHC: Chronic hepatitis C; APRI: Aspartate aminotransferase to platelet ratio index; CPT: Child-Pugh-Turcotte; SVR: Sustained viral response; AFP: Alpha-fetoprotein; WFA⁺M2BP: Wisteria floribunda agglutinin positive Mac-2 binding protein; HCV: Chronic hepatitis C; GGTP: Gamma-glutamyl transpeptidase.

occurrence and recurrence are male gender, older age, non-SVR, advanced liver fibrosis and higher post-treatment AFP levels, in agreement with those identified by prior studies of the IFN era^[25,72].

CONCLUSION

Data provided by the most recent and relevant articles sustain a reduced incidence rate of both *de novo* and recurrent HCC after achieving SVR with DAA therapy, therefore we consider that the debate regarding the impact of DAAs on HCC risk is drawing to an end.

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Basic Study

***Lactobacillus bulgaricus* inhibits colitis-associated cancer via a negative regulation of intestinal inflammation in azoxymethane/dextran sodium sulfate model**

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Abstract

BACKGROUND

Colitis-associated cancer (CAC) accounts for 2%-3% of colorectal cancer (CRC) cases preceded by inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis. Intestinal microbiota has been reported to play a central role in the pathogenesis of IBD and CAC. Recently, numerous prebiotics and probiotics have been investigated as antitumor agents due to their capacity to modulate inflammatory responses. Previous studies have indicated that lactic acid bacteria could be successfully used in managing sporadic CRC, however little is known about their role in CAC.

AIM

To investigate the effect of the probiotic *Lactobacillus bulgaricus* (*L. bulgaricus*) during the development of an experimental model of colitis associated colon

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Institutional review board

statement: All authors declare that the Institutional Review Board approval was not applicable for this manuscript, once this study does not involve human beings.

Institutional animal care and use

committee statement: All animal experiments conformed to the National Council for Animal Experiment Control accepted principles for the care and use of laboratory animals [ethics committee on the use of animals (CEUA), protocol No. 14.1.418.53.1].

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cancer (CAC).

METHODS

C57BL/6 mice received an intraperitoneal injection of azoxymethane (10 mg/kg), followed by three cycles of sodium dextran sulphate diluted in water (5% w/v). Probiotic group received daily *L. bulgaricus*. Intestinal inflammation was determined by scoring clinical signs. Cytokines levels were determined from colon and/or tumor samples by ELISA BD OptEIA™ kits. The level of significance was set at $P < 0.05$. Graphs were generated and statistical analysis performed using the software GraphPad Prism 6.0.

RESULTS

L. bulgaricus treatment inhibited of total tumor volume and mean size of tumors. In addition, the probiotic also attenuated the clinical signs of intestinal inflammation inducing a decrease in intestinal and tumor levels of IL-6, TNF- α , IL-17, IL-23 and IL-1 β .

CONCLUSION

Our results suggest a potential chemopreventive effect of probiotic on CAC. *L. bulgaricus* regulates the inflammatory response and preventing CAC.

Key Words: *Lactobacillus bulgaricus*; Colitis-associated cancer; Colorectal cancer; Carcinogenesis; Probiotics; Inflammation

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Core Tip: Recent studies suggested that consideration of the intestinal microbiota has an essential role in carcinogenesis. Probiotic supplementation is an alternative means of favourably modulating the intestinal microbiota. In this study, we investigate the effect of *Lactobacillus bulgaricus* (*L. bulgaricus*) during the development of an experimental model of colitis-associated colon cancer. Our results evidence an anti-inflammatory role and consequent antitumor effect of *L. bulgaricus* on colitis-associated cancer that may be used as a promising tool for the prevention and treatment of colitis-associated cancer.

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INTRODUCTION

Colorectal cancer (CRC) remains one of the most incident type of cancer worldwide, being the third and second most frequently cancer diagnosed in men and women, respectively^[1]. Colitis-associated cancer (CAC) specifically accounts for 2%-3% of CRC cases preceded by inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis^[2]. The link between inflammation and cancer was firstly recognized in 1863 and has been recently exemplified by CAC. Patients with IBD have a higher risk for developing CRC and are affected by the disease earlier than patients with sporadic CRC^[3,4].

Currently, it has become increasingly evident that intestinal microbiota plays a crucial role in the pathogenesis of IBD and CRC. Changes in intestinal microbiota have been reported in patients with colon cancer, supporting this idea^[5]. Among the gut microorganisms, probiotic bacteria may be defined as live microbial food supplements that confer benefits to the health of the consumer (WHO), including reduction of pathogen colonization by competition^[6], improvement in vitamin synthesis and nutrients absorption, stimulation of epithelial cell proliferation and differentiation,

quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

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fortification of intestinal barrier and optimization of intestinal transit^[7].

In addition to the direct benefit of probiotics on the improvement of the host gut microbiota, probiotics have received considerable attention due to their anti-carcinogenic activities, mainly in CRC^[8]. The underlying mechanisms for their anti-tumor effects are versatile and include: Modulation of host immune responses, such as proliferation of regulatory T cells, activation of macrophages and dendritic cells, and production of immunoglobulins and cytokines^[9]; alteration of intestinal microbiota metabolism^[10]; regulation of cell death, apoptosis, cell cycle, proliferation, invasion and metastasis^[11]; competition with pathogenic bacteria^[11]; and inactivation of carcinogenic compounds^[12].

The most common microorganisms used as probiotics comprise a group of bacteria named lactic acid bacteria (LAB) that produces lactic acid as the primary metabolite of sugar metabolism, such as *Lactobacillus* and *Bifidobacterium*^[13,14]. Although previous studies have indicated that LAB could be successfully used in managing food allergies, diarrhea, IBDs and sporadic CRC^[15-17], little is known about its role in CAC. In this study, we sought to investigate the effects of the probiotic *Lactobacillus bulgaricus* (*L. bulgaricus*) in colitis-associated carcinogenesis.

MATERIALS AND METHODS

Mice and treatment protocol

In the present study, we used male C57BL/6 wild type (WT) mice, between 4-6 wk old and weighing between 20-25 g. The animals were purchased from the Animal Facility of the University of São Paulo (USP) and housed at the facility of Ribeirão Preto College of Nursing - EERP/USP (Ribeirão Preto, SP, Brazil) under controlled temperature conditions (25 ± 2 °C) with 12/12 photoperiod hours. Water and food were available ad libitum. All experiments were handled in accordance with institutional ethical guidelines, and the study was approved by the Ethics Committee on Animal Research from the University of São Paulo (CEUA PUSP-RP: No. 14.1.418.53.1).

L. bulgaricus and treatment

Lactobacillus delbrueckii ssp *bulgaricus*, LOT No. FK0201, identification LB-G040, Chinese origin, was purchased from Liane Drugstore, Ribeirão Preto, SP, Brazil and stored in at 4 °C. For mice treatment, 1×10^9 CFU were diluted in 200 µL of PBS and orally given to each mouse, 3 times a week during all experimental period. Prior to tumor induction, mice were randomly distributed in 2 groups ($n = 10$) and treated with PBS (control group) or *L. bulgaricus* (Lb group) by gavage (0.2 mL/mouse) for one week.

CAC induction

For CAC induction, mice were intraperitoneally (i.p.) injected with a single dose (10 mg/kg in 300 µL solution) of azoxymethane (AOM, Sigma-Aldrich), followed by 3 cycles of one week of 2.5% dextran sulfate sodium (DSS) diluted in drinking water intercalated for 2 wk of normal water^[18]. Mice were euthanized 12th week after CAC induction (Figure 1A).

Disease score evaluation

Intestinal inflammation *in vivo*, or disease score, was determined by scoring clinical signs as previously described^[19]. Briefly, we used a scoring system in which one point (1.0) was attributed to each signal presented by the mouse, including: Weight loss $\geq 5\%$ and $< 10\%$ of body weight compared to the previous day; presence of humid perianal region; presence of diarrhea; blood in the stool or perianal region; hyporactivity and piloerection. When weight loss was $\geq 10\%$ 2 points were attributed to the "weight loss" signal. Finally, the final sum of these points determined the clinical score of the disease.

Determination of colon length

The severity of intestinal inflammation was also assessed by measuring the length of the entire large intestine. After euthanasia, colons were collected, carefully placed on a clean surface and photographed. The images were calibrated by the presence of a graduated ruler that served as a scale for the analyzes. Subsequently, images of the large intestine were evaluated using ImageJ software.

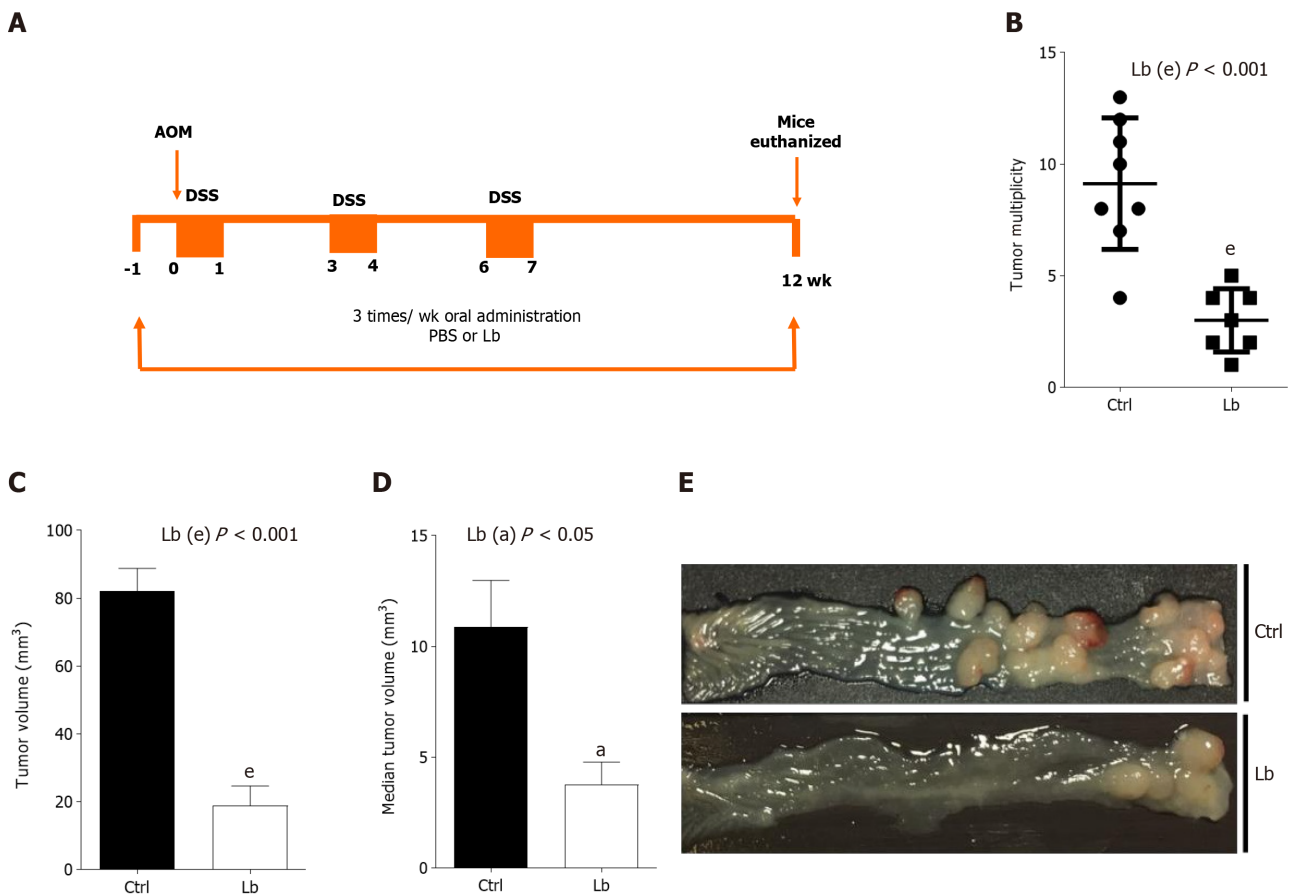


Figure 1 *Lactobacillus bulgaricus* inhibits tumor progression in azoxymethane/dextran sulfate sodium-exposed mice. A: Prior to tumor induction, mice were treated 3 times with PBS or the probiotic by gavage (0.2 mL/mouse) for one week. After that, colitis-associated cancer was induced by intraperitoneal injection of a single dose of azoxymethane (AOM), followed by 3 cycles of 2, 5% dextran sulfate sodium (DSS) in drinking water for one week and normal drinking water for 2 wk; B: After euthanasia at 12th week, colons were longitudinally opened, washed and examined for multiplicity; C: Total tumor volume; D: Mean tumor volume; E: The dimensions of colorectal tumors were measured with pachymeter and the volumes calculated as: $(\text{Width})^2 \times \text{length} / 2$. Illustrative and endoscopic images of AOM/DSS-induced tumors. ^a $P < 0.05$; ^e $P < 0.001$ vs controls. AOM: Azoxymethane; Ctrl: Controls; DSS: Dextran sulfate sodium; Lb: *Lactobacillus bulgaricus*; PBS: Phosphate-buffered saline.

Determination of tumor volume and multiplicity

After euthanasia, the colons were longitudinally opened, washed and examined with regards to presence of tumors. The multiplicity of tumors was verified for each animal in the experimental groups. The dimensions of the colorectal tumors were measured with pachymeter and the volumes were calculated by the formula: $(\text{Width})^2 \times \text{length} / 2$ ^[20]. Total tumor volume, indicates the sum of the volumes of all tumors found in each colon. Mean tumor volume refers to the mean tumor size, i.e., total tumor volume divided by the number of tumors of each colon.

Histological analysis

Distal colon parts were fixed in 4% p-formaldehyde in phosphate-buffered formalin and unblocked in paraffin. Tissue sections (4.0 μm) were prepared from the paraffin-embedded tissue blocks, stained with hematoxylin and eosin and evaluated in a blinded fashion by an experienced pathologist (MOB). Normal colon, polyp without dysplasia, adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and invasive adenocarcinoma were identified in the different groups.

Cytokine quantification

Cytokines levels were determined from colon and/or tumor samples. Tissues were collected, weighed and immediately homogenized in PBS in the presence of protease inhibitors (Roche) using a tissue homogenizer (Polytron System PT 1200E). The material was then centrifuged at 6000 rpm for 15 min at 4 °C and the supernatant collected, aliquoted and stored at -80 °C until the time of use. The concentrations of TNF- α , IL-6, IL-12 (p70), IL-17, IFN- γ , IL-1 β , IL-10, TGF- β , IL-23 were determined by ELISA BD OptEIA™ kits (BD Biosciences Pharmingen) or DuoSet (R&D Systems).

The protocol was performed according to the manufacturers' instructions. Cytokine concentrations were determined with reference to the linear regression line obtained with the serial dilution data of each recombinant mouse cytokine.

Statistical analysis

Data were analyzed using the statistical program GraphPad Prism version 6. Parametric and non-parametric samples were analyzed by one-way Analysis of Variance (ANOVA test) and Kruskal-Wallis test followed by Dunn's test, respectively. The probability was considered statistically significant if $P < 0.05$. Results were expressed as mean \pm SEM.

RESULTS

L. bulgaricus treatment inhibited tumor progression in AOM/DSS-induced model of colon carcinogenesis

To investigate whether *L. bulgaricus* is able to inhibit the progression of CAC, we compared tumor development in AOM/DSS-induced mice treated or not with the probiotic (Figure 1A). As shown in Figure 1, animals from both groups developed tumors at the end of the experimental protocol, whereas those treated with the probiotic developed fewer and smaller tumors. Control mice developed between 4-13 colorectal tumors, whereas animals treated with *L. bulgaricus* developed only 1-5 (Figure 1B). *L. bulgaricus*-treated animals showed a total tumor volume (Figure 1C) and a mean tumor volume (Figure 1D and E) about 4.4-fold and 3-fold lower, respectively. However, no difference was observed in the incidence of tumors (data not shown).

L. bulgaricus attenuated intestinal inflammation in AOM/DSS-induced model of colon carcinogenesis

Once inflammation plays a critical role in CAC carcinogenesis, we evaluated intestinal inflammation in AOM/DSS-induced mice treated with *L. bulgaricus* by three different parameters: Body weight, disease score and colon length. Although we did not observe differences in body weight loss between control and *L. bulgaricus*-treated (Figure 2A), we found differences in clinical signals in *L. bulgaricus*-treated mice, which showed a lower clinical score on the 13th and 15th days after tumor initiation (Figure 2B).

In addition to the attenuation of intestinal inflammation score, we observed that the treatment with *L. bulgaricus* reduced the DSS-induced shortening of the colon (Figure 2C and D) so that the control group had a shorter colon extension when compared to Lb group (Figure 2C and D). After histopathological evaluation of the tumor sections, we observed that, regardless of treatment, both groups of mice presented morphologically similar neoplastic lesions. In general, colorectal tumors were lesions of the polypoid adenoma type with variation between low and high degrees of dysplasia and mixed inflammation (Supplementary Figure 1).

L. bulgaricus inhibits the production of proinflammatory cytokines in tumors and colons of AOM/DSS-induced mice

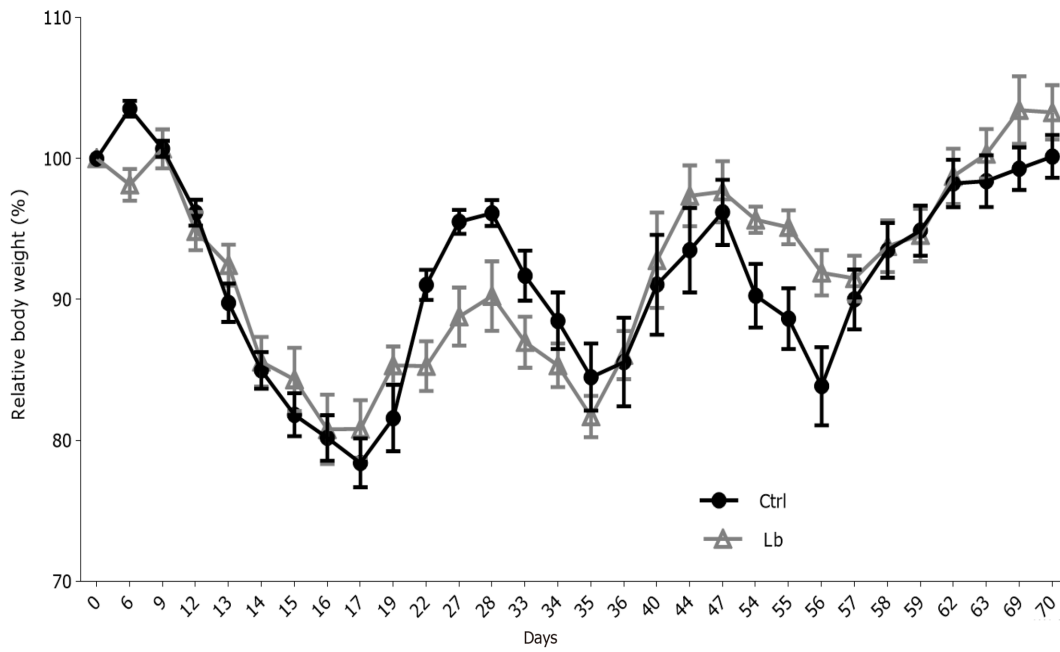
Once we observed that *L. bulgaricus* regulates gut inflammation, we also measured the intestinal concentration of inflammatory mediators involved in CAC pathogenesis. In segments of the large intestine that did not present tumors (inflamed colon) we observed a reduction of at least 2-fold in the levels of the cytokines TNF- α , IL-1 β , IL-23 and IL-17 in *L. bulgaricus*-treated mice in comparison to controls (Figure 3). In contrast, increased concentrations of IFN- γ were also observed in Lb group (Figure 3). We did not observe differences in IL-6 levels (Figure 3).

Regarding the cytokines measured in tumor tissues, we observed a pattern similar to that found in the inflamed colon (Figures 3 and 4). We observed a negative regulation of all analyzed cytokines, including IL-6, in mice treated with the probiotic (Figure 4), and an increase in IFN- γ levels in this group (Figure 4).

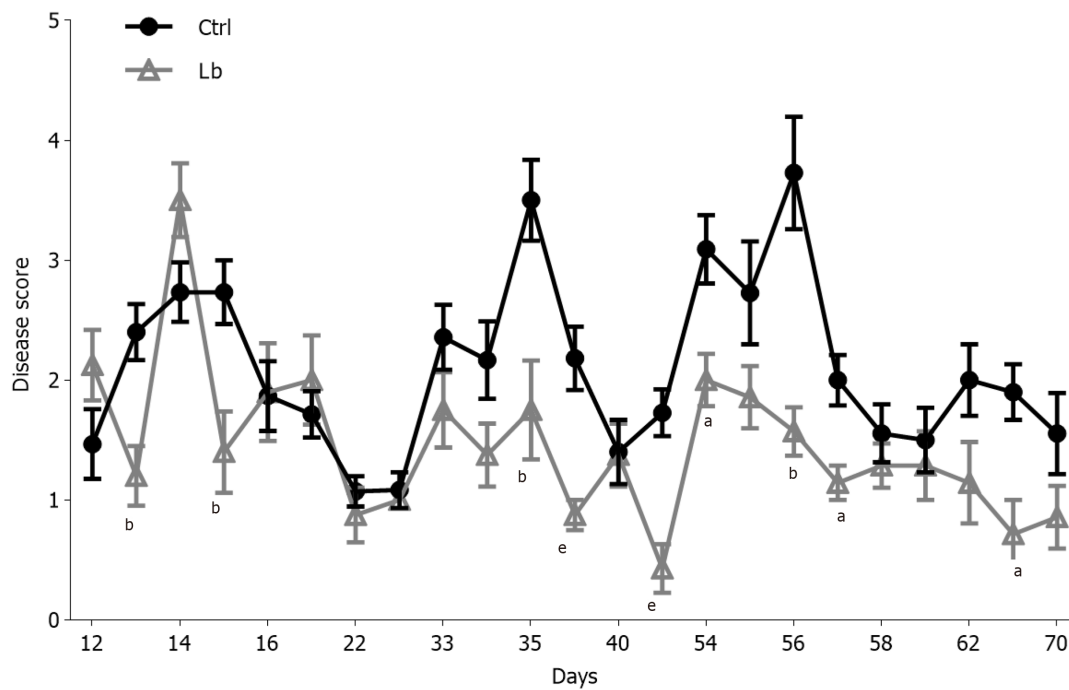
DISCUSSION

Recently, prebiotics and probiotics are being investigated as antitumor agents due to their capacity to modulate inflammatory responses. Studies have shown that

A



B



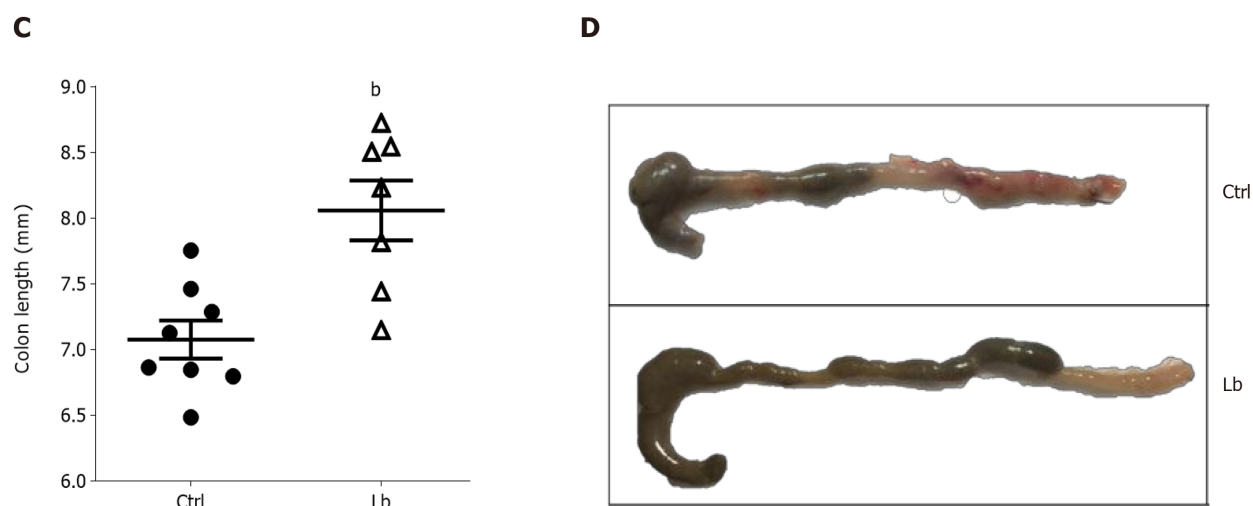


Figure 2 *Lactobacillus bulgaricus* attenuates intestinal inflammation in azoxymethene/dextran sulfate sodium-exposed mice. A: Relative body weight of azoxymethene/dextran sulfate sodium-induced mice treated or not with probiotic; B: Disease score determined by a scoring system based on clinical signs, such as weight loss, humid perianal region, presence of diarrhea, blood in the stool or perianal region, hyporeactivity and piloerection; C: Colon length was determined using graduated images processed in ImageJ; D: Illustrative images of colon extension. Results are expressed as mean \pm EPM. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs controls. Ctrl: Controls; Lb: *Lactobacillus bulgaricus*.

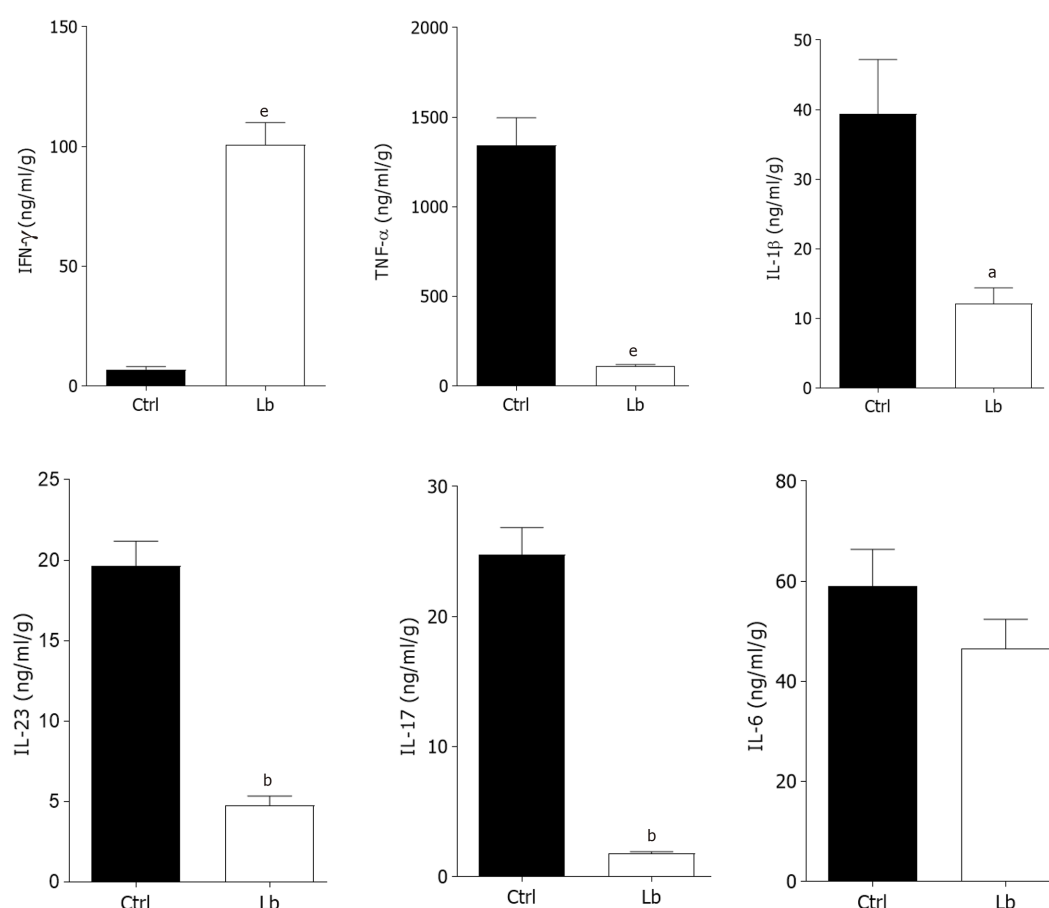


Figure 3 *Lactobacillus bulgaricus* regulates the production of intestinal proinflammatory cytokines in azoxymethene/dextran sulfate sodium-exposed mice. Segments of the colon which did not present tumors (inflamed colon) were homogenized and the levels of TNF- α , IL-6, IL-1 β , IL-17, IFN- γ e IL-23 per gram of tissue were determined by ELISA ($n = 10$ mice per group). Results expressed as mean \pm SEM. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs controls. Ctrl: Controls; Lb: *Lactobacillus bulgaricus*.

probiotics may exert positive effects at different stages of colorectal carcinogenesis: Antimutagenic activity; inactivation of mutagens or carcinogens; reduction of

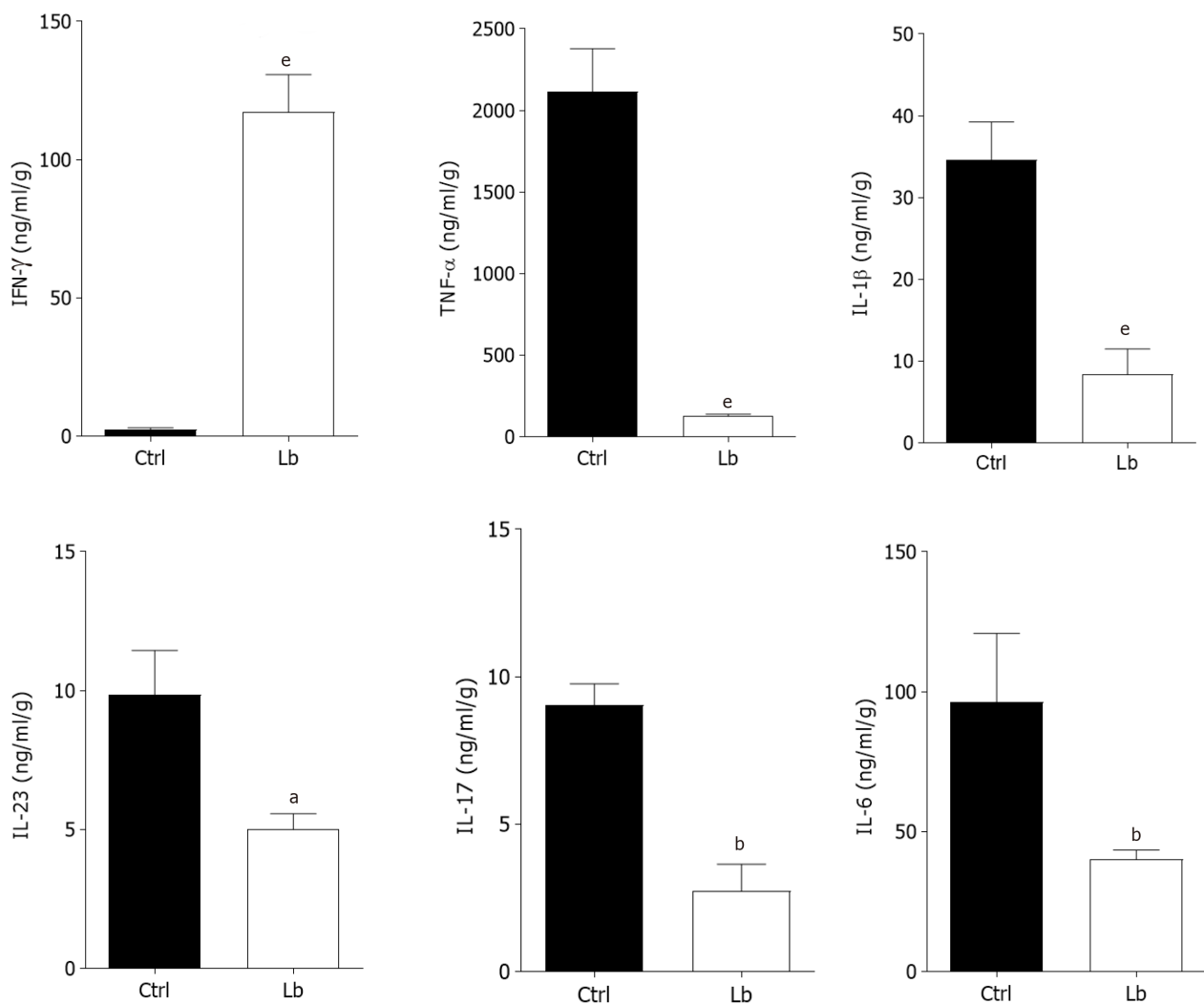


Figure 4 *Lactobacillus bulgaricus* regulates the production of tumors proinflammatory cytokines in azoxymethene/dextran sulfate sodium-exposed mice. Tumor tissues were homogenized and the concentration of TNF- α , IL-6, IL-1 β , IL-17, IFN- γ e IL-23 per gram of tissue (colon) were determined by ELISA ($n = 10$ mice per group). Results expressed as mean \pm SEM. ^a $P < 0.05$, ^b $P < 0.01$, ^e $P < 0.001$ vs controls. Ctrl: Controls; Lb: *Lactobacillus bulgaricus*.

intestinal pH; immunomodulatory effects; intestinal microbiota modulation; regulation of apoptosis and cell differentiation; and tyrosine kinase signaling pathway inhibition^[21]. In addition, among probiotics, the genera *Lactobacillus* has been reported to exert immuno-regulatory effects, including modulation of innate immune responses and promotion of humoral and cellular immunity^[22], suppression of pathogens and restoration of gut microbiota homeostasis^[23] and improvement of IBD^[24]. In the present study, we used an experimental model of CAC to investigate the effects of the probiotic *L. bulgaricus* on colon carcinogenesis. We showed that *L. bulgaricus* negatively regulated tumor progression, resulting in an expressive reduction of total tumor volume and mean size of tumors. Furthermore, the probiotic also attenuated the clinical signs of intestinal inflammation inducing a decrease in intestinal and tumor levels of IL-6, TNF- α , IL-17, IL-23 and IL-1 β .

Similarly, it has been recently observed that *L. salivary* and *L. fermentum* reduced the proliferation of colon cells in sporadic CRC^[25,26]. Given that cell proliferation defines the speed of cancer development^[27], probiotics capable of modulating cell proliferation are of great interest to prevent tumor growth and/or metastasis.

Our results also demonstrated that the probiotic *L. bulgaricus* attenuated intestinal inflammation by decreasing intestinal and tumor levels of IL-6, TNF- α , IL-17, IL-23 and IL-1 β . Finally, we also demonstrated an increase in IFN- γ levels in animals treated with *L. bulgaricus*. Due to their involvement in the pathogenesis of IBDs and CAC, the development of strategies that target the inflammatory cytokines IL-6, TNF- α , IL-17, IL-23 and IL-1 β is of potential interest in the therapeutic field. In a clinical trial with CRC patients a significant reduction in the blood levels of the proinflammatory

cytokines TNF- α , IL-12, IL-1 β , IL-6, IL-17 and IL-22, was observed after six months of a mix of probiotics consumption^[28].

The cytokine IL-1 β is found at high levels in several types of cancers and in CRC its expression is increased throughout the tumor progression^[29]. IL-1 β activates Wnt pathway in colon cancer cells promoting their growth and invasion^[30]. TNF- α is an important inflammatory mediator whose effects have been implicated in several cellular events, such as cell proliferation, differentiation and cell death^[31]. Anti-TNF therapies have been successfully used in IBD patients which confirms the crucial role of this cytokine in IBD and CAC development^[32]. Increased expression of TNF- α promotes cancer development through both leukocytic and nonhematopoietic cell TNFR1 expression in colonic tissue has been reported in studies using CAC model induced by AOM and DSS^[33,34].

Up-regulation of IL-17 has also been reported in colitis and colorectal tumors^[35]. The differentiation of Th17 cells may occur in the presence of different combinations of the cytokines TGF- β , IL-6, IL-1 β and/or IL-23, while its maintenance requires only IL-23 and/or IL-1 β ^[36]. Although we have not attempted to elucidate the molecular mechanisms that mediate the inhibitory role of *L. bulgaricus* in the regulation of IL-17 and IL-23 in our study, we hypothesize that the reduced expression of TNF- α is involved. In fact, previous studies have clearly shown that NF- κ B, a critical mediator of TNF- α signaling, regulates the transcription of the IL-23p19 gene^[37]. A recent finding showed that the probiotics *Bifidobacterium breve* and *Lactobacillus rhamnosus* GG inhibit LPS-induced expression of IL-23 in intestinal cells cultured in a condition of histone acetylation inhibition and increased DNA methylation^[38]. This finding might provide another potential mechanism for the *L. bulgaricus*-mediated negative regulation of IL-23.

Only a few trials in IBD patients have examined the composition of intestinal microbiota before and after supplementation therapy, so the effect (if any) of administering probiotics to the resident microbiota is not fully understood. However, it has been suggested that probiotics can change the intestinal ecosystem by generating an ecological environment that is unfavorable to the growth of noxious species, increasing the number of *Lactobacillus* and *Bifidobacteria* and stabilizing the intestinal microbiota^[39,40].

Dysregulation of gut microbiota has been associated with increased inflammation and the administration of probiotics have been reported to prevent chronic inflammatory diseases^[41]. In recent years there has been growing interest in the possible application of probiotics as a part of combination therapy with conventional treatment of cancer^[41-43]. However, studies investigating probiotics effects in patients with CRC are still very limited. For clinical application in humans many other studies, mainly randomized controlled trials would be needed to better evaluate the dosage, duration of the intervention and host physiology for confirm these findings^[41].

Several researches have indicated that the use of probiotics might improve beneficial microbiota, induce the release of antimicrobials and anticarcinogenic agents that help to remove carcinogens, and modulate immune responses that decrease intestinal inflammation in CRC patients^[41,44,45]. Here, we have shown that *L. bulgaricus* inhibited CAC *via* a negative regulation of intestinal inflammation. Although a deeper characterization of the molecular mechanisms underlying *L. bulgaricus* anti-inflammatory activity, the strength of our findings indicates a relevant and evidenced phenotypic pattern, which may be important in IBD field to prevent inflammation-associated tumorigenesis. To the best of our knowledge this is the first study to investigate and provide promising evidences of a preventive effect of the probiotic *L. bulgaricus* in cancer development in an experimental model of CAC.

CONCLUSION

In conclusion, our results show an anti-inflammatory and antitumor role of *L. bulgaricus* in colitis-associated carcinogenesis which may play an important role in prevention and treatment of CAC in the future. However, although the antitumor effects of this probiotic are promising, the mechanisms by which they occur still need to be better elucidated. Nevertheless, our study is extremely important as regards to the potential use of *L. bulgaricus* as a new therapeutic agent that can mediate and/or regulate the progression of CAC.

ARTICLE HIGHLIGHTS

Research background

Intestinal inflammatory disorders are associated with the infiltration of immune cells and the proinflammatory release of cytokines that play a critical role in the onset and progression of colitis-associated cancer (CAC). Recent studies suggested that the intestinal microbiota has an essential role in carcinogenesis. Probiotic supplementation is an alternative means of favorably modulating the intestinal microbiota. Currently, it has become increasingly evident that intestinal microbiota plays a crucial role in the pathogenesis of inflammatory bowel diseases (IBD) and colorectal cancer. Moreover, increasing evidence suggests that probiotics prevent inflammation and carcinogenesis and several bacteria strains have been used for the prevention and treatment of the infectious colitis, IBD. Thus, probiotic modulation of intestinal microbiota has emerged as a potential chemo-preventive agent.

Research motivation

Although supplementation with probiotics have been reported to prevent CAC, little is known about the administration of strains of *Lactobacillus bulgaricus* (*L. bulgaricus*), as well as their impact on neoplastic changes in the intestinal mucosa. Our study may contribute to address the gaps in the literature of how this probiotic, dose and supplementation time used for this experimental model impact on colitis, serum cytokines and neoplastic development.

Research objectives

The purpose of this study is to investigate the effect of the probiotic *L. bulgaricus* during the development of an experimental model of CAC. Overall, this study intends to strengthen data from preclinical studies, encouraging clinical trials to investigate their role in preventing colitis and CAC in humans.

Research methods

We used an experimental model of CAC. For mice treatment, 1×10^9 CFU were diluted in 200 μ L of PBS and orally given to each mouse, 3 times a week during all experimental period. Prior to tumor induction, C57BL/6 mice were randomly distributed in 2 groups ($n = 10$) and treated with PBS (control group) or *L. bulgaricus* (Lb group) by gavage (0.2 mL/mouse) for one week. For CAC induction, mice were intraperitoneally (i.p.) injected with a single dose (10 mg/kg in 300 μ L solution) of azoxymethene (Sigma-Aldrich), followed by 3 cycles of one week of 2.5% dextran sulfate sodium (DSS) diluted in drinking water intercalated for 2 wk of normal water. Mice were euthanized 12th week after CAC induction. Intestinal inflammation *in vivo*, or disease score, was determined by scoring clinical signs. The severity of intestinal inflammation was assessed by measuring the length of the entire large intestine. Also, the dimensions of the colorectal tumors were measured with pachymeter and the volumes were calculated by the formula: $(\text{width})^2 \times \text{length} / 2$. For histological analysis, distal colon parts were fixed in 4% p-formaldehyde in phosphate-buffered formalin and unblocked in paraffin. Tissue sections (4.0 μ m) were prepared from the paraffin-embedded tissue blocks, stained with hematoxylin and eosin and evaluated in a blinded fashion by an experienced pathologist. Cytokines levels were determined from colon and/or tumor samples by ELISA. Statistical analyses were performed using GraphPad Prism version 6.0. A 2-tailed *P* value < 0.05 was considered to be statistically significant.

Research results

We have shown that *L. bulgaricus* treatment inhibited the total tumor volume and mean size of tumors. Although we did not observe differences in body weight loss between control and *L. bulgaricus*-treated, we found differences in clinical signals in *L. bulgaricus*-treated mice, which showed a lower clinical score on the 13th and 15th days after tumor initiation. In addition to the attenuation of intestinal inflammation score, we observed that the treatment with *L. bulgaricus* reduced the DSS-induced shortening of the colon. In segments of the large intestine that did not present tumors (inflamed colon) we also observed a reduction of at least 2-fold in the levels of the cytokines TNF- α , IL-1 β , IL-23 and IL-17 in *L. bulgaricus*-treated mice in comparison to controls. In contrast, increased concentrations of IFN- γ were also observed in Lb group. Regarding the cytokines measured in tumor tissues, we observed a pattern similar to that found in the inflamed colon with a negative regulation of proinflammatory cytokines in mice

treated with the probiotic and an increase in IFN- γ levels in this group. Overall, these findings highlight the protective effect of *L. bulgaricus* in the regulation of gut inflammation and preventing CAC development. Thus, further clinical trials are needed to confirm these preclinical insights.

Research conclusions

We found an anti-inflammatory role and consequent antitumor effect of *L. bulgaricus* on CAC that may be used as a promising tool for the prevention and treatment of CAC. In summary, *L. bulgaricus* treatment during colitis-associated colorectal carcinogenesis model may be responsible for anti-inflammatory and antitumor role by lowering proinflammatory cytokine expression.

Research perspectives

The present study has shown that *L. bulgaricus* inhibited CAC via a negative regulation of intestinal inflammation. Hence, has demonstrates promising evidence on *L. bulgaricus* probiotic has a preventive potential in CAC development. Therefore, clinical trials are needed to confirm this hypothesis and increase the therapeutic arsenal against CAC.

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Basic Study

Antifungal activity and antidiarrheal activity via antimotility mechanisms of (-)-fenchone in experimental models

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Institutional review board

statement: The study was reviewed and approved by the Commission for Ethics in Animal Experimentation (CEUA) of the

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Abstract

BACKGROUND

(-)-Fenchone is a bicyclic monoterpene present in essential oils of plant species, such as *Foeniculum vulgare* and *Peumus boldus*, used to treatment of gastrointestinal diseases. Pharmacological studies report its anti-inflammatory, antioxidant, and antinociceptive activity.

AIM

To investigate antidiarrheal activity related to gastrointestinal motility, intestinal secretion and antimicrobial activity.

METHODS

A castor oil-induced diarrhea model was used to evaluate antidiarrheal activity. Intestinal transit and gastric emptying protocols were used to assess a possible antimotility effect. Muscarinic receptors, presynaptic α_2 -adrenergic and tissue adrenergic receptors, K_{ATP} channels, nitric oxide were investigated to uncover antimotility mechanisms of action and castor oil-induced enteropooling to elucidate antisecretory mechanisms. The antimicrobial activity was evaluated in the minimum inhibitory concentration model, the fractional inhibitory

Research Ethics Committee of the Federal University of Paraíba.

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concentration index using the (-)-fenchone association method with standard antifungal agents.

RESULTS

(-)-Fenchone (75, 150 and 300 mg/kg) showed antidiarrheal activity, with a significant decrease in the evacuation index. This activity is possibly related to a percentage of reduced intestinal transit (75, 150 and 300 mg/kg). The antimotility effect of (-)-fenchone decreased in the presence of pilocarpine, yohimbine, propranolol, L-NG-nitroarginine methyl ester or glibenclamide. In the enteropooling model, no reduction in intestinal fluid weight was observed. (-)-Fenchone did not show antibacterial activity; on the other hand, inhibits the growth of strains of fungi with a minimum fungicide concentration of 32 µg/mL. However, when it was associated with amphotericin B, no synergism was observed.

CONCLUSION

The antidiarrheal effect of (-)-fenchone in this study involves antimotility effect and not involve antisecretory mechanisms. (-)-Fenchone presents antifungal activity; however, it did not show antibacterial activity.

Key Words: (-)-Fenchone; Monoterpenes; Diarrhea; Motility; Antifungal

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Core Tip: (-)-Fenchone is a bicyclic monoterpene present in essential oils of plant species, such as *Foeniculum vulgare* and *Peumus boldus*, used to treatment of gastrointestinal diseases. Diarrhea is a pathological condition characterized by an increase in three or more defecations in 24 h, being of multiple origins, whether infectious or not. In the search for new therapeutic alternatives, natural products, and medicinal plants are of great relevance. Many species of plants and their isolated compounds, including terpenes, have shown promising antidiarrheal and motility, based on this result, the monoterpene (-)-fenchone was selected for this study.

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INTRODUCTION

Diarrhea is one of the most prevalent conditions affecting the gastrointestinal tract worldwide, contributing significantly to morbidity and mortality of the population^[1]. The prevalence of chronic diarrhea is estimated at 1%-5% of the adult population, and in developed countries^[2]. This condition can be defined as water and electrolyte loss caused by an increase in defecation frequency and abnormality in stool consistency throughout three or more times over 24 h^[3-5].

The etiology of diarrheal disorders is multifactorial, attributed to factors such as infectious agents^[6], food allergies, disturbances in intestinal function^[7], alcohol intake^[8], malabsorption of bile salts^[9] and use of some medications, *e.g.*, antimicrobials, antineoplastic, antiretrovirals, oral hypoglycemic agents, β-blockers, nonsteroidal anti-inflammatory drugs and proton pump inhibitors^[10].

In adults, the most common causes of diarrhea include irritable bowel syndrome, inflammatory bowel disease, celiac disease, malabsorption of syndromes and microscopic colitis. The assessment and management of this condition can be a challenge, since the diagnosis is quite broad, especially about the differentiation between organic or functional causes that may be involved in its etiology^[2-11].

Treatment of this disorder aims to reduce the dehydration and discomfort caused by

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frequent bowel movements through pharmacological and non-pharmacological actions^[12,13]. Oral rehydration therapy is the primary non-pharmacological approach used for fluid and electrolyte replacement through the use of an oral rehydration solution, which may be associated with zinc supplementation^[14]. Pharmacological therapy is nonspecific and indicated for the reduction of persistent and clinically significant symptoms. Among the main classes of drugs used are antisecretory and motility suppressing agents, probiotics, enkephalinase inhibitors, bismuth compounds and α_2 -adrenergic receptor agonists^[12,15,16].

However, current therapy is limited due to side effects, *e.g.*, dry mouth, distension and abdominal cramps, severe constipation, nausea and vomit^[17]. Besides, respiratory depression and paralytic ileus are among the most dangerous side effects of loperamide in children^[18].

In the search for new therapeutic alternatives, natural products and medicinal plants are of great relevance. Plant extracts, their semi-synthetic derivatives and synthetic compounds inspired by natural products make up the majority of medicines in use currently^[19]. Thus, natural products are good starting points for the development of new drugs used in the treatment of gastrointestinal disorders such as diarrhea^[20,21].

In fact, in recent years many species of plants and their isolated compounds, including terpenes, have shown promising antidiarrheal and motility effects, such as the friedelan-3 β -ol, friedelin, volvalerenol A triterpenes^[22,23] and the monoterpene carvone^[24] and α -terpineol^[25].

Based on this criterion, the monoterpene (-)-fenchone (1,3,3-trimethylbicyclo[2.2.1]heptane-2-one) was selected for this study. This monoterpene is made up of two enantiomers, (+)-fenchone and (-)-fenchone, obtained from fennel oil (*Foeniculum vulgare*) and thuya oil (*Thuja occidentalis* L.), respectively^[26,27]. Research shows that the (-)-fenchone isomer has antinociceptive activity^[28], antimicrobial^[29], anti-inflammatory and antioxidant^[30,31].

From this perspective, the present study aimed to evaluate the antidiarrheal and antimicrobial activity of (-)-fenchone using experimental models.

MATERIALS AND METHODS

Animals

Swiss adult male (*Mus musculus*), weighing between 25-35 g, were obtained from the Animal Production Unit of the Institute for Research on Drugs and Medicines of Federal University of Paraíba (IPeFarM/UFPB), protocol No. 035/2017 and No. 4996090518/2018. They were housed under 23 \pm 1 °C, with a 12/12 h light/dark cycle, fed with Purina® chow and water ad libitum for two weeks before experimentation. Intragastric gavage administration was carried out with conscious animals, using straight gavage needles appropriate for the animal size. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection, following internationally accepted principles for the use of laboratory animals.

Substances

The following drugs were used: Carboxymethylcellulose (Formula Brasil®, Brazil); castor oil (Tayuyna Lab Ltda®, Brazil); loperamide hydrochloride (2 mg; Janssen Cilag Farmacêutica Ltda®, Brazil); activated charcoal (Proquímios®, Brazil); glibenclamide, L-NG-nitroarginine methyl ester (L-NAME), propranolol and yohimbine (Sigma Aldrich®, Brazil).

Study material

(-)-Fenchone was purchased from SIGMA-ALDRICH Brasil Ltda (product reference: 196436) 98% purity, density: 0.948 g/cm³ at 25 °C, CAS: 7787-20-4, molecular mass 152.23, boiling point: 192-194 °C and melting point 5-6 °C.

Culture mediums

The culture media used were Brain Heart Infusion (BHI), Sabouraud Dextrose Agar purchased from Difco Laboratories Ltd, United States, France, RPMI 1640 with L-glutamine, and bicarbonate (Difco Laboratories Ltd, United States, France, and INLAB, San Paulo, Brazil). All media were prepared as per the manufacturer's descriptions.

Microorganisms

For the biological activity assays of the test products, strains of *Staphylococcus aureus* ATCC-25923 and LM-177, *Pseudomonas aeruginosa* ATCC-25853 and LM-297, *Escherichia coli* ATCC-18739 and LM-39, *Candida albicans* ATCC-76645 and LM-05; *Candida tropicalis* ATCC-13803 and LM-20; *Candida Krusei* ATCC-6258 and LM-13; obtained from the Laboratory of Mycology, Department of Pharmaceutical Sciences (DCF), Health Sciences Center (CCS) of the Federal University of Paraíba (UFPB). For inoculum preparation, the colonies of microorganisms were suspended in 0.85% sterile 0.9% NaCl solution and adjusted according to the 0.5 scale of McFarland standard to obtain an inoculum of 1×10^6 - 5×10^6 colony-forming units per milliliter (CFU/L) for fungi and 1×10^8 - 2×10^8 CFU/mL for bacteria^[32].

Pharmacological assays

Effects of (-)-fenchone on castor oil-induced diarrhea: For the evaluation of (-)-fenchone antidiarrheal activity, the protocol described by Awouters *et al*^[33] was used. Male Swiss mice ($n = 7$ per group), fasting 12 h, were treated orally with vehicle (5% tween 80, 10 mL/kg), loperamide (5 mg/kg) or (-)-fenchone (37.5, 75, 150 or 300 mg/kg). After 1 h, 10 mL/kg of castor oil was administered orally. The animals were placed separately in boxes with a paper-lined floor for analysis of fecal cakes. The severity of diarrhea was observed during 4 h, analyzing the following parameters: Evacuation index (formed or solid, semi-solid or pasty and liquid), percentage of liquid stools, and percentage of diarrhea inhibition. Evacuation index: $\sum (\text{Solid stool} \times 1) + (\text{Liquid stool} \times 2) + (\text{Liquid stool} \times 3)$ % ID = (Tween group average - Treated group average) / Tween group average $\times 100$.

Effect of (-)-fenchone on gastric emptying: The effect on gastric emptying was evaluated according to experiments conducted by Scarpignato *et al*^[34]. Male Swiss mice ($n = 7$ per group), fasted for 24 h, orally received the vehicle (5% tween 80; 10 mL/kg), loperamide (5 mg/kg) or (-)-fenchone (37.5, 75, 150, 300 mg/kg). After 1 h the animals received the 10 mL/kg phenol red-colored marker (0.05% phenol red-colored in 1.5% carboxymethylcellulose-thickening agent). To the non-treated control group (the zero-time control group), the phenol red-colored marker was administered and the mice were immediately euthanized. The treated groups received the same marker and were euthanized 30 min after administration. The abdominal cavity was opened; the pylorus and distal esophagus were clamped. The stomach was removed, opened, and washed with 7 mL of distilled water. Gastric contents were collected and centrifuged at 3000 rpm for 15 min. Then, 1 mL of the supernatant was collected, and 1 mL of 1 mol/L NaOH (pH = 12) was added. The results were obtained by spectrophotometry with a wavelength reading of 560 nm and expressed as a percentage of gastric emptying, calculated by the formula: % Gastric emptying = $(100 - \text{mean absorbance of sample}) / \text{mean absorbance of zero-time control group} \times 100$.

Effect of (-)-fenchone on castor oil-induced intestinal transit: The effect on intestinal transit was evaluated according to the methodology described by Stickney *et al*^[35]. Male Swiss mice ($n = 7$ per group) were fasted for 24 h and orally received the vehicle (5% tween 80; 10 mL/kg), loperamide (5 mg/kg) or (-)-fenchone (37.5, 75, 150, 300 mg/kg). After 1 h the oral administration of castor oil (1 mL/100 g) was performed. After 30 min, 10 mL/kg of activated charcoal marker [activated charcoal (10%) in gum arabic (5% thickening agent)] was orally administered to the animals. After 30 min, the animals were euthanized, the intestine removed from the pylorus to the ileocecal junction and using a ruler the total length of the intestinal segment and the distance covered by the activated charcoal were measured to calculate the percentage of intestinal transit: % Intestinal transit = (distance traveled by phenol red)/total intestine length $\times 100$.

(-)-Fenchone ant motility mechanisms of action

Evaluation of the participation of muscarinic, adrenergic, nitrergic pathway and ATP-dependent potassium channels (K_{ATP}) in (-)-fenchone ant motility mechanisms in the intestinal transit model: To investigate the ant motility mechanisms involved in the antidiarrheal activity fenchone were evaluated according to the model described by Santos *et al*^[36]. The role of muscarinic receptors, alpha and beta-adrenergic receptors, nitric oxide (NO) and K_{ATP} were evaluated. The mice were fasted for 24 h and distributed in different groups ($n = 7$ per group). Two groups received intraperitoneal NaCl solution 0.9% (10 mL/kg), the other two received pilocarpine (non-selective muscarinic receptor agonist, 1 mg/kg), yohimbine (α_2 -adrenergic presynaptic receptor antagonist, 1 mg/kg), propranolol (non-selective β -adrenergic

receptor antagonist), L-NAME (NO synthase activity inhibitor, 25 mg/kg) or glibenclamide (K_{ATP} channel blocker, 1 mg/kg). These drugs were dissolved in NaCl 0.9% and given intraperitoneally. After 30 min, the animals were treated orally with 5% tween 80 (control group), or fenchone 150 mg/kg (most effective dose). After 60 min, 10 mL/kg (p.o.) of the black marker (10% activated charcoal suspension in 5% arabic gum) was administered and 30 min later, the animals were euthanized for removal of the small intestine to calculate the percentage of intestinal transit.

Evaluation of (-)-fenchone antisecretory mechanism in castor oil-induced intraluminal fluid accumulation (enteropooling) model: The effects on intestinal enteropooling were evaluated according to protocols described by Ezeja *et al.*^[37] adapted. Mice ($n = 7$ per group), after 24 h fasting, were orally pretreated with 5% tween 80 (vehicle 10 mL/kg), loperamide (5 mg/kg) or (-)-fenchone (150 mg/kg). After 60 min, the animals received orally 10 mL/kg of castor oil to induce diarrhea. After 1 h, the mice were euthanized, their abdomens were opened, and their intestines removed (pylorus ileocecal junction) and weighed with the intestinal contents (full intestine). The intestinal contents were removed and weighed again (empty intestine). Difference between the first and second weight is considered as the weight of the intestinal content of each animal.

Evaluation of antibacterial and antifungal activity

Minimum inhibitory concentration: Antimicrobial activity assays were performed according to the protocols of Cleeland *et al.*^[38], Eloff^[39], and CLSI^[32]. Minimum inhibitory concentration (MIC) determination of the (-)-fenchone on bacterial and fungal strains was performed by the microdilution technique in 96 well plates. Initially, 100 μ L of double concentrated RPMI/BHI broth was distributed to the wells of the microdilution plates. Then, 100 μ L of (-)-fenchone was dispensed into the wells of the first line of the plate and by serial dilution at a ratio of two concentrations of 1024 μ g/mL to 16 μ g/mL were obtained. Finally, 10 μ L of bacterial and fungal inoculum (strains of *Staphylococcus aureus* ATCC-25923 and LM-177, *Pseudomonas aeruginosa* ATCC-25853 and LM-297, *Escherichia coli* ATCC-18739 and LM-39, *Candida albicans* ATCC-76645 and LM-05, *Candida tropicalis* ATCC-13803 and LM-20, *Candida Krusei* ATCC-6258 and LM-13) were added to the wells. Controls performed: Microorganisms and culture medium to check the viability of the strains and the sterility of the medium and control with Gentamicin (64 μ g/mL) and amphotericin B (32 μ g/mL). The prepared plates were sealed aseptically and incubated at 35 ± 2 °C for 24-48 h. The antimicrobial activity of the products was interpreted and considered as active or inactive according to the following criteria: Up to 600 μ g/mL = strong activity; 600-1500 μ g/mL = moderate activity; > 1500 μ g/mL = weak activity or inactive product^[40-42].

Minimum fungicide concentration: After MIC, 10 μ L aliquots of the supernatant from the wells in which complete fungal growth inhibition (MIC, MIC \times 2, MIC \times 4, and MIC \times 8) was observed on the microdilution plates were added to 100 μ L RPMI broth contained in new culture plates. Plates were incubated for 24-48 h at 35 ± 2 °C. Minimum fungicide concentration (MFC) was considered as the lowest concentration of the product that was able to inhibit the growth of microorganisms.

Association test: The effect of the association of (-)-fenchone with amphotericin B was determined from the checkerboard method for derivation of the fractional inhibitory concentration index (FICI) against *C. albicans* strains ATCC-76645 and LM-05. Solutions of the tested products were used at concentrations determined from their MICs. Initially, 100 μ L RPMI broth was added to the 96 well plates. Then, 50 μ L of each product tested at several levels (1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, MIC \times 2 and MIC \times 4) were added vertically (amphotericin B) and horizontally (-)-fenchone. Finally, 20 μ L of the fungal suspension was added after the incubation period of the microplates at 35 ± 2 °C for 24-48 h. The fractional inhibitory concentration (FIC) was calculated by summing the FICA + FICB (FICA = FIC of the test product; FICB = FIC of the standard antifungal). The FICA, in turn, is calculated using the combined MICA / MICA isolated, while FICB = combined MICB / isolated MICB. This index is interpreted as follows: Synergism (FICI \leq 0.5), antagonism (FICI > 4.0) and indifference ($0.5 < \text{FICI} \leq 4$).

Statistical analyses

Data were expressed as mean \pm standard deviation from the mean for parametric data and median (minimum-maximum values) for nonparametric data. These values were

statistically analyzed by one-way analysis of variance (parametric data) or Kruskal-Wallis (nonparametric data), followed by Dunnet or Dunn's posttests, respectively. Results were considered significant when $P < 0.05$. GraphPad Software® 5.0 (United States) was used for data processing. The statistical methods applied in this study were reviewed by Gessenildo P. Rodrigues, PhD, Padronize - Academic Consulting Firm.

RESULTS

Effect of (-)-fenchone in diarrhea induced by castor oil

According to the observed results, a negative control group treated with the vehicle (5% tween 80, 10 mL/kg) presented diarrhea, with evacuation index 23 (20-24) and 81% of liquid feces. Pre-treatment with (-)-fenchone (75, 150 and 300 mg/kg, p.o.) reduced the evacuation by 7 (6-7) with 51% inhibition of diarrhea ($P < 0.05$), 4 (3-6) and 81% ($P < 0.01$), 3 (1-4) and 88% ($P < 0.001$), respectively, when compared to the negative control group. A standard antidiarrheal drug, loperamide (5 mg/kg, p.o.), produced an 87% inhibition of diarrhea (Table 1).

Effect of (-)-fenchone on gastric emptying

Results of this model showed that the group treated with the vehicle (5% tween 80, 10 mL/kg) presented 89% gastric emptying. The groups treated with loperamide (5 mg/kg, p.o.) and (-)-fenchone at doses of 75, 150 and 300 mg/kg reduced gastric emptying to 54% ($P < 0.01$), 76% ($P < 0.01$), 61% ($P < 0.001$) and 57% ($P < 0.001$), respectively, when compared to the negative control group (Figure 1).

Effect of (-)-fenchone on intestinal transit

Results of this study showed that the distance travelled by a marker (activated charcoal) in terms of percentage of the total length of the intestine was 97% in the negative control group. Treatment with loperamide (5 mg/kg, p.o.) and (-)-fenchone at doses of 75, 150 and 300 significantly reduced ($P < 0.001$) the percentage of intestinal transit to 26%, 48%, 38% and 37%, respectively, when compared to the control group (Figure 2).

Mechanisms of antimotility action of (-)-fenchone

Treatment with (-)-fenchone at its most effective dose (150 mg/kg, p.o.) significantly reduced the percentage of intestinal transit to 37% when compared to the negative control group (85%). In the group pretreated with pilocarpine (non-selective muscarinic receptor agonist, 1 mg/kg, i.p.), fenchone significantly reduced muscarinic action in intestinal transit to 75% (Figure 3).

(-)-Fenchone (150 mg/kg, orally), reduced the percentage of intestinal transit to 34% compared to the control group (78%). In the presence of yohimbine (α_2 -adrenergic antagonist 1 mg/kg, i.p.) or propranolol (non-selective β -adrenergic receptor antagonist, 1 mg/kg, i.p.) the inhibitory effect was significantly reduced to 98% and 88%, respectively, when compared to the group (-)-fenchone (unlocked, Figure 4). Pre-treatment with L-NAME (inhibitor of NO synthase activity, 25 mg/kg, i.p.) or glibenclamide (K_{ATP} channel blocker, 1 mg/kg, i.p.) reversed the inhibitory effect of this monoterpene on intestinal transit to 85% and 92%, respectively, when compared to the unblocked group (-)-fenchone (Figure 5).

Mechanisms of anti-secretion of (-)-fenchone (enteropooling)

Treatment with (-)-fenchone (150 mg/kg, p.o.) showed no significant reduction in intestinal fluid accumulation (0.53 ± 0.04) when compared to the control group (0.51 ± 0.01), treatment with loperamide (5 mg/kg, p.o.) reduced the weight of intestinal fluid (0.28 ± 0.01 g) significantly when compared to the control group (Figure 6).

Antibacterial and antifungal activity

(-)-Fenchone did not show inhibitory activity against any of the bacterial strains in the concentration range used. Thus, for bacterial strains, it was not possible to determine the MIC and minimum bactericidal concentration. The results of the antifungal activity (Table 2) shown that (-)-fenchone inhibited the growth of 4 (66%) of the six fungal strains studied up to a concentration of 32 μ g/mL, with only two strains being inhibited to a concentration of 64 μ g/mL. Therefore, the MIC₅₀ (level capable of inhibiting the growth of up to 50% of the species) was considered to be 32 μ g/mL.

Table 1 Effect of oral (-)-fenchone and loperamide administration on castor oil-induced diarrhea in mice

Tratament (p.o.)	Dose (mg/kg)	EI	Liquid stools (%)	Diarrheal inhibition (%)
Tween 80 5%		23 (20-24)	81	
Loperamide	5	7 (4-7) ^e	8	87
(-)-Fenchone	37, 5	16 (14-17)	52	28
(-)-Fenchone	75	7 (6-7) ^a	19	51
(-)-Fenchone	150	4 (3-6) ^b	4	81
(-)-Fenchone	300	3 (3-4) ^e	4	88

Data expressed as median (minimum-maximum values) and analyzed by Kruskal-Wallis test followed by Dunn's test (

^a $P < 0.05$,

^b $P < 0.01$,

^e $P < 0.001$, compared to the 5% tween 80 group) ($n = 7$ /per group). EI: Evacuation index.

Table 2 Effect of the evaluation of the minimum inhibitory concentration and minimum fungicide concentration ($\mu\text{g/mL}$) of (-)-fenchone against fungal strains

Species	Strains	(-)-Fenchone	
		MIC ($\mu\text{g/mL}$)	MFC ($\mu\text{g/mL}$)
<i>Candida albicans</i>	ATCC-76645	32	32
<i>Candida albicans</i>	LM-05	32	32
<i>Candida tropicalis</i>	ATCC-13803	32	32
<i>Candida tropicalis</i>	LM-20	32	256
<i>Candida kruzei</i>	ATCC-6258	64	256
<i>Candida kruzei</i>	LM-13	64	256

MIC: Minimum inhibitory concentration; MFC: Minimum fungicide concentration.

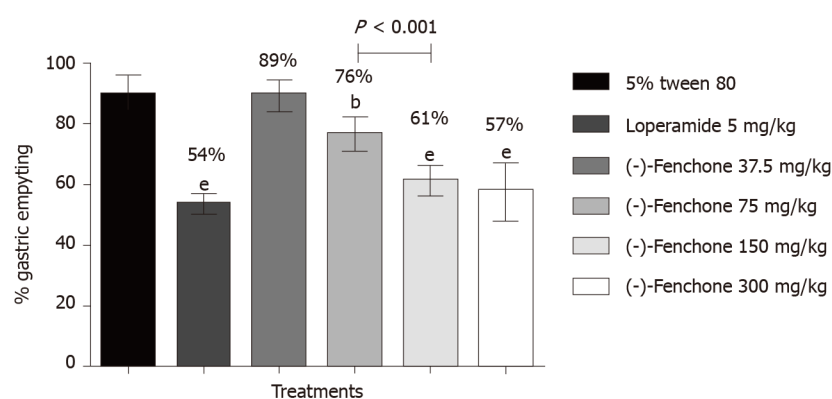


Figure 1 Effect of oral administration of (-)-fenchone and loperamide on gastric emptying in male Swiss mice. Data expressed as mean \pm standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey's multiple comparison tests (^b $P < 0.01$ and ^e $P < 0.001$, compared to the 5% tween 80 group) ($n = 7$ /per group).

MFC of the product against fungal strains varied between 32 $\mu\text{g/mL}$ and 256 $\mu\text{g/mL}$, with the concentration of 32 $\mu\text{g/mL}$ fungicidal for 3 (50%) of the strains tested. In this way, the MFC50 was established as 32 $\mu\text{g/mL}$.

Association assay

Tests demonstrated that (-)-fenchone maintained the MIC of 32 $\mu\text{g/mL}$ and amphotericin B the MIC of 0.2 $\mu\text{g/mL}$ for the strains tested, maintaining the MIC at

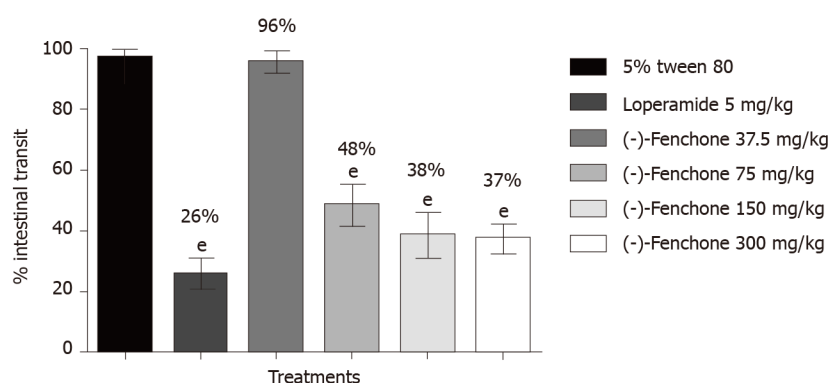


Figure 2 Effect of oral administration of (-)-fenchone and loperamide in intestinal transit in male Swiss mice. Data expressed as mean \pm standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey's multiple comparison tests ($^eP < 0.001$, compared to the 5% tween 80 group) ($n = 7$ /per group).

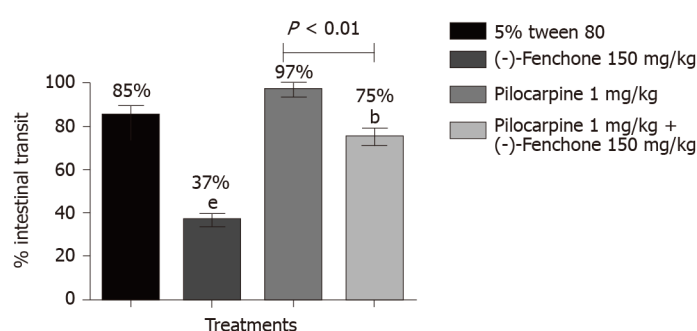


Figure 3 Effect of oral administration of (-)-fenchone after treatment with pilocarpine and yohimbine, and on intestinal transit of mice. Data expressed as mean \pm standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey's tests ($^bP < 0.01$ and $^eP < 0.001$, compared whit tween 5% tween 80) ($P < 0.01$ compared to the pilocarpine whit pilocarpine + (-)-fenchone group) ($n = 7$ /per group).

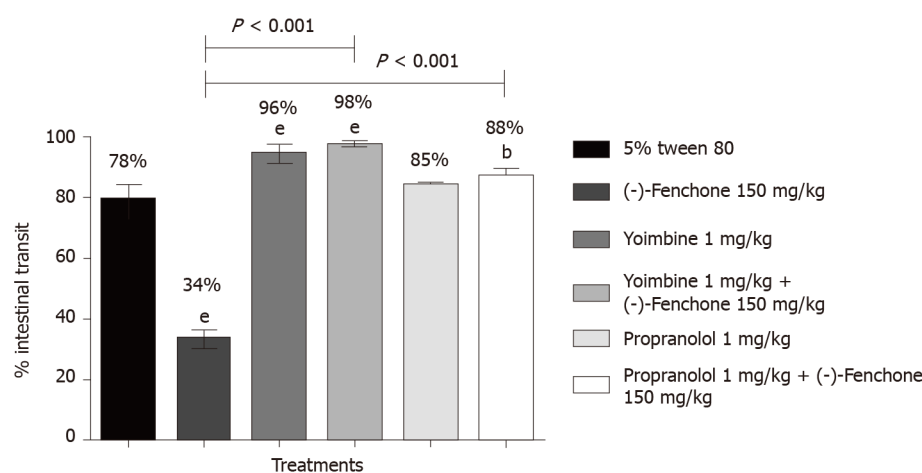


Figure 4 Effect of oral administration of (-)-fenchone after treatment with propranolol on intestinal transit of mice. Data expressed as mean \pm standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey's tests ($^bP < 0.01$ and $^eP < 0.001$, compared whit tween 5% tween 80) ($P < 0.001$ compared to the unblocked (-)-fenchone group) ($n = 7$ /per group).

equal values both individually and in an association. Since the FIC, that is, the ratio of the combined inhibitory concentrations between the isolated inhibitory concentrations, for each substance was equivalent to 1, the FICI was equal to 2 (Table 3).

Table 3 Determination of the fractional inhibitory concentration index of the association between (-)-fenchone and amphotericin B on *Candida albicans* strains

Strains	FICA, (-)-fenchone	FICB, Amphotericin B	FICI	Interaction type
<i>Candida albicans</i> (ATCC-76645)	1	1	2	Indifferent
<i>Candida albicans</i> (LM-05)	1	1	2	Indifferent

FICA: Fractional inhibitory concentration of the test product; FICB: Fractional inhibitory concentration of the standard antifungal; FICI: Fractional inhibitory concentration index.

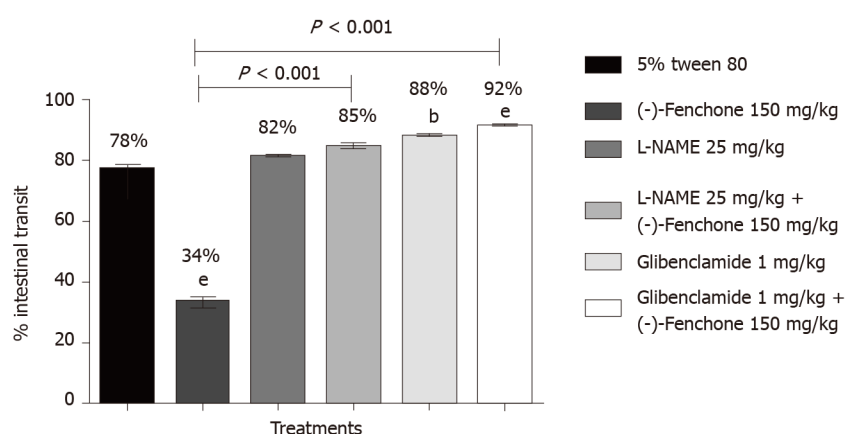


Figure 5 Effect of oral administration of (-)-fenchone after treatment with L-NG-nitroarginine methyl ester, and glibenclamide on intestinal transit of mice. Data expressed as mean \pm standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey's tests (^b $P < 0.01$ and ^e $P < 0.001$, compared with 5% tween 80 groups) ($P < 0.001$ compared to the unblocked (-)-fenchone group) ($n = 7$ /per group). Statistical analysis: ANOVA followed by the Tukey test.

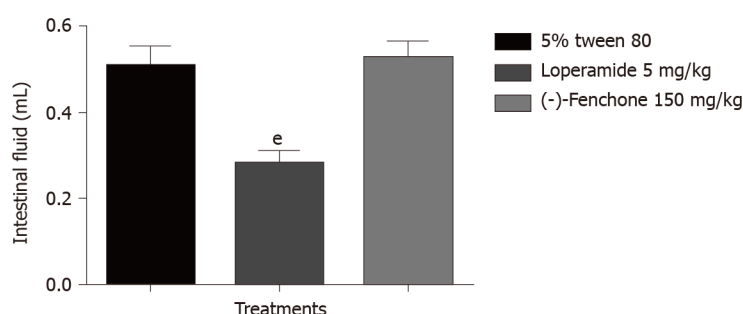


Figure 6 Effect of oral administration of (-)-fenchone and loperamide on castor oil-induced enteropooling in mice. Data expressed as mean \pm standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey's tests (^e $P < 0.001$, compared with 5% tween 80 groups) ($n = 7$ /per group).

DISCUSSION

To evaluate the antidiarrheal activity of (-)-fenchone the castor oil-induced diarrhea model was used in mice. Castor oil is a natural product obtained from the seeds of *Ricinus communis*, that when ingested is metabolized by intestinal lipases, releasing ricinoleic acid in the intestinal lumen, creating extensive contractions in the transverse and distal colon^[43]. This substance causes a reduction in Na⁺, K-ATPase activity, inhibition of sodium, chloride, and water absorption, increased contractions of smooth intestine muscles, produces a cytotoxic effect on enterocytes, resulting in abundant watery diarrhea with an accumulation of intestinal fluid^[44].

(-)-Fenchone (75, 150, and 300 mg/kg, p.o.) presented antidiarrheal activity, decreasing ($P > 0.05$) the evacuation index (evacuation index, percentage of liquid stools, and percentage of diarrhea inhibition). These results corroborate a study by Dos Santos Negreiros *et al.*^[25] with the monoterpene α -terpineol showed a reduction in the total number of stools and diarrheal stools in the castor oil-induced diarrhea model.

Study with essential oil of *Mentha longifolia* L., whose main components are monoterpenes pulegone and 1,8-cineol showed antidiarrheal activity these same model^[45].

To evaluate if (-)-fenchone influenced gastrointestinal motility, the evaluation of gastric emptying and intestinal transit protocols were assessed. The findings suggested an antimotility activity mediated by (-)-fenchone since it was efficient in decreasing gastric emptying and intestinal transit. Similar results were found for monoterpene 1,8-cineol showed a reduction in gastric emptying^[46]. (-)-Fenchone declined the propulsion of the marker (activated charcoal suspension) through the intestine. It suggests that (-)-fenchone influenced peristaltic movements of the intestine, characterizing an antimotility activity. Silva *et al*^[24] shown that the monoterpene carvone also reduced the percentage of intestinal transit in this model.

An investigation was then carried out to determine whether fenchone can act through cholinergic mechanisms. Acetylcholine (ACh) exerts an excitatory effect on the gastrointestinal smooth muscle by activating muscarinic M_3 receptors (coupled to $Gq/11$), there is an increase in the cytosolic concentration of Ca^{2+} , which results in contraction of the smooth muscle and increased intestinal transit^[47]. Therefore, a reduction in the release of this neurotransmitter, as well as the inhibition of its action on its respective receptors, can delay intestinal transit.

Pilocarpine, a cholinergic agonist, was used to induce intestinal motility. It can be seen that fenchone significantly reduced the stimulating effects promoted by pilocarpine resulting in a decrease of intestinal transit. From this result, we can infer that (-)-fenchone may be competing for GIT M_3 receptors, acting as a partial antagonist of muscarinic receptors and that its antimotility involves the cholinergic pathway. This result corroborates the study by Dos Santos Negreiros *et al*^[25], with the monoterpene α -terpineol, which presented the same behavior when associated with bethanechol (muscarinic agonist), suggesting the participation of the muscarinic pathway in its antimotility effect.

Sympathetic innervation (*via* noradrenaline) acts as inhibitory feedback modulating the release of ACh in the myenteric plexus (*via* presynaptic α_2 -adrenergic receptors coupled to Gi/Go), and also by its action on receptors present in the intestinal smooth muscle (*via* post receptors β_2 -adrenergic synaptic coupled to Gs)^[48]. Both actions result in inhibition of peristaltic activity and decreased tone of intestinal smooth muscle, leading to reduced intestinal motility^[49]. Therefore, a blockade of pre or postsynaptic receptors can increase intestinal transit.

The presynaptic α_2 -adrenergic receptor antagonist yohimbine or postsynaptic β -adrenergic receptor antagonist propranolol were used to induce intestinal motility. It can be observed that in the presence of blockers the antimotility effect of (-)-fenchone was significantly reduced, with this, it can be inferred that the adrenergic pathway is related to the antimotility effect of fenchone.

NO is a neurotransmitter released by inhibitory enteric neurons and causes post-junction hyperpolarization responses resulting in smooth muscle relaxation^[50]. The K_{ATP} channels are expressed in large quantities in the intestinal smooth muscle modulating gastrointestinal motility in physiological and pathophysiological states^[51]. NO activates soluble guanylyl cyclase, leading to the production of cyclic guanosine monophosphate (cGMP) that enables its dependent protein kinase, which activates potassium channels^[50]. Activation of K_{ATP} promotes hyperpolarization of the cell membrane, reduction of Ca^{2+} influx and inhibition of cellular excitability, thus generating smooth muscle relaxation^[51].

L-NAME an inhibitor of NO synthase or glibenclamide a K_{ATP} channel blocker was used as blockers of this pathway. From the results, it was observed that in the presence of the blockers the antimotility effect of fenchone was significantly reduced; thus, it can be suggested that the antimotility effect to fenchone may involve the NO-cGMP- K_{ATP} pathway.

These results corroborate with the study by Formiga *et al*^[52] with the ethanolic extract of *Maytenus erythroxylon* Reissek, which has triterpenes in its composition, the administration of the extract associated with propranolol, L-NAME or glibenclamide reduced its antimotility effect, suggesting the participation of adrenergic and nitrergic pathways and K_{ATP} channels in their effect.

To evaluate the antisecretory effect of (-)-fenchone was used the intestinal fluid accumulation model (enteropooling) induced by castor oil. The results show that (-)-fenchone did not reverse the accumulation of fluid caused by the inducing agent. It can suppose that the antidiarrheal effect of this monoterpene does not involve antisecretory and pro-absorptive mechanisms. This result differs the Sisay *et al*^[53], in which the extract of *Verbena officinalis* rich in terpenoids, had an inhibitory effect on enteropooling induced by castor oil.

According to the results obtained in the tests, it was found that (-)-fenchone at concentrations of 1024 to 16 µg/mL does not show antibacterial activity against any of the tested bacterial strains. A study conducted by Balogun *et al.*^[54] demonstrated that the essential oil of *Moringa oleifera* at a concentration of 25 mg/mL. With monoterpenes as the most abundant class of compounds, exhibited relatively weak antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Several studies have reported a variety of fungus can cause diarrhea and opportunistic infections, such as genus *Candida* (*C. albicans*, *C. tropicalis*, and *C. krusei*)^[55,56]. Many essential oils already have known antifungal activity^[57]. The results obtained in this study showed that monoterpene (-)-fenchone in concentrations of 32 µg/mL and 64 µg/mL, inhibited the growth of all tested strains (*Candida albicans* ATCC-76645 and LM-05; *Candida tropicalis* ATCC-13803 and LM-20; *Candida Krusei* ATCC-6258 and LM-13).

The results obtained corroborate the study by Dias *et al.*^[58] the antifungal activity of monoterpene linalool in clinical strains of *C. albicans*, *C. krusei*, and *C. tropicalis* was evaluated. The results showed that this monoterpene inhibited the growth of all strains of fungi at the concentrations evaluated.

Several studies have demonstrated the antifungal potential of products of natural origin used alone or in combination with medications already used. It was evaluated the association between (-)-fenchone and amphotericin B, an antifungal agent standard, utilizing the checkerboard method^[59]. *Candida albicans* is still the most prevalent species in infections caused by fungi, bringing an even more worrying scenario due to the high rates of resistance to antifungals^[60]. Amphotericin B is an antifungal of the polyenes class, and its mechanism of action involves interaction with ergosterol. It leads to the formation of pores in the membrane, loss of integrity, rapid extravasation of potassium and other ions causing cell death^[61].

When tested in combination (-)-fenchone and amphotericin B, they did not show any synergism or antagonism effect, indicating that the combination between these two substances does not present significant interaction, that is, indifferent. The antimicrobial action of essential oils and their constituents such as terpenes is being associated with their high lipophilicity, which facilitates access to the lipid layer of the bacterial cell membrane and fungal mitochondria. It acts to increase the permeability of these structures, resulting in extravasation cellular contents and ions, leading to cell lysis^[62-64]. The lack of synergism may be related to the fact that (-)-fenchone and amphotericin B possibly act by the same mechanism of action, competing for the same binding site, which limits an effect potentiation.

Another association study was carried which the monoterpene geraniol, showed varying degrees of interaction with antifungal patterns fluconazole and amphotericin B, demonstrating synergism for some strains of *C. albicans* and indifference for others, showing that there is no pattern of the synergistic effect of monoterpenes^[65].

CONCLUSION

In brief, it can be observed that (-)-fenchone presents antidiarrheal activity related to an antimotility effect. This antimotility effect involves anticholinergic mechanisms, which can be partially reversed in the presence of a muscarinic agonist. It can be blocked by α_2 - and β -adrenergic receptor antagonists, suggesting the participation of the adrenergic pathway. It can also be blocked by L-NAME and glibenclamide, indicating a possible involvement of NO and K_{ATP} channels. Not related to antisecretory or pro-absorption activities. In the evaluation of antimicrobial activity, monoterpene inhibited the growth of fungal strains, being considered a product with intense antifungal activity, but without antibacterial activity. Besides, it has no synergistic or antagonistic effect in combination with the standard antifungal.

ARTICLE HIGHLIGHTS

Research background

Pharmacological therapy for diarrhea is associated with contraindications and side effects. In the search for new therapeutic alternatives, natural products and medicinal plants are of great relevance, plant extracts, their semi-synthetic derivatives and synthetic compounds inspired by natural products make up the majority of drugs in use today. Many plant species and their isolated compounds, including terpenes,

showed promising effects in the context of diarrhea, based on this criterion, the monoterpene (-)-fenchone was selected for this study.

Research motivation

(-)-Fenchone is a bicyclic monoterpene present in essential oils of plant species, such as *Foeniculum vulgare* and *Peumus boldus*, used in the treatment of gastrointestinal diseases. It has relevant pharmacological activities described in the literature. Many species of plants and their isolated compounds, including terpenes, have shown promising antidiarrheal and motility, based on this result, the monoterpene (-)-fenchone was selected for this study.

Research objectives

The main objective of our study was to evaluate the antidiarrheal activity related to gastrointestinal motility, intestinal secretion and antimicrobial and antifungal activity of (-)-fenchone.

Research methods

In this study, antidiarrheal activity was evaluated *in vivo*, using male Swiss mice. The effects of (-)-fenchone in the castor oil-induced diarrhea model. Intestinal transit and gastric emptying protocols were used to evaluate a possible antimotility impact. Muscarinic receptors, presynaptic α_2 -adrenergic and tissue adrenergic receptors, K_{ATP} channels, nitric oxide were investigated to uncover antimotility mechanisms of action and castor oil-induced enteropooling to elucidate antisecretory mechanisms. The antimicrobial activity was evaluated in the minimum inhibitory concentration model, the fractional inhibitory concentration index using the (-)-fenchone association method with standard antimicrobial agents.

Research results

(-)-Fenchone at doses (75, 150 and 300 mg/kg) has antidiarrheal activity, with a significant decrease in the evacuation index. This activity is possibly related to a percentage of reduced intestinal transit (75, 150 and 300 mg/kg). The antimotility effect of (-)-fenchone decreased in the presence of pilocarpine, yohimbine, propranolol, L-NG-nitroarginine methyl ester or glibenclamide. In the enteropooling model, no reduction in intestinal fluid weight was observed. (-)-Fenchone did not show antibacterial activity, inhibits the growth of strains of fungi with a minimum fungicidal concentration of 32 μ g/mL. As for the association between (-)-fenchone and amphotericin B in strains of *Candida albicans*, it was observed that the association was indifferent.

Research conclusions

The antidiarrheal effect of (-)-fenchone found in this study involves antimotility and not involve antisecretory mechanisms. (-)-Fenchone has antifungal activity; however, it did not show antibacterial activity.

Research perspectives

The main limitations of our study include strains of tested bacteria that are not the most prevalent in infectious diarrhea, as well as other *in vivo* models of diarrhea and post-exposure treatment. The prospects are to perform other models of diarrhea *in vivo* that can help to reinforce these data, as well as other analyzes of molecular markers to characterize mechanisms.

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Basic Study

Effects of Yue-Bi-Tang on water metabolism in severe acute pancreatitis rats with acute lung-kidney injury

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Abstract

BACKGROUND

The complications acute lung injury and acute kidney injury caused by severe inflammation are the main reasons of high mortality of severe acute pancreatitis (SAP). These two complications can both lead to water metabolism and acid-base balance disorders, which could act as additional critical factors affecting the disease trend. Aquaporins (AQPs), which can regulate the transmembrane water transport, have been proved to participate in the pathophysiological process of SAP and the associated complications, such as acute lung injury and acute kidney injury. Thus, exploring herbs that can effectively regulate the expression of AQP in SAP could benefit the prognosis of this disease.

AIM

To determine whether Yue-Bi-Tang (YBT) can regulate the water metabolism in rats with severe acute pancreatitis *via* regulating the expression of aquaporins.

METHODS

Sprague-Dawley rats were randomly divided into three groups, sham operation group (SOG), model group (MG), and treatment group (TG). SAP was induced with 3.5% sodium taurocholate in the MG and TG. Rats in the TG were administered with YBT while SOG and MG rats were given the same volume of saline. Blood and tissue samples were harvested to detect serum inflammatory cytokines, histopathological changes, malondialdehyde and superoxide dismutase

Institutional animal care and use

committee statement: The study was reviewed and approved by the University Guidelines and the Animal Care Committee Guidelines of West China Hospital (Chengdu, China) (protocol number, 2018167A).

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in the lung, and protein and mRNA expression of kidney injury molecule-1, α -smooth muscle actin, and vimentin in the kidney, and AQP1 and 4 in the lung, pancreas, and kidney.

RESULTS

The serum interleukin-10, tumor necrosis factor α , and creatinine levels were higher in the MG than in the SOG. Tumor necrosis factor α level in the TG was lower than that in the MG. Malondialdehyde level in lung tissues was higher than in the SOG. The pathological scores and edema scores of the pancreas, lung, and kidney tissues in the MG were all higher than those in the SOG and TG. The protein expression of AQP4 in lung tissues and AQP1 in kidney tissues in the MG were higher than those in the SOG and TG. The expression of vimentin was significantly higher in the MG than in the SOG. The expression of AQP1 mRNA in the lung and kidney, and AQP4 mRNA in the kidney was up-regulated in the MG compared to the SOG.

CONCLUSION

YBT might regulate water metabolism to reduce lung and kidney edema of SAP rats *via* decreasing AQP expression, and alleviate the tissue inflammatory injury.

Key Words: Yue-Bi-Tang; Aquaporins; Severe acute pancreatitis; Acute lung injury; Acute kidney injury; Water metabolism

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Core Tip: This is the first study to verify the effects of Yue-Bi-Tang (YBT) in regulating water metabolism and reducing tissue injury in rats with severe acute pancreatitis complicated with acute lung injury and acute kidney injury. We demonstrated the protective effect of YBT on lung and renal injury associated with severe acute pancreatitis from two aspects, alleviation of inflammatory injury and reduction of edema. Furthermore, the edematous elimination effect of YBT is achieved by regulating water metabolism *via* decreasing the expression of aquaporins.

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INTRODUCTION

The mortality rate of severe acute pancreatitis (SAP) is as high as 35%-50% in the first week of SAP onset, due to acute lung injury (ALI) and acute kidney injury (AKI) caused by serious inflammation^[1,2]. As is known, acute respiratory failure, which is the first organ failure from SAP that takes place after ALI, leads to hypoxia, and has a serious influence on the clinical development and prognosis of SAP patients^[3]. Furthermore, ALI and AKI may cause water metabolism and acid-base balance disorders, which could act as additional critical factors affecting the disease trend^[4]. As shown in some studies, hypoxemia caused by respiratory failure would lead to or aggravate renal injury^[5]. On the other hand, the retention of water and sodium caused by kidney failure or aggressive fluid resuscitation can cause or worsen acute lung edema^[6]. The interrelationship between ALI and AKI reveals that water circulation may be the key point in treating SAP. Therefore, finding suitable methods for regulating the water mechanism and electrolyte balance may alleviate the condition of patients with SAP and mitigate the associated complications.

Aquaporins (AQPs), which are proteins in the cell membrane that are part of family of major intrinsic proteins constituting water channels in the cell membrane, facilitate water transportation between cells^[7] and are expressed in many organs including the pancreas, lung, and kidney^[8-12]. It has been proved that AQPs could participate in the

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pathophysiological process of SAP and the associated complications, such as ALI and AKI^[13]. In an ALI rat model, high expression of AQP4 has been found on alveolar type II cells^[14]. The increase in the extravascular lung water volume was consistent with ascension of AQP5, and increasing AQP5 was detected in alveolar lavage fluid in rats with SAP^[15]. Lung fluid transport also apparently decreased in *AQP5* knockout mice, and the same association was found for AQP1 and AQP4^[16]. In renal tissues, AQP1 and 4, which display very high water osmotic permeability, were also found^[17]. AQP2 also plays a role in the membrane effector of antidiuretic hormones to promote the reabsorption of water from urine as it is removed from the blood by the kidney^[18]. These results prove the effect of AQPs on the water metabolism of the lung and kidney. Some other studies showed that AQP4 can be up-regulated by tumor necrosis factor α (TNF- α), which is an inflammatory factor during SAP, *via* activation of TNF-receptor-1^[19]. Based on these results, it can be inferred that the systemic water metabolic disorder caused by SAP may be related to the abnormal function of AQPs. Thus, exploring herbs that can effectively regulate the expression of AQPs in SAP could benefit the prognosis of this disease.

Yue-Bi-Tang (YBT) was first described in “Jin-Gui-Yao-Lue”, a classical traditional Chinese medicine work. It contains five herbal medicines: Mahuang (Ephedra), shigao (gypsum), shengjiang (ginger), dazao (Chinese dates), and gancao (liquorice). YBT has been widely used as a diaphoretic and edema-clearing decoction to treat edema generated from some respiratory diseases based on the theory of traditional Chinese medicine for ages. In modern Chinese medicine treatments, YBT is directly used to treat some edema conditions resulting from kidney injury, such as acute glomerulonephritis^[20]. However, there is no study discussing the effect of YBT on ALI-AKI in rats with SAP. In this paper, we designed an experiment to verify whether YBT could treat lung and kidney injury simultaneously in SAP and whether the therapeutic mechanism functions by regulating the expression of AQPs in SAP.

MATERIALS AND METHODS

Animals

Male specific pathogen free Sprague-Dawley rats ($n = 36$) weighing 220 ± 15 g were purchased from Chengdu Dossy Experimental Animals Co. Ltd. (Chengdu, China). All animals were fed and treated in accordance with the Guidelines described in previous articles of our group^[21]. After one week of adaptive feeding, these animals were fasting for 24 h before SAP modelling and anesthesia during the experiment. All rats were handled according to the University Guidelines and the Animal Care Committee Guidelines of West China Hospital (Chengdu, China) (protocol number, 2018167A).

Preparation of YBD

Particles obtained by spray drying of mahuang (NO: 17110037), shigao (NO: 18010165), shengjiang (NO: 17120189), dazao (NO: 17120063), and gancao (NO: 18010126) were purchased from Sichuan Hospital of Traditional Chinese Medicine (Chengdu, China). They were stored at 4 °C before use. YBT comes from Jin-Gui-Yao-Lue, in which the herb dosages of YBT are described as mahuang 18 g, shengjiang 9 g, shigao 24 g, dazao 9 g, and gancao 6 g. The method to mix the powders of YBT was as previously described^[21]. Rats in YBT-treatment group (TG) were intragastrically administered with YBT at a dosage of 5.63 g/kg BW, calculated following the Method of Pharmacology that the equivalent dosage for rats is 6.3 times more than that for human being^[22].

Induction of AP and intervention

The rats were divided into three groups randomly ($n = 12$ each): Sham operation group (SOG), model group (MG), and TG. All the rats would receive 12 h of pre-operation fasting. After anesthetization with 2% sodium pentobarbital (intraperitoneal injection, 40 mg/kg BW), SAP was induced in rats by retrograde injection of 3.5% sodium taurocholate (Sigma, St. Louis, MO, United States) into the biliopancreatic duct (1 mL/kg BW) at a velocity of 6 mL/h using a micro-infusion pump. The SOG received a saline injection replacing 3.5% sodium taurocholate. YBT was administered intragastrically to rats in the TG 12 h after operation. Meanwhile, the rats in the SOG and MG were given equal volume of saline. All the rats received subcutaneous injection of 1 mL/100 g weight body of saline q2h after operation. Twenty-four hours after YBT administration, all rats were euthanized. Serum samples were obtained and used to detect amylase (AMY) and inflammatory mediators. Pancreas, lung, and

kidney tissues were harvested for histological analysis, Western blot analysis, and mRNA detection.

Serum amylase and inflammatory mediator measurements

AMY, creatinine (Cr), and blood urea nitrogen (BUN) levels were detected in blood collected from the heart, with a spectrophotometer following the manufacturer's instructions. Levels of inflammatory cytokines, such as TNF- α , interleukin (IL)-6, and IL-10, were measured with immunoassay kits (Millipore Corporation, Billerica, MA, United States) that we used formerly^[21].

Histological analysis

Pieces of pancreas, lung, and kidney tissues were fixed in 4% formaldehyde overnight, and then embedded in paraffin after dehydration. Sections (6 μ m) were used for hematoxylin and eosin staining and then examined by light microscopy. The severity of pancreatitis was evaluated in the same way that our previous study used^[22]. Lung injury was scored with a previous scoring system in a blinded manner, with thickening of the septum, edema, congestion, and intestinal leukocyte infiltration scored on a 0 (absent) to 4 (extensive) scale^[23,24]. The grade of tubulointerstitial damage was scored semi-quantitatively on a 0 to 5+ scale, based on the percentage of the outer medulla area affected by necrosis and/or apoptosis of tubules, tubular atrophy, brush border loss, and tubular dilation (0 = none, + = < 25%, ++ = 25% to 50%, +++ = > 50% to 75%, ++++ = > 75% to < 100%, and +++++ = 100%)^[25].

Detection of malondialdehyde and superoxide dismutase in the lung

One hundred milligrams of fresh lung tissues from rats were cut into small pieces, and mixed with 100 μ L of saline. After 5 min of homogenization and 2 min of ultrasonic breakage, the suspensions were obtained and centrifuged at 10000 rpm for 10 min. Supernatants were collected and stored at -80 °C in a refrigerator first, and latter detected using malondialdehyde (MDA) (cargo number: A003-1, batch number: 20170916) and superoxide dismutase (SOD) kits (WST-1 method, item No: A001-3, batch number: 20170919) purchased from Nanjing Institute of Biotechnology following the instructions.

Western blot analysis

Western blot was used to detect the protein content of AQP1-4, kidney injury molecule-1 (KIM-1), α -smooth muscle actin (α -SMA), and vimentin. The steps were as previously described^[26]. The primary antibodies used for Western blot were purchased from Abcam (KIM-1: ab190696; α -SMA: ab32575; vimentin: ab92547; AQP1: ab168387; AQP4: ab46182). Grey bands on the picture were semi-quantitatively analyzed with ImageJ, with GAPDH used as the internal control.

KIM-1 and AQP mRNA expression

Trizol reagent (Cargo No. 15596026, Life Technologies) was used to isolate total RNA from tissues following the standard protocol. Total RNA (9 μ g) was reverse transcribed using Revert Aid First Stand complementary deoxyribonucleic acid Synthesis Kit (No: K1622, Thermo scientific). Quantitative polymerase chain reaction was performed using SYBR Premix Ex Taq II (NO: RR820A) for detecting the mRNA expression of KIM-1 and AQP1 and 4. The internal control used was GAPDH. The primer sequences used are: AQP1 R: 5'- ACCTGCTGGCCATTGACTAC-3' and F: 5'- CCAGGGCACTCCCAATGAAT-3' (129 bp); AQP3 R: 5'- GAGTTGATG AACCGTTGCGG-3' and F: 5'- TTGATGGTGAGGAAGCCACC-3' (164 bp); AQP4 R: 5'- GGAAGGCATGAGTGACGGAG-3' and F: 5'- CAGACGCCTTTGAAAGCCAC-3' (95 bp); Kim-1 R: 5'- GTTAAACCAGAAATTTCCCAACAAG-3' and F: 5'- TCTCATGGGCATAAAATGTAGTG-3' (191 bp); GAPDH R: 5'- GGTGAAGGTCGGTGTGAACG-3' and F: 5'- CTCGCTCCTGGAAGATGGTG-3'.

Statistical analysis

Statistical software SPSS 23.0 (IBM SPSS Statistics 23.0) was used to process the data. All the values are expressed as the mean \pm SD. One-way ANOVA was used when data had homogeneity of variance and a normal distribution. The least-significance-difference method was used for pairwise comparison of independent samples. The Kruskal-Wallis *H* test with multiple independent samples was used when data did not have homogeneity of variance and a normal distribution. The significance level was set at *P* < 0.05 for comparison.

RESULTS

Effect of YBD on serum AMY, Cr, BUN, and inflammatory mediators

The rats in the MG had higher serum AMY, Cr, and BUN levels than those in the SOG ($P < 0.05$), and the AMY level in the TG was lower than those in the other two groups ($P < 0.05$) (Table 1). As seen from the above results, we have successfully built a model of SAP with AKI, and it can be treated with YBT. However, there was no statistical difference in Cr or BUN levels between the MG and TG. The serum levels of IL-10 and TNF- α in the TG were lower than those in the MG, but higher than those in the SOG ($P < 0.05$) (Table 2). This result showed an anti-inflammatory effect of YBT, but no effect on kidney function.

Histopathological effect of YBD on lung, pancreatic, and renal tissues

Pancreas: The SOG presented mild edema with few tissue hemorrhages and no necrosis; the MG group had serious edema, inflammatory cells infiltration, and obvious tissue hemorrhage and necrosis (MG = 19.83 ± 3.71 vs SOG = 10.17 ± 2.40 , $P < 0.05$); the TG group had milder hemorrhage, necrosis, and interstitial edema in the tissues than the MG group, but it produced no difference in inflammatory infiltration (TG = 17.33 ± 1.63 vs MG = 19.83 ± 3.71 , $P > 0.05$).

Lungs: The SOG showed some edema and infiltration of neutrophils and lymphocytes, but no congestion, hyaline membrane, or cystic dilatation; distinct interstitial edema, inflammatory cell infiltration, hemorrhage, and necrosis were found in the tissue of MG rats (MG = 46.00 ± 3.16 vs SOG = 2.33 ± 1.51 , $P < 0.05$); the tissue injuries in the TG were milder (TG = 5.67 ± 2.80 vs MG = 46.00 ± 3.16 , $P < 0.05$).

Kidneys: Loss of the brush border in a few areas, with no atrophy or dilation, was found in the SOG; vacuolar degeneration and loss of brush border were found in many regions, along with periodic tubular hemorrhage and necrosis, in the MG (MG = 11.17 ± 2.64 vs SOG = 3.00 ± 1.10 , $P < 0.05$); mild injury was found in the TG (TG = 5.00 ± 2.10 vs MG = 11.17 ± 2.64 , $P < 0.05$) (Figure 1A and B).

Tissue edema: The edema degree of the three organs in the MG was significantly higher than that in the SOG (pancreas: MG = 7.83 ± 2.41 vs SOG = 1.67 ± 0.52 , $P < 0.05$; lung: MG = 7.67 ± 1.63 vs SOG = 1.50 ± 0.84 , $P < 0.05$; kidney: MG = 4.00 ± 1.55 vs SOG = 1.17 ± 0.41 , $P < 0.05$), while that in the TG was lower than the degree in the MG (Pancreas: TG = 5.33 ± 1.63 vs MG = 7.83 ± 2.41 , $P < 0.05$; lung: TG = 3.67 ± 0.82 vs MG = 7.67 ± 1.63 , $P < 0.05$; kidney: TG = 1.33 ± 0.82 vs MG = 4.00 ± 1.55 , $P < 0.05$) (Figure 1C).

Effect of YBD on the contents of MDA and SOD in the lungs

MDA content in the lung tissue in the MG was higher than that in the SOG (MG = 72.41 ± 5.52 vs SOG = 52.07 ± 14.96 , $P < 0.05$), but there was no significant difference in the MDA and SOD contents between MG and TG (Figure 2). As is known, MDA is a marker for oxidative stress, so those result showed that the rat model of SAP combined with ALI-AKI was in a high oxidative stress state.

Effect of YBD on the expression of AQPs and acute renal injury biomarker proteins

The expression of AQP4 in the lungs of MG rats was significantly higher than that in the SOG and TG (MG = 1.651 ± 0.471 vs SOG = 0.375 ± 0.271 , $P < 0.05$; MG = 1.651 ± 0.471 vs TG = 0.958 ± 0.360 , $P < 0.05$), but AQP4 in the pancreas and kidneys showed no significant differences among the three groups. The expression of AQP1 in the kidneys of MG rats (MG = 3.811 ± 0.714 vs SOG = 2.044 ± 0.677 , MG = 3.811 ± 0.714 vs TG = 2.221 ± 0.032 , $P < 0.05$) was significantly higher than that in the other groups. In the lungs, AQP1 had no significant differences among the three groups. The expression of vimentin, one of the biomarkers of AKI, was significantly higher in the MG than in the SOG (MG = 3.549 ± 0.795 vs SOG = 1.643 ± 0.104 , $P < 0.05$), but had no significant difference from that in the TG. Another two biomarkers of AKI, KIM-1 and α -SMA, showed no significant differences among the three groups (Figure 3).

Results of reverse transcription-polymerase chain reaction

The AQP1 mRNA expression in the lungs and kidneys of MG rats was significantly higher than that in the SOG ($P < 0.05$), but had no significant differences from that in the TG. Meanwhile, the mRNA expression of AQP4 in the kidneys of MG rats was significantly higher than that in the SOG. No significant differences in the mRNA expression of pancreatic AQP1 and 4, or pulmonary AQP4 or KIM-1 were found

Table 1 Serum amylase, creatinine, and blood urea nitrogen in three groups

Parameter	SOG	MG	TG
AMY (U/L)	1309.73 ± 252.61	1943.27 ± 517.39 ^a	1243.30 ± 203.68 ^b
Cr (μmol/L)	25.47 ± 1.65	34.02 ± 4.05 ^a	33.88 ± 3.65
BUN (mmol/L)	7.36 ± 1.07	9.27 ± 1.00 ^a	9.18 ± 0.59

The results are represented as the mean ± SD.

^a*P* < 0.05 model group *vs* sham operation group.

^b*P* < 0.05 treatment group *vs* model group (*n* = 12). SOG: Sham operation group; MG: Model group; TG: Treatment group; MDA: Malondialdehyde; AMY: Amylase; Cr: Creatinine; BUN: Blood urea nitrogen.

Table 2 Serum interleukin-6, interleukin-10, and tumor necrosis factor α in three groups

Parameter	SOG	MG	TG
IL-6	33.79 ± 2.69	32.77 ± 1.29	41.48 ± 28.88
IL-10	74.49 ± 42.19	122.31 ± 29.88 ^a	85.02 ± 1.61
TNF-α	76.19 ± 3.42	84.88 ± 5.02 ^a	76.68 ± 3.39 ^b

The results are represented as the mean ± SD.

^a*P* < 0.05 model group *vs* sham operation group.

^b*P* < 0.05 treatment group *vs* model group (*n* = 12). SOG: Sham operation group; MG: Model group; TG: Treatment group; MDA: Malondialdehyde; IL: Interleukin; TNF-α: Tumor necrosis factor α.

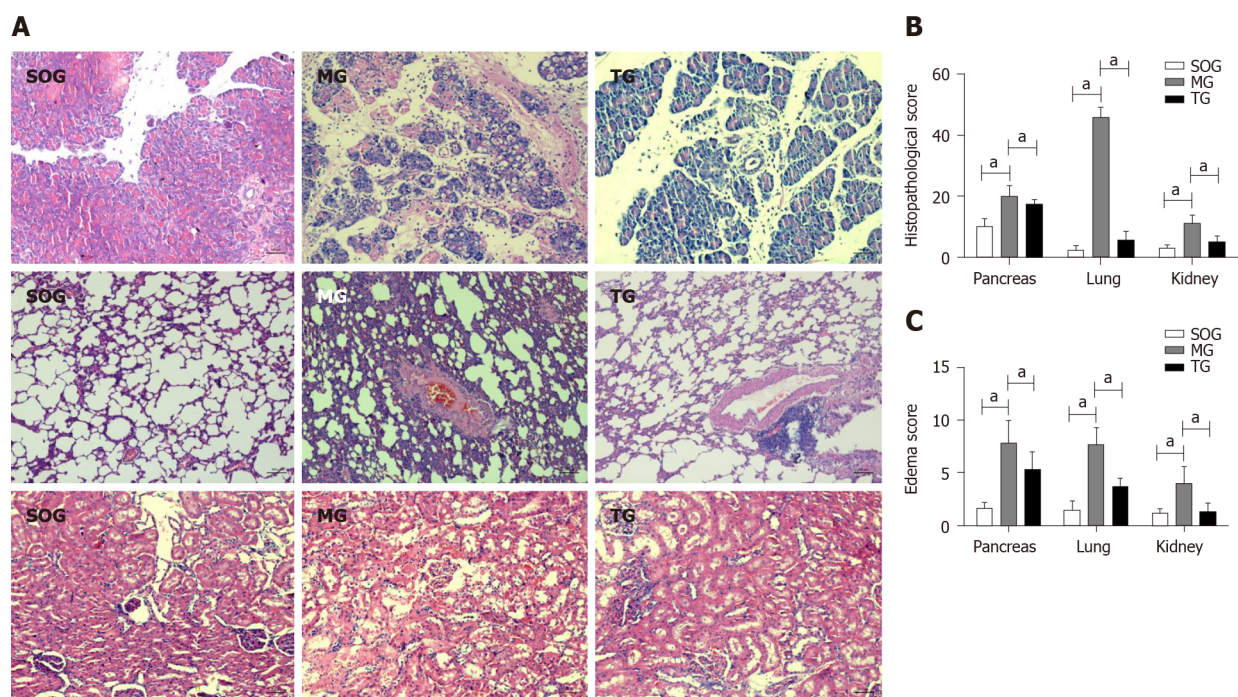


Figure 1 Histological images and pathologic and edema scores of pancreatic, lung, and kidney tissues in the three study groups. A: Pathological images of the pancreatic, lung, and kidney tissues (hematoxylin-eosin staining, magnification: pancreas and kidney × 200; lung × 100); B: Histological scores of the three types of tissues; C: Edema scores of the three types of tissues. The results are represented as the mean ± SD. ^a*P* < 0.05 (*n* = 12). SOG: Sham operation group; MG: Model group; TG: Treatment group.

among the three groups (Figure 4).

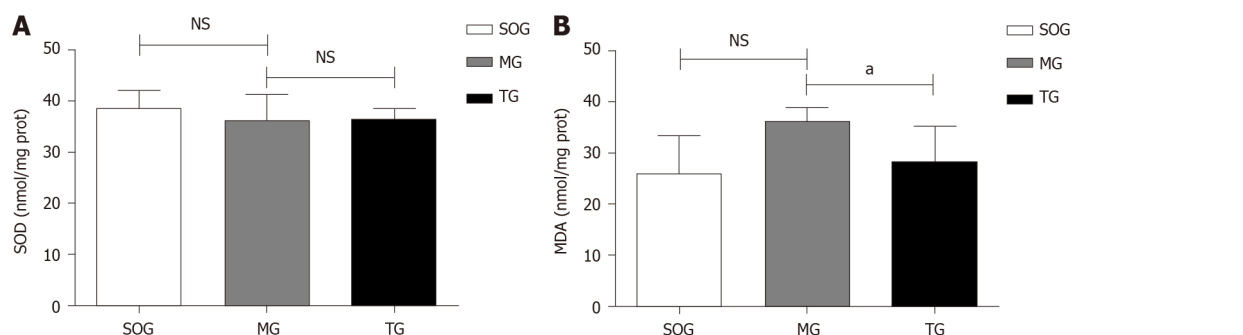


Figure 2 Levels of lung injury factors. A: Levels of superoxide dismutase in three groups; B: Levels of malondialdehyde in three groups. The results are represented as the mean \pm SD. $^aP < 0.05$, NS: $P > 0.05$ ($n = 12$). SOG: Sham operation group; MG: Model group; TG: Treatment group; SOD: Superoxide dismutase; MDA: Malondialdehyde.

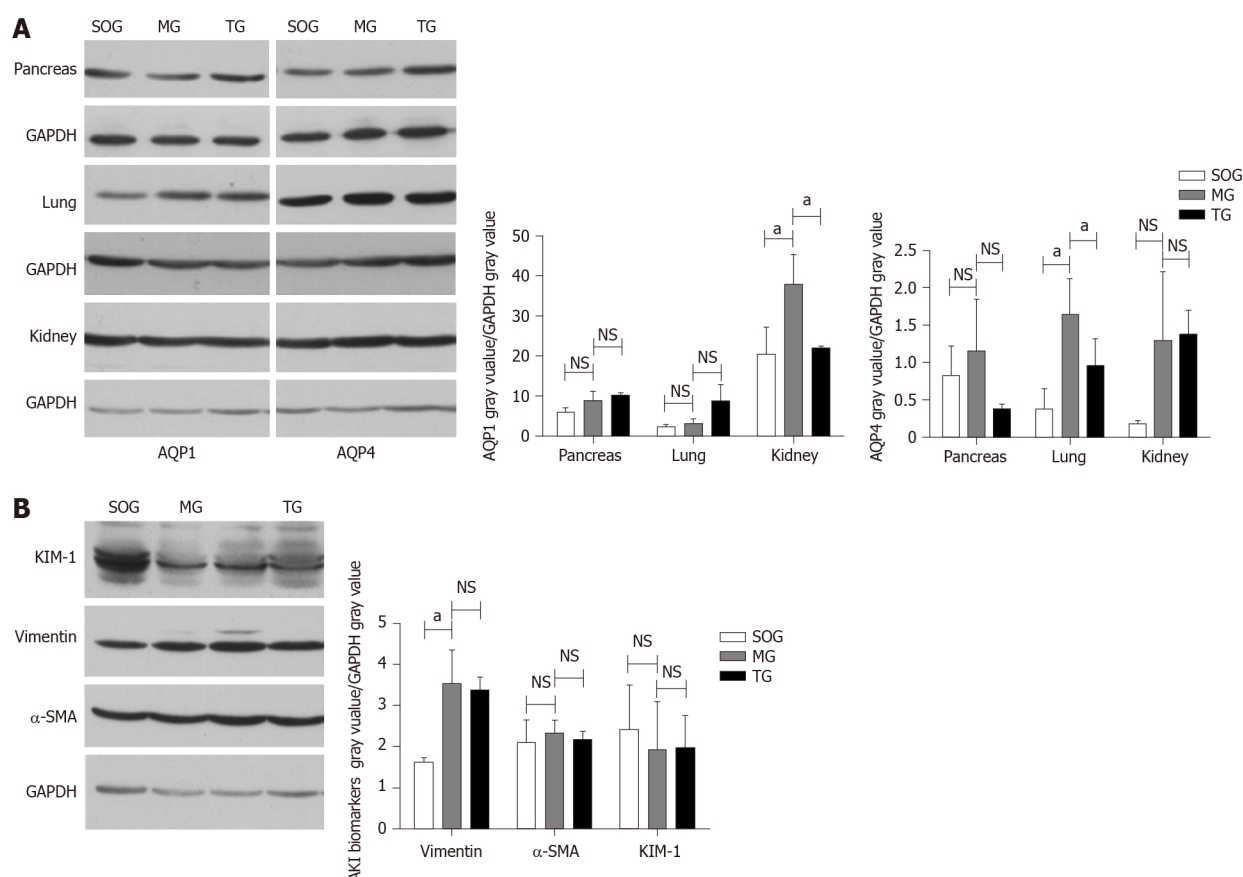


Figure 3 Western blot analysis of expression of aquaporins and acute renal injury biomarkers. A: Grayscale images and relative expression of aquaporins in three groups; B: Grayscale images and relative expression of acute renal injury biomarkers in three groups. The results are represented as the mean \pm SD. $^aP < 0.05$, NS: $P > 0.05$ ($n = 12$). SOG: Sham operation group; MG: Model group; TG: Treatment group; MDA: Malondialdehyde; KIM-1: Kidney injury molecule-1; α -SMA: α -smooth muscle actin.

DISCUSSION

In this study, some molecular changes reflecting tissue inflammatory injury were found in rats with SAP, such as TNF- α in serum, the MDA contents and SOD activity in the lungs, both of which indicated high oxidative stress in this animal model. The expression of vimentin in renal tissue and creatinine level in serum also indicated kidney injury. As a key point of our study, AQPs in lung and kidney tissues of SAP rats changed considerably. The protein expression of AQP4 in the lungs and AQP1 in the kidneys of MG rats was obviously higher than that in SOG rats. Treatment with YBT could regulate AQP4 protein expression in the lungs and AQP1 protein expression in the kidney. Furthermore, YBT treatment can also alleviate the histopathological lesion and edema in the pancreas, lung, and kidney of SAP rats

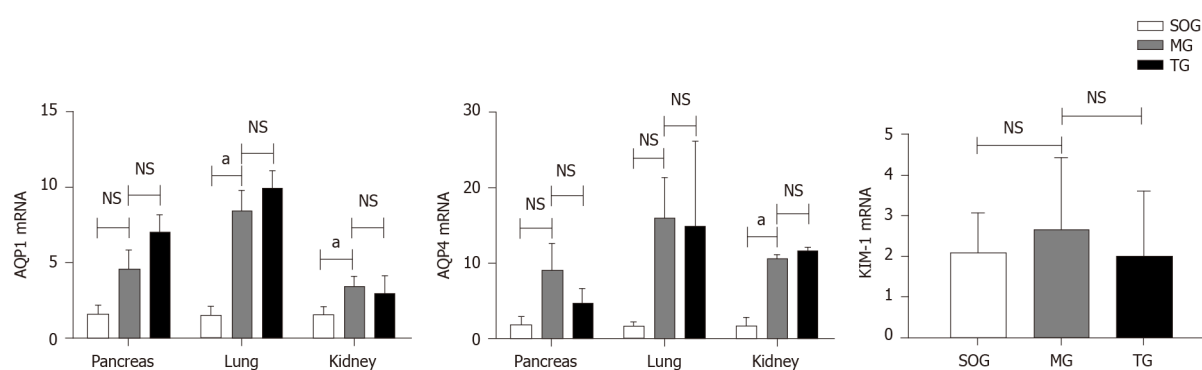


Figure 4 Aquaporins and kidney injury molecule-1 mRNA expression in three groups. The results are represented as the mean \pm SD. ^a $P < 0.05$; NS: $P > 0.05$ ($n = 12$). MG: Model group; TG: Treatment group; KIM-1: Kidney injury molecule-1; AQP1: Aquaporin 1.

complicated with ALI-AKI.

In the development of SAP, inflammatory injury is the main and lethal factor that damages the organism^[27]. Besides inflammatory injury of tissue and organs, fluid loss into the third space contributes to many of the early systemic complications in SAP^[28], leading to pleural effusion, seroperitoneum and skin swelling, and hypovolemic shock, all of which are fatal complications of SAP^[29]. Meanwhile, water metabolic disorder also exists in tissues when SAP occurs, the irritation of inflammatory substances and overload of the infused fluid while resuscitation result in edema of the cells within the tissue, which could cause organ dysfunction, particularly acute renal failure and respiratory failure^[30]. Based on the results of our study, we can deduce that there were tissue inflammatory injury and water metabolic disorder caused by abnormal expression of AQPs in SAP. Studies have shown that both Chinese herb prescription Da-Cheng-Qi decoction and patent medicine Shenfu injection could treat SAP rats with AKI, but the mechanism of these formulas is to ameliorate inflammatory injury not to regulate water metabolism^[31,32]. However, the present study found that the effect of YBT on SAP rats with ALI-AKI was achieved not only by decreasing inflammation but also by regulating water metabolism to reduce tissue edema.

The pro-inflammatory cytokine TNF- α and anti-inflammatory cytokine IL-10 are usually regarded as early predictors of SAP severity and prognoses^[33]. The results showed the serum TNF- α and IL-10 increased significantly when SAP occurred, while YBT intervention could reduce TNF- α but had no effect on IL-10 levels. From these results, we can deduce that YBT could decrease the production of TNF- α and inhibit inflammation, but did not interfere with anti-inflammatory ability of SAP rats. Ephedra, the herb component of YBT, and its extract ephedrine had been proven to reduce TNF- α in serum and lung tissue of mice with pneumococci induced lung injury^[34]. The values of MDA and SOD can reflect the oxidative stress injury of organism, therefore they are often used to evaluate the body's inflammatory levels^[35]. The MDA in lung tissue rose after YBT treatment in SAP rats, which means that YBT could protect the lungs from inflammatory injury. Abnormal expression of vimentin and creatinine, which serve as biomarkers for human renal injury, revealed the existence of AKI in our model^[36]. Thus, by combining these results with those of our study, we believe that the SAP rat model had complicated lung and kidney injury, and YBT could alleviate the tissue inflammatory injury in rats with SAP.

Recent studies showed that AQPs play a key role in liquid metabolism in organisms, because the AQP family, which is expressed on the endothelial cells of the capillaries and cell membranes of many tissues, can regulate transmembrane transport of water^[37]. In lung tissue, AQP1, AQP3, AQP4, and AQP5 were found, among which AQP1 has high water permeability and is mainly expressed in pulmonary bronchial capillary endothelial cells and alveolar type I epithelial cells^[38,39]. AQP4 is mainly distributed in airway epithelial of different sizes; it is partially expressed in the alveolar type I epithelial cells, ciliary tubes, and basal membranes of acinar cells. AQP1 and AQP4 play an important role in the airspace-to-capillary transport of water and elimination of lung interstitial edema^[40]. Thus, they were employed to evaluate the disorder of lung water metabolism of SAP rats and the effect of YBT on lung edema in our study. The results showed the upregulation of AQP4 protein expression and more serious edema in the MG than in the SOG. After treatment with YBT, the AQP4 in the lungs was down-regulated, and the tissue edema was reduced significantly. It has been proven that AQP4 has a positive correlation with the tissue moisture content^[41],

and increasing the expression of AQP4 would exacerbate tissue edema. Thus, one important effect of YBT in SAP treatment is to decrease AQP4 to alleviate the lung edema.

In the kidneys, AQP1 is present in the brush border apical membrane and basal lateral membrane of proximal renal tubules in the wall segment of the descending branch of the medullary loop, and it undertakes the major role in water reabsorption in the kidneys. AQP4 is expressed at the basolateral membrane of renal collecting duct cells and provides entrance to the blood when water enters a renal collecting duct cell^[37]. Those two proteins could illustrate the entire process of water translocation from the kidneys to vascular system, which is the pathway of edema elimination. Apparently increased expression of AQP1 in kidney tissue in the MG, which was the group with more serious edema, indicated that up-regulation of the protein could lead to tissue edema. After treatment with YBT, the expression of AQP1 declined, and the tissue edema of the kidneys in the TG decreased significantly. Studies also have found that suppression of AQP1 could facilitate water and sodium excretion^[42]. Thus, we can deduce that YBT might reduce the urine reabsorption and produce diuretic effects *via* regulating AQP1 expression in the kidneys, thereby regulating water metabolism and reducing tissue edema. However, YBT seemed to have no effect on AQP1 and 4 in the pancreas, though there might be some other mechanisms responsible for the edema elimination function in the pancreas.

The mRNA expression of lung AQP1 and kidney AQP4 in the MG increased, but the protein expression was hardly changed. One reason for the inconsistent expression of mRNA and protein may be related to the changes in the factors affecting protein transcription caused by SAP-related pathophysiological changes^[43]. Another reason is that the mRNA expression of AQPs in SAP rats can be increased by glucocorticoid, which would increase in the early stage of SAP, and the complications of SAP, such as intra-abdominal hypertension and abdominal compartment syndrome^[44,45]. Therefore, there would be an increase in mRNA expression of AQPs in MG, but no significant increase in protein expression or functional changes.

Some studies report that AQP expression can be regulated by TNF- α signaling^[46] through the TNF-receptor-1 pathway^[47]. The TNF- α level in serum exhibited the same changes as AQP expression in the MG, and YBT could reduce the level. Based on these results, we can deduce that YBT could affect the expression of AQPs in the lungs and kidneys by reducing TNF- α , thereby reducing tissue edema.

Determining whether YBT could treat SAP-induced water metabolic disorders can provide some new insights for clinical treatments. This is the first time that YBT has been used in an animal model to treat SAP, though many methods are being tried and need further exploration and improvement. The still-unknown mechanisms of YBT in regulating AQPs also need to be verified in future research.

CONCLUSION

In conclusion, lung and kidney edema of SAP rats may relate to disorder of water metabolism caused by up-regulation of AQP expression. YBT might regulate water metabolism to reduce lung and kidney edema in SAP rats *via* decreasing AQP expression, and finally alleviate the tissue inflammatory injury.

ARTICLE HIGHLIGHTS

Research background

The complications acute lung injury (ALI) and acute kidney injury (AKI) caused by severe inflammation are the main reasons for high mortality of severe acute pancreatitis (SAP). These two complications can both lead to water metabolism and acid-base balance disorders, which could act as additional critical factors affecting the disease trend. Aquaporins (AQPs), which can regulate the transmembrane water transport, have been proved to participate in the pathophysiological process of SAP and the associated complications, such as ALI and AKI. Thus, exploring herbs that can effectively regulate the expression of AQPs in SAP could benefit the prognosis of this disease.

Research motivation

Our previous studies have demonstrated that SAP rats would be complicated with

ALI, AKI, and some other manifestations of fluid metabolism disorders, such as pulmonary edema, pleural effusion, and seroperitoneum. Yue-Bi-Tang (YBT) has been widely used as a diaphoretic decoction to treat edema generated from some respiratory diseases based on the theory of traditional Chinese medicine for ages. In modern Chinese medicine treatments, YBT is directly used to treat some edema diseases resulting from kidney injury, such as acute glomerulonephritis. The question is whether YBT could alleviate ALI and AKI by regulating AQP expression in SAP rats. Therefore, this study aimed to verify the effect of YBT treatment in SAP rats complicated with ALI-AKI and explore the underlying mechanism.

Research objectives

To determine whether YBT can regulate the water metabolism in rats with severe acute pancreatitis *via* regulating expression of aquaporins.

Research methods

Healthy male Sprague-Dawley rats were randomly divided into a sham-operated group (SOG), model group (MG), and YBT-treatment group (TG), with 12 rats in each group. SAP was induced with 3.5% sodium taurocholate in the MG and TG. Rats in the TG were administered with YBT while SOG and MG rats were given the same volume of saline. Blood and tissue samples were harvested to detect serum inflammatory cytokines, histopathological changes, malondialdehyde and superoxide dismutase in the lung, protein and mRNA expression of kidney injury molecule-1, α -smooth muscle actin, and vimentin in the kidney, protein and mRNA expression of AQP1 and 4 in the lung, pancreas, and kidney.

Research results

The serum IL-10, TNF- α , and creatinine levels in the MG were higher than those in the SOG. Treatment with YBT could decrease TNF- α level. Malondialdehyde level in the lung was higher than that in SAP model rats. Rats of the MG had more serious pathological injury and edema in the pancreas, lung, and kidney, and higher protein expression of AQP4 in the lung and AQP1 in the kidney than those of the other two groups. The expression of vimentin was significantly higher in the MG than in the SOG. The expression of AQP1 mRNA in the lung and kidney, and AQP4 mRNA in the kidney in the MG were all up-regulated compared to that of the SOG.

Research conclusions

YBT might regulate water metabolism to reduce lung and kidney edema in SAP rats *via* decreasing AQP expression, and alleviate the tissue inflammatory injury.

Research perspectives

As we observed that YBT might regulate water metabolism to reduce lung and kidney edema in SAP rats *via* decreasing AQP expression, and alleviate the tissue inflammatory injury, further investigation of the underlying molecular mechanisms of YBT in regulating AQP is required to provide experimental evidence for wider clinical usage.

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Basic Study

Tissue microarray-chip featuring computerized immunophenotypical characterization more accurately subtypes ampullary adenocarcinoma than routine histology

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Abstract

BACKGROUND

Ampullary adenocarcinomas (AACs) are heterogeneous tumors currently classified into three important sub-classes (SC): Intestinal (INT), Pancreato-Biliary (PB) and Mixed-Type (MT). The different subgroups have similar clinical presentation and are treated by pancreatoduodenectomy with curative intent. However, they respond differently to chemotherapy and have different prognostic outcomes. The SC are often difficult to identify with conventional histology alone. The clinical outcome of all three remains unclear, particularly for MT.

AIM

To identify two main subtypes of AACs, using an immunohistochemical (IHC) score based on CDX2, CK7 and CK20.

METHODS

Tissue samples from 21 patients who had undergone resection of AAC were classified by HE histology and IHC expression of CDX2, CK7 and CK 20. An IHC

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score was obtained for each marker by counting the number of positive cells (0 = no stained cells; 1 < 25%; 2 < 50% and 3 > 50%) and their intensity (1 = weak; 2 = moderate and 3 = strong). A global score (GS) was then obtained by summation of the IHC scores of each marker. The MT tumors were grouped either with the INT or PB group based on the predominant immuno-molecular phenotype, obtaining only two AACs subtypes. The overall survival in INT and PB patients was obtained by Kaplan-Meier methods.

RESULTS

Histological parameters defined the AACs subtypes as follows: 15% INT, 45% PB and 40% MT. Using IHC expression and the GS, 75% and 25% of MT samples were assigned to either the INT or the PB group. The mean value of the GS was 9.5 (range 4-16). All INT samples had a GS above the average, distinct from the PB samples which had a GS score significantly below the average ($P = 0.0011$). The INT samples were identified by high expression of CDX2 and CK20, whereas PB samples exhibited high expression of CK7 and no expression of CK20 ($P = 0.0008$). The INT group had a statistically significant higher overall survival than in the PB group (85.7 mo *vs* 20.3 mo, HR: 8.39; 95%CI: 1.38 to 18.90; $P = 0.0152$).

CONCLUSION

The combination of histopathological and molecular criteria enables the classification of AACs into two clinically relevant histo-molecular phenotypes, which appear to represent distinct disorders with potentially significant changes to the current therapeutic strategies.

Key Words: Ampullary adenocarcinoma; Histo-molecular phenotype; Prognostic; CK7; CK20; CDX2

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Core Tip: Ampullary adenocarcinomas are heterogeneous tumors with different responses to specific chemotherapy regimens and prognosis, probably because they are a heterogeneous group including differing ampullary growth and overlapping histological phenotypes. Conventional histology does not allow a definitive identification of the three subgroups. We used an immunohistochemical score based on CDX2, CK7 and CK20 and identified only two sub-types, representing two groups of apparently separate neoplastic disorders with different oncological outcomes.

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INTRODUCTION

The ampullary region is located at the confluence of the main biliary and pancreatic ducts in the second part of the duodenum. The ampulla itself is composed of several cell types^[1]. It includes different epithelial lining originating from the pancreatic duct, distal common bile duct and duodenum^[1,2]. Hence, ampullary adenocarcinomas (AACs) can exhibit either an intestinal (INT) or pancreatobiliary (PB) histology or a mixture of both (MT)^[1-5]. Neoplasms arising from the ampulla of Vater are rare, with an estimated incidence of 5 to 7 cases per million per year, representing 0.2% of gastrointestinal tract cancers and 6% to 20% of periampullary tumors^[6,7].

Kimura *et al*^[2] (1994), were the first to propose a subclassification of AACs, based exclusively on histological features. The authors reported different prognoses between the two subtypes, *i.e.*, long-term survival after excision being significantly greater in patients with intestinal type AACs compared to the PB type. Several authors have

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since tried to better understand the histological phenotypes by proposing various immune-histochemical panels to improve the histological classification^[1,8-16].

The INT phenotype usually stains for CK20, CDX2 (caudal-type homeodomain transcription factor 2), MUC2 and occasionally CEA and CD10^[9,16,17]. While, the PB phenotype is positive for CK7, MUC1, and MUC5A^[16].

However, in each of these studies, different and sometimes complex combinations of immune-histochemical markers have been used, many of which were arbitrarily defined, with their primary validation using histomorphologic classification as the “gold standard”^[10,11,15].

Furthermore, many of these immunohistochemical markers are not widely available for use in common practice in many parts of the world (at least not yet). Additionally, some problems in some of the proposed immunohistochemical panels have been advocated. For example, CDX2 and CK20, the most useful markers to establish intestinal lineage, can be expressed not uncommonly by pancreatobiliary adenocarcinomas, albeit often more focally, as several studies have recently shown^[18-22]. Moreover, due to the mixed/hybrid nature of ampullary tumors, a frequent finding in some studies is a mixed histomorphologic phenotype of the two subgroups^[15,21]. Thus, even with the most complex immunohistochemical panels created to try to accurately define most cases, a significant proportion (18%-39%) of AACs remains without a distinct subclassification^[10,11,18,19].

Some authors have promoted a new classification of AACs consisting of only two AACs sub-classes (SC)^[15]. However, in these reports the histological have been insufficient to reach identify the phenotype. Furthermore, immune-phenotypical classification remains controversial, since there is no standardized method to define the type and the number of markers needed to assess the AACs SC. Hence, several authors have used 2 to 6 cluster of markers, while others have not used immune-phenotypical support to distinguish between the AACs SC^[15,17,22,23].

All the subgroups have a similar clinical presentation, mostly with jaundice and are treated by pancreatoduodenectomy with curative intent. However, because they comprise different phenotypes, they exhibit different responses to chemotherapy, *e.g.*, although both gemcitabine and fluorouracil may be effective in pancreatic cancer, gemcitabine is ineffective in carcinomas of intestinal origin^[24].

According to current guidelines, only patients with pancreatic and duodenal adenocarcinomas should be treated with adjuvant therapy^[5,25]. Nevertheless, adjuvant chemotherapy for ampullary tumors has been confirmed to improve overall survival when compared to surgery alone^[3,9,11-13,26-29]. There is evidence that the histological subtype may have a stronger impact than the anatomical origin of the primary tumor on survival^[8,14,30,31].

Due to these considerations, an accurate histological classification of AACs is crucial for management by a tailored therapeutic strategy and valid prognostic assessment^[32,33].

The aim of this study is to establish a reliable, inexpensive method for a more accurate histological definition of the AACs subtypes, using an immunohistochemistry score based on CDX2, CK7 and CK20, and correlating this score value with overall survival (OS). This will provide a valid prognostic stratification for patients after resection of AACs.

MATERIALS AND METHODS

Patient selection

From January 2009 to July 2016, 21 patients with AACs underwent pancreaticoduodenectomy at our Institution. The study population was obtained from the electronic institutional prospectively maintained database of patients undergoing pancreatic surgery. The data were analyzed retrospectively. This study was approved by the institutional review board.

Data collection and histopathological analysis

Preoperative evaluation included demographic information (age, gender), body mass index, American Society of Anesthesiologists score, comorbidities (cardiovascular diseases, hypertension, diabetes mellitus, COPD), presence of symptoms (jaundice, pain, nausea and vomiting, loss of weight), preoperative placement of external biliary drainage or endoprosthesis, level of carbohydrate antigen 19.9 (CA 19.9), and neoadjuvant chemotherapy and / or radiotherapy.

Postoperative data included length of hospital stay (LOS), postoperative

complications (using the Clavien-Dindo Classification), re-intervention rate and mortality^[34].

Pathological data were obtained from the final pathological reports and tumor specimens were staged according to the 8th edition of the American Joint Committee on Cancer–Union for International Cancer Control, in order to assess their dimensions (T), nodes status (N) and presence of metastases (M), according to TNM classification^[35,36]. The study included only AACs, as defined by the 2010 World Health Organization classification^[37]. For all patients, the original hematoxylin and eosin stained slides from each specimen were examined to confirm the diagnosis of ACC. The specimens were then analyzed by two specialists in pancreatic histopathology and/or translational researchers experienced in peripancreatic pathologies (LEP and NF). All tumors were categorized according to the appearance of intestinal-like (INT; tall columnar cells with elongated nuclei), pancreaticobiliary-like (PB; rounded cells with rounded nuclei with scant fibrous cores) or mixed [Mixed-type (MT); representing both of the previous mentioned characteristics] (Figure 1). Histological evaluations of MT subtypes were re-classified based on the predominant cytological features (Table 1).

After surgery, all patients underwent oncological evaluation before proceeding with adjuvant treatment.

Follow-up information was obtained during ambulatory visit or by examination of outpatient records. Follow-up data included the recurrence rate, any further treatments, disease-free survival, and OS. Disease-free survival was defined as the length of time from surgical resection to disease recurrence. Recurrence was defined as radiological evidence of intra-abdominal soft tissue around the surgical site or of distant metastasis. OS was defined as the length of time from the pancreatic surgical resection to the patient's death (or, if not available, last follow-up). Patients who died within 30 d from surgery were not included in the survival analysis.

Tissue microarrays

Three tissue microarrays (TMA) were designed by computer in order to contain 7 different samples each, with normal controls representing either mucosa of the duodenum, bile duct, pancreatic duct and normal pancreas. All TMAs were constructed from formalin-fixed, paraffin-embedded blocks, with the histological features and their phenotypical subclassifications in three groups: INT, PB or MT types. Four cores of tissue (1.5 mm) were obtained from each patient. Two cores were obtained from normal tissues detailed above. A total of 8 dots for normal controls were placed in each TMA. The TMA Tissues-Chip were built by a totally automated procedure performed through the instrument TMA Grand Master (Hungary). The machine was connected with the computer by a dedicated software to render the TMAs. Immunohistochemistry was performed on 4-µm serial sections mounted on Super Frost slides (Menzel-Glaser, Braunschweig, Germany).

Immunohistochemistry staining

Immunohistochemical (IHC) stains were performed on 5-mm unstained sections from the TMA blocks. To retrieve the antigenicity, the tissue sections were treated at 100°C in a steamer containing 10 mmol citrate buffer (pH 6.0) for 60 min. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 min to block the endogenous peroxidase activity and incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated for 90 min at 37°C with primary antibodies: CDX2, CK7 and CK20 (Table 2). Standard avidin–biotin conjugated complex and DAB staining were sequentially performed through the automated system based on the surgical pathology guideline (BenchmarkDX, Ventana systems, United States). The positive tumor cells were highlighted by the brown precipitate on the membranes, the cytoplasm or the nuclei, according to the specificity of the antibody used. Normal tissues of duodenal mucosa or normal pancreatic ducts were used as positive controls of the INT subtype or PB subtype, respectively. Negative controls were obtained by subtraction of primary antibody, during standard procedure IHC (Figure 2A).

Immuno-histochemistry scoring

All stained sections were evaluated by computerized software connected to a digital scanner (D-sight, Menarini, Italy). For each staining different values were acquired: (1) The number of stained tumor cells (STC) (0 = STC; 1 = less than 25% of STC; 2 = STC ranging from 25% to 50%; 3 = more than 50% of STC; (2) The intensity of stained tumor cells was as follows: 0 = no staining; 1 = weak; 2 = moderate; and 3 = strong.

Table 1 Categories used to classify ampullary adenocarcinomas

	Pure histological classification	Basal histological classification	Molecular and histological classification
AACs	5 Categories	4 Categories	3 Categories
Tubular	Pure INT	Pure INT	INT
	Mixed INT-predominant	Mixed	
	Mixed PB-predominant		PB
	Pure PB	Pure PB	
Non-tubular	Other	Other	Other

AACs: Ampullary adenocarcinomas; INT: Intestinal; PB: Pancreato-Biliary.

Table 2 Immunophenotypical characterization of ampullary adenocarcinomas

Ref.	Markers						Number
	CK7	CK17	CK20	CDX2	MUC1	MUC2	
Chang <i>et al</i> ^[11] , 2013				X	X		2
Kumari <i>et al</i> ^[22] , 2013	X	X	X	X	X	X	6
Ang <i>et al</i> ^[18] , 2014			X	X	X	X	4
Fernández Moro <i>et al</i> ^[16] , 2016				X	X		2
Reid <i>et al</i> ^[15] , 2016							0
Liu <i>et al</i> ^[24] , 2019	X	X	X	X	X	X	6
Moekotte <i>et al</i> ^[31] , 2018			X	X	X	X	4
Al Abbas <i>et al</i> ^[30] , 2019			X	X	X	X	4
Abraham <i>et al</i> ^[17] , 2020	X		X				2
Total	33%	22%	67%	78%	78%	56%	Mean 3.33

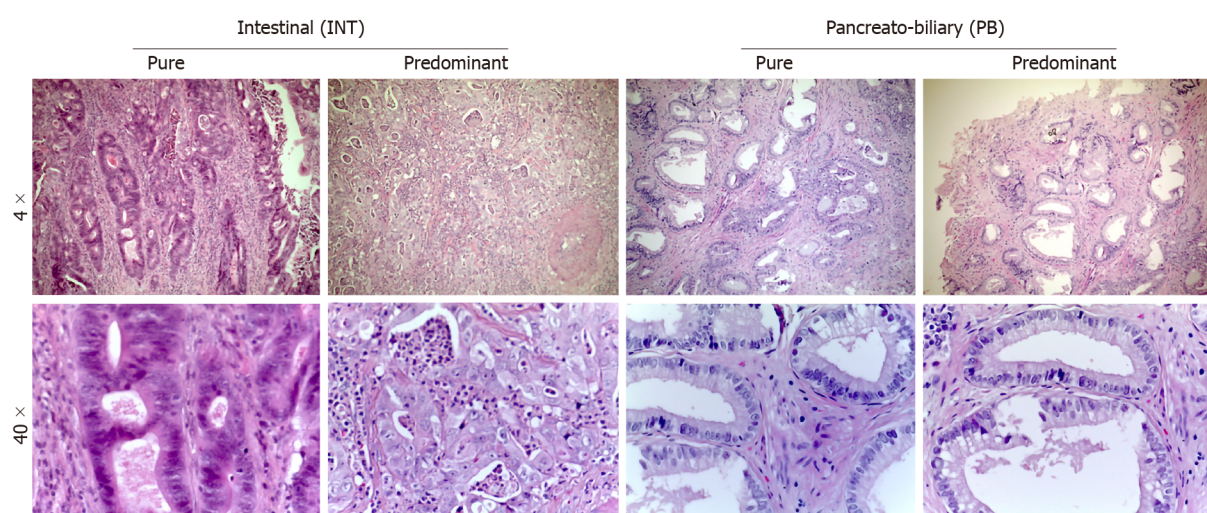


Figure 1 Representation of predominant histological sub-types of ampullary adenocarcinomas. Right side: Pancreatobiliary types; Left side: Intestinal types (Up magnification 4 ×, down magnification 40 ×). PB: Pancreato-Biliary; INT: Intestinal.

Calibration of the system was assessed based on positive control results. The total score (TS) for each antibody was evaluated by summing the values of percentage of tumor cells and their positivity. A Global Score (GS) for IHC evaluation was obtained for each sample, by adding the three TSs obtained for CDX-2, CK7 and CK 20. All specimens were defined as: "high level Marker x.", if the TS > 3; or "low level Marker x." if the TS < 3. The heat-maps were generated from the total GS and the protein expression of markers (Figure 2B and C). The color scale was generated automatically based on the total score obtained (range: 0 - 6) for each marker. The 3D heat-map showed a complete image of each marker in each TMA. Statistical analyses of TS and GS were used to set the molecular cut-off for INT or PB subtype attribution (Figure 3). The analysis of OS was performed in relation to both histological and IHC evaluations.

Statistical analysis

Survival analysis was performed using the Kaplan-Meier estimator. All experiments were performed in triplicate and repeated 3 times. Data were expressed as mean values \pm SE and analyzed by the Student's *t*-test or ANOVA followed by Tukey's multiple comparison. Data were analyzed using SPSS/PC+17 (SPSS Inc., Chicago, IL, United States). Statistical significance was set at $P < 0.05$. The statistical review of the study was performed by a biomedical statistician.

RESULTS

Patient data are summarized in Table 3. Males represented 52.3%. The mean age was 72.9 ± 8.1 years. The overall median follow-up was 32.4 (0.3–189.5) mo. Postoperative data are summarized in Table 4. The median OS was 87.7 (95%CI: 42.9 to 109.5) mo. The 3- and 5-year OS estimates were 62.8% (95%CI: 54.4 to 70.1%) and 54.4% (95%CI: 45.6 to 62.2%), respectively, for all patients.

Histological parameters defined AAC subtype samples as follows: 15% INT, 45% PB and 40% MT. Using the IHC expression and the GS, 75% and 25% of MIX samples were assigned to INT and PB, respectively. The mean value of the GS was 9.5 (range 4–16). All INT samples had a GS higher than the mean, while all PB samples had a GS below the mean ($P = 0.0011$). The INT samples were identified by high expression of CDX2 and CK20, while PB samples exhibited high expression of CK7 and negative expression of CK20 (Figure 4A, $P = 0.0008$). The GS profiles completely separated and relocated all MT type samples into the other two groups. The mean value of the GS was higher in the INT patients (mean 4.111 ± 0.577) compared to PB patients (mean 2.717 ± 0.760 ; $P = 0.0018$; Figure 4B). The analysis revealed that the GS value of 3.500 completely discriminated the INT from PB. The mean value of the GS in all three groups was 3.530, indicating a non-significant difference between this value and the "cut-off" (3.500) and the mean GS in the MT group (3.762; Figure 4C). However, the GS computerized IHC evaluation confirmed the first histological HE examination by the pathologist (Chi-Test; $P = 0.0350$; Figure 4D).

The OS of the molecular intestinal histo-molecular phenotype was significantly better than that of PB phenotype patients (72.92 mo *vs* 23.08 mo, HR: 4.62; 95%CI: 1.21 to 17.36; $P = 0.0259$; Figure 5C). The re-classification of AACs based on GS only, altered the Kaplan-Meier analysis considerably as the two different curves were more obviously separated (87.5 mo *vs* 20.35 mo, HR: 8.39; 95%CI: 1.38 to 18.90; $P = 0.0152$). Finally, the IHC effect of CDX-2 expression seems to play a pivotal role in the attribution of histological subtype in AAC patients. Higher protein expression of CDX-2 was always present in INT patients, while lower protein levels of CDX-2 were associated with PB sub-type. The complete analysis of all markers in all TMAs was carried-out in only four days (Figure 5A and B).

DISCUSSION

In the most recent classification, AACs are classified according to histological appearance into either PB or INT type^[2,8,15]. However, due to ampullary growth and overlapping histological epithelia, an accurate histological classification of these tumors may be difficult^[38,39].

Obviously, conventional histology alone is insufficient for valid classification of AAC phenotypes. Nonetheless, histo-morphologic classification of AACs is a requirement by the College of American Pathologists' reporting guidelines. In

Table 3 Patient characteristics

Patient characteristics	<i>n</i>
Age, mean \pm SD (yr)	72.9 \pm 8.1
M/F	11/10
BMI, mean \pm SD (kg/m ²)	22.9 \pm 3.9
ASA I, <i>n</i> (%)	3 (14.2)
ASA II, <i>n</i> (%)	6 (28.6)
ASA III, <i>n</i> (%)	11 (52.4)
ASA IV, <i>n</i> (%)	1 (4.8)
Comorbidity, <i>n</i> (%)	16 (76.1)
Cardiovascular disease, <i>n</i> (%)	6 (28.6)
COPD, <i>n</i> (%)	6 (28.6)
Hypertension, <i>n</i> (%)	10 (47.6)
Diabetes mellitus, <i>n</i> (%)	3 (14.2)
Symptomatic, <i>n</i> (%)	12 (57.1)
Jaundice, <i>n</i> (%)	7 (58.3)
Pain, <i>n</i> (%)	3 (25.1)
Nausea or Vomiting, <i>n</i> (%)	1 (8.3)
Loss of weight, <i>n</i> (%)	1 (8.3)
Placement of PTBD or biliary endoprosthesis, <i>n</i> (%)	4 (33.3)
CA 19.9, mean \pm SD (U/mL)	37.1 \pm 53.2
Neoadjuvant therapy, <i>n</i> (%)	0 (0)

ASA: American Society of Anesthesiologists; COPD: Chronic obstructive pulmonary disease; PTBD: Percutaneous transhepatic biliary drainage.

Table 4 Post-operative data

LOS, mean \pm SD (d)	15.4 \pm 6.2
Overall complications, <i>n</i> (%)	7 (33.3)
Clavien-Dindo > III, <i>n</i> (%)	3 (14.2)
Reoperation, <i>n</i> (%)	2 (9.5)
30-d mortality, <i>n</i> (%)	0 (0)

LOS: Length of hospital stay.

addition, MT AACs often present therapeutic dilemmas to the oncologists in deciding on the treatment that provides the “best supportive care” for these patients^[25,38], aside from which, the scientific community shares the view that the sub-classifications of AACs should be revised into two types only: INT and PB^[15]. This simpler classification is important not only for pathologists, but to clinicians (surgeons and oncologists) involved in the treatment of patients harboring AACs. In our series, 40% of patients had AACs, illustrating that the diagnosis and management issues raised by mixed AACs is relatively common.

Reid *et al*^[15] reanalyzed the “mixed” cases and concluded that the hybrid nature of ampullary cancers can manifest in different ways; in some, the mixed nature is manifest in different zones of the same tumor exhibiting distinctive morphologic patterns, specifically small tubular units with different cytomorphology within the tumor’s advancing edge^[15]. In others, the tumor cells within the same region appeared chimeric, with some features resembling intestinal and others, pancreatobiliary

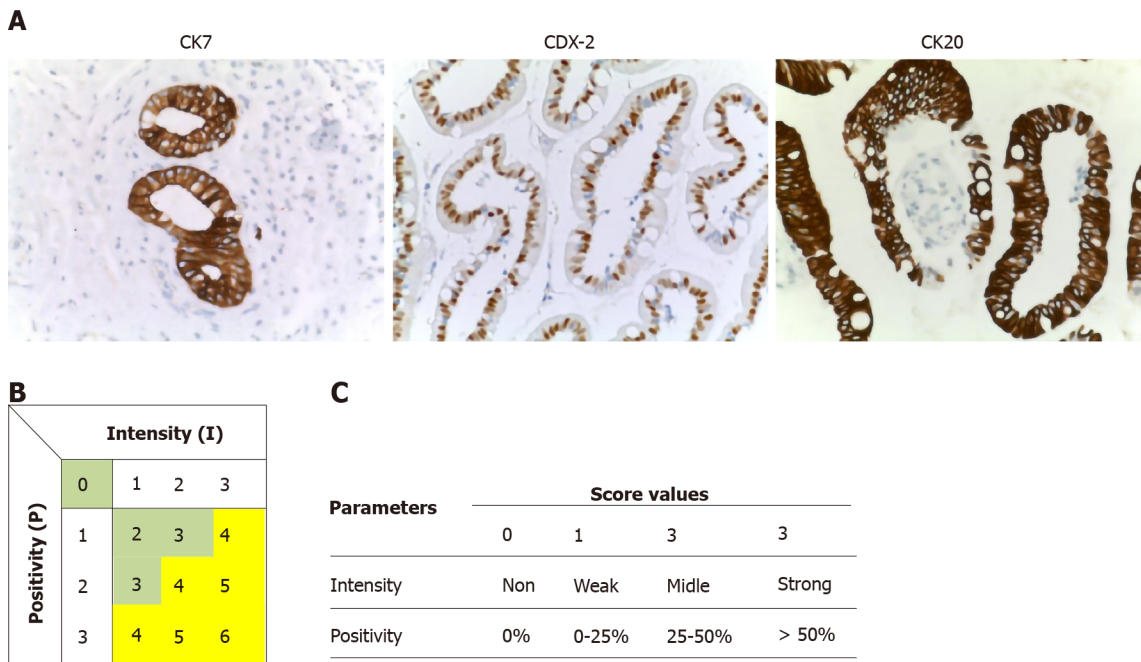


Figure 2 The analyses and quantification of three markers was based on the expression in normal controls. A: Normal control for CK7, CDX-2 and CK20; B: The total score was established before the analyses; and C: Intervals of values associated with each parameter of the immunohistochemical score.

lineage.

Several studies on ampullary adenocarcinomas have demonstrated that the histological type (PB *vs* INT) rather than primary tumor location determines survival^[40-42]. There is growing evidence that the intestinal type is associated with a less aggressive tumor biology and a better prognosis, which is indicative of two distinct different subtypes^[38,39].

In this study, using a panel of immunohistochemical markers, we distinguished different tumor types based on their marker profile. The use of a specific histo-molecular panel classification resulted in the identification of a particularly aggressive cohort of patients with PB. These comprised 45% of our patient cohort and provided an improved classification by re-allocating the MT AACs, comprising 40% of the total to either the INT or PB phenotypes.

By means of IHC expression and the GS, it was possible to re-assign the MT group to the INT and the PB group in 75% and 25% of cases, respectively. The literature confirms high expression of CDX2 and CK20 in INT lesions, while PB lesions exhibited high expression of CK7 with negative expression of CK20 ($P = 0.0008$). Both epigenetic and epigenomic analyses might explain the “ambiguous” molecular morphology of MT AACs. The micro-RNAs, play a significant role in molecular suppression and/or modulation, probably by displacement of the markers used for AACs classification^[43,44]. According to the suggested classification, patients belonging to the PB group had a median OS of 20.3 mo *vs* 85.7 mo in patients belonging to the INT group ($P = 0.0152$). In a recent study including 163 ampullary carcinomas, Schueneman *et al*^[23] validated the histo-molecular classification by Chang *et al*^[11] (2013) using a large data set^[11,15]. Their results supported the clinical use of this new classification for AACs. They evaluated CDX2 and MUC1 expression using an IHC parameter of “all positive staining” along with their data demonstrating improved prognostication with MUC1 positivity defined as 10% staining. These authors proposed this definition for MUC1 positivity when applied to histo-molecular subtyping of AACs. In this assessment, 25.2% of their population exhibited a PB sub phenotype with a median OS of 21.1 mo compared to 108.3 mo for the INT sub phenotype ($P < 0.0001$). The present study supports the use of CDX2, CK7 and CK20 expression associated with the GS, which together define only two AACs subtypes and thus eliminate the issues associated with MT AACs^[15].

The selection of these panels of specific markers (*i.e.*, CDX-2, CK7 and CK20) seems to prove the correct SC of AACs. The TMA platform, combined with: (1) Automatic staining of histological core samples; (2) Automatic detection of their staining; and (3) Automatic analysis of the score, represent a robust tailored flow-chart for both diagnostic and clinical management of these patients. At the same time, the automatic

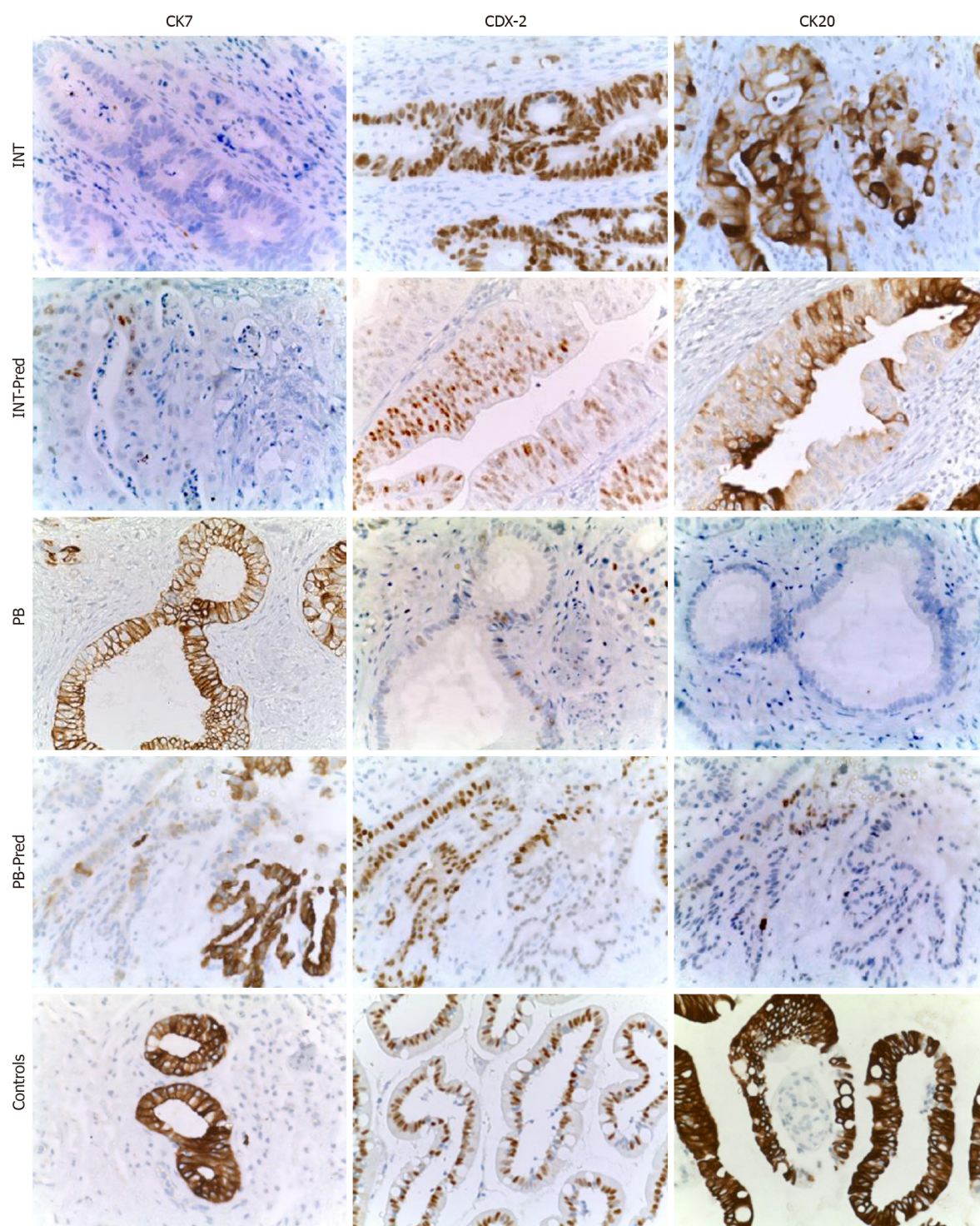


Figure 3 Representation of immunohistochemical staining in all sub-types observed in ampullary adenocarcinoma samples. All images were acquired using a magnification 40 ×. IHC: Immunohistochemical; PB: Pancreato-Biliary; INT: Intestinal.

platform represents a valid tool to screen the more representative markers for pancreatic pathologies, in which the distinction of histological sub categories, may reflect their different clinical behavior^[45,46].

This study has clinical implications by improving prognosis and therapeutic decision-making to provide an individualized treatment, especially whether the patients should undergo primary surgery or neoadjuvant treatment.

The main limitations of the study are its retrospective nature, the small cohort of patients and the lack of data on the type of adjuvant therapy and possible interaction between the type of chemotherapy response and histo-molecular subtype.

Further prospective randomized or observational studies are needed to validate these results in a larger cohort to address this controversial issue.

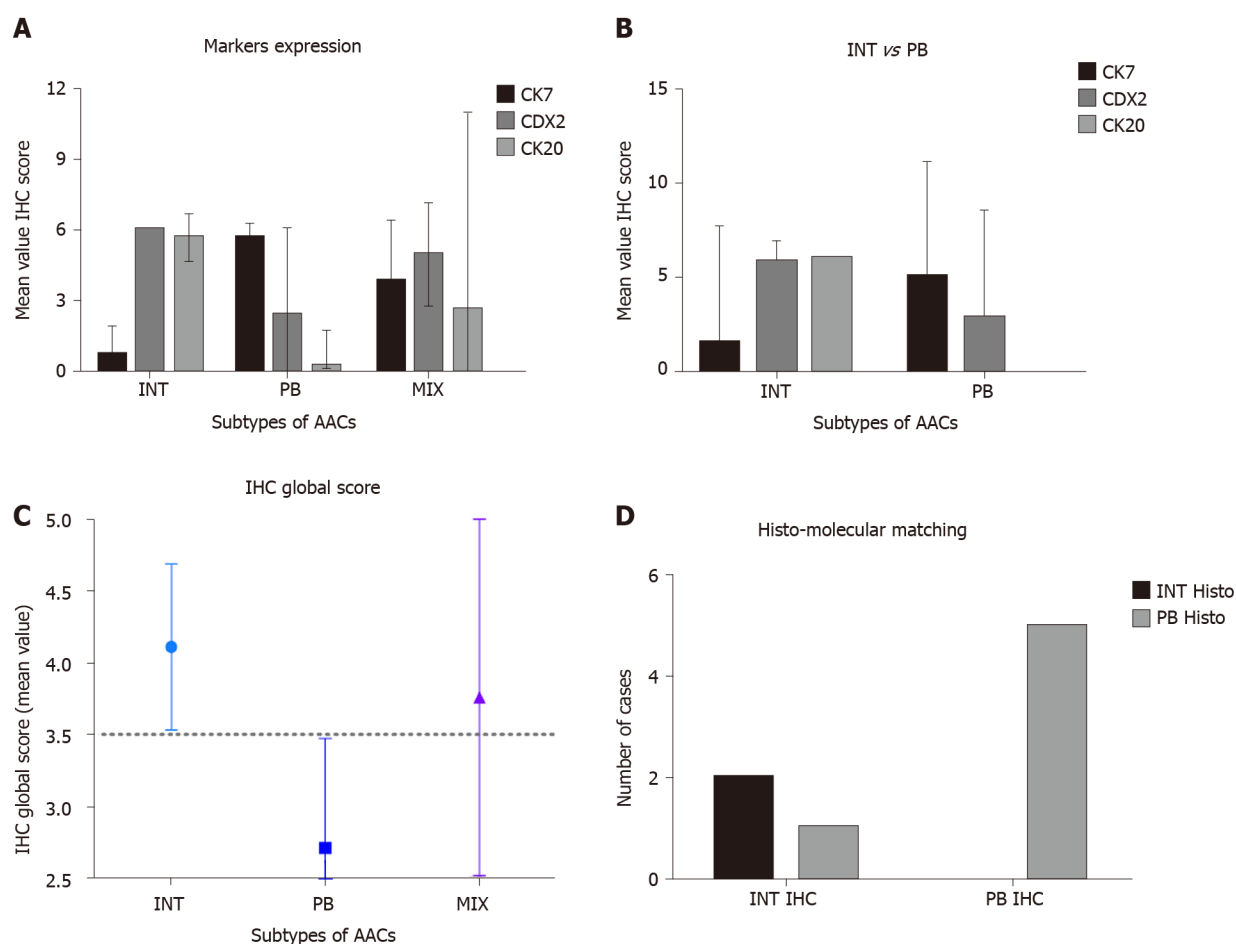


Figure 4 Statistical analyses of immunohistochemical markers and their values. A: Mean immunohistochemical value for each marker in INTestinal, Pancreato-Biliary and MIX types of ampullary adenocarcinomas, according to histological analyses (hematoxylin-eosin staining); B: Mean immunohistochemical value to compare intestinal vs Pancreato-Biliary; C: Global Score mean value in all sub-types of AACs; and D: Chi-square test to compare the histological vs molecular subtypes. AACs: Ampullary adenocarcinomas; IHC: Immunohistochemical; INT: Intestinal; PB: Pancreato-Biliary.

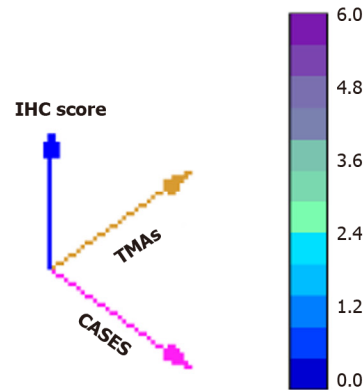
CONCLUSION

The combination of histopathological and molecular criteria (three markers panel) evaluated through the TMA platform defines two clinically relevant histo-molecular sub-phenotypes of AACs. This molecular classification appears able to predict the clinical outcome and to indicate the best adjuvant treatment for these patients. The two AACs SC identified seem to represent distinct diseases with significant implications for current therapeutic strategies; hence, a useful tool for both surgeons and oncologists. A preoperative biopsy of the ampulla could provide an AACs subtype classification, enabling tailored oncological treatment to the tumor phenotype and planning the extent of the surgical resection^[47-49].

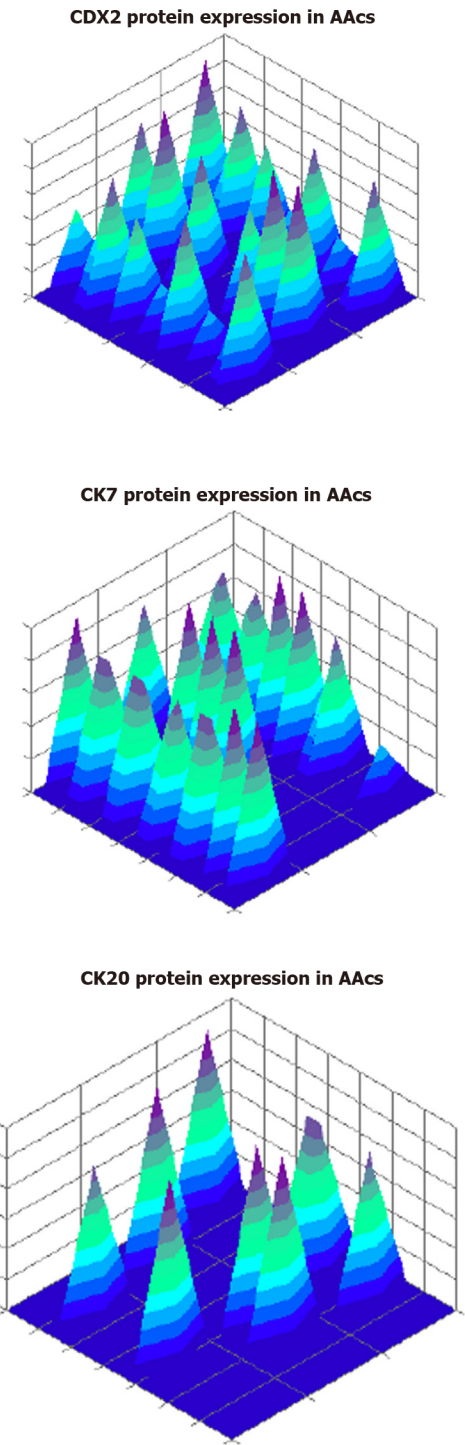
A

HD	TS	GS	CDX2	CK7	CK20	GSV
INT	INT	INT				12
INT	INT	INT				12
INT	INT	INT				12
MIX	INT	INT				16
MIX	INT	INT				12
MIX	INT	INT				10
MIX	INT	INT				10
MIX	PB	INT				10
MIX	INT	INT				16
MIX	PB	INT				10
MIX	INT	PB				4
PB	PB	PB				9
PB	PB	PB				9
PB	PB	PB				6
PB	PB	PB				8
PB	PB	PB				8
PB	PB	PB				8
PB	PB	PB				8
PB	PB	PB				6
PB	PB	PB				8
PB	PB	PB				6

Scoring	0	2	3	4	5	6
Expression	Low					High



B



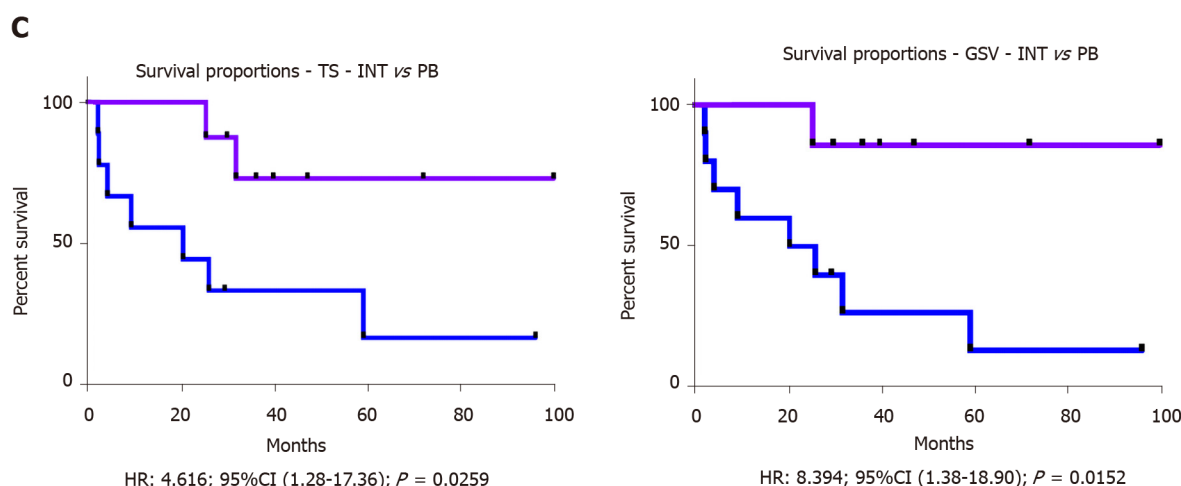


Figure 5 Results elaborating the computerized analyses. A: Classification according to the three different data sets obtained: Histology, Total score and Global score; B: 3D representation of immunohistochemical analyses of all samples; C: Kaplan–Meier curves of INTestinal vs Pancreato-Biliary according to the molecular partition by Total score (above) or Global Score (below). IHC: Immunohistochemical; TS: Total score; INT: Intestinal; PB: Pancreato-Biliary; AACs: Ampullary adenocarcinomas; TMA: Three tissue microarrays; INT: Intestinal.

ARTICLE HIGHLIGHTS

Research background

Ampullary adenocarcinomas (AACs) are heterogeneous tumors currently classified into the three most important sub-classes (SC): Intestinal (INT), Pancreato-Biliary (PB) and Mixed-Type (MT). The different subgroups have similar clinical presentation and are treated by pancreatoduodenectomy with curative intent; however, they have different responses to specific chemotherapeutics, with different prognoses.

Research motivation

Conventional histology does not allow a definitive identification of the three subgroups.

Research objectives

In this study using an immunohistochemical (IHC) score based on CDX2, CK7 and CK20 evaluation through three tissue microarray platforms, we identified two clinically relevant histo-molecular sub-phenotypes of AACs.

Research methods

Tissue samples from 21 patients who had undergone AAC resection were arranged on three tissue microarray platforms and were classified by histology and IHC expression of CDX2, CK7 and CK20. An IHC score was obtained for each marker summing the number of positive cells (0 = no stained cells; 1 < 25%; 2 < 50% and 3 > 50%) and their intensity (1 = weak; 2 = moderate and 3 = strong). A global score (GS) was then obtained summing the IHC scores of each marker. The MT tumors were re-located to either the INT or PB group on the basis of the predominant immune-molecular phenotype, identifying only two AACs subtypes. The overall survival of INT and PB patients was obtained by Kaplan-Meier methods.

Research results

Histological parameters defined the AACs subtypes as follows: 15% INT, 45% PB and 40% MT. Using the IHC expression and the GS, 75% and 25% of MT samples were assigned to the INT and PB group, respectively. The mean value of GS was 9.5 (range 4-16). All INT samples had a GS above the average, while all PB sample had a GS below the average ($P = 0.0011$). In particular, the INT samples were identified by high expression of CDX2 and CK20, while PB samples showed high expression of CK7 and negative expression of CK20 ($P = 0.0008$). The overall survival analysis was statistically significantly better for INT than PB patients (85.7 vs 20.3 mo, HR: 8.39; 95%CI: 1.38 to 18.90; $P = 0.0152$).

Research conclusions

The combination of histopathological and molecular criteria enables the definition of only two clinically relevant histo-molecular phenotypes of AACs that potentially represent distinct disorders with different management and chemotherapeutic strategies.

Research perspectives

A preoperative biopsy of the ampulla could provide a AACs subtype classification, allowing the tailoring of oncological treatment and planning the extension of surgical resection.

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Retrospective Cohort Study

Feasibility and nutritional impact of laparoscopic assisted tailored subtotal gastrectomy for middle-third gastric cancer

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Abstract

BACKGROUND

Laparoscopic assisted total gastrectomy (LaTG) is associated with reduced nutritional status, and the procedure is not easily carried out without extensive expertise. A small remnant stomach after near-total gastrectomy confers no significant nutritional benefits over total gastrectomy. In this study, we developed a modified laparoscopic subtotal gastrectomy procedure, termed laparoscopic-assisted tailored subtotal gastrectomy (LaTSG).

AIM

To evaluate the feasibility and nutritional impact of LaTSG compared to those of LaTG in patients with advanced middle-third gastric cancer (GC).

METHODS

We retrospectively analyzed surgical and oncological outcomes and postoperative nutritional status in 92 consecutive patients with middle-third GC who underwent radical laparoscopic gastrectomy at Department of Pancreatic Stomach Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College between 2013 and 2017. Of these 92 patients, 47 underwent LaTSG (LaTSG group), and the remaining underwent

Written informed consent was obtained from all patients before the procedure. The data were anonymously analyzed.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: Some or all data, code generated or used during the study are available from the corresponding author by request.

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LaTG (LaTG group).

RESULTS

Operation time (210 ± 49.8 min *vs* 208 ± 50.0 min, $P > 0.05$) and intraoperative blood loss (152.3 ± 166.1 mL *vs* 188.9 ± 167.8 mL, $P > 0.05$) were similar between the groups. The incidence of postoperative morbidities was lower in the LaTSG group than in the LaTG group (4.2% *vs* 17.8%, $P < 0.05$). Postoperatively, nutritional indices did not significantly differ, until postoperative 12 mo. Albumin, prealbumin, total protein, hemoglobin levels, and red blood cell counts were significantly higher in the LaTSG group than in the LaTG group ($P < 0.05$). No significant differences in Fe or C-reaction protein levels were found between the two groups. Endoscopic examination demonstrated that reflux oesophagitis was more common in the LaTG group (0% *vs* 11.1%, $P < 0.05$). Kaplan-Meier analysis showed a significant improvement in the overall survival (OS) and disease free survival (DFS) in the LaTSG group. Multivariate analysis showed that LaTSG was an independent prognostic factor for OS ($P = 0.048$) but not for DFS ($P = 0.054$). Subgroup analysis showed that compared to LaTG, LaTSG improved the survival of patients with stage III cancers, but not for other stages.

CONCLUSION

For advanced GC involving the middle third stomach, LaTSG can be a good option with reduced morbidity and favorable nutritional status and oncological outcomes.

Key Words: Gastric cancer; Laparoscopy assisted tailored gastrectomy; Nutritional status; Morbidity; Reflux oesophagitis; Resection margin

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Core Tip: We developed a modified the laparoscopic subtotal gastrectomy procedure termed laparoscopic-assisted tailored subtotal gastrectomy (LaTSG) to treat advanced middle-third gastric cancer. Compared with laparoscopic assisted total gastrectomy (LaTG), LaTSG is a safer procedure in terms of both short and long-term outcomes. The long-term survival of patients who underwent LaTSG was better than that of patients who underwent LaTG. Furthermore, LaTSG may have an advantage over LaTG by improving the postoperative nutritional status and preventing reflux oesophagitis.

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INTRODUCTION

In recent decades, the incidence of gastric cancer (GC) has declined worldwide, however, it remains the second most common cancer among men and the third most common among women. According to data from the National Central Cancer Registry of China, in 2015, there were 677000 new cases of GC in China, which accounted for half of all incident cases globally^[1].

In resectable advanced GC, the range of resection is determined by tumor characteristics, including its size, location, clinical stage, and distance from the proximal resection margin. According to the latest Japanese GC treatment guidelines^[2], tumors located in the upper or low-third of the stomach have a definite range of gastric resection. However, consensus on the optimal surgical treatment strategy for advanced GC located in the middle of the stomach is yet to be established. Such cancer is not easily cured by distal or proximal gastrectomy, and most patients ultimately undergo total gastrectomy (TG). However, numerous studies have



demonstrated that TG was more traumatic than partial gastrectomy, and it is further suggested that TG could result in higher rates of postoperative complications^[3-5]. Moreover, patients who underwent TG showed significant nutritional deficits, including a higher incidence of anemia and low serum vitamin E levels^[6,7].

A recently reported procedure^[8], laparoscopic subtotal gastrectomy with a very small remnant stomach, has demonstrated advantages over laparoscopic-assisted total gastrectomy (LaTG), showing reduced postoperative morbidity and improved nutritional status, when used to treat patients with early GC located in the upper-third of the stomach. Recently, several large clinical studies have demonstrated that laparoscopy can be used for advanced GC^[9,10]. We developed a modified laparoscopic subtotal gastrectomy procedure termed laparoscopic-assisted tailored subtotal gastrectomy (LaTSG) to treat advanced middle-third GC. In the present study, we evaluated short-term postoperative patient outcomes, nutritional status, and long-term oncological outcomes to assess the safety and efficacy of LaTSG compared to those of LaTG.

MATERIALS AND METHODS

Patients

This retrospective study assessed patient outcomes in 47 patients who received LaTSG and 45 patients who received LaTG for preoperatively diagnosed GC. Patients (aged 18-75 years) were enrolled and treated at the Department of Pancreatic Stomach Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College from February 2013 to November 2017. Preoperative contrast-enhanced computed tomography (CT) and endoscopic ultrasonography were performed to assess the tumor.

LaTSG was performed in patients who fulfilled the following criteria: (1) Tumor identified by clinical staging as T2-4bN0-3M0 based on the 8th American Joint Committee on Cancer staging system; (2) Non-Borrmann type IV tumor located in the middle-third of the stomach, or non-Borrmann type IV lesion in the lower-third of the stomach that extends into the middle-third of the stomach, with no distant metastasis. In view of the susceptibility to No. 2 lymph node (LN) metastasis, TG was performed for the tumors located in the greater curvature of the stomach; (3) The proximal margin was at least 3 cm from the tumor with a non-infiltrative growth pattern; and (4) Intraoperative peritoneal washing cytology was negative. In patients selected to receive LaTSG, an additional intraoperative frozen pathology was performed. If the margin of the biopsy was positive for tumor cells, LaTG instead of LaTSG was performed. Standard D2 lymph node dissection was performed in all cases. All methods were carried out in accordance with Japanese GC treatment guidelines 2014 (ver. 4)^[2].

Patients were excluded from the study if they met any of the following criteria: (1) Contemporaneous existence of other malignancies; (2) Gastric stump cancer; (3) Received preoperative radiotherapy; (4) Existence of distant metastasis including No. 16 LN, left supraclavicular lymph node, liver, lung, or bone metastasis; (5) Peritoneal dissemination or positive intraoperative peritoneal washing cytology; (6) Borrmann type IV tumor; and (7) Existence of enlarged or bulky regional lymph nodes, larger than 3 cm in the long diameter according to preoperative imaging.

Evaluation of clinical parameters

Early postoperative complications (occurring on postoperative days 0-30) were graded using the Clavien-Dindo classification. To evaluate the postoperative nutritional status, body mass index (BMI) and serum concentrations of hemoglobin (HGB), ferrous iron (Fe), albumin (ALB), prealbumin (PALB), total protein (TP), and red blood cell counts (RBC) were measured 1 d, 1 mo, and 12 mo after the procedure was conducted. Gastro-esophageal reflux was evaluated *via* endoscopy based on the Los Angeles classification system^[11] 12 mo after the surgery. For patients with pathologic stage II or higher tumors, adjuvant chemotherapy with 6 mo of a fluorouracil-based chemotherapy was mandated. All patients were followed regularly by clinic visits and telephone. Chest, abdominal, and pelvic contrast-enhanced CT was performed every 3 mo for the first 3 years and 6 mo thereafter.

Surgical procedure

Endotracheal intubation was conducted under general anesthesia, and the laparoscopic surgery was performed with the patient placed in the split-leg reverse

Trendelenburg position. A 10-mm flexible laparoscope was used, and CO₂ pressure was maintained at 13–15 mmHg. During surgery, the operator was positioned on the left side of the patient, the first assistant was on the right side, and the cameraman stood between the legs of the patient.

A five-port system (two 5-mm ports and three 12-mm ports) was used for the laparoscopic assisted gastrectomy (Figure 1). The location of the observation port was determined based upon the distance between the patient's xiphoid and umbilicus.

A 12-mm trocar could be inserted into the umbilicus with sufficient distance from the xiphoid. Our additional ports (two ports with 12-mm diameter and two with 5-mm diameter) were inserted under direct visualization into the upper abdomen. Trocars were separated by a width of at least four fingers which helped prevent each trocar from impacting the others *via* the “chopstick effect”.

LaTSG procedure

After dissociating the distal stomach and completing D2 LN dissection (D1+7+8a+9+11p+12a), at least two branches of the short gastric artery near the gastric cardia were preserved. An endoscopic liner stapler was used to cut and close the duodenum.

Different resection lines were selected based on different locations of the tumors (Figure 1). In tumors located within the lesser curvature of the stomach, a liner stapler was used to make a curved transaction line 3 cm from the lateral edge of the tumor, thereby retaining the fundus and cardia, which were used to form a proximal tube-shaped stomach (Figure 1A, Figure 2 and Figure 3). In tumors located in the middle of the stomach, a liner stapler was used to make a curved transection line 3 cm from the upper edge of the tumor (Figure 1B). After removing the specimen and ensuring that a negative frozen margin was obtained, conventional Billroth II reconstruction, alongside either Braun's or Roux-en-Y anastomosis, was performed with a circular stapler.

LaTG procedure

Patients with positive tumor indications in their proximal margin frozen sections were transitioned to undergo LaTG. After dissecting the LNs, the duodenum was transected using a linear stapler. The jejunum was subsequently extracorporeally transected 20 cm distal to the ligament of Treitz using another linear stapler. Jejunojejunostomy was performed using a liner stapler, 44 cm from the cutting end. The ensuing mesenteric hole was closed using a 3-0 absorbable suture. Oesophagojejunostomy was performed with a circular stapler. The reverse piercing method was used to put the nail base into the oesophagus and to perform esophagojejunal end-to-side anastomosis.

Statistical analysis

Clinical data were obtained from patients' records. Statistical analyses were performed using SPSS.20 (SPSS Inc., Armonk, NY, United States). All values are expressed as the mean \pm SD. We compared categorical and continuous variables using the χ^2 test and Student's *t* test, respectively. Kaplan–Meier estimation and log-rank test were performed to compare survival. A Cox proportional hazards regression model was used to verify independent prognostic factors through univariate and multivariate analyses. A *P* value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Table 1 details the clinical and nutritional characteristics of patients undergoing LaTSG and LaTG. Between the LaTSG and LaTG groups, no significant differences were observed in patients' BMI, ASA-PS, the number of patients with a previous abdominal operation, or the number of patients receiving neoadjuvant chemotherapy. Although the pT stage was significantly different between the two groups (*P* = 0.027), there were no significant intergroup differences in the pTNM stage (*P* = 0.217).

Operative findings

Operative outcomes are summarized in Table 2. Mean operation time and mean estimated blood loss were similar between the groups. There were no significant differences between the two groups in the levels of intraoperative blood transfusion. Proportionately, more LNs were retrieved in the LaTG group than in the LaTSG group

Table 1 Clinical characteristics of patients in the laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy groups

Characteristic	LaTSG (n = 47)	LaTG (n = 45)	P value
Sex (M/F)	11/36	17/28	0.175
Age	57.0 ± 11.1	58.0 ± 9.9	0.571
BMI (kg/m ²)	23.6 ± 3.4	23.7 ± 3.0	0.559
ASA-PS (1/2/3)	2/44/1	2/42/1	0.998
pT stage			0.027
pT1	18 (38.3)	10 (22.2)	
pT2	5 (10.6)	4 (8.9)	
pT3	8 (17.0)	22 (48.9)	
pT4a	16 (34.0)	9 (20.0)	
pN stage			0.371
pN0	23 (48.9)	14 (31.2)	
pN1	10 (21.3)	14 (31.2)	
pN2	6 (12.8)	7 (15.6)	
pN3	8 (17.0)	10 (22.2)	
pTNM stage			0.217
I	18 (38.3)	11 (24.4)	
II	10 (21.3)	16 (35.6)	
III	19 (40.4)	18 (40.0)	
Previous abdominal operation	4 (8.5)	1 (2.2)	0.362
Neoadjuvant chemotherapy	9 (19.1)	6 (13.3)	0.575
Histological type			0.237
Signet-ring cell carcinoma	20 (42.6)	12 (26.7)	0.187
Blood vessel invasion	30 (63.9)	30 (66.6)	0.829
Neural infiltration	26 (55.4)	27 (60.0)	0.532
Tumor location			0.604
Lesser curvature	25 (53.2)	24 (53.4)	
Middle	3 (6.4)	5 (11.1)	
Middle and low	19 (40.4)	16 (35.6)	

LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy; BMI: Body mass index; ASA-PS: American Society of Anesthesiologists Physical Status Classification.

($P < 0.05$). Tumor size was larger in the LaTG group than in the LaTSG group, while there were no significant differences between the two groups in the proximal and distal margins. Neural infiltration and lymph vessel invasion were more common in the LaTG group than in the LaTSG group, however, these differences were not significant ($P > 0.05$).

Short-term outcomes

Postoperative patient complications are listed in Table 3. The mean postoperative hospital stay was significantly shorter in the LaTSG group (8.9 ± 5.0 d) than in the LaTG group (12.0 ± 7.3 d) ($P < 0.05$). No mortality was recorded for the LaTSG group; however, one patient in the LaTG group died from acute kidney failure due to intraabdominal bleeding. The overall postoperative complication rate was 4.25% in the LaTSG group, and 17.8% in the LaTG group ($P < 0.05$). The frequency of anastomotic complications was significantly higher in the LaTG group (8.9%) than in the LaTSG

Table 2 Operative findings in the laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy groups

Variable	LaTSG (n = 44)	LaTG (n = 58)	P value
Operation time (min)	210.0 ± 49.8	208.0 ± 50.0	0.684
Estimated blood loss (mL)	152.3 ± 166.1	188.9 ± 167.8	0.141
Intraoperative blood transfusion	6/47	8/45	0.570
Tumor size	3.4 ± 1.2	4.5 ± 1.8	0.020
No. of retrieved lymph nodes	31.8 ± 10.1	44.0 ± 16.5	0.008
Proximal margin	3.7 ± 1.6	3.9 ± 2.0	0.233
Distal margin	5.0 ± 2.6	6.2 ± 3.2	0.149

LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy.

Table 3 Postoperative complications in the laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy groups, n (%)

Complication	LaTSG (n = 47)	LaTG (n = 45)	P value
Anastomotic leakage	0 (0)	3 (6.7)	
Anastomotic stricture	0 (0)	1 (2.2)	
Anastomotic bleeding	0 (0)	0 (0)	
Duodenal stump leakage	1 (2.1)	0 (0)	
Intra-abdominal bleeding	0 (0)	0 (0)	
Intra-abdominal abscess	1 (2.1)	3 (6.7)	
Lymph leak	0 (0)	1 (2.2)	
Ileus	0 (0)	0 (0)	
Incision	0 (0)	1 (2.2)	
Total	2 (4.2)	8 (17.8)	0.048
Mortality	0 (0)	1 (2.2)	
Postoperative hospital stay	8.9 ± 5.0	12.0 ± 7.3	0.025
Reflux esophagitis (LA)			0.002
LA -B	2 (4.2)	5 (11.1)	
LA-C	0 (0)	5 (11.1)	

LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy; LA: Los Angeles classification grade (A/B/C/D).

group (0%) ($P < 0.05$). Los Angeles Grade B or more severe reflux oesophagitis was observed in ten (17.2%) patients in the LaTG group, while such severe reflux oesophagitis was observed in only two (4.5%) patients in the LaTSG group ($P = 0.002$).

Nutritional indexes

Figure 4 shows the pre- and postoperative mean levels of ALB, TP, PALB, RBC, HGB, C-reactive protein (CRP), and Fe in patients who underwent LaTSG and LaTG. On the first day postoperatively, these nutritional indices did not significantly differ between the groups. However, at 12 mo post-surgery, ALB, TP, HGB, and RBC levels were significantly higher in the LaTSG group than in the LaTG group ($P < 0.05$). No significant differences in Fe, PALB, or CRP levels were found between the groups ($P > 0.05$) (Table 4).

Table 4 Nutritional indexes of laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy at 1 year postoperatively

Variable	LaTSG (n = 47)	LaTG (n = 45)	P value
ALB (g/L)	45.2 ± 3.7	45.1 ± 7.2	0.005
PALB (g/L)	21.6 ± 4.7	18.5 ± 5.3	0.015
TP (g/L)	71.0 ± 4.2	67.6 ± 4.6	0.002
Fe	15.6 ± 5.7	13.4 ± 6.9	0.194
HGB (g/L)	133 ± 20.7	120.0 ± 20.1	0.007
RBC(10 ¹² /L)	4.5 ± 0.7	4.0 ± 0.4	0.003
CRP	0.37 ± 0.97	0.05 ± 0.25	0.187

LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy; HGB: Hemoglobin; Fe: ferrous iron; ALB: Albumin; PALB: Prealbumin; TP: Total protein; CRP: C-reaction protein.

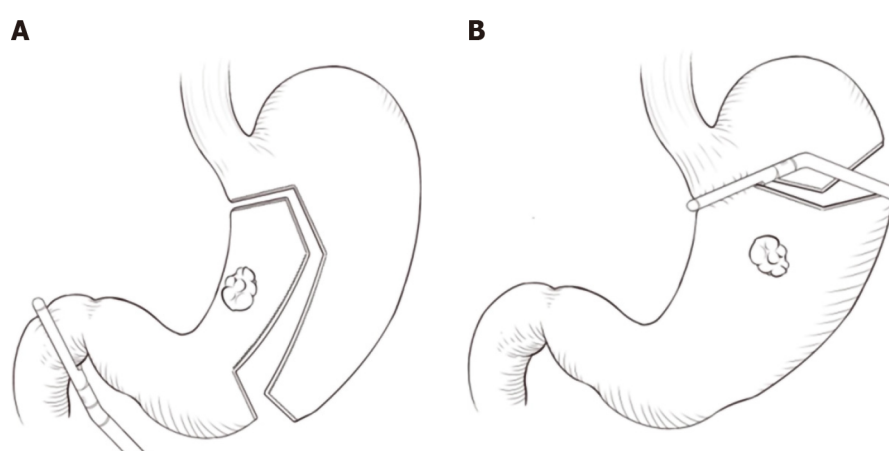


Figure 1 Selected resection lines based on the tumor's location. A: Lesser curvature; B: Middle.

Survival results

The median follow-up time was 41 mo (range, 37-46 mo) in this study. Kaplan-Meier analysis showed a significant difference in overall survival between the two groups ($P = 0.020$) (Figure 5). The 3-year overall survival rates in the LaTSG and LaTG groups were 85.6% and 67.4%, respectively ($P < 0.05$). Subgroup analysis showed that compared to LaTG, LaTSG improved the survival of patients with stage III cancer, but not for other stages. Univariate analysis identified tumor size ($P = 0.036$), TNM stage ($P = 0.002$), and surgical type ($P = 0.036$) to be prognostic factors for overall survival. Multivariate analysis revealed that pTNM stage ($P = 0.004$) and surgical type ($P = 0.048$) were independent prognostic factors for the overall survival (Table 5).

Kaplan-Meier analysis also showed a significant difference in disease-free survival between the two groups ($P = 0.022$) (Figure 6). The 3-year disease-free survival rates in the LaTSG and LaTG groups were 86.7% and 57.3%, respectively ($P < 0.05$). Multivariate analysis shows that pTNM stage ($P = 0.046$) was the only independent prognostic factor for disease-free survival (Table 6). Subgroup analysis showed that compared to LaTG, LaTSG improved the disease free survival of patients with stage III cancer, but not for other stages.

DISCUSSION

In the present study, we evaluated the efficacy of LaTSG compared to LaTG in patients with advanced middle-third GC, and the findings demonstrated that long-term survival was better for those who underwent LaTSG than for those who underwent LaTG.

Table 5 Univariate and multivariate analyses of overall survival for included patients

Variable	Univariate	P value	Multivariate	P value
	HR (95%CI)		HR (95%CI)	
Age, yr (≤ 65)	0.831 (0.301-2.293)	0.721	-	-
Sex (female)	0.493 (0.204-1.192)	0.117	-	-
BMI (≤ 25 kg/m ²)	0.703 (0.270-1.832)	0.471	-	-
ASA (I/II/III)	1.440 (0.256-8.086)	0.679	-	-
Previous abdominal operation	0.046 (0.000-250.286)	0.482	-	-
Primary site (L/M/LM)	0.981 (0.707-1.360)	0.908	-	-
Tumor size (> 4 cm)	2.607 (1.064-6.390)	0.036	1.180 (0.906-1.538)	0.219
Neoadjuvant chemotherapy (Yes)	1.369 (0.454-4.129)	0.577	-	-
Tumor grade (W/M/P/U)	0.851 (0.578-1.253)	0.415	-	-
Lauren classification (I/D/M)	0.949 (0.513-1.758)	0.869	-	-
pTNM stage (I/II/III)	3.195 (1.518-6.724)	0.002	3.170 (1.461-6.878)	0.004
Lymphovascular invasion (Yes)	2.203 (0.906-5.358)	0.081	-	-
Neural infiltration (Yes)	1.755 (0.715-4.304)	0.219	-	-
Surgical type (LaTSG/LaTG)	2.977 (1.073-8.263)	0.036	2.876 (1.009-8.195)	0.048
Operation time (> 210 min)	1.130 (0.456-2.803)	0.791	-	-
Estimated blood loss (> 200 mL)	1.261 (0.522-3.048)	0.606	-	-
Intraoperative blood transfusion (Yes)	0.933 (0.273-3.192)	0.912	-	-
No. of retrieved lymph nodes (> 30)	1.631 (0.590-4.511)	0.346	-	-
Proximal margin (> 3 cm)	0.876 (0.362-2.120)	0.770	-	-
Distal margin (> 3 cm)	0.500 (0.203-1.121)	0.132	-	-

BMI: Body mass index; ASA: American Society of Anesthesiologists; L: Lesser curve; M: Middle; ML: Middle and low; W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated; U: Undifferentiated; I: Intestinal type; D: Diffuse type; M: Mixed type; LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy.

In this study, our inclusion criteria included non-Bormman type IV tumors, in which the lateral resection margin was at least 3 cm from the tumor. Bormman type IV tumors were excluded because of difficulty in obtaining a negative margin. It is advisable to utilize frozen pathology to obtain complementary diagnostic information, despite the risk for false-negatives. Preoperative markers or intraoperative guidance is necessary so that the tumor does not penetrate any serous membranes. If the margin biopsy was positive for tumor, LaTG, instead of LaTSG, was performed. In this study, all patients received R0 resection, and whether proximal or distal margin was greater than 3 cm was not a prognostic factor for overall survival, as has also been reported in previous reports^[12-14].

With respect to the number of retrieved LNs, the LaTG group had a significantly larger number than the LaTSG group ($P < 0.05$). The main reason for this difference is that the LN dissection extent differed between the two groups. In the LaTSG group, we did not resect the No.2, No.4sa, and No.11d LNs, which were dissected in the LaTG group, in accordance with the Japanese GC treatment guidelines^[2]. With respect to No. 4sa, it is possible to remove most of the lymph nodes along the short gastric vessels. However, a complete dissection, which also includes the nodes around the superior pole of the spleen, should be avoided, so as to preserve blood supply to the remnant stomach. This is especially important when the tumor is located within the lesser curvature of the stomach.

Many patients undergoing TG suffer from iron and/or vitamin B12 deficiencies due to malabsorption. The absence of gastric acid secretion and a lack of intrinsic factor have been reported to cause poor absorption of these nutrients, resulting in clinically evident anemia or neuropathy. Previous retrospective studies have documented that distal gastrectomy has advantages over TG in postoperative nutritional status and

Table 6 Univariate and multivariate analyses of disease free survival for included patients

Variable	Univariate	P value	Multivariate	P value
	HR (95%CI)		HR (95%CI)	
Age, yr (≤ 65)	0.505 (0.209-1.221)	0.129	-	-
Sex (female)	0.795 (0.288-2.192)	0.658	-	-
BMI (≤ 25 kg/m ²)	0.675 (0.259-1.761)	0.422	-	-
ASA (I/II/III)	1.864 (0.277-12.523)	0.522	-	-
Previous abdominal operation	0.045 (0.000-188.621)	0.467	-	-
Primary Site (L/M/LM)	0.993 (0.715-1.377)	0.964	-	-
Tumor size, (> 4 cm)	2.535 (1.035-6.208)	0.042	1.740 (0.706- 4.294)	0.229
Neoadjuvant chemotherapy (Yes)	1.338 (0.442-4.051)	0.606	-	-
Tumor grade (W/M/P/U)	0.852 (0.580-1.252)	0.416	-	-
Lauren classification (I/D/M)	0.970 (0.523-1.798)	0.923	-	-
pTNM stage (I/II/III)	3.317 (1.566-7.023)	0.002	3.353 (1.558-7.214)	0.002
Lymphovascular invasion (Yes)	2.209 (0.906-5.383)	0.081	-	-
Neural infiltration (Yes)	1.729 (0.703-4.253)	0.233	-	-
Surgical type (LaTSG/LaTG)	2.859 (1.027-7.958)	0.044	2.776 (0.981-7.854)	0.054
Operation time (> 210 min)	1.115 (0.450-2.761)	0.814	-	-
Estimated blood loss (> 200 mL)	1.273 (0.526-3.080)	0.592	-	-
Intraoperative blood transfusion (Yes)	0.938 (0.274-3.208)	0.919	-	-
No. of retrieved lymph nodes (> 30)	1.643 (0.593-4.553)	0.339	-	-
Proximal margin (> 3 cm)	0.904 (0.373-2.192)	0.823	-	-
Distal margin (> 3 cm)	0.537 (0.219-1.319)	0.175	-	-

BMI: Body mass index; ASA: American Society of Anesthesiologists; L: Lesser curve; M: Middle; ML: Middle and low; W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated; U: Undifferentiated; I: Intestinal type; D: Diffuse type; M: Mixed type; LaTSG: Laparoscopic assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy.

quality of life of patients^[15,16]. Moreover, previous studies have indicated that the gastrectomy itself may induce iron deficiency, because less oral intake of food, and thereby of dietary Fe, occurs due to the small stomach volume.

Patients who underwent LaTSG showed significantly higher HGB and RBC levels at 12 mo post-surgery than those who underwent LaTG. Furthermore, significant differences in ALB, TP, and PLAB levels were found between the two groups. In contrast, we did not find a significant difference between the LaTG and LaTSG groups with respect to Fe absorption. Nevertheless, in aggregate, our data suggested that partial preservation of the remnant stomach increased the absorption of some nutrients compared to total resection.

LaTSG is a feasible and safe technique in terms of the operation time, estimated blood loss, and intraoperative blood transfusion. In our analysis, the frequency of anastomotic complications was significantly lower in the LaTSG group than in the LaTG group, suggesting that LaTSG has advantages over LaTG by reducing the incidence of postoperative anastomotic complications. Our results are supported by the study of Lee *et al*^[17], who reported a higher rate of postoperative complications with LaTG than with laparoscopic-assisted distal gastrectomy, especially with respect to the incidence of anastomotic stricture. This reduced incidence of postoperative complications could be related to the fact that LaTG is more complicated than LaTSG. In particular, when using a tubular stapler, sophisticated purse-string suture and anvil placement were severely limited by the narrow space.

Studies largely agree that subtotal gastrectomy for distal gastric adenocarcinoma improves the quality of life without causing any adverse effects on long-term survival^[15,18,19]. Likewise, in this study, 1 year postoperatively, the anti-reflux effect was much better in patients who underwent LaTSG than in those who underwent LaTG.

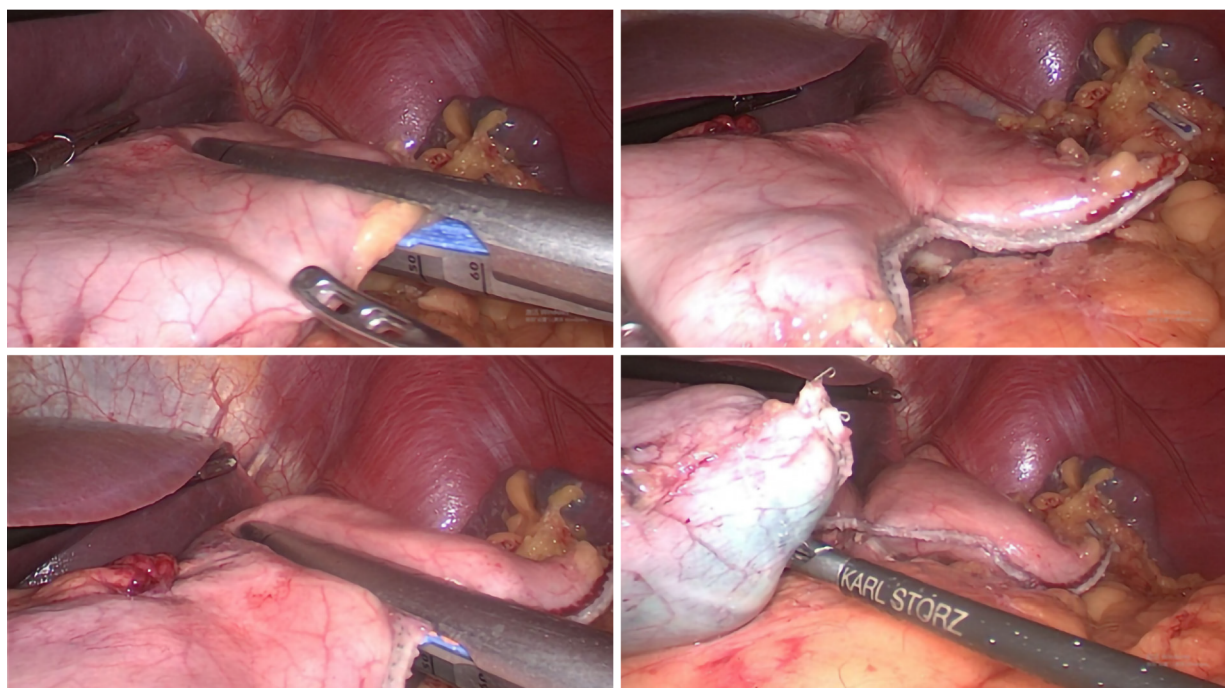


Figure 2 In a tumor located in the lesser curvature of the stomach, a liner stapler was used to make a curved transection line 3 cm from the lateral edge of the tumor.

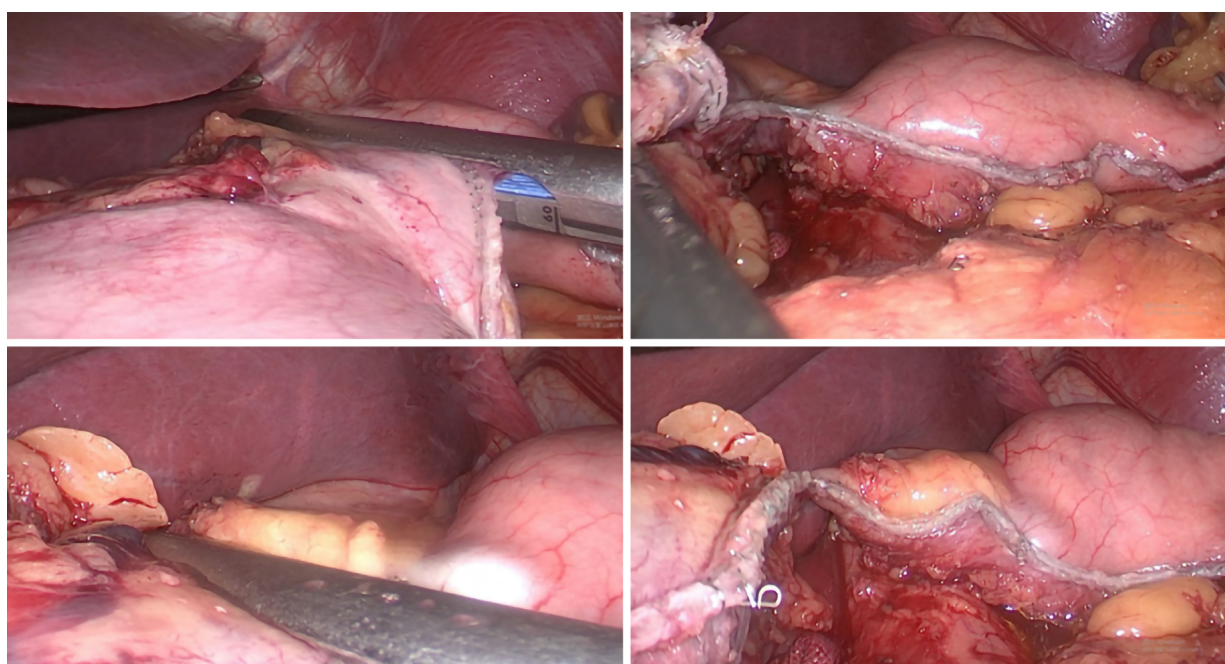


Figure 3 The stomach was resected while the fundus and cardia were retained and used to form a larger proximal remnant stomach than subtotal gastrectomy.

Indeed, LaTSG retains the complete cardia structure and further preserves a larger remnant stomach than subtotal gastrectomy, which collectively greatly reduces the incidence of postoperative reflux oesophagitis, without any anastomotic stricture.

For the treatment of malignant tumors, the focus has always been on survival. Other studies have showed a survival advantage for subtotal procedures^[20]. In our study, the overall survival was better for patients in the LaTSG group than those in the LaTG group, and the 3-year survival rates in the LaTSG and TG groups were 85.6% and 67.2%, respectively ($P < 0.05$).

Specifically, in previous studies as well as in our study, subgroup analysis showed

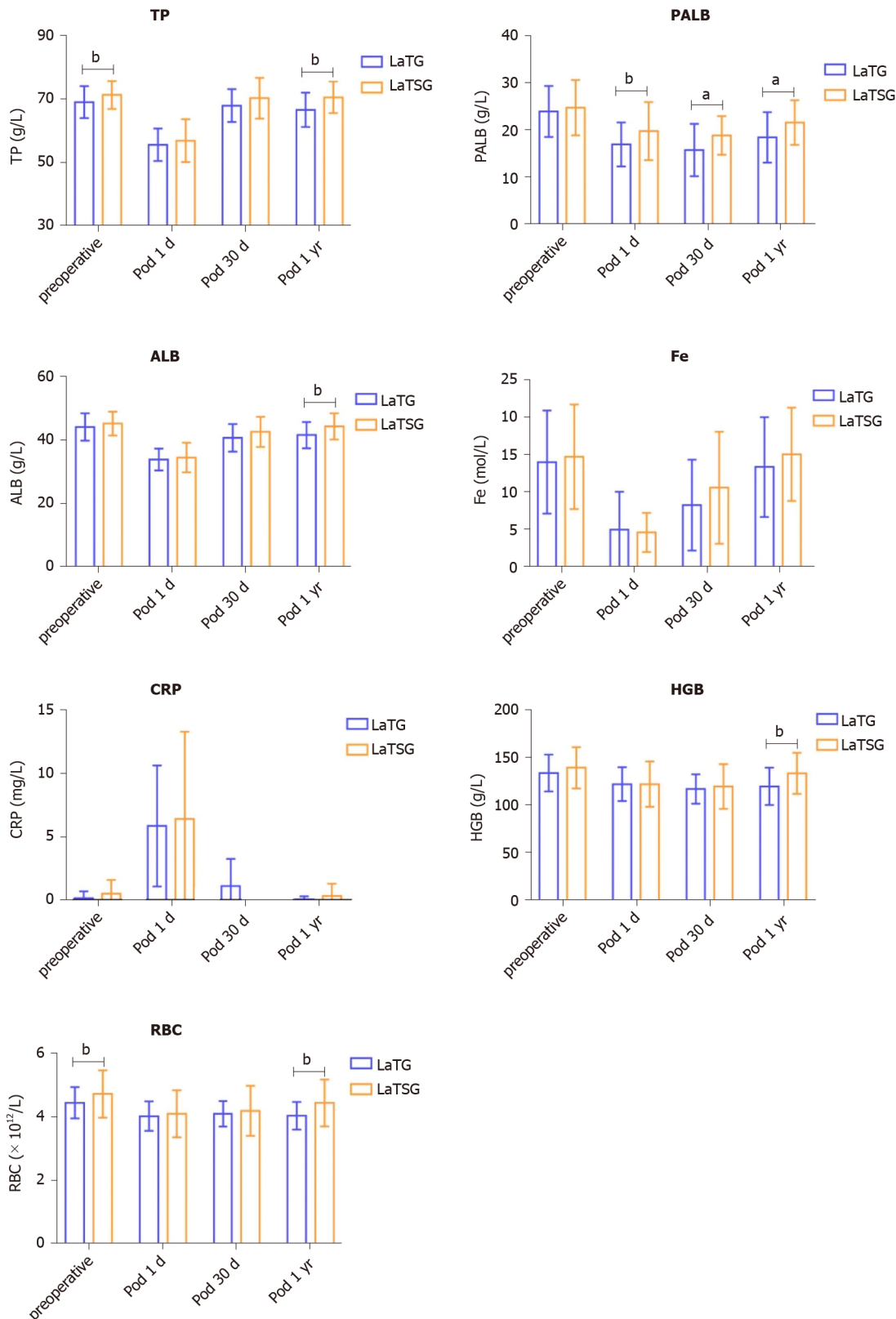


Figure 4 Mean preoperative and postoperative levels of albumin, total protein, prealbumin, red blood cell counts, hemoglobin, C-reaction protein, and ferrous iron in patients undergoing laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy. ^a $P < 0.05$; ^b $P < 0.01$. On the first day postoperatively, these nutritional indices did not significantly differ between the groups. However, at 12 mo post-surgery, albumin, total protein, hemoglobin, and red blood cell levels were significantly higher in the laparoscopic-assisted tailored subtotal gastrectomy group than in the laparoscopic assisted total gastrectomy group ($P < 0.05$). No significant differences in ferrous iron, prealbumin, or C-reaction protein levels were found between the two groups ($P > 0.05$) (Table 4). TP: Total protein; PALB: Prealbumin; ALB: Albumin; Fe: Ferrous iron; CRP: C-reaction protein; HGB: Hemoglobin; RBC: Red blood cell; LaTG: Laparoscopic assisted total gastrectomy; LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy.

that compared to TG, distal gastrectomy was shown to improve the survival of patients with stage III cancer, but not for other stages^[16,18]. According to the above analyses, compared with LaTG, LaTSG did not increase local recurrence of advanced middle-third stomach carcinoma, but prolonged the overall survival.

Limitations

There is some bias in this study. This study was a retrospective study and involved only a few patients. Further randomized control trials and more enrolled patients are needed to help validate our findings. We have yet to assess the patients' quality of life with a long-term follow-up duration. Furthermore, we did not test vitamin B12 and vitamin E levels, which could better reflect the nutritional status of patients after surgery.

CONCLUSION

Our results suggest that LaTSG is a safer procedure than LaTG in both short and long-term outcomes. The long-term survival of patients who undergo LaTSG is better than that of patients who undergo LaTG. Furthermore, LaTSG may have an advantage over LaTG in improving the postoperative nutritional status and preventing reflux oesophagitis.

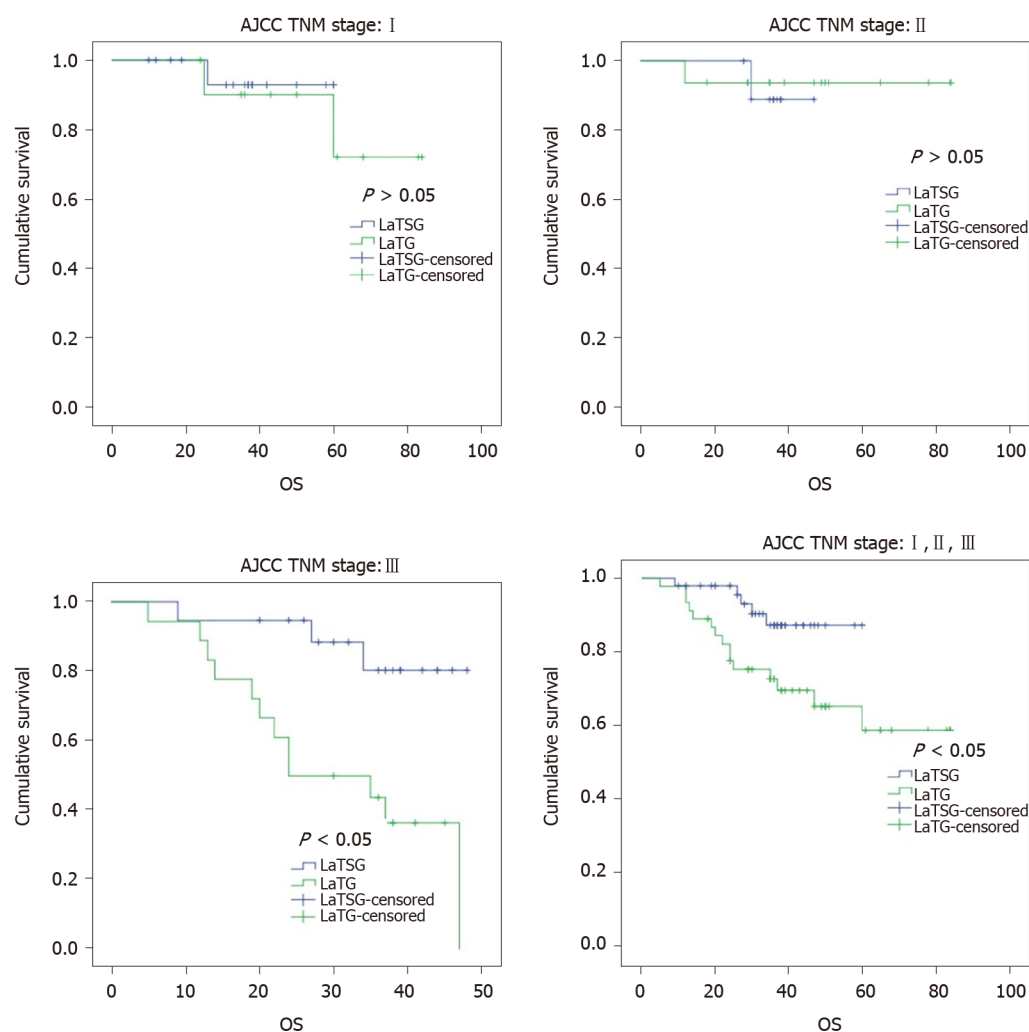


Figure 5 Overall survival curves of patients in the laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy groups. Overall survival was better in the laparoscopic-assisted tailored subtotal gastrectomy (LaTSG) group than in the laparoscopic assisted total gastrectomy (LaTG) group. Subgroup analysis showed that compared to LaTG, LaTSG improved the survival of patients with stage III cancer, but not for other stages (stages I and II). LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy.

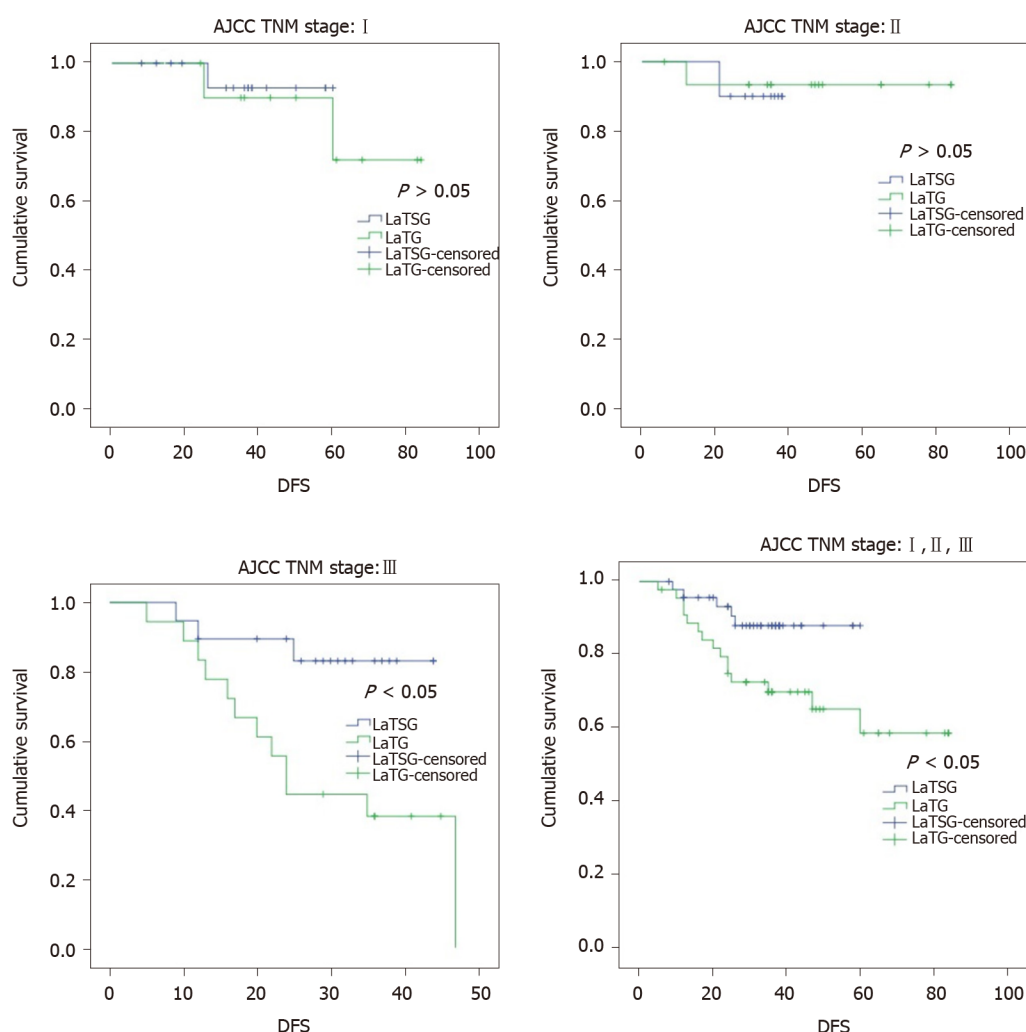


Figure 6 Disease-free survival curves of patients in the laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy groups. Disease-free survival rate was better in the laparoscopic-assisted tailored subtotal gastrectomy (LaTSG) group than in the laparoscopic assisted total gastrectomy (LaTG) group. Subgroup analysis showed that compared to LaTG, LaTSG improved the disease free survival of patients with stage III cancer, but not for other stages. LaTSG: Laparoscopic-assisted tailored subtotal gastrectomy; LaTG: Laparoscopic assisted total gastrectomy.

ARTICLE HIGHLIGHTS

Research background

Consensus on the optimal surgical treatment strategy for advanced gastric cancer located in the middle of the stomach is yet to be established. Most patients ultimately undergo total gastrectomy (TG).

Research motivation

TG is associated with reduced nutritional status, and higher rates of postoperative complications. We modified the laparoscopic subtotal gastrectomy procedure to treat advanced middle-third gastric cancer.

Research objectives

This study aimed to evaluate short-term postoperative patient outcomes, nutritional status, and long-term oncological outcomes to assess the safety and efficacy of laparoscopic-assisted tailored subtotal gastrectomy (LaTSG) compared to those of laparoscopic-assisted total gastrectomy (LaTG).

Research methods

This study retrospectively analyzed surgical and oncological outcomes and postoperative nutritional status in 92 consecutive patients with middle-third gastric cancer who underwent LaTSG (47 cases) or LaTG (45 cases) at Department of

Pancreatic Stomach Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College between 2013 and 2017.

Research results

The incidence of postoperative morbidities was lower in the LaTSG group than in the LaTG group (4.2% *vs* 17.8%, $P < 0.05$). At postoperative 12 mo, albumin, prealbumin, total protein, hemoglobin levels, and red blood cell counts were significantly higher in the LaTSG group than in the LaTG group ($P < 0.05$). Endoscopic examination demonstrated that reflux oesophagitis was more common in the LaTG group (0% *vs* 11.1%, $P < 0.05$). Kaplan–Meier analysis showed a significant improvement in the overall survival (OS) and disease free survival (DFS) in the LaTSG group.

Research conclusions

LaTSG is a safer procedure than LaTG in terms of both short and long-term outcomes. The long-term survival of patients who undergo LaTSG is better than that of patients who undergo LaTG.

Research perspectives

Further randomized control trials and more enrolled patients are needed to help validate our findings.

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Retrospective Cohort Study

Role of doublecortin-like kinase 1 and leucine-rich repeat-containing G-protein-coupled receptor 5 in patients with stage II/III colorectal cancer: Cancer progression and prognosis

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Abstract

BACKGROUND

Cancer stem cells (CSCs) are a subpopulation of cancer cells with the potential of self-renewal and differentiation. CSCs play critical roles in tumorigenesis, recurrence, metastasis, radiation tolerance and chemoresistance.

AIM

To assess the expression patterns and clinical potential of doublecortin-like kinase 1 (DCLK1) and leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), as prognostic CSC markers of colorectal cancer (CRC).

METHODS

The expression of DCLK1 and Lgr5 in CRC tissue sections from 92 patients was determined by immunohistochemistry. Each case was evaluated using a combined scoring method based on signal intensity staining (scored 0-3) and the

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proportion of positively stained cancer cells (scored 0-3). The final staining score was calculated as the intensity score multiplied by the proportion score. Low expression of DCLK1 and Lgr5 was defined as a score of 0-3; high expression of DCLK1 and Lgr5 was defined as a score of ≥ 4 . Specimens were categorized as either high or low expression, and the correlation between the expression of DCLK1 or Lgr5 and clinicopathological factors was investigated.

RESULTS

DCLK1 and Lgr5 expression levels were significantly positively correlated. CRC patients with high DCLK1, Lgr5 and DCLK1/Lgr5 expressions had poorer progression-free survival and overall survival. Moreover, high expression of DCLK1 was an independent prognostic factor for recurrence and overall survival in patients with CRC by multivariate analysis ($P = 0.026$ and $P = 0.049$, respectively).

CONCLUSION

DCLK1 may be a potential CSC marker for the recurrence and survival of CRC patients.

Key Words: Colorectal cancer; Cancer stem cells; Doublecortin-like kinase 1; Leucine-rich repeat-containing G-protein-coupled receptor 5; Cancer prognosis; Cancer progression

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Core Tip: The role of doublecortin-like kinase 1 (DCLK1) and leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) in patients with stage II/III colorectal cancer (CRC) remains uncertain. In this study, we found a positive correlation between the expression of DCLK1 and Lgr5, suggesting that DCLK1 and Lgr5 were involved in the malignant pathological development of CRC. High DCLK1 expression could predict the risk of recurrence and survival in CRC patients after surgery, which may be used as a potential cancer stem cells marker for the recurrence and survival of stage II/III CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common malignant tumor worldwide, with 1.8 million new cases annually. In China, CRC has caused more than 800000 deaths, and its incidence is increasing every year^[1,2]. Despite significant improvements in the management of CRC, distant metastases and relapse remain the major causes of patient mortality. Cancer stem cells (CSCs) are a subpopulation of cancer cells with the potential of self-renewal and differentiation. CSCs play critical roles in tumorigenesis, recurrence, metastasis, radiation tolerance and chemoresistance.

Doublecortin-like kinase 1 (DCLK1), a microtubule-associated protein, has been regarded as a CSC marker receiving considerable attention. Nakanishi *et al*^[3] showed that DCLK1-positive CRC cells mark a subset of tumor cells with higher potential for tumor initiation, sphere formation and *in vivo* tumorigenicity. DCLK1 distinguishes CSCs from normal stem cells in CRC. Combination treatment with fluorouracil (5-FU) and the DCLK1 inhibitor, LRRK2-IN-1 (LRRK), decreased 5-FU-induced phosphorylation of Chk1 and canceled 5-FU-induced cell-cycle arrest at the S phase. Suehiro *et al*^[4] suggested that a combination of 5-FU and LRRK may be an effective, novel approach for the treatment of CRC. DCLK1-positive tumor cells exhibited spheroid formation and tumorigenesis in mouse pancreas^[5]. Targeting DCLK1-expressing cells in hepatocellular and pancreatic carcinoma revealed that this marker

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may be a reliable molecule in targeted therapeutic strategies^[6,7]. However, contradictory observations have been reported in which patients with CRC exhibiting high DCLK1 expression had longer survival times than patients with low DCLK1 expression^[8]. Further study may be needed to define the role of DCLK1 in CRC development and progression.

Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) belongs to the family of G-protein-coupled receptors, which contain 17 Leucine-rich repeats and a transmembrane domain containing an α -helix. Lgr5 is a marker of normal intestinal stem cells and colorectal CSCs^[9,10]. Lgr5 plays an important role in the pathogenesis of gastric cancer and CRC^[11-13], and Lgr5 expression is closely related to tumorigenesis, chemotherapy resistance and recurrence of gastric cancer and CRC^[14,15]. High Lgr5 expression is associated with a poor prognosis in stage IV CRC^[16]. Therefore, Lgr5 is considered an indicator of poor outcome and is a potential target of CRC; however, other reports have shown increased Lgr5 expression in well-differentiated and early-stage gastric carcinomas^[17,18].

Designing novel targeting drugs based on specific CSC markers is the goal of CSC therapy. Due to the lack of clear understanding of the potency of DCLK1 in CRC in previous studies, its expression and clinical significance were determined in an extensive collection of CRC samples. Additionally, we evaluated the potential CSC marker, Lgr5, in the same series of CRC samples, considering the possible similarities between gastric cancer and CRC. The aim of this study was to identify the relationship between DCLK1 and Lgr5 in CRC, determine their clinical significance as CSC markers for the recurrence and survival of stage II/III CRC patients, and lay the foundation for further study on the role of DCLK1 and Lgr5 in CRC stem cells. To our knowledge, this is the first study to investigate the relationship between clinicopathological parameters and the prognostic value of these CSC markers in CRC.

MATERIALS AND METHODS

Clinical data

In total, 92 patients with CRC from Peking University, Shenzhen Hospital, from August 2007 to February 2016 were studied. All patients were pathologically confirmed to have stage II/III CRC and had surgical tumor or nontumor tissues stored before therapy. Clinicopathological parameters, including age, gender, and depth of penetration, lymph node metastasis, pathological tumor-node-metastasis (TNM) stage, tumor differentiation, and primary tumor site were documented in a database and were fully anonymous throughout the study (Table 1). We excluded patients if they had previous chemoradiotherapy treatment or metachronous or synchronous cancers. Patients' clinical data were retrospectively obtained from their medical records, and the last follow-up was in March 2019.

Immunohistochemistry

A total of 92 formalin-fixed, paraffin-embedded samples were cut into sections 5- μ m thick and stained using a standard-chain polymer-conjugated technique. The tissue sections were dewaxed, antigens were retrieved in an autoclave for 10 min, and the sections were then cooled to room temperature. Endogenous peroxidases were blocked by incubating the sections in 3% hydrogen peroxide (kit-0014, Maxim, Fuzhou, China). The sections were then incubated overnight at 4 °C with the following primary antibodies: Rabbit polyclonal anti-DCLK1 (1:100 dilution, ab31704; Abcam, United Kingdom) and rabbit polyclonal anti-Lgr5 (1:50 dilution, ab75732; Abcam, United Kingdom). The slides were stained and visualized with a standard immunohistochemistry kit (kit-0014, Maxim, Fuzhou, China). Colorectal cancer tissues with intense immunoreactivity to DCLK1 and Lgr5 were used as positive controls; in the negative control, the primary antibody was replaced with phosphate-buffered saline (PBS).

Scoring system

DCLK1 and Lgr5 staining was evaluated using a coded semiquantitative scoring system, and the evaluator was blinded to the clinical and pathological parameters^[19]. A pathologist diagnosed the samples, and two observers scored the immunostained slides semiquantitatively after examining a series on a double-headed microscope. A pathologist also confirmed the results to provide a comprehensive review of section staining. Initially, the slides were scanned at 10 \times magnification to obtain a general impression of the overall tumor cell distribution^[20]. Positive cells were then assessed

Table 1 Correlation of immunoreactivity of doublecortin-like kinase 1 and leucine-rich repeat-containing G-protein-coupled receptor 5 with clinicopathological features

Clinicopathologic characteristics	n (%)	DCLK1		P value	Lgr5		P value
		Low (n = 48)	High (n = 44)		Low (n = 62)	High (n = 30)	
Age (yr)							
≤ 49	49 (53)	26 (53)	23 (47)	0.856	33 (67)	16 (33)	0.992
> 49	43 (47)	22 (51)	21 (49)		29 (67)	14 (33)	
Gender							
Male	52 (57)	29 (56)	23 (44)	0.431	37 (71)	15 (29)	0.380
Female	40 (43)	19 (47)	21 (53)		25 (63)	15 (37)	
Depth of penetration							
T3	18 (20)	6 (33)	12 (67)	0.074	13 (72)	5 (28)	0.626
T4	74 (80)	42 (57)	32 (43)		49 (66)	25 (34)	
Lymph node							
N0	38 (41)	20 (53)	18 (47)	0.892	27 (71)	11 (29)	0.787
N1	41 (45)	22 (54)	19 (46)		27 (66)	14 (34)	
N2	13 (14)	6 (12)	7 (16)		8 (62)	5 (38)	
Tumor stage (TNM)							
II	38 (41)	20 (55)	18 (45)	0.941	27 (71)	11 (29)	0.530
III	54 (59)	28 (52)	26 (48)		35 (65)	19 (35)	
Differentiation							
Well/moderate	66 (72)	37 (56)	29 (44)	0.234	48 (73)	18 (27)	0.082
Poor/mucinous	26 (28)	11 (42)	15 (58)		14 (54)	12 (46)	
Tumor site							
Left	42 (46)	24 (57)	18 (43)	0.537	32 (76)	10 (24)	0.032
Right	17 (18)	7 (41)	10 (59)		7 (41)	10 (59)	
Rectum	33 (36)	17 (52)	16 (48)		23 (70)	10 (30)	
Postoperative adjuvant chemotherapy							
Xeloda monotherapy	7 (7)	4 (57)	3 (43)	0.783	5 (71)	2 (29)	0.763
FOLFOX regimen	32 (35)	18 (56)	14 (44)		20 (63)	12 (37)	
XELOX regimen	53 (58)	26 (49)	27 (51)		37 (70)	16 (30)	
CEA							
Normal	68 (74)	38 (56)	30 (44)	0.231	45 (66)	23 (34)	0.676
High	24 (26)	10 (42)	14 (58)		17 (71)	7 (29)	
CA19-9							
Normal	66 (72)	35 (53)	31 (47)	0.793	45 (68)	21 (32)	0.797
High	26 (28)	13 (50)	13 (50)		17 (67)	9 (33)	
MSI							
MSS	89 (97)	46 (52)	43 (48)	0.609	60 (67)	29 (33)	0.978
MSI	3 (3)	2 (67)	1 (33)		2 (67)	1 (33)	
Recurrence							
Absent	67 (73)	37 (55)	30 (45)	0.338	44 (66)	23 (34)	0.565

Present	25 (27)	11 (44)	14 (56)		18 (72)	7 (28)	
Lung metastasis							
Absent	80 (87)	40 (50)	40 (50)	0.281	52 (65)	28 (35)	0.206
Present	12 (13)	8 (67)	4 (33)		10 (83)	2 (17)	
Liver metastasis							
Absent	83 (90)	42 (51)	41 (49)	0.359	55 (66)	28 (34)	0.484
Present	9 (10)	6 (67)	3 (33)		7 (78)	2 (22)	

DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5; TNM: Tumor-node-metastasis.

semiquantitatively at higher magnifications, and the final scores were determined. DCLK1 and Lgr5 expression levels in CRC were assessed using three scoring methods: Staining intensity, proportion of positive cells and final score. The immunostaining intensity was divided into four categories: 0 (no immunostaining), 1 (weak immunostaining), 2 (moderate immunostaining) and 3 (strong immunostaining). Using the proportion of positive cells, the protein expression levels were semiquantitated and scored from 0 to 3 as follows: 0 (positive cells < 5%), 1 (positive cells 5%-30%), 2 (positive cells 31%-60%), and 3 (positive cells > 60%). The final staining score was calculated as the intensity score multiplied by the proportion score, and a score of 0, 1, 2, 3, 4, 6 and 9 was given^[21]. Low expression of DCLK1 and Lgr5 was defined as a score of 0-3; high expression of DCLK1 and Lgr5 was defined as a score of ≥ 4 .

Statistical analysis

The software SPSS (version 16, United States) was used to analyze the findings. Pearson's Chi-square and Spearman's correlation tests were applied to evaluate the correlation between DCLK1 and Lgr5 expression and clinicopathological parameters. Cumulative survival of the patients was estimated using the Kaplan-Meier method, and the significance of the survival differences was tested using the log rank test. Multivariate analysis was performed using a Cox proportional hazards regression model to examine the interaction between DCLK1 and Lgr5 expression and other clinicopathological variables and to estimate the independent prognostic effect of DCLK1 and Lgr5 on survival by adjusting for confounding factors. A difference of $P < 0.05$ between the groups was considered statistically significant.

RESULTS

Patient and clinicopathological characteristics

The mean age of the 92 CRC patients was 49 years (range 15-79 years). Fifty-two patients were male (57%). Eighteen CRC patients (20%) showed T3 depth of penetration, whereas 74 patients (80%) showed T4. For the patients with available regional lymph node metastasis, 38 (41%) were category N0, 41 (45%) were N1, and 13 (14%) were N2. Based on tumor distant metastasis staging, 38 CRC cases (41%) were stage II and 54 (59%) were stage III. In terms of tumor differentiation, 66 (72%) and 26 (28%) cases had well/moderate and poor/mucinous differentiation, respectively. Forty-two tumors (46%) were in the left colon, 17 (18%) were in the right colon, and 33 (36%) were in the rectum. Table 1 summarizes the clinicopathological characteristics of these patients. The relationships between preoperative CEA/CA19-9/adjuvant chemotherapy/MSI/recurrence/lung metastases/liver metastases and DCLK1/Lgr5 are shown in Tables 1 and 2.

DCLK1 and Lgr5 expression and association with clinicopathological parameters

Immunohistochemical findings showed that DCLK1 expression was mainly localized in the membranous area of CRC cells. Low DCLK1 expression was found in 48 cases (52%), while high DCLK1 expression was seen in 44 cases (48%) (Figure 1). DCLK1 expression and clinicopathological parameters were not significantly correlated (Table 1). Immunodetection of Lgr5 expression showed that it was generally localized in the cytoplasmic area of tumor cells. In the light of the final score, 67% of cases (62/92) displayed low Lgr5 expression (score of 0-3), and 33% showed high Lgr5

Table 2 Correlation of immunoreactivity of doublecortin-like kinase 1/leucine-rich repeat-containing G-protein-coupled receptor 5 with clinicopathological features

Clinicopathologic characteristics	n (%)	DCLK1/Lgr5 phenotypes				P value
		DCLK1 ^{Low} /Lgr5 ^{Low}	DCLK1 ^{Low} /Lgr5 ^{High}	DCLK1 ^{High} /Lgr5 ^{Low}	DCLK1 ^{High} /Lgr5 ^{High}	
Age (yr)						
≤ 49	49 (53)	23 (47)	3 (6)	10 (20)	13 (27)	0.890
> 49	43 (47)	18 (42)	4 (9)	11 (26)	10 (23)	
Gender						
Male	52 (57)	25 (48)	4 (8)	12 (23)	11 (21)	0.348
Female	40 (43)	16 (40)	3 (7)	9 (23)	12 (30)	
Depth of penetration						
T3	18 (20)	5 (28)	1 (6)	8 (44)	4 (22)	0.223
T4	74 (80)	36 (49)	6 (8)	13 (17)	19 (26)	
Lymph node						
N0	38 (41)	17 (45)	3 (8)	10 (26)	8 (21)	0.858
N1	41 (45)	18 (44)	4 (10)	9 (22)	10 (24)	
N2	13 (14)	6 (46)	0 (0)	2 (15)	5 (39)	
Tumor stage (TNM)						
II	38 (41)	17 (45)	3 (8)	10 (26)	8 (21)	0.774
III	54 (59)	24 (45)	4 (7)	11 (20)	15 (28)	
Differentiation						
Well/moderate	66 (72)	32 (48)	5 (8)	16 (24)	13 (20)	0.115
Poor/mucinous	26 (28)	9 (35)	2 (8)	5 (19)	10 (38)	
Tumour site						
Left	42 (46)	22 (52)	2 (5)	10 (24)	8 (19)	0.187
Right	17 (18)	4 (23)	3 (18)	3 (18)	7 (41)	
Rectum	33 (36)	15 (46)	2 (6)	8 (24)	8 (24)	
Postoperative adjuvant chemotherapy						
Xeloda monotherapy	7 (7)	3 (43)	1 (14)	21 (29)	22 (14)	0.875
FOLFOX regimen	32 (35)	15 (47)	3 (9)	5 (16)	9 (28)	
XELOX regimen	53 (58)	23 (43)	3 (6)	14 (26)	13 (25)	
CEA						
Normal	68 (74)	33 (49)	5 (7)	12 (18)	18 (26)	0.238
High	24 (26)	8 (33)	2 (8)	9 (38)	5 (21)	
CA19-9						
Normal	66 (72)	29 (44)	6 (9)	16 (24)	15 (23)	0.710
High	26 (28)	12 (46)	1 (4)	5 (19)	8 (31)	
MSI						
MSS	89 (97)	39 (44)	7 (8)	21 (25)	22 (23)	0.713
MSI	3 (3)	2 (67)	0 (0)	0 (0)	1 (33)	
Recurrence						
Absent	67 (73)	30 (45)	7 (10)	14 (21)	16 (24)	0.374

Present	25 (27)	11 (44)	0 (0)	7 (28)	7 (28)	
Lung metastasis						
Absent	80 (87)	33 (41)	7 (9)	19 (24)	21 (26)	0.365
Present	12 (13)	8 (66)	0 (0)	2 (17)	2 (17)	
Liver metastasis						
Absent	83 (90)	35 (42)	7 (9)	20 (24)	2 (25)	0.478
Present	9 (10)	6 (67)	0 (0)	1 (11)	2 (22)	

DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5; TNM: Tumor-node-metastasis.

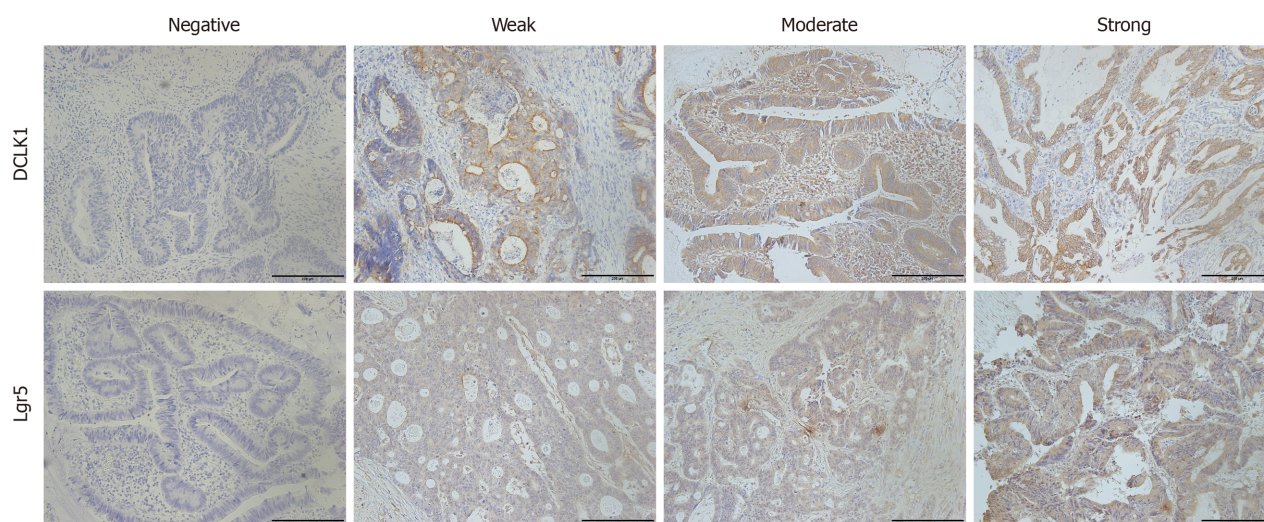


Figure 1 Representative doublecortin-like kinase 1 and leucine-rich repeat-containing G-protein-coupled receptor 5 immunohistochemical staining in colorectal cancer samples. Doublecortin-like kinase 1 (DCLK1) was expressed at the membrane, and leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) was expressed in the cytoplasm of colorectal cancer cells. Expression levels of DCLK1 and Lgr5 were scored as negative (0), weak (1), moderate (2), or strong (3) staining intensity (original magnification 200 ×). DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5.

expression (Figure 1). Our analysis indicated that patients with primary tumor in the left colon had higher Lgr5 expression levels ($P = 0.032$) (Table 1). Lgr5 expression and other clinicopathological parameters were not significantly correlated (Table 1).

Combined analysis of DCLK1 and Lgr5 expression in CRC

A comparison of the expression patterns of DCLK1 and Lgr5 markers showed that these two markers had reciprocal significant correlation in the same series of CRC samples ($P < 0.001$, Figure 2). Among the 92 CRC samples, 41 (44%) showed the DCLK1^{Low}/Lgr5^{Low} phenotype, 7 (8%) showed the DCLK1^{Low}/Lgr5^{High} phenotype, 21 (23%) showed the DCLK1^{High}/Lgr5^{Low} phenotype, and 23 (25%) showed the DCLK1^{High}/Lgr5^{High} phenotype (Table 2). One-way analysis of variance (ANOVA) and Tukey's post hoc analysis were used to calculate the correlation between DCLK1/Lgr5 phenotypic expression and clinicopathological parameters. It was found that the DCLK1/Lgr5 phenotypic expression was not significantly associated with clinicopathological variables (Table 2).

Progression-free and overall survival analysis in CRC patients

Progression-free survival (PFS) and overall survival (OS) differed significantly according to the immunoreactivity of DCLK1 and Lgr5 expression levels. Analysis using the Kaplan-Meier method and log-rank tests showed a lower survival rate in the DCLK1^{High} group compared with the DCLK1^{Low} group (PFS: $P = 0.003$, OS: $P = 0.003$, Figure 3A and B). The prognostic impact of Lgr5 expression in CRC was similar to that of DCLK1 (PFS: $P = 0.037$, OS: $P = 0.036$, Figure 3C and D). Moreover, patients with co-expression of DCLK1^{High}/Lgr5^{High} had a poorer prognosis than the other groups (PFS: $P = 0.003$, OS: $P = 0.008$, Figure 3E and F), including the DCLK1^{Low}/Lgr5^{Low}, DCLK1^{Low}

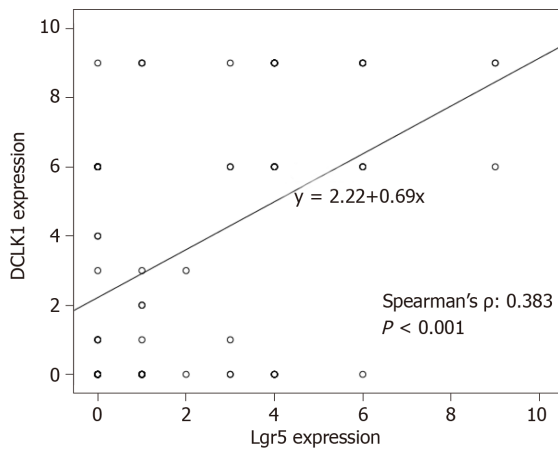


Figure 2 Correlation between doublecortin-like kinase 1 and leucine-rich repeat-containing G-protein-coupled receptor 5 immuno-histochemical expression in colorectal cancer specimens. Doublecortin-like kinase 1 (DCLK1) and leucine-rich repeat-containing G-protein-coupled receptor 5 expression were positively significantly correlated ($r = 0.206$, $P < 0.001$). DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5.

/Lgr5^{High}, and DCLK1^{High}/Lgr5^{Low} phenotypes.

Predictive value of DCLK1 and Lgr5 expression levels for recurrence and survival

A Cox proportional hazards model was used to estimate the effect of DCLK1 and Lgr5 expression on recurrence and survival. Univariate analysis showed that the following factors were significantly related to postoperative recurrence and OS: Tumor stage, differentiation, CEA, CA19-9, DCLK1, Lgr5 and DCLK1/Lgr5 expression level ($P = 0.022$, 0.037 , 0.001 , 0.002 , 0.006 , 0.044 , and 0.006 for recurrence, $P = 0.017$, 0.031 , 0.002 , 0.005 , 0.006 , 0.043 , and 0.012 for OS, respectively). Multivariate analysis confirmed that DCLK1 expression level was an independent predictor of recurrence and OS in patients with CRC (hazard ratio = 4.656, 95% confidence interval = 1.207-17.956, $P = 0.026$ for recurrence, hazard ratio = 4.272, 95% confidence interval = 1.005-18.167, $P = 0.049$ for OS; Tables 3 and 4).

DISCUSSION

Few studies have reported the correlation between DCLK1 and Lgr5 in CRC. The results of this study showed that DCLK1 and Lgr5 immunoreactivity was observed in 53% and 41% of CRC cases, respectively, using standard immunostaining. DCLK1 and Lgr5 expression were positively correlated in CRC tissues. Therefore, DCLK1 may affect Lgr5 expression *via* an unknown mechanism^[3,22,23], and the two complement each other to participate in CRC development, invasion and metastasis^[3,24,25]. It was found that patients with DCLK1^{High}, Lgr5^{High} and DCLK1^{High}/Lgr5^{High} expression had poorer PFS and OS, which implied that high DCLK1 and Lgr5 expression may specifically predict the most aggressive and fatal types of primary CRC in cases with stage II/III disease^[12,26-29]. DCLK1 and Lgr5 are targets of Wnt signaling, which has emerged as a critical regulator of stem cells, and its pathway is integral in both stem cell and cancer cell maintenance and growth^[30,31]. Gastric cancer patients with high DCLK1 expression were shown to have significantly shorter PFS and OS^[26]. Uchida confirmed that Lgr5 was observed in both early and late events in colorectal tumorigenesis^[12]. DCLK1 may be associated with Lgr5 during CRC development. Conversely, contradictory evidence has shown that DCLK1 overexpression is significantly associated with better PFS and OS^[32], and increased Lgr5 expression in well-differentiated and early-stage gastric carcinomas^[17,18]. The exact regulatory mechanism between DCLK1 and Lgr5 requires further exploration.

Despite no association between DCLK1 expression and TNM stage or degree of tissue differentiation being found, DCLK1 was shown to be a potential predictor of tumor recurrence and survival in patients with stage II/III CRC by univariate and multivariate Cox regression analyses, reflecting a more invasive tumor phenotype^[23,33]. The discovery of CSCs suggests intratumoral heterogeneity^[34]. High DCLK1 expression levels indicate more invasive CRC, resulting in recurrence and metastasis, which may be related to the CSC characteristics of some cancer cells^[35]. Other

Table 3 Prognostic value, univariate and multivariate analyses of potential predictor for recurrence

Variables	HR	95%CI	P value
Univariate			
Age (≤ 49 vs > 49 yr)	0.742	0.303-1.818	0.515
Gender (Male vs Female)	0.758	0.302-1.901	0.555
Depth of penetration (T3 vs pT4)	1.310	0.383-4.476	0.006
Lymph node			
N0	1 (ref)		
N1	2.590	0.795-8.439	0.114
N2	7.562	2.189-26.115	0.001
Tumor stage (TNM) (II vs III)	3.610	1.201-10.849	0.022
Differentiation (well/moderate vs poor/mucinous)	2.561	1.058-6.198	0.037
Tumor site			
Left	1 (ref)		
Right	0.735	0.205-2.636	0.637
Rectum	0.834	0.307-2.263	0.721
CEA (normal vs high)	4.722	1.933-11.535	0.001
CA19-9 (normal vs high)	3.961	1.633-9.606	0.002
MSI (MSS vs MSI)	0.047	0.000-1327.112	0.559
Lung metastasis (absent vs present)	2.325	0.844-6.405	0.103
Liver metastasis (absent vs present)	2.351	0.785-7.038	0.127
DCLK1 (low vs high)	3.958	1.496-10.472	0.006
Lgr5 (low vs high)	2.521	1.025-6.199	0.044
DCLK1/Lgr5 (others ¹ vs high/high)	3.808	1.471-9.854	0.006
Multivariate			
Tumor stage (TNM) (II vs III)	6.846	1.918-24.445	0.003
Differentiation (well/moderate vs poor/mucinous)	1.864	0.699-4.971	0.241
CEA (normal vs high)	4.835	1.651-14.154	0.004
CA19-9 (normal vs high)	4.102	1.529-11.005	0.005
DCLK1 (low vs high)	4.656	1.207-17.956	0.026
Lgr5 (low vs high)	0.764	0.076-7.691	0.819
DCLK1/Lgr5 (others ¹ vs high/high)	3.429	0.212-55.583	0.386

¹Low/low low/high and high/low. HR: Hazard ratio; CI: Confidence interval; DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5; TNM: Tumor-node-metastasis.

candidate CSC markers, such as Lgr5, ALDH1, and Musashi-1, also suggest a correlation between tumor expression and poor prognosis in CRC^[36-38]. The present study results are based only on immunohistochemical analysis, which does not quantify RNA or protein expression. Therefore, future studies should quantitatively evaluate DCLK1 using reverse transcriptase polymerase chain reaction or fluorescence-activated cell sorting in both normal and CRC tissues.

Although several putative CRC stem cell markers have been identified, how these markers can be used clinically remains unclear. One study applied nanoparticle-based DCLK1 small interfering RNA in colorectal tumor xenografts, which inhibited tumor growth and downregulated c-Myc and Notch1 expression^[24]. Sureban found that small interfering RNA blockage of DCLK1 reduced *in vivo* tumorigenic potential in CRC. This function was mediated by reducing the primary transcript of MIRLET7 and

Table 4 Prognostic value, univariate and multivariate analyses of potential predictor for survival

Variables	HR	95%CI	P value
Univariate			
Age (≤ 49 vs > 49 yr)	0.681	0.276-1.684	0.406
Gender (Male vs Female)	0.795	0.316-2.001	0.626
Depth of penetration (T3 vs pT4)	0.772	0.226-2.640	0.680
Lymph node			
N0	1 (ref)		
N1	2.774	0.839-9.170	0.094
N2	8.899	2.496-31.723	0.001
Tumor stage (TNM) (II vs III)	3.880	1.274-11.871	0.017
Differentiation (well/moderate vs poor/mucinous)	0.376	0.155-0.913	0.031
Tumour site			
Left	1 (ref)		
Right	0.798	0.221-2.880	0.731
Rectum	0.872	0.319-2.384	0.790
CEA (normal vs high)	0.245	0.101-0.595	0.002
CA19-9 (normal vs high)	3.569	1.477-8.621	0.005
MSI (MSS vs MSI)	0.047	0.000-3055.106	0.588
Recurrence (absent vs present)	8.038	3.070-21.050	0.001
Lung metastasis (absent vs present)	2.141	0.776-5.911	0.142
Liver metastasis (absent vs present)	2.216	0.734-6.689	0.158
DCLK1 (low vs high)	4.167	1.491-11.644	0.006
Lgr5 (low vs high)	2.575	1.030-6.438	0.043
DCLK1/Lgr5 (others ¹ vs high/high)	3.273	1.297-8.262	0.012
Multivariate			
Tumor stage (TNM) (II vs III)	6.087	1.647-22.499	0.007
Differentiation (well/moderate vs poor/mucinous)	2.706	0.989-7.406	0.053
CEA (normal vs high)	4.363	1.346-14.136	0.014
CA19-9 (normal vs high)	1.078	0.362-3.208	0.893
Recurrence (absent vs present)	12.002	3.066-46.988	0.001
DCLK1 (low vs high)	4.272	1.005-18.167	0.049
Lgr5 (low vs high)	7.088	0.582-86.342	0.125
DCLK1/Lgr5 (others ¹ vs high/high)	0.593	0.039-9.085	0.708

¹Low/low low/high and high/low. HR: Hazard ratio; CI: Confidence interval; DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5; TNM: Tumor-node-metastasis.

increasing c-Myc expression, both of which are related to the loss of epithelial differentiation^[39]. CSCs are widely chemoresistant and radioresistant, which is a key factor in treatment resistance and cancer recurrence^[40-42]. These studies suggest that DCLK1 may be a CSC marker with a functional role and thus may be an important therapeutic target.

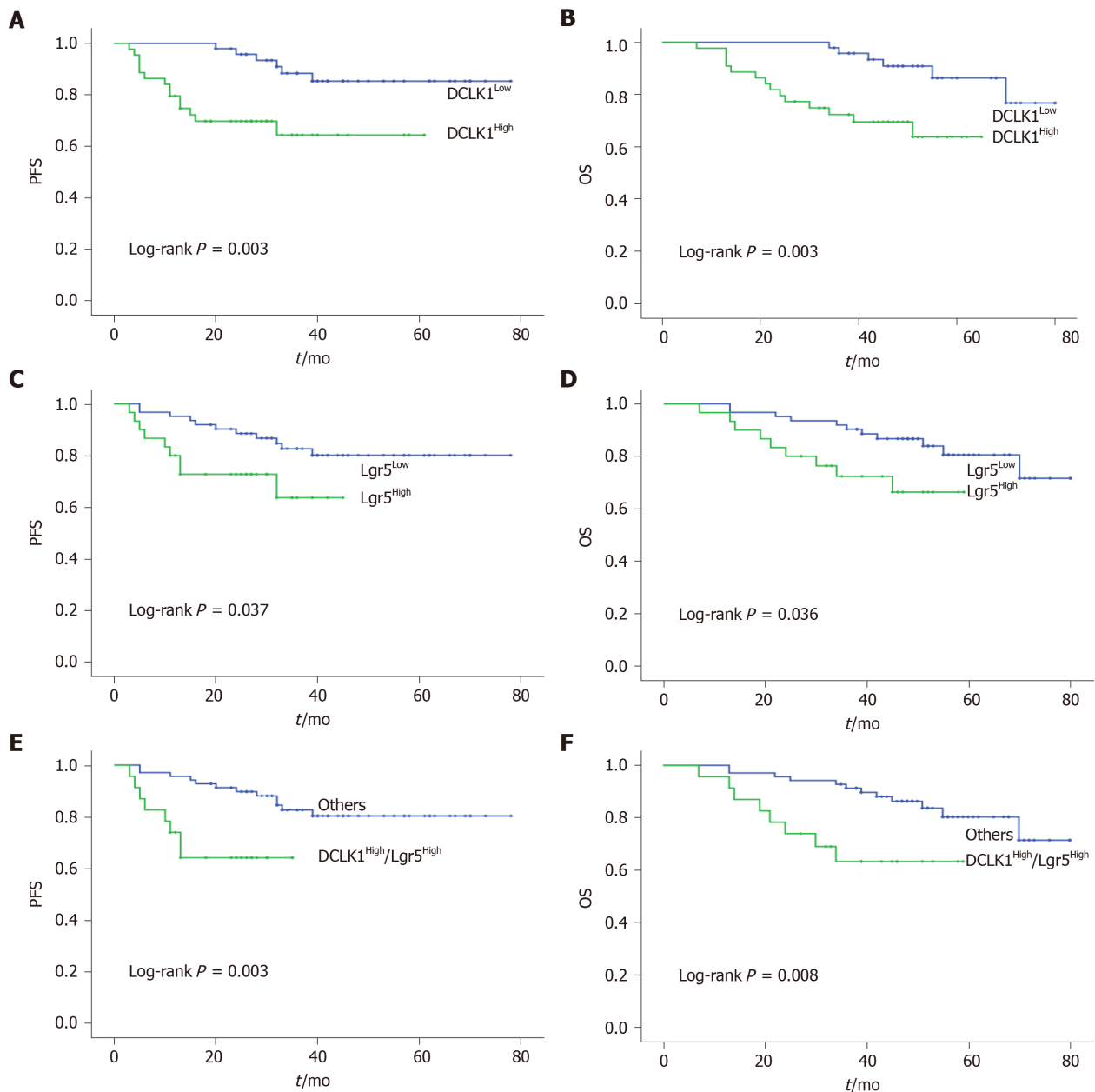


Figure 3 High expression of doublecortin-like kinase 1 and leucine-rich repeat-containing G-protein-coupled receptor 5 was significantly associated with poorer progression-free survival and overall survival in patients with colorectal cancer. Cumulative progression-free survival and overall survival of colorectal cancer patients in different groups were compared. A and B: Doublecortin-like kinase 1 (DCLK1)^{High} and DCLK1^{Low}; C and D: Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5)^{High} and Lgr5^{Low}; E and F: DCLK1^{High}/Lgr5^{High} and others. DCLK1: Doublecortin-like kinase 1; Lgr5: Leucine-rich repeat-containing G-protein-coupled receptor 5; PFS: Progression-free survival; OS: Overall survival.

CONCLUSION

In summary, this study found a positive correlation between the expression of DCLK1 and Lgr5, suggesting that DCLK1 and Lgr5 are involved in the malignant pathological development of CRC. DCLK1^{High}, Lgr5^{High} and DCLK1^{High}/Lgr5^{High} expression resulted in poorer PFS and OS in patients with stage II/III CRC. High DCLK1 expression could predict the risk of recurrence and survival in CRC patients after surgery, which may be used as a potential CSC marker for the recurrence and survival of stage II/III CRC patients. However, further analysis is required to investigate the CSC marker, DCLK1, as a potential early diagnostic and therapeutic target for CRC.

ARTICLE HIGHLIGHTS

Research background

The role of doublecortin-like kinase 1 (DCLK1) and leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) in patients with stage II/III colorectal cancer (CRC) remains uncertain.

Research motivation

Designing novel targeting drugs based on specific cancer stem cell (CSC) markers is the goal of CSC therapy. Due to the lack of clear understanding of the potency of DCLK1 in CRC in previous studies, its expression and clinical significance were determined in an extensive collection of CRC samples.

Research objectives

We determined the clinical significance of DCLK1 and Lgr5 as CSC markers for the recurrence and survival of stage II/III CRC patients.

Research methods

The expression of DCLK1 and Lgr5 in CRC tissue sections from 92 patients was detected by immunohistochemistry.

Research results

In this study, we have found a positive correlation between the expression of DCLK1 and Lgr5, suggesting that DCLK1 and Lgr5 are involved in the malignant pathological development of CRC. High DCLK1 expression could predict the risk of recurrence and survival in CRC patients after surgery.

Research conclusions

DCLK1 may be a potential CSC marker for the recurrence and survival of CRC patients.

Research perspectives

Our research raises the possibility that treatments targeting DCLK1 could be beneficial in CRC patients.

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Clinical Trials Study

Comparison of two supplemental oxygen methods during gastroscopy with propofol mono-sedation in patients with a normal body mass index

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Abstract

BACKGROUND

Hypoxemia due to respiratory depression and airway obstruction during upper gastrointestinal endoscopy with sedation is a common concern. The Wei nasal jet tube (WNJT) is a new nasopharyngeal airway with the ability to provide supraglottic jet ventilation and oxygen insufflation *via* its built-in wall channel. The available evidence indicates that with a low oxygen flow, compared with nasal cannula, the WNJT does not decrease the occurrence of hypoxemia during upper gastrointestinal endoscopy with propofol sedation. To date, there has been no study assessing the performance of WNJT for supplemental oxygen during upper gastrointestinal endoscopy with sedation when a moderate oxygen flow is used.

AIM

To determine whether the WNJT performs better than the nasal prongs for the prevention of hypoxemia during gastroscopy with propofol mono-sedation when a moderate oxygen flow is provided in patients with a normal body mass index.

METHODS

This study was performed in 291 patients undergoing elective gastroscopy with propofol mono-sedation. Patients were randomized into one of two groups to receive either the WNJT (WNJT group, $n = 147$) or the nasal cannula (nasal cannula group, $n = 144$) for supplemental oxygen at a 5-L/min flow during gastroscopy. The lowest SpO₂ during gastroscopy was recorded. The primary endpoint was the incidence of hypoxemia or severe hypoxemia during gastroscopy.

RESULTS

Ethics Committee of Beijing Friendship Hospital, China (Ethics Committee number: 2017-P2-009-02).

Clinical trial registration statement:

This study is registered with the Chinese Clinical Trial Registry (registration No. ChiCTR-IOR-17013089).

Informed consent statement: The written informed consent was obtained from each patient included in the study.

Conflict-of-interest statement: No external funding or competing interests declared.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The authors have read the CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 statement.

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The total incidence of hypoxemia and severe hypoxemia during gastroscopy was significantly decreased in the WNJT group compared with the nasal cannula group ($P = 0.000$). The lowest median SpO₂ during gastroscopy was significantly higher (98%; interquartile range, 97-99) in the WNJT group than in the nasal cannula group (96%; interquartile range, 93-98). Epistaxis by device insertion in the WNJT group occurred in 7 patients but stopped naturally without any treatment. The two groups were comparable in terms of the satisfaction of physicians, anesthetists and patients.

CONCLUSION

With a moderate oxygen flow, the WNJT is more effective for the prevention of hypoxemia during gastroscopy with propofol mono-sedation compared with nasal prongs, but causing slight epistaxis in a few patients.

Key Words: Gastroscopy; Hypoxemia; Wei nasal jet tube; Nasal cannula; Supplemental oxygen; Adverse outcomes

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Core Tip: This study is a prospective randomized controlled trial aimed to determine whether the Wei nasal jet tube (WNJT) performs better than the nasal prongs for the prevention of hypoxemia during gastroscopy with propofol mono-sedation when a moderate oxygen flow is provided in patients with a normal body mass index. Our results show that compared with nasal prongs for supplemental oxygen, the WNJT is more effective for the prevention of hypoxemia during gastroscopy with propofol mono-sedation when a moderate oxygen flow is provided. However, the WNJT caused slight epistaxis in a few patients.

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INTRODUCTION

Endoscopy is an effective method for early detection of gastrointestinal (GI) cancers, as well as a standard technique for the diagnosis and treatment of many GI diseases^[1,2]. However, patients are often reluctant to undergo GI endoscopy while awake due to its uncomfortable nature and adverse reactions, such as nausea, vomiting, anxiety, throat bleeding, and others^[3]. As the use of sedation may significantly reduce patients' discomfort during GI endoscopy, it can improve patient satisfaction, acceptance and compliance with repeated GI endoscopy screening^[4]. Thus, sedation using short-acting intravenous anesthetics, such as propofol and remifentanyl, has been recommended by the international guidelines for upper GI endoscopy^[5-8]. However, hypoxemia due to respiratory depression and airway obstruction during GI endoscopy with sedation is a common concern^[9]. In particular, severe hypoxemia not only requires emergent airway management, such as mask ventilation and even endotracheal intubation, but can also result in an interruption of the endoscopic procedure^[10]. Therefore, prevention of hypoxemia is essential in safe and effective sedation for GI endoscopy.

Currently, both the American Society of Anesthesiologists and the American Society for Gastrointestinal Endoscopy recommend the use of supplemental oxygen to reduce the occurrence of hypoxemia during GI endoscopy with sedation. Furthermore, the nasal cannula is one of most convenient tools for providing supplemental oxygen. It has been shown that compared with patients not receiving supplemental oxygen, those receiving supplemental oxygen using a nasal cannula exhibit a significantly decreased incidence of hypoxemia during GI endoscopy with sedation^[9]. However, the use of nasal cannula cannot overcome upper airway obstruction due to soft tissue collapse and tongue falling, which is a major cause of hypoxemia during GI endoscopy

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in patients receiving deep sedation^[11]. For this condition, a supraglottic airway device, such as the nasopharyngeal airway, might be a good solution because it can be conveniently inserted to ensure upper airway opening without interfering with the gastroscopic procedure^[12]. It has been shown that compared with nasal prongs in obese patients undergoing gastroscopy with intravenous anesthesia, the use of a convenient nasopharyngeal airway for supplemental oxygen results in attenuated SpO₂ reduction and improves the satisfaction of physicians and anesthetists^[13]. However, the insertion of a convenient nasopharyngeal airway is an invasive procedure with the potential risk of airway injury^[13,14].

The Wei nasal jet tube (WNJT; Well Lead Medical Co. Ltd., Guangzhou, China; **Figure 1**) is a new design of special nasopharyngeal airway with two additional channels built inside the wall for jet oxygen supplementation and monitoring of the end-tidal partial concentration of carbon dioxide. In contrast to the convenient nasopharyngeal airways, the WNJT can directly connect to an anesthesia machine to deliver oxygen into the upper airway through its jet ventilation channel. Furthermore, the end-tidal partial concentration of carbon dioxide monitored continuously by another channel of the WNJT can be used as a sign to observe regular breathing airflow in the upper airway and the occurrence of respiratory depression during GI endoscopy with sedation^[15,16]. Currently, there are two sizes of WNJTs commercially available for adult patients with inner diameters of 5.0 mm and 7.0 mm, outer diameters of 7.3 mm and 10.0 mm, and lengths of 145 mm and 155 mm.

Recently, one multicenter, randomized controlled trial assessing the influence of supplemental oxygen with the WNJT on respiration and ventilation during gastroscopy with propofol sedation showed that with a low oxygen flow of 2 L/min, compared with the nasal cannula, the WNJT only decreased use of the jaw-thrust maneuver for upper airway opening but did not reduce the incidences of total adverse events, subclinical respiratory depression, hypoxemia, severe hypoxemia, or facemask ventilation^[15]. It is believed that increasing oxygen flow will improve the efficacy of supplemental oxygen in the upper airway^[17]. However, no study has assessed the performance of WNJT for supplemental oxygen during GI endoscopy with sedation when a moderate oxygen flow is used. Most importantly, the insertion of the WNJT is an invasive procedure with a potential risk of epistaxis. To help anesthesiologists choose appropriate supplemental oxygen methods based on the risk-benefit ratio, we conducted a prospective randomized controlled trial to compare the efficacy and safety of WNJT and nasal prongs for supplemental oxygen during gastroscopy with sedation in patients with a normal body mass index (BMI) when a moderate oxygen flow was used.

MATERIALS AND METHODS

Patient population and study design

After the study protocol was approved by the Institutional Ethics Committee of Beijing Friendship Hospital, China (Ethics Committee number: 2017-P2-009-02) and registered with the Chinese Clinical Trial Registry (registration No. ChiCTR-IOR-17013089), adult patients scheduled for gastroscopy with propofol mono-sedation between November 2017 and December 2018 were recruited. The inclusion criteria were male or female patients aged 18 to 65 years, a BMI of 18 to 25 kg/m², American Society of Anesthesiologists physical status classification 1-2 and the ability to provide informed consent. Exclusion criteria were a history of coagulopathies or nose bleeding; severe cardiac, pulmonary, hepatic or renal diseases; infection of the mouth, the nose, or the throat; and allergy to propofol, eggs, soybean, or albumin.

Written informed consent was obtained from each patient included in the study. According to a random number table generated by a computer, patients were randomly assigned to receive either the WNJT (WNJT group) or the nasal prongs (nasal cannula group) for supplemental oxygen during gastroscopy. All patients fasted for 8 h before gastroscopy. After patients entered the examination room, topical anesthesia of the oral cavity and pharynx was administered by gargling a 2% lidocaine gel (10 g; 0.2 g; Jumpcan Pharmaceutical Group, China), and routine monitoring, including heart rate, noninvasive blood pressure, and pulse oxygen saturation (SpO₂), was performed. After intravenous access was established, the patient was placed in the lateral position, and preoxygenation was performed until an end-tidal oxygen concentration of 88%-90% was reached. To facilitate preoxygenation, patients were asked to take 8 deep breaths in 60 s with 100% oxygen.

After adequate preoxygenation, sedation was induced with slow intravenous

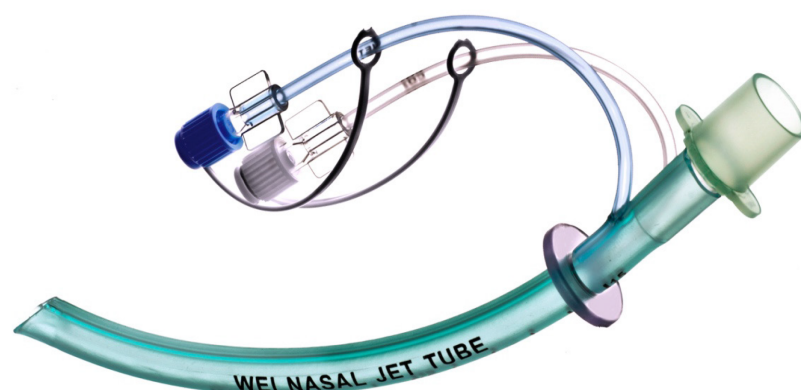


Figure 1 Wei nasal jet tube.

injection of propofol (10 mg/1 mL; Diprivan, Astrazeneca, United Kingdom). Sedation depth was evaluated according to the modified observer's assessment of alertness/sedation (MOAA/S) score^[18]: 5: Responds readily to spoken name; 4: Lethargic response; 3: Response after name called loudly; 2: Response after mild to moderate shaking; 1: Response to trapezius squeeze. The depth of sedation was assessed by an anesthesiologist blinded to the group assignment who was well trained to master the application of the MOAA/S scoring system before the initiation of this study. According to our routine practice, deep sedation with a MOAA/S score of 1 was first obtained to ensure successful gastroscope insertion and to decrease adverse responses to the insertion of the gastroscope into the upper airway. During gastroscopy, moderate sedation with a MOAA/S score of 2 or 3 was maintained with 20-30 mg additional propofol as needed.

Before sedation, two sprays of ephedrine were applied to each nostril in all patients. The selected naris for the placement of the studied devices was the one that the patient thought was more patent. By examining the outer diameter of the WNJT and the size of patients' nostrils, an appropriate WNJT size was selected for each patient in the WNJT group. Using the scale on the exterior wall of the WNJT, the distance between the tip of the nose and earlobe on one side was measured in each patient and was used as the predicted insertion depth of the WNJT. After adequate depth of sedation was obtained before gastroscopy was initiated, the oxygen facemask was removed. Then, both the WNJT and nasal prongs were placed for supplemental oxygen. After inserting the WNJT, its position in the upper airway was re-examined by gastroscopy and adjusted if needed. If the insertion of the WNJT was difficult *via* the selected nasal passage, the other side was tried. If it was still unsuccessful after three attempts, the insertion of the WNJT was regarded as a failure.

During gastroscopy, a moderate oxygen flow of 5 L/min was continuously delivered through the WNJT and nasal cannula in both groups. After gastroscopy, the WNJT and nasal cannula were removed before full recovery from sedation. Consequently, patients were also blinded to their group assignment. The duration of gastroscopy and total dosage of propofol were recorded.

Observed variables

The lowest SpO₂ during gastroscopy was noted. If hypoxemia (SpO₂ < 90%) occurred, we implemented the following measures: (1) No additional drug was administered; (2) Audio or painful stimulation was applied; (3) Oxygen flow was increased from 5 to 8 L/min; (4) Airway opening was performed using conventional maneuvers, including jaw thrust, head extension and head position change; (5) The gastroscope tube was removed and facemask ventilation was performed if necessary; and (6) Endotracheal intubation for artificial ventilation was performed if the above measures were unsuccessful.

The primary outcome was the incidence of hypoxemia (SpO₂ = 75%-89% for < 60 s) and severe hypoxemia (SpO₂ < 75% at any time or < 90% for > 60 s)^[15]. Secondary outcomes included (1) The lowest SpO₂ during gastroscopy; (2) Interventions to manage hypoxemia, including jaw thrust, facemask ventilation, and endotracheal intubation; (3) Adverse events, such as epistaxis, body movement and cough during gastroscopy, and postoperative sore throat; and (4) Satisfaction of anesthetists,

physicians, and patients. Epistaxis was assessed by gastroscopy using a subjective scale: 0, no bleeding; 1, minimal bleeding not requiring suctioning; 2, moderate bleeding requiring suctioning but not hampering visualization; and 3, severe bleeding requiring suctioning and hampering visualization^[19]. If severe epistaxis occurred in the WNJT group, compression hemostasis was first performed; if it did not work, other medical or surgical measures were considered. Postoperative sore throat was assessed at the time of consciousness recovery and 30 min later. At 30 min after the procedure, satisfaction of anesthetists, physicians, and patients was evaluated using a 10-point scale and classified as follows: Poor, 1-4; Fair, 5-7; and Good, 8-10^[20].

Randomization and sample size estimation

Computer-generated randomization sequences were used for group assignment in our study. The randomization sequence was generated by a research assistant who was independent of the study and did not have contact with the study participants. Randomization was performed using opaque sealed envelopes before sedation induction.

Sample size was calculated using Pass software (version 11.0, NCSS, LLC, Kaysville, UT, United States). Two independent proportions of procedures were used. Based on our preliminary study, the incidence of hypoxemia during gastroscopy with propofol mono-sedation was approximately 30%. Thus, a 30% of patients in the nasal cannula group were expected to develop hypoxemia. P1 and P2 were calculated from the assumption that the WNJT would achieve a reduction from 30% to 15% in the incidence of hypoxemia. With $\alpha = 0.05$ and a power of 90%, we estimated that 131 patients per group would be required for our study. If the dropout rate was approximately 10%, a total of 288 patients (144 in each group) would be required.

Statistical analysis

Statistical analysis of data was performed using Statistical Product and Service Solutions (23.0) by a blinded statistician from the Clinical Research Institute of Beijing Friendship Hospital. Data are summarized as the mean \pm SD or median (25th and 75th percentile) for continuous data and as frequency and percentages for categorical data. For continuous data, the characteristics and outcomes of the two groups were compared using Student's *t* test or Wilcoxon-Mann-Whitney test based on viability of the normality assumption. Chi-squared or Fisher's exact tests were used to compare two groups with categorical characteristics and outcomes. A *P* value < 0.05 was considered statistically significant.

RESULTS

The flow chart of included and excluded patients in this study is shown in **Figure 2**. A total of 303 patients were enrolled, and 12 were excluded. Of the 12 excluded patients, 3 were allergic to eggs, 2 had chronic obstructive pulmonary disease, 2 had uremia, one had nasal bone fracture, 3 had incomplete consent forms, and one had missing basic data. Thus, a total of 291 subjects were randomized into the two groups. After randomization, however, 3 patients in the WNJT group were further excluded due to a failed WNJT insertion. Finally, 144 patients in each group were included for data analysis. The baseline characteristics of patients were not significantly different between the groups, but the procedure time and total propofol dosage were significantly lower in the nasal cannula group than in the WNJT group (**Table 1**).

Adverse events and interventions related to hypoxemia are listed in **Table 2**. The median lowest SpO₂ during gastroscopy was 98% (interquartile range, 97, 99) and 96% (interquartile range, 93, 98) in the WNJT and nasal cannula groups, respectively, with a significant difference between the groups. The incidence of severe hypoxemia (0.7% *vs* 1.4%) during gastroscopy was comparable between the two groups (*P* = 1.000), but the incidence of hypoxemia (1.4% *vs* 13.2%, respectively) and the total incidence of hypoxemia and severe hypoxemia (2.1% *vs* 14.6%, respectively) during gastroscopy were significantly lower in the WNJT group than in the nasal cannula group (*P* = 0.000). Furthermore, the use of airway opening maneuvers to correct hypoxemia was reduced in the WNJT group compared with the nasal cannula group (2.8% *vs* 25%, respectively, *P* = 0.000). In the WNJT group, epistaxis occurred in 7 patients, but visible epistaxis was uncommon. In the nasal cannula group, no case experienced epistaxis, but 4 patients required an interruption of the endoscopic procedure for facemask ventilation to correct hypoxemia. In addition, the incidence of epistaxis was significantly higher in the WNJT group than in the nasal cannula group, but other

Table 1 Baseline characteristics of patients, procedure time and total propofol dosage

	WNJT group (n = 144)	Nasal cannula group (n = 144)	P value
Age (yr)	53 (40, 60)	55 (41, 60)	0.537
Gender (M/F)	43/101	46/98	0.702
Body mass index (kg/m ²)	23 (21,24)	22 (21,24)	0.086
ASA physical classification (1/2)	(32/112)	(41/103)	0.223
Baseline SpO ₂ (%)	98 (97, 99)	98 (97, 98)	0.061
Procedure times (min)	5.0 (4.4, 5.2)	4.8 (4.5, 5.0)	0.002
Total propofol dosages (mg)	160 (140, 188)	140 (120, 160)	0.000

Data are presented as median (interquartile range) or number of patients. SpO₂: Pulse oxygen saturation; ASA: American Society of Anesthesiologists; WNJT: Wei nasal jet tube.

Table 2 Hypoxemia and lowest pulse oxygen saturation during the gastroscopy, and interventions related to hypoxemia and adverse events

	WNJT group (n = 144)	Nasal cannula group (n = 144)	P value
Total occurrence of hypoxemia	3 (2.1)	21 (14.6)	0.000
Hypoxemia	2 (1.4)	19 (13.2)	0.000
Severe hypoxemia	1 (0.7)	2 (1.4)	1.000
Lowest SpO ₂	98 (97, 99)	96 (93, 98)	0.000
Airway opening maneuvers	4 (2.8)	36 (25)	0.000
Facemask ventilation	0 (0)	4 (2.8)	0.131
Body movement	8 (5.6)	9 (6.3)	0.803
Cough	10 (6.9)	11 (7.6)	0.821
Epistaxis (0/1/2/3)	0/7/0/0 (4.9)	0 (0)	0.022
Sore throat	2 (1.4)	3 (2.1)	1.000

Data are presented as median (interquartile range) or number of patients (percent). SpO₂: Pulse oxygen saturation; WNJT: Wei nasal jet tube.

adverse events were not significantly different between the two groups.

The two groups were comparable in terms of the satisfaction of physicians, anesthetists and patients (Table 3).

DISCUSSION

Hypoxemia is common during GI endoscopy with sedation^[10]. Although this issue is commonly transient and may spontaneously recover, it can occasionally lead to the need for urgent airway management and interruption of the endoscopic procedure^[11]. It has been reported that propofol deep sedation for GI endoscopy is associated with an increased risk of airway adverse events compared to general anesthesia administered in the operating room^[21]. Most importantly, it is difficult for anesthetists to identify early signs of hypoxemia and manage it promptly, as sedated patients do not exhibit a proper response to hypoxia^[9]. Thus, there is increasing enthusiasm worldwide for exploring effective measures to prevent or decrease the occurrence of hypoxemia during GI endoscopy with sedation.

Given that intravenous propofol alone is often the preferred sedation method for the vast majority of patients undergoing GI endoscopy with a short procedure time^[22], propofol mono-sedation was selected in this study. By comparing the efficacy and safety of WNJT and nasal prongs for supplemental oxygen during gastroscopy with propofol mono-sedation, the primary purpose of the present study was to determine

Table 3 Satisfaction of physicians, anesthetists and patients

	WNJT group (n = 144)			Nasal cannula group (n = 144)			P value
	Good	Fair	Poor	Good	Fair	Poor	
Physicians	143 (99.3)	1 (0.7)	0	139 (96.5)	4 (2.8)	1 (0.7)	0.214
Anesthetists	140 (97.2)	4 (2.8)	0	134 (93.1)	8 (5.6)	2 (1.4)	0.196
Patients	142 (98.6)	2 (1.4)	0	144 (100)	0	0	0.498

Data are presented as number of patients (percent). WNJT: Wei nasal jet tube.

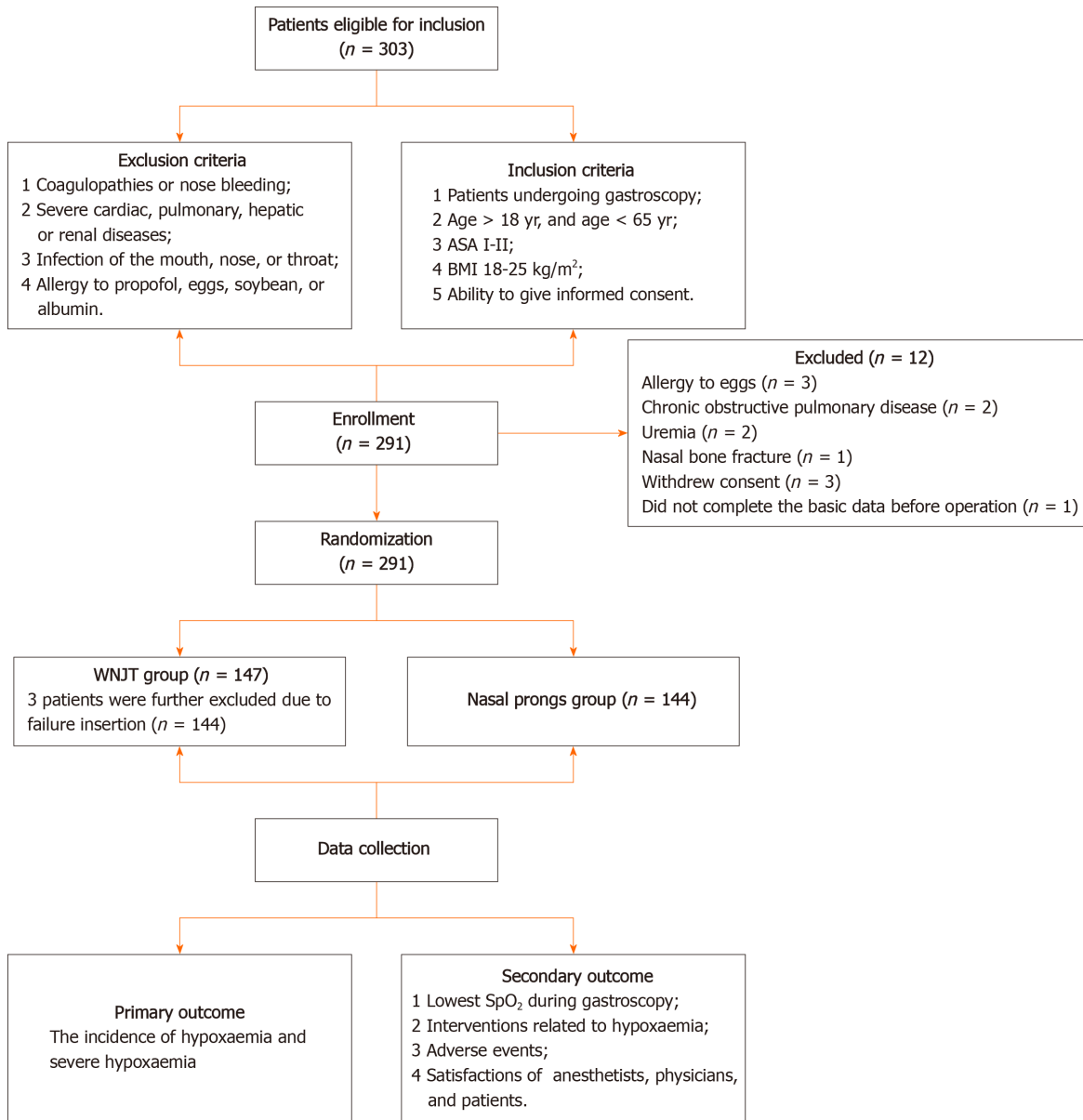


Figure 2 Flow chart of included and excluded patients in this study. ASA: American Society of Anesthesiologists; BMI: Body mass index; WNJT: Wei nasal jet tube.

whether the WNJT performed better than the convenient nasal prongs for providing supplemental oxygen during gastroscopy with propofol mono-sedation in patients with a normal BMI. The primary findings included the following: (1) The use of the WNJT significantly decreased the occurrence of hypoxemia and improved arterial oxygenation level; (2) The incidence of postoperative adverse events was similar in the two groups, but epistaxis by device insertion only occurred in the WNJT group; and

(3) The two devices provided the same satisfaction of physicians, anesthetists and patients.

Qin *et al*^[15] previously assessed the performance of WNJT for supplemental oxygen during gastroscopy with propofol mono-sedation. They showed that compared to nasal prongs, the WNJT only decreased use of the jaw-thrust maneuver but did not decrease the incidences of hypoxemia/severe hypoxemia or the use of facemask ventilation. We noted that the incidence of severe hypoxemia in the WNJT group was comparable between their study and our study (0.2% *vs* 0.7%), but the incidence of hypoxemia in the WNJT group was significantly higher in their study than in our study (8% *vs* 1.4%). This may be due to the following factors. First, a low oxygen flow of 2 L/min was used in their study, while a moderate oxygen flow of 5 L/min was implemented in our study. It is generally believed that increasing oxygen flow improves the efficacy of supplemental oxygen in the upper airway^[17]. Second, a wealth of evidence indicates that adequate preoxygenation enables patients to tolerate a prolonged period of apnea and provides an increased margin of safety^[23,24]. In our center, preoxygenation aimed at obtaining an expiratory oxygen concentration of 88% to 90% is routinely performed before sedation for GI endoscopy. This has been considered a particularly meaningful process for patients who are unable to obtain immediate facemask ventilation when hypoxemia occurs in some conditions, for example, in the course of gastroscopy. Third, in our study, an initial deep sedation was induced by the use of propofol alone to facilitate gastroscope insertion and decrease the adverse events induced by gastroscope insertion. In contrast, a moderate sedation level with an MOAA/S score of 2 or 3 was used in the Qin *et al*^[15] study. The available evidence indicates that when propofol is intravenously injected as a single agent and administered to the level of moderate sedation, patients often present significant responses to GI endoscope insertion, which may interfere with the endoscopic procedure^[4]. If airway topical anesthesia or other drugs are not combined, upper GI endoscope insertion under moderate sedation with propofol can induce significant airway reflexes and increase the occurrence of adverse events^[25].

The WNJT is a new supraglottic airway device that can be used for supraglottic jet oxygenation and ventilation (SJOV), as shown in other studies^[15,16]. Furthermore, Qin *et al*^[15] found that compared to supplemental oxygen using the WNJT, SJOV with the WNJT decreased the occurrence of hypoxemia and adverse events during gastroscopy with propofol mono-sedation when an oxygen flow of 2 L/min was provided. However, SJOV with the WNJT was not applied in our study due to the following factors: (1) Gastroscopy is commonly a short procedure with a duration of less than 5 min in our center; (2) The need for a manual jet ventilator for SJOV increases the complexity of supplemental oxygen; (3) SJOV may result in complications, such as barotrauma, gastric distension, and xerostomia; and (4) Propofol mono-sedation used in our study has been shown to produce reduced respiratory suppression during GI endoscopy compared to sedation schemes combining propofol with other anesthetics^[26]. Actually, the incidence of hypoxemia during gastroscopy was 3% when SJOV with the WNJT was used in the study by Qin *et al*^[15], while the incidence of hypoxemia during gastroscopy was only 1.4% in the WNJT group without SJOV in our study. These results indicate that with a moderate oxygen flow of 5 L/min, the use of the WNJT alone as a nasopharyngeal airway for supplemental oxygen effectively decreases the occurrence of hypoxemia during gastroscopy with propofol mono-sedation, and SJOV is not necessary.

Our results demonstrated that the use of airway opening maneuvers and facemask ventilation to correct hypoxemia was less common in the WNJT group compared to the nasal cannula group. This further supports the effectiveness of WNJT in preventing hypoxemia during gastroscopy with propofol mono-sedation. To determine the risk-benefit ratio of the studied devices, however, adverse effects should always be considered in a clinical trial. Thus, our study compared the complications of two tested devices to enable clinicians to fully consider the study results. Our results showed that adverse events were not significantly different between the groups, but the WNJT occasionally resulted in the occurrence of epistaxis. It has been reported that the incidence of epistaxis caused by inserting the convenient nasopharyngeal airway is approximately 5%-12.5%^[27-29]. In contrast, the incidence of epistaxis by the WNJT insertion was only 0.7%-2% in the study by Qin *et al*^[15] and 4.9% in our study. The low incidence of epistaxis with the WNJT may be due to its soft material texture. However, the incidence of epistaxis with the WNJT was significantly higher in our study than in the Qin *et al*'s^[15] study. As the details of the assessment criteria for epistaxis in their study are not provided, the exact reason for these different findings is unclear. In our experience, after placement of the WNJT, bleeding due to nasal mucosa injury in some patients was noted by gastroscopy, as shown in **Figure 3**, but no visible epistaxis

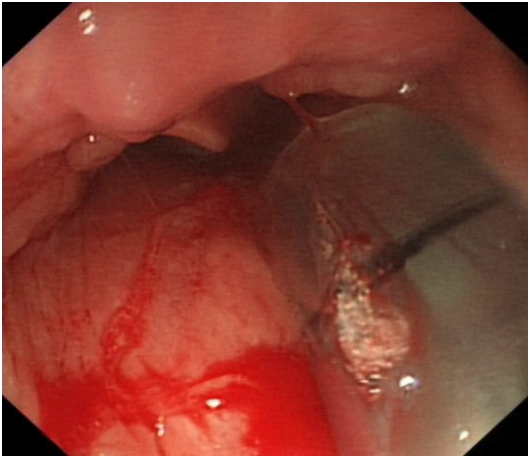


Figure 3 Epistaxis observed by gastroscopy in patients with Wei nasal jet tube placement.

occurred. Thus, we infer that epistaxis defined by gastroscopy may be one of the important reasons for the higher incidence of epistaxis with the WNJT in our study. Moreover, we recommend that when epistaxis is used as an outcome variable in airway studies, the use of endoscopy or laryngoscopy to define the occurrence of nasal mucosa injury may be a more reliable assessment method than visible observation of epistaxis.

Although all nasal bleeding due to the WNJT insertion in our study stopped naturally without any treatment and none of the patients with epistaxis complained of sore throat 30 min after the procedure, care should still be taken to avoid violent insertion of the WNJT. Due to possible stenosis of the nasal cavity, insertion failure of the WNJT occurred in 3 (2.1%) of our patients. The incidence of failed WNJT insertion in our study was similar to findings using a convenient nasopharyngeal airway in the study by Gasparović *et al*^[21].

In our study, both the procedure time and total propofol dosage were significantly decreased in the nasal cannula group compared to the WNJT group. This may be because the insertion of the WNJT requires additional time and can produce stronger stimuli to the nasal passage and the upper airway. Despite all this, the total incidence of hypoxemia and severe hypoxemia was still significantly decreased in the WNJT group. This may primarily be attributed to the upper airway opening and periglottic oxygen delivery by the WNJT. Moreover, the difference in median propofol dosages between groups was only 20 mg. For most adult patients, this small difference in median propofol dosages would not be clinically significant.

Our study also showed that satisfactions of physicians, anesthetists, and patients were not significantly different between groups. These findings may be due to the following factors. (1) The occurrence of adverse events and the use of interventions during GI endoscopy with sedation are the primary determinants for the satisfaction of anesthetists^[30]. Other than a higher incidence of hypoxemia and increased use of convenient airway maneuvers during gastroscopy in the nasal cannula group, the incidences of other adverse events were very low in the two groups and were not significantly different between groups. When hypoxemia occurred during gastroscopy with propofol mono-sedation, the six-step intervention mentioned above was performed in our study. By increasing oxygen flow and opening the upper airway with a jaw-lift maneuver in most patients with hypoxemia, SpO₂ rapidly increased. In addition, the use of a six-step intervention to correct hypoxemia is easy to perform. These may explain why the two methods are comparable with respect to the satisfaction of anesthetists. (2) The use of the WNJT was an invasive procedure, but it was inserted after adequate sedation and removed before full recovery of sedation. Furthermore, epistaxis was only noted by gastroscopy in a few patients using the WNJT, with no visible epistaxis after the procedure noted by patients, and the incidence of postoperative sore throat was the same between the two groups. These factors might be attributed to the same satisfaction of patients in the two groups. And (3) Multiple attempts at gastroscopy insertion by significant body movement and interruption of endoscopy by urgent airway management were the main reasons for the dissatisfaction of physicians^[30,31]. As an initial deep sedation was routinely used in our practice, all body movements observed in this study were slight and did not affect the gastroscopy insertion procedure. Furthermore, the incidence of body movement

was not significantly different between groups. In addition, only 4 patients in the nasal cannula group required an interruption of the gastroscopic procedure for facemask ventilation to correct hypoxemia. Differences in satisfaction of physicians due to this low-incidence event should be further determined.

The main strength of this study is the inclusion of a large sample with consistent GI endoscopic procedures and the use of a prospective randomized controlled design. However, there are still some limitations in our study design that deserve special attention. First, the insertion of the WNJT after sedation and removal of the WNJT before full recovery of sedation blinds patients to the group assignment, but the investigators were not blinded to the studied devices. Thus, this study is only a single-blinded trial and may result in biases in outcome assessment, affecting the power of the results. Second, the duration of gastroscopy in our study was relatively short at approximately 5 min. Therefore, our results should not be extrapolated to other settings with a long duration of endoscopic procedures, such as endoscopic retrograde cholangiopancreatography. Third, the subjects of this study were healthy adults aged 18–65 years with a normal BMI. Thus, our findings are not applicable for older patients and those with an abnormal BMI, as these patients often have comorbidities, increased sensitivity to sedatives and anesthetics, and limited physiological reserves^[13,32]. Further clinical trials are needed to address the above issues.

CONCLUSION

In summary, for patients with a normal BMI, compared to nasal prongs used for supplemental oxygen, the WNJT is more effective for the prevention of hypoxemia during gastroscopy with propofol mono-sedation when a moderate oxygen flow of 5 L/min is provided. However, the WNJT results in slight epistaxis in a few patients. When making a decision about the choice of supplemental oxygen methods for gastroscopy with propofol mono-sedation in patients with a normal BMI, the risk-benefit ratio of using the WNJT should also be considered.

ARTICLE HIGHLIGHTS

Research background

Hypoxemia by respiratory depression and airway obstruction during upper gastrointestinal endoscopy with sedation is a common concern. The nasal cannula is one of most convenient tools for supplemental oxygen, but it cannot overcome upper airway obstruction. Compared to the nasal prongs, the convenient nasopharyngeal airway provides improved efficiency of supplemental oxygen delivery during upper gastrointestinal endoscopy with sedation, but the insertion of the nasopharyngeal airway is an invasive procedure with a potential risk of airway injury.

Research motivation

In view of the significant limitations of available supplemental oxygen methods, it is necessary to identify new effective measures for supplemental oxygen during upper gastrointestinal endoscopy with sedation. The Wei nasal jet tube (WNJT) is a new design of special nasopharyngeal airway made of soft material. The available evidence indicates that with a low oxygen flow, compared to nasal cannula, the WNJT does not decrease the occurrence of hypoxemia during upper gastrointestinal endoscopy with propofol sedation. Given that increasing oxygen flow improves the efficacy of supplemental oxygen in the upper airway, we designed this study to compare the efficacy and safety of WNJT and nasal prongs for supplemental oxygen delivery during gastroscopy with sedation in patients with a normal body mass index when a moderate oxygen flow was provided.

Research objectives

In this study, we aimed to determine whether the WNJT performs better than the nasal prongs for the prevention of hypoxemia during gastroscopy with propofol mono-sedation when a moderate oxygen flow is provided.

Research methods

To address whether the WNJT performs better than the nasal prongs for the

prevention of hypoxemia during gastroscopy with propofol mono-sedation when a moderate oxygen flow is provided, we designed this study as a prospective randomized controlled trial in which patients undergoing elective gastroscopy with propofol mono-sedation were randomized into one of two groups to receive either the WNJT or the nasal cannula supplemental oxygen with a 5-L/min flow. The primary endpoint was the incidences of hypoxemia or severe hypoxemia during gastroscopy.

Research results

A total of 291 subjects were randomized into two groups, but a total of only 144 patients were used for data analysis because 3 patients in the WNJT group were excluded. The total incidence of hypoxemia and severe hypoxemia during gastroscopy was significantly lower in the WNJT group than in the nasal cannula group. In the WNJT group, however, epistaxis by device insertion occurred in 7 patients.

Research conclusions

With a moderate oxygen flow of 5 L/min, compared to nasal prongs, the WNJT is more effective for the prevention of hypoxemia during gastroscopy with propofol mono-sedation, but resulted in slight epistaxis in a few patients.

Research perspectives

With a moderate oxygen flow of 5 L/min, the WNJT performs better than the nasal prongs for the prevention of hypoxemia during gastroscopy with propofol mono-sedation in patients with a normal body mass index. Thus, the WNJT may represent a useful tool for supplemental oxygen during gastroscopy with propofol mono-sedation. Because the WNJT insertion results in a risk of slight epistaxis in a few patients, the risk-benefit ratio of using the WNJT should be considered.

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Observational Study

Barriers for resuming endoscopy service in the context of COVID-19 pandemic: A multicenter survey from Egypt

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Institutional review board

statement: The study was approved by the Institutional Review Board of the National Liver Institute, Menoufia University, Egypt (NLI IRB 00003413) in June 2020, protocol number 00203/2020.

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Abstract

BACKGROUND

The current coronavirus disease 2019 (COVID-19) pandemic has affected routine endoscopy service across the gastroenterology community. This led to the suspension of service provision for elective cases.

AIM

To assess the potential barriers for resuming the endoscopy service in Egypt.

METHODS

A national online survey, four domains, was disseminated over a period of 4 wk in August 2020. The primary outcome of the survey was to determine the impact of the COVID-19 pandemic on the endoscopy service and barriers to the full resumption of a disabled center(s).

RESULTS

A hundred and thirteen Egyptian endoscopy centers participated in the survey. The waiting list was increased by $\geq 50\%$ in 44.9% of areas with clusters of COVID-19 cases ($n = 49$) and in 35.5% of areas with sporadic cases ($n = 62$). Thirty nine (34.8%) centers suffered from staff shortage, which was considered a barrier against service resumption by 86.4% of centers in per-protocol analysis. In multivariate analysis, the burden of cases in the unit locality, staff shortage/recovery and the availability of separate designated rooms for COVID-19 cases could markedly affect the resumption of endoscopy practice ($P = 0.029$, < 0.001 and 0.02 , respectively) and Odd's ratio (0.15, 1.8 and 0.16, respectively).

CONCLUSION

The COVID-19 pandemic has led to restrictions in endoscopic volumes. The staff shortage/recovery and the availability of COVID-19 designed rooms are the most important barriers against recovery. Increasing working hours and dividing endoscopy staff into teams may help to overcome the current situation.

Key Words: COVID-19; Endoscopy; Practice; Pandemic; Egypt; Barriers

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Core Tip: This survey study included 113 endoscopy centers from all over Egypt. Our findings highlighted the barriers for resuming endoscopy in different types of health care facilities in Egypt as well as reporting the current practice in Egyptian endoscopy units during the pandemic. Our study offers real life based snapshot of the current practice and recommendations for routine endoscopic practice during the second wave.

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INTRODUCTION

In December 2019, a novel an enveloped Ribonucleic acid (RNA) beta coronavirus caused an outbreak called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) - resulted in coronavirus disease 2019 (COVID-19) began in Wuhan^[1,2]. The virus rapidly spread throughout the country, then throughout the whole world, and was declared a global pandemic by the World Health Organization in March 2020^[3]. As of September 23, 2020, it has infected over 31 million people worldwide and caused more than 965000 deaths^[3,4].

The clinical gastrointestinal manifestations are present and less severe when compared with SARS^[1,5,6]. Early reports from Wuhan stated that around 10% of COVID-19 cases had diarrhea and nausea, 1 to 2 d before the beginning of respiratory symptoms^[6], and some cases may be missed if screening was applied only to those with respiratory symptoms. It is widely believed that SARS-CoV-2 spreads *via* droplets and contact (especially if within one meter of distance), but there is evidence that airborne spread is also possible during aerosol-generating procedures (AGPs)^[2].

In addition, new emerging evidence that SARS-CoV-2 can be found in the feces of patients, giving the possibility of fecal-oral route transmission^[7,8]. This can be explained by the excessive expression of angiotensin-converting enzyme 2 protein, a receptor for SARS-CoV-2 required for cell entry in the epithelial layer of the gastrointestinal tract^[9].

Being highly infective, SARS-CoV-2 put the healthcare workers (HCWs) in clinical departments performing endoscopy in great challenges during this pandemic as being highly susceptible to getting the infection^[10]. Endoscopy could be considered a high-risk procedure as pulmonary and gastric secretions, as well as fecal material, may contain high viral loads. So, infection to HCWs occurs either due to direct contact with the infected patients or to the lack or improper use of the personal protective equipment (PPE)^[11]. In fact, several studies have discussed infection prevention and control measures that must be implemented to increase patient safety, prevent nosocomial outbreaks, protect HCWs, and ensure the rational use of limited PPE^[11,12].

Several endoscopy societies and expert groups have offered recommendations and position statements for endoscopy during the COVID-19 pandemic^[11,13-16]. Several GI society guidelines recommended ceasing elective procedures during the pandemic to decrease the risk of infection^[17-19]. Different recommendations/guidelines have been recently released to ensure the smooth reopening of endoscopy units and resuming elective procedures^[17]. Whether or not endoscopy units in different parts of the world are ready to resume service based on these recommendations is not fully known.

This study is a multicenter study to discuss the barriers of resuming endoscopic maneuvers in different centers and governorates in Egypt as the coronavirus infection rate has remarkably decreased and there are international calls for resuming all the activities with caution and different precautions, and to assess whether the endoscopic service has returned to its near normal standard level.

MATERIALS AND METHODS

Methods

We designed an online survey that was based on four domains (Supplementary Figure 1). The first domain included the demographic data as regards the governorate, the type of health care facility, and the type(s) of endoscopy service(s) provided. The second domain was for the status of the COVID-19 pandemic and its impact on the endoscopy service(s). The third domain was directed to the readiness of the health care facility for dealing with COVID-19 cases (infrastructure, working staff, PPEs, case stratification, *etc.*), and the fourth domain was about the ability of the facility to resume the endoscopy service(s) and the different barriers which preclude service resumption. The whole survey included 20 main questions. All the questions were closed-ended questions that could be answered by selecting the appropriate answer(s).

The questionnaire was set-up by a nationally representative group of endoscopy physicians covering different Egyptian governorates. The survey was designed using the RedCap platform (v9.1.0., United States). The study was approved by the Institutional Review Board of the National Liver Institute, Menoufia University, Egypt (NLI IRB 00003413) in June 2020, protocol number 00203/2020. The questionnaire was distributed online for 4 wk (starting from July 30, 2020 till August 28, 2020). The primary outcome of the survey was to identify the current impact of the COVID-19 pandemic on the endoscopy service and the barriers against the full resumption of the participating centers.

Statistical analysis

Data were collected and entered into personal computers and analyzed using Statistical Package for Social Sciences (SPSS version 26.0) software (IBM SPSS Inc. Chicago, United States). The incomplete responses were excluded from the analysis. For simplification, we divided the responding governorates according to the administrative division of the governorates of Egypt (see below in discussion). The data were expressed as numbers (proportions). Comparisons between groups were made by the χ^2 or Fisher's test (FET) for the qualitative variables. A uni- and multivariate regression analysis was done to identify the parameters which determine the decision making as regards the resumption of endoscopy service if not working. Correlation analysis also was done to explore the correlation between the current status of endoscopy service and the different domains.

RESULTS

Characteristics of the endoscopy Centers

The survey was sent to 115 endoscopy centers all over Egypt, of which 113 (98.3%) responded to the survey in complete response and two centers didn't give a full response, so they were excluded from the analysis. Most respondents ($n = 67$, 59.3%) were from high-volume endoscopy units either University or teaching facility general hospitals providing emergency endoscopy. Cairo and Giza regions have participated with 30 (26.5%) centers, Lower Egypt (Alexandria, Beheira, Sharqia, Qalyubia, Damietta, and Ismailia) and Upper Egypt (Assiut, Fayoum, Sohag, Qena, Aswan and Luxor) have participated through 34 (30.1%) centers, and the Delta region (Menoufia, Kafr El-sheik, Gharbia, and Dakahlia) have participated through 13 (11.5%) centers. COVID-19 designated facilities were present among 48 (42.5%) centers (Figure 1).

Patient selection and classification

On the day of the planned endoscopic procedures patients were checked for being suspected cases of COVID-19 in 95 (84.1%) of centers. The process of selection and screening was based on symptoms among 90 centers (79.6%), temperature check among 82 centers (72.6%), non-contrast multi spiral computed tomography (MSCT) chest among 50 centers (44.2%), polymerase chain reaction (PCR) testing among 11 centers (9.7%), and antibody testing among 3 centers (2.7%).

Impact of the current status of the pandemic on the waiting lists

Endoscopy practice was almost resuming or resumed in about 70% of the centers (Figure 2A). In general, there was an expansion of the waiting lists all over the country. The Quantification of the expansion is shown in Figure 2B. We found significant variation in the waiting lists according to the distribution of COVID-19 pandemic

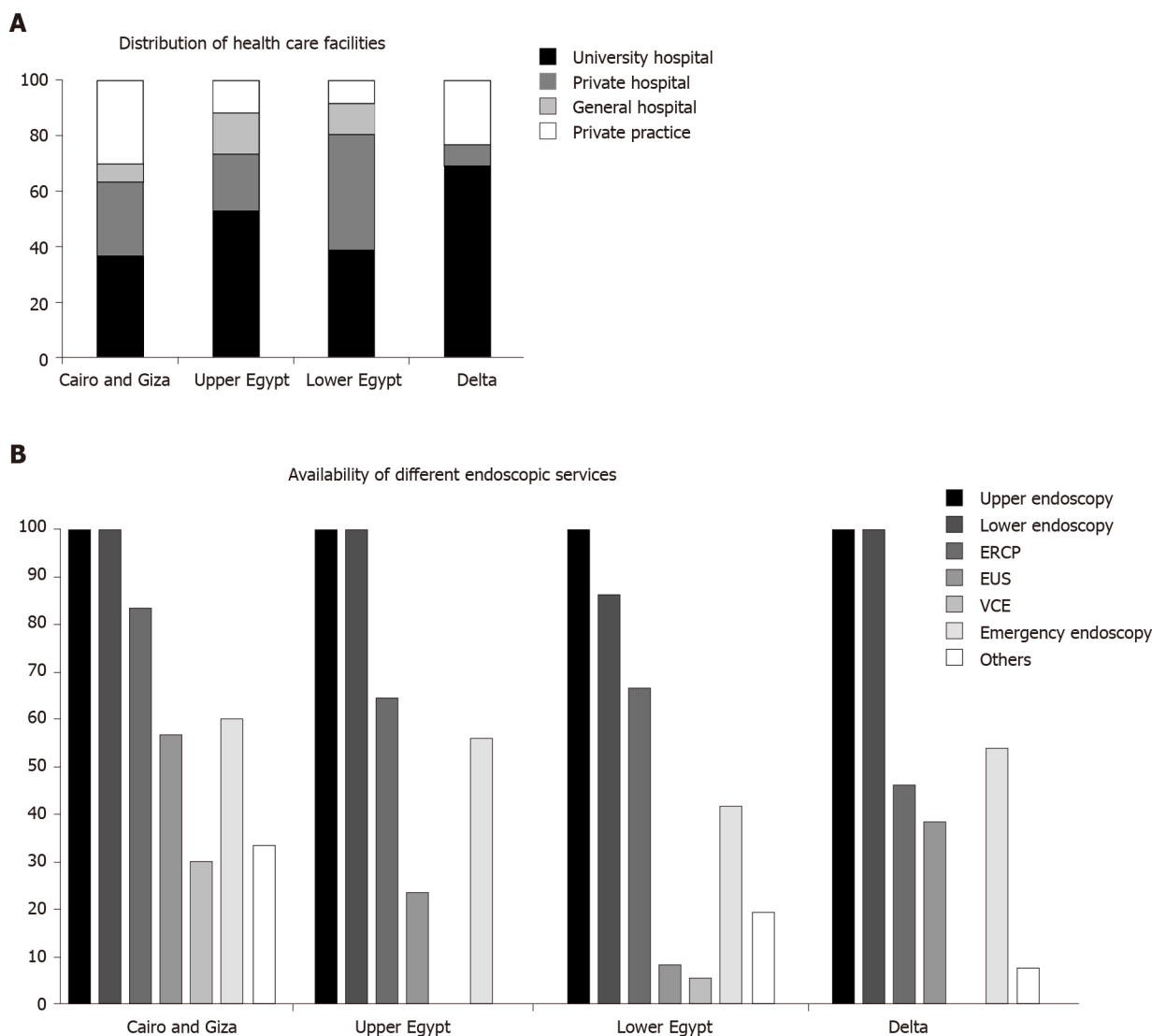


Figure 1 Distribution of different types of healthcare facilities and endoscopic services according to different regions. A: Distribution of health care facilities; B: Availability of different endoscopic services. ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound; VCE: Video capsule endoscopy (Y-axis is standing for percentage).

status among different centers (Figure 2B and C). In areas with clusters of cases ($n = 49$), the waiting lists increased by 0%-25% in 14.29%, 26%-50% in 32.65%, 51%-75% in 34.69%, 76%-100% in 10.2%, and by more than 100% in 8.17% of these centers. While in areas with sporadic cases ($n = 62$), 45.16% of the centers had their waiting list increased by 0%-25%, however, in 19.35% and 20.9% of the centers the list increased by 26%-50%, and 51%-75%, respectively. In about 14.59% of the centers, the waiting list increased by more than 75%, meanwhile, areas with no new cases ($n = 2$), 50% of centers had their list increased by only 0%-25% (FET = 19.1, $P = 0.005$).

Shortage in staff

During the COVID-19 outbreak, endoscopy staff has been deployed to COVID-19 designated wards and hospitals. In addition, a significant number of healthcare providers were infected. The shortage of endoscopy staff was present among 39 (34.8%) of centers (Figure 2D). Specialists shortage was present in 88 (77.9%) of centers, nursing staff shortage in 90 (79.6%) of centers, and trainees shortage in 110 (97.3%) of centers. This shortage was considered as a barrier against resuming endoscopy service among 38 (33.6% in intention to treat analysis, 86.4% in per-protocol analysis; 69 centers didn't answer). The shortage in specialists was more pronounced in Delta and lower Egypt than upper Egypt and Cairo and Giza (61.5% and 91.3% vs. 64.7% and 83.3% respectively, $P = 0.019$). However, the shortage of nursing staff and trainees were not significantly different among different centers, $P = 0.93$ and $P = 0.24$, respectively).

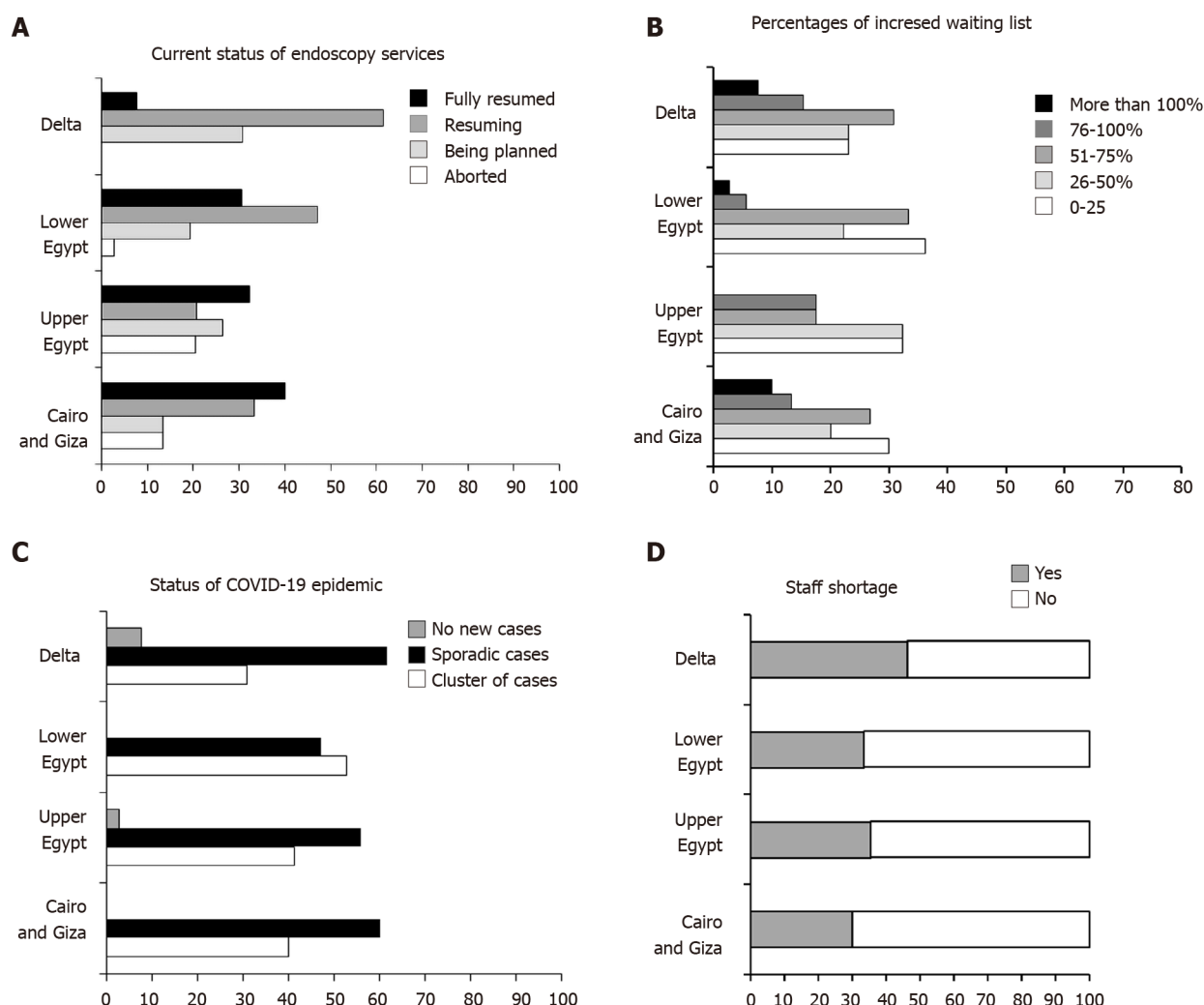


Figure 2 Impact of the status of the pandemic in different regions on the increase of waiting lists as well as resumption of endoscopic services and the impact of staff shortage. A: Current status of endoscopy services; B: Percentages of increased waiting list; C: Status of coronavirus disease 2019 epidemic; D: Staff shortage. COVID-19: Coronavirus disease 2019 (X-axis is standing for percentage).

Only 41 units (35%) had to increase the working hours to compensate for this shortage. Seventy-six units (67.3%) recovered their staff either from sick leave or COVID-19 wards.

Pre-procedural precautions applied by different Centers during the COVID-19 pandemic

During the current COVID-19 epidemic in Egypt, 72 (63.7%) of endoscopy centers have provided suitable waiting areas for appropriate social distancing precautions, 65 (57.5%) of centers could provide an adequate number of recovery rooms to keep the social distancing strategy to the optimum, and 40 (35.4%) of centers have increased their working hours to accommodate the extra-burden of increased cases volume; at the same time, 77 (68.1%) of centers have adopted selection strategy to select the endoscopist and the assisting team based on the presence of a suspected/confirmed COVID-19 cases. Sixty-three (55.7%) units were planning to resume the pre-pandemic capacity with a designated track for confirmed COVID-19 cases. However, only 24 (21.2%) of centers could provide special endoscopy rooms for confirmed or suspected COVID-19 cases. Of note, training was already resumed in 16 units (14.2%).

The Intra-procedural precautions applied during the COVID-19 pandemic in Egypt varied according to the availability of PPE in each facility locality as shown in [Table 1](#) (more details in [Supplementary Table 1](#)).

Table 1 Intra-procedural precautions applied by different Centers during the coronavirus disease 2019 pandemic in Egypt, n (%)

Governorates	Usage of PPE				Action taken by centers	
	For all cases	For suspected cases	For confirmed cases only	Vary accordingly	Delay of turnover for cleaning	Increased time/case
Cairo and Giza	5 (16.7)	13 (43.3)	12 (40)	8 (26.7)	27 (90)	21 (70)
Upper Egypt	10 (29.4)	7 (20.6)	13 (38.2)	7 (20.6)	32 (94.1)	27 (79.4)
Lower Egypt	13 (36.1)	10 (27.8)	10 (27.8)	8 (22.2)	32 (88.9)	29 (80.6)
Delta	7 (53.8)	4 (30.8)	3 (23.1)	3 (23.1)	13 (100)	13 (100)

PPE: Personal protective equipment.

Post-procedural precautions applied by different Centers during the COVID-19 pandemic

Post-procedural patient follow-up for the development of fever or suspected COVID-19 symptoms for 14 d after discharge was a strategy adopted by 18 (15.9%) of centers only. The follow up was performed by either a resident doctors (8%), endoscopy nurses (5.3%), administrative staff (6.2%), and/or specialist physicians (1.8%).

Multivariate regression analysis showed that burden of cases in the unit locality, staff shortage and their recovery and the availability of separate designated rooms for COVID-19 cases could markedly affect the resumption of endoscopy practice ($P = 0.029$, < 0.001 and 0.02 , respectively) and Odd's ratio (0.15, 1.8 and 0.16, respectively) (Table 2).

DISCUSSION

The prolonged suspension of routine endoscopic services during the SARS-CoV-2 pandemic has significant implications on diagnostic endoscopic services such as delay in diagnosis and management of cancer patients as well as the expansion of waiting lists worldwide. Therefore, this encouraged the international gastrointestinal endoscopic societies to release position statements, recommendations, and guidance for the rapid and safe resumption of endoscopic services commensurate with facilities and pandemic situations of each country^[14,17-19]. To our knowledge, whether or not endoscopy centers in Egypt are ready to resume elective services has not been studied. Accordingly, the results of the current survey represent the current status of resuming routine endoscopic services in Egypt and determine the barriers of resuming such services.

Our results showed that 70% of different centers all over Egypt have resumed routine endoscopic services as is illustrated in Figure 2A. This is in parallel to the current situation of early recovery phases of the pandemic and the decline in the number of new COVID-19 cases in Egypt and other countries worldwide^[3]. Expansion of elective endoscopy waiting lists is one of the challenges to resume the full capacity of endoscopic services. According to the British Society of Gastroenterology guidance in the early recovery stages, triage mechanisms are needed to prioritize patients scheduling according to the indications^[20].

The highest percentages of waiting lists expansion are observed in areas with clusters of COVID-19 cases which indicate the awareness of senior decision-makers of endoscopy centers in Egypt with the importance of triaging and prioritization of patients scheduling in the light of clinical need with the available capacity. Also, this reflects their compliance with GI endoscopy societies' guidance. In addition, this observation complies with the current SARS-CoV-2 pandemic situation in Egypt, as there is a decrease in the number of areas with new cases allowing endoscopy centers to restore their full capacity as soon as possible.

Regarding the shortage in endoscopy staff, 34.5% of the centers reported a significant shortage of staff during the SARS-CoV-2 pandemic in nursing staff (79.6%) and specialists (77.9%). This is related to two factors; one was the reallocation of staff and medical equipment to the surge in demand to manage suspected and confirmed COVID-19 cases and the other was some of them get infected caused by frequent exposure to infected patients. The shortage in specialists was more pronounced in

Table 2 Regression analysis for factors that determine decision making as regard resuming endoscopy service

Factor	Univariate		Multivariate		
	P value	OR	P value	OR	95%CI
Status of pandemic	0.03	1.5	0.029	0.15	0.20-0.9
Shortage of PPE	0.04	4.2	0.42	2.24	0.31-16.1
Staff recovery	0.05	1.4	< 0.001	1.8	5.1-6.7
Having a COVID designed unit	0.04	4.2	0.01	0.5	0.11-0.82
Having separate endoscopy room	0.04	1.01	0.02	0.16	0.03-0.7
Patient classified before procedure	0.03	1.7	0.007	0.7	1.1-1.18

OR: Odd's ratio; COVID: Coronavirus disease; PPE: Personal protective equipment.

Delta and Lower Egypt geographically which may be related to the geographic distribution of a large number of confirmed COVID-19 cases and isolation hospitals that need more medical staff.

This shortage in endoscopy staff is considered as a barrier against resuming endoscopy service among 38 centers in the current study (33.6% in intention to treat analysis where $n = 113$, 86.4% in the per-protocol analysis where $n = 44$). Regarding patient selection before endoscopy, a large number of participating centers (84.1%) screened their patients. The most common method used was symptoms-based screening for COVID-19 risk stratification (79.6%) of the centers and fewer centers used PCR testing (9.7%).

Similarly, Alboraie *et al*^[21] reported that most worldwide centers (93.9%) screened patients for possible COVID-19 disease prior to the procedure and 54 centers (33.13%) used PCR testing. However, asymptomatic infected patients are a known source for transmission^[2,22], accordingly, this type of screening is not sufficient to guide for COVID-19 risk stratification. A recent study from China, employed both symptoms-based screening and PCR in patient's screening, reported no cases of endoscopy-related nosocomial COVID-19 disease transmission in 1361 cases^[23].

Regarding pre-procedural precautions and endoscopy centers designation, our results show that most participating centers so far follow the different international GI endoscopy societies guidelines^[14,17-19] including appropriate social distancing precautions or increase working hours to accommodate the extra-burden of increased cases volume. More than two-thirds of our centers have adopted a selection strategy to select the endoscopist and the assisting team based on the presence of a suspected/confirmed COVID-19 case. Also, Alboraie *et al*^[21] stated that the majority (78.5%) of the centers considered dedicated teams for the delivery of endoscopic services after the onset of the pandemic.

To reduce the risk of infection spread most of the international society's recommendations are in agreement with the existence of a restricted protocol for infection control and the awareness of medical staff for the donning and doffing of PPE. According to the results of our study, there is wide variability between different centers regarding intra-procedural precautions depending upon the availability of PPE and type of procedures. We can overcome those types of barriers by reuse some of PPE components such as respirator masks in case of shortage as it was reported by many studies as the methods of decontamination were explained^[23-25].

Most of the international recommendations on endoscopy encourage follow-up of patients after endoscopic procedures^[11]. Unfortunately, in our study few participating centers (15.9%) are practicing the strategy of following their patients for the development of any suspected symptoms post-procedure. A similar study showed that 18.4% of centers only called patients back two weeks after procedures^[21]. According to multivariate regression analysis that was done in our study, we found that endoscopy staff shortage, availability of separate designated rooms for COVID-19 cases, and the burden of cases in each locality are the most obvious barriers for the resumption of routine endoscopy practice in Egypt.

Although there are many barriers that may interfere with the complete restoration of endoscopy services in Egypt, according to the results of our study, most participating centers are preparing their facilities for the resumption of full endoscopy services.

We recommend increasing working hours and dividing endoscopy staff into teams

to overcome the shortage of endoscopy staff. Also, follow up of patients for two weeks after endoscopic procedures to detect any possible transmission of SARS-CoV-2 infection in endoscopy centers as this strategy may help in tracing the source of nosocomial transmission.

CONCLUSION

In conclusion, the current study represents an important national multicenter survey addressing crucial information about resuming regular gastrointestinal endoscopy services and its barriers in Egypt.

ARTICLE HIGHLIGHTS

Research background

An outbreak of coronavirus disease 2019 (COVID-19) has hit the world and disturbed the whole healthcare system, including endoscopic practices which are a very risky procedures in terms of exposure to infection.

Research motivation

Smooth resumption of routine endoscopic service has to be guaranteed as well as decreasing the burden of exposing patients and endoscopy staff to infection during endoscopic procedures. Many strategies have to be implemented in endoscopy units, however, these strategies face many barriers as shortage of personal protective equipment, working staff and post procedure tracing of infection and follow-up of patients.

Research objectives

This survey study was designed to evaluate the feasibility and the difficulty of resumption of routine endoscopic service in the context of COVID-19 pandemic.

Research methods

We conducted a survey study that included 20 questions to be answered by the head of endoscopy units in 113 units from all over Egypt.

Research results

One hundred and thirteen centers participated in the study from all over Egypt. Due to halting the routine endoscopic services during the pandemic, the waiting lists were doubled in most of the centers. Third of the centers experienced significant shortage of endoscopy staff. The lack of dedicated endoscopy rooms for infected patients and the staff shortage were the main barriers to resume routine services smoothly.

Research conclusions

We recommend increasing working hours and dividing endoscopy staff into teams to overcome the shortage of endoscopy staff. Also, follow up of patients for two weeks after endoscopic procedures to detect any possible transmission of infection in endoscopy centers as this strategy may help in tracing the source of nosocomial transmission.

Research perspectives

For smooth resumption of endoscopic service between two waves of the current pandemic, several strategies have to be applied in a uniform manner in all endoscopy units. Our study showed how different centers acted differently during this pandemic in terms of their plan to safely getting routine endoscopy service on track. Future studies should propose modalities to guarantee uniform application of determined strategies that overcome the current barriers.

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Crohn's disease in low and lower-middle income countries: A scoping review

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Abstract

BACKGROUND

While Crohn's disease has been studied extensively in high-income countries, its epidemiology and care in low and lower-middle income countries (LLMICs) is not well established due to a lack of disease registries and diagnostic capacity.

AIM

To describe the published burden, diagnostic/treatment capacity, service utilization, challenges/barriers to individuals with Crohn's in LLMICs and their providers.

METHODS

We conducted a scoping review utilizing a full search strategy was developed and conducted in PubMed, Embase and World Health Organization Global Index Medicus. Two independent reviewers screened the titles and abstracts of all of the publications found in this search, reviewed selected publications, and extracted relevant data, which underwent descriptive review and was analyzed in Excel.

RESULTS

The database search yielded 4486 publications, 216 of which were determined to

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be relevant to the research questions. Of all 79 LLMICs, only 21 (26.6%) have publications describing individuals with Crohn's. Overall, the highest number of studies came from India, followed by Tunisia, and Egypt. The mean number of Crohn's patients reported per study is 57.84 and the median is 22, with a wide range from one to 980.

CONCLUSION

This scoping review has shown that, although there is a severe lack of population-based data about Crohn's in LLMICs, there is a signal of Crohn's in these settings around the world.

Key Words: Crohn's disease; Low and lower-middle income countries; Scoping review; Service utilization; Diagnostic/Treatment capacity

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Core Tip: This scoping review demonstrates the lack of epidemiologic data on Crohn's disease in low and lower-middle income countries, but that it does exist in these settings and presents unique challenges. There is a need for population-based research to fully understand the its burden among the world's poorest people.

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INTRODUCTION

Crohn's disease is a chronic gastrointestinal disease which appears to be only moderately heritable^[1,2], and multiple possible environmental and behavioral causes have been invoked^[3], including insufficient contact with infectious diseases in childhood (the hygiene hypothesis), antibiotic exposure, tobacco use, and consumption of highly processed foods. Crohn's has historically been regarded as a "lifestyle" disease of industrialized countries^[4,5]. First described in the United States in 1932, Crohn's was increasingly diagnosed in Europe and North America during the 20th century, where around 0.5% of the population is now thought to be affected^[6,7]. More recently, Crohn's has been recognized in the rapidly developing upper-middle income countries of East Asia^[8] and South America^[9], with prevalence rates as high as 24 per 100000 in Brazil^[10], and 11 per 100000 in South Korea^[11].

In contrast, previous systematic reviews of the published literature on Crohn's epidemiology have found few studies on either the prevalence or incidence of Crohn's coming from the low- and lower-middle income countries (LLMICs)^[12,13]. A review of population-based studies that were published between 1990 and 2016 found data from only four LLMIC studies^[14]. These LLMIC studies were all in Asia (the Gampaha district of Sri Lanka, the Hyderabad district of India, Manila city in the Philippines, and Central Jakarta in Indonesia), had all come out of the prospective Asia-Pacific Crohn's and Colitis Epidemiologic Study^[14-17], and were all focused on urban areas. An earlier systematic review of incidence and prevalence studies (both population and facility-based) published between 1950 and 2010 only identified data from three countries that were classified as LLMICs at the time the research was conducted^[18]. These countries were Sri Lanka (prospective study of Columbo and Gampaha districts)^[19], Panama (restrospective review of hospital data from the Colon district)^[20], and China (review of published reports from all hospitals)^[21]. Currently, two of these countries – China and Panama are classified as upper-middle income and high income respectively. As a result, the 2017 Global Burden of Disease study largely based its inflammatory bowel disease (IBD) rate estimates for LLMICs on global trends^[22].

The vast majority of the world's poorest billion people live in the rural areas of LLMICs in sub-Saharan Africa and South Asia^[23]. In the absence of primary population

data regarding Crohn's in these countries, there has been a perception that the burden of Crohn's remains low among the global poor. Prior systematic reviews on Crohn's have been limited, however, by narrow inclusion criteria focused on epidemiology (incidence and prevalence). Furthermore, the lack of reports regarding Crohn's in LLMICs may be due to limitations in access to diagnosis and treatment for Crohn's rather than the absence of disease. The correct diagnosis of Crohn's requires a complex chain of events beginning with patients seeking care and ending with colonoscopy and histology. A break in any part of this chain resulting from gaps in financing, education, equipment, or supplies on the part of the patient or provider can result in a missed diagnosis. Even the pathological diagnosis of Crohn's may be confused with intestinal tuberculosis in the absence of experienced healthworkers^[24]. Crohn's may also have an impact on patients living in extreme poverty that is out of proportion with the disease prevalence. Follow-up care for Crohn's, like many other chronic diseases, requires frequent visits to health facilities, a steady supply of medications (including biologics), and often surgery^[24]. The absence of the services could result in a high rates of disability and death among the poor affected by Crohn's in LLMICs.

As part of an effort to understand the non-communicable burden of disease among the world's poorest, we have conducted a scoping review with broad inclusion criteria focused on the experience with Crohn's in LLMICs^[25]. Specifically, this review seeks to answer the following research questions: (1) What is the published evidence regarding the burden of Crohn's disease in communities and health facilities in LLMICs? Is underdiagnosis a problem? (2) What is the diagnostic and treatment capacity for Crohn's disease in LLMICs? What services, equipment, and medications are used to diagnose and manage Crohn's in LLMICs? (3) What challenges and barriers are there to providers and patients with Crohn's disease in LLMICs in terms of diagnosis, treatment, and long-term management? And (4) What is known from the published literature regarding the social and demographic characteristics of patients with Crohn's disease in LLMICs?

MATERIALS AND METHODS

Inclusion criteria

This review considered studies that describe cases of individuals with Crohn's disease in an LLMIC as defined by The World Bank^[17]. The World Bank categorizes the world's countries into four income groups based on gross national income per capita: low-income countries, lower-middle income countries, upper-middle income countries, and high-income countries. The group of interest, LLMIC, includes both low-income countries and lower-middle income countries, or countries with a gross national income per capita of United States \$3895 or less. To capture possible undiagnosed or misdiagnosed cases of Crohn's in LLMICs that do not have any published Crohn's data, as well as to understand their diagnostic and treatment capacity for Crohn's, studies that mention the use of diagnostics (*i.e.*, colonoscopy, small bowel follow-through, stool calprotectin), findings (*i.e.*, skip lesions, cobblestone, small bowel obstruction), and treatments (*i.e.*, colectomy, small bowel resection, infliximab) utilized in managing Crohn's disease were also included. See [Supplementary Table 1](#) for a full list of search terms. Publications that do not describe cases of individuals diagnosed with Crohn's disease were excluded from this review. Relevant secondary sources (*i.e.*, reviews, editorials, and commentaries) were excluded and used as background information. Studies that are based on cases of Crohn's in middle, upper-middle, or high-income countries were excluded. Studies that are published in a language other than English were excluded. Studies describing non-human animals were excluded.

Search strategy

The search strategy aimed to locate published studies from all years. An initial limited search of PubMed was undertaken to identify articles on the topic. The text words contained in the titles and abstracts of relevant articles, and the index terms used to describe the articles were used to develop a full search strategy for PubMed in collaboration with an experienced medical librarian (see [Supplementary Table 1](#) for full search strategy). The search strategy, including all identified keywords and index terms, was adapted for Embase and World Health Organization (WHO) Global Index Medicus. The team used free text and Medical Subject Headings, when applicable. Searches were conducted on publications in English for all years.

Information sources

The information sources for this review were the databases PubMed, Embase, and WHO Global Index Medicus, which includes AMRO (Africa), IMEMR (Eastern Mediterranean), IMSEAR (South East Asia), LILACS (Americas), and WPRIM (Western Pacific), as well as MEDLINE and SciELO.

Study selection

Following the search on May 14, 2019, all identified citations were collated and uploaded into EndNote X9 2018 (Clarivate Analytics, PA, United States) and duplicates removed. The study selection process consisted of two parts. First, two independent reviewers (SB and SM) screened the title and abstracts of all of the initially selected publications and included all of the studies that indicated a signal of Crohn's disease or any related Crohn's diagnostics or Crohn's treatment in an LLMIC. Studies that met or could potentially meet the inclusion criteria were saved for full text review in EndNote. Any disagreements that arise between the reviewers were resolved through discussion, or with a third reviewer.

Next, a full text review was conducted to categorize the studies based on country and relevance to the research question. Two independent reviewers (KN and SM) assessed the full text of selected citations in detail against the inclusion criteria. Reasons for exclusion of full text studies that did not meet the inclusion criteria were recorded and are reported in a Preferred Reporting Items for Systematic Reviews and Meta-analyses flow diagram (Figure 1). Any disagreements that arose between the reviewers were resolved through discussion, or with a third reviewer (SB).

Data extraction

Full text of the publications selected were reviewed by two independent researchers (KN and SM) and data was extracted using a pre-structured and tested data collection form in Microsoft Excel. The data extracted includes specific details about the state of Crohn's disease burden and care in LLMICs according to the review questions and specific objectives of the study. Any disagreements that arose between the reviewers were resolved through discussion, or with a third reviewer (SB). Authors of papers were contacted to request missing or additional data, where required.

A charting table was developed (see Supplementary Table 2, data extraction instrument) to record key information of the source, including the title, author, journal, date of publication, country, study years, study design, and results relevant to the review questions. In relation to burden, we collected data on number of cases reported, prevalence, incidence, odds, mortality rate, disability-adjusted life year rate, and average disease duration. Patient characteristics include both sociodemographic characteristics (age, residency, socioeconomic status, insurance coverage, out of pocket expenses) and clinical features of their disease (age at diagnosis, sex, risk factors, disease severity, disease behavior, and disease location, Crohn's Disease Activity Index, extraintestinal manifestations, comorbidities, and disease outcomes). Qualitative information about disease diagnosis, management, long-term and follow-up care, and complications was documented to understand care pathways. Availability of diagnostic and treatment services included blood tests, stool tests, tissue pathology, Tuberculosis (TB) testing, endoscopy, radiology/imaging, other equipment, providers, and financing. Qualitative information about provider challenges (diagnostic and management) and patient barriers (access and financial) were collected in the table.

Data analysis

The descriptive findings extracted from the studies identified were charted to summarize the results of the research objectives of this review. The charted data then underwent a narrative review and descriptive analysis to identify emerging themes found in the data in terms of Crohn's care pathways and availability of diagnostic and treatment services. Quantitative data regarding the burden of Crohn's were analyzed in Excel.

RESULTS

The initial database search of studies that describe Crohn's disease and related diagnostics and findings in LLMICs found 4480 publications after removing duplicates, 702 of which were kept after title and abstract screening (Figure 1). Of

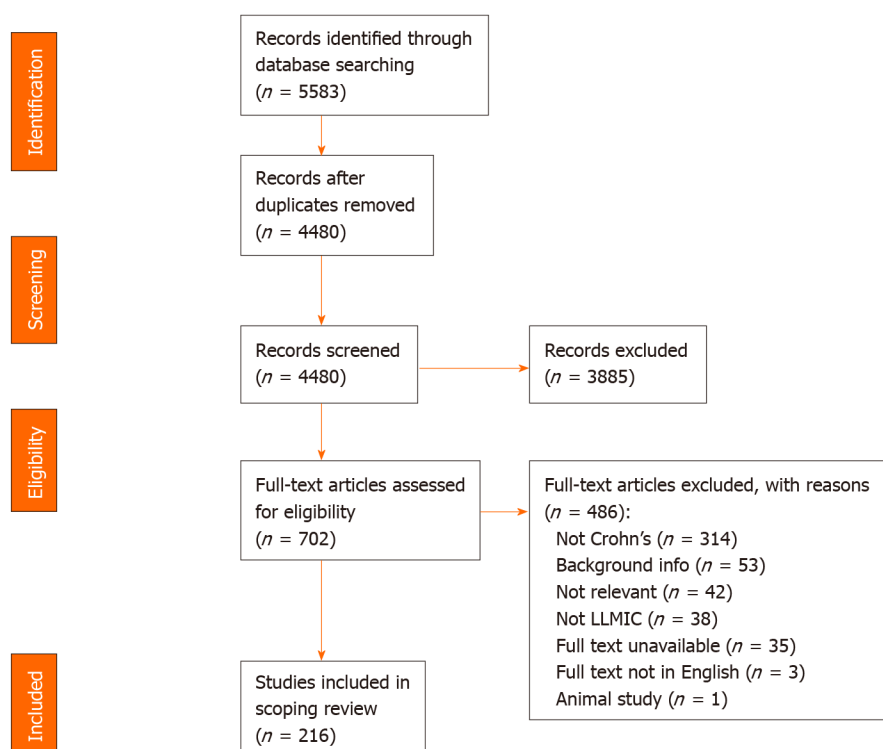


Figure 1 Preferred reporting items for systematic reviews and meta-analyses flow diagram depicting the number of studies identified and excluded at each stage of the review process. LLMIC: Lower-middle income countries.

those 702 publications, 216 were relevant to the research questions, 208 (96.3%) of which were based in lower-middle income countries and 8 (3.7%) of which were based in low income countries (see [Supplementary Table 3](#) for a summary of all studies included in review by country). Of all 79 LLMICs, we only found 21 (26.6%) with studies describing individuals with Crohn's disease. Most (73.4%) of the LLMICs do not have any studies describing individuals with Crohn's disease identified through our search ([Figure 2A](#)).

Of the relevant articles, 129 (59.7%) were based in LLMICs in South Asia, 67 (31.0%) were from the Middle East and North Africa, 16 (7.4%) were from sub-Saharan Africa, 5 (2.3%) were from East Asia and Pacific, and 1 (0.5%) was from Latin America (see [Supplementary Table 3](#), [Figure 2B](#)). The majority of Crohn's studies identified are from India (49.5%), followed by Tunisia (19.0%), Egypt (8.3%), and Sri Lanka (5.1%). Bolivia, Cameroon, Ghana, Malawi, the Philippines, Syria, Uganda, and Vietnam each had one Crohn's disease study (0.5%) (see [Supplementary Table 3](#), [Figure 2C](#)).

Crohn's disease burden in LLMICs

Overall, the mean number of cases of Crohn's disease reported per study is 57.84 and the median is 22, but ranges widely from single-patient case studies to cohorts of as many as 980 individuals with Crohn's, and varies from country to country ([Table 1](#)). Countries in South Asia (63.42%) and the Middle East and North Africa (61.73%) regions reported substantially higher mean numbers of Crohn's cases per study compared to sub-Saharan Africa (3.25%) and East Asia and Pacific (2.67%). Syria (106) and Morocco (102) reported the highest mean number of cases per study, while Bangladesh, Cameroon, Ghana, Malawi, and Uganda, each only have one single-patient case study ([Table 1](#)).

Of the 21 LLMICs included in the full-text review, only two countries had studies estimating prevalence of Crohn's, India and Sri Lanka ([Table 2](#)). Two studies reported prevalence of Crohn's in Sri Lanka, ranging from 1.2 per 100000 in the Colombo and Gampaha Districts in 2010^[19], to 2.33 per 100000 in the Central Province in 2018^[26]. Four of the 21 included countries—India, Indonesia, Sri Lanka, and the Philippines—reported incidence of Crohn's disease, with most of these data coming from one multi-country study published in 2019^[14] ([Table 2](#)). This study reported annual incidence of Crohn's ranging from 0.14 per 100000 in the Philippines to 3.91 per 100000 in India^[14]. The 2010 study from Sri Lanka reported an annual incidence of

Table 1 Mean and range number of cases of Crohn's reported by each study included in the review, overall, by region, and by low and lower-middle income countries

Region/Country	n ¹	Total cases	Mean	Median	Range
Overall	220 ¹	12725	57.84	22.00	1-980
South Asia	131	8485	64.77	17.00	1-980
India	107	8054	75.27	22.00	1-980
Sri Lanka	10	332	33.20	6.00	1-153
Pakistan	9	82	9.11	3.00	1-52
Nepal	4	16	4.00	2.00	1-11
Bangladesh	1	1	1.00	1.00	1
Middle East and North Africa	67	4165	62.16	39.00	1-226
Tunisia	41	2984	72.78	45.00	1-226
Egypt	18	361	20.06	12.50	1-100
Morocco	7	714	102.00	101.00	68-136
Syria	1	106	106.00	106.00	106
sub-Saharan Africa	16	52	3.25	1.00	1-17
Nigeria	5	15	3.00	1.00	1-8
Sudan	3	23	7.67	8.00	3-12
Ethiopia	2	8	4.00	4.00	1-7
Kenya	2	2	1.00	1.00	1-1
Cameroon	1	1	1.00	1.00	1
Ghana	1	1	1.00	1.00	1
Malawi	1	1	1.00	1.00	1
Uganda	1	1	1.00	1.00	1
East Asia and Pacific	5	15	3.00	8.00	1-6
Indonesia	2	6	3.00	3.00	1-5
Philippines	2	3	1.50	1.50	1-2
Vietnam	1	6	6.00	6.00	6
Latin America and the Caribbean	1	8	8.00	8.00	8
Bolivia	1	8	8.00	8.00	8

¹Higher than actual number of studies due to two multi-country papers reporting cases of Crohn's.

Crohn's disease of 0.09 per 100000 in the Colombo and Gampha Districts^[19].

Diagnostic and treatment capacity and utilization of services

Of the 216 studies included in the review, 112 discussed the utilization of diagnostic and treatment services, all of which include cases that were confirmed *via* both colonoscopy and histology (Table 3). Of the 21 LLMICs included, all but Bolivia and Syria had at least one study discussing the utilization of Crohn's diagnostic services: Blood testing, stool testing, TB testing, radiology/imaging, endoscopy, and pathology services. South Asian countries reported the highest utilization of diagnostic services, with only Nepal lacking studies mentioning stool testing and TB testing (Table 3, see Supplementary Table 4 for numbers of studies reporting utilization of Crohn's disease diagnostic and treatment services from each country). Included LLMICs in the Middle East and North Africa all had multiple studies reporting the utilization of endoscopy, radiology, and stool testing, however only one study from Egypt mentioned TB testing (see Supplementary Table 4)^[27]. Studies from sub-Saharan Africa also mentioned TB testing as a tool for diagnosing Crohn's less frequently compared to those from South

Table 2 Prevalence and incidence of Crohn's disease reported by each study included in the review, by low and lower-middle income countries

Country	Prevalence	Incidence
India	-	3.91 per 100000 (Ng <i>et al</i> ^[14] , 2019)
Sri Lanka	1.2 per 100000 (Niriella <i>et al</i> ^[19] , 2010)	0.52 per 100000 (Ng <i>et al</i> ^[14] , 2019)
	2.33 per 100000 (Kalubowila <i>et al</i> ^[25] , 2018)	0.09 per 100000 (Niriella <i>et al</i> ^[19] , 2010)
Indonesia	-	0.27 per 100000 (Ng <i>et al</i> ^[14] , 2019)
Philippines	-	0.14 per 100000 (Ng <i>et al</i> ^[14] , 2019)

Asia, with only two of the 16 included sub-Saharan African countries having studies discussing it in this context^[28,29]. Twelve studies (five from India^[30-34], two from Tunisia^[35,36], and one each from Egypt^[37], Nepal^[38], Sudan^[28], Ethiopia^[39], and Uganda^[40]) described Crohn's being diagnosed surgically or on autopsy. Two studies, one a multi-country study from Asia and one from Nigeria, mentioned that only a clinical diagnosis of Crohn's disease was made without endoscopic and pathologic investigation unless multiple diseases were suspected. In India, a failed trial of anti-tubercular therapy was mentioned in 11 studies as an important part of diagnosing Crohn's disease. Two studies from Pakistan, and one study each from Ethiopia and Malawi, also discussed first treating their patients with anti-tubercular therapy to aid in Crohn's disease diagnosis. It is important to note that many countries only have one or two included studies, so the absence of diagnostics mentioned might reflect a lack of academic research rather than a true lack of diagnostic capacity in those countries (see [Supplementary Table 4](#)).

Of the 21 LLMICs included, all but Bolivia, Ghana, and Syria had at least one study discussing the utilization of one or more Crohn's medications or surgical treatments ([Table 3](#)). Corticosteroids, aminosalicylates, and immunomodulators are the most frequently reported medications overall, while biologic agents are the least available (see [Supplementary Table 4](#)). Studies from India, Pakistan, Sri Lanka, Tunisia, and Egypt, report the use of medications in all major Crohn's medication categories, while those from Bangladesh, Morocco, Kenya, Uganda, and Vietnam did not mention any Crohn's medications ([Table 3](#)). The use of biologics were only discussed in 26 of the 216 studies, and were not mentioned in any studies from sub-Saharan Africa or East Asia and Pacific (see [Supplementary Table 4](#)). Nutritional therapy was also scarcely mentioned, with only six studies discussing dietary changes as a treatment for IBD: four from India^[41-44], one from Egypt^[45], and one from Malawi^[46] (see [Supplementary Table 4](#)).

The most frequently discussed Crohn's surgery overall is colectomy, followed closely by small bowel resection (see [Supplementary Table 4](#)). Ileoanal pouches were not specifically described in any of the included studies, but several studies described other or unspecified anal surgery for Crohn's disease. Studies from Ghana, Malawi, and the Philippines did not mention any Crohn's surgeries, and those from Bangladesh, Morocco, Sudan, and Kenya discussed surgery but did not specify which types (see [Supplementary Table 4](#)). Again, it is important to note that those countries with higher numbers of included studies also report the greatest use of diagnostics, medications, and surgeries.

Socioeconomic characteristics

Of the 216 studies in 21 countries included in this review, only 29 studies in 11 countries discussed patient geographic, socioeconomic, or cost information (see [Supplementary Table 5](#) for a summary of patient geographic residency, socioeconomic characteristics, insurance coverage, and out of pocket costs). India had the most information due to the large number of available publications. All eight^[14,32,47-52] of the studies from India that discussed patients' geography reported that more individuals with Crohn's resided in urban areas compared to rural areas (see [Supplementary Table](#)). Similarly, studies from Egypt^[53], Ethiopia^[54], and Indonesia^[14,55] report individuals with Crohn's coming from cities more frequently. Sri Lanka, on the other hand, has a recent study reporting more cases among rural communities (73.9%) than urban (26.1%)^[53]. Two older studies from Steury in 1975^[54] and Bhatt in 1980^[55] also describe more rural Crohn's patients than urban.

Only eight studies from three of the included countries reported on socioeconomic characteristics, either income level, education level, or employment status: India,

Table 3 Utilization of Crohn's disease diagnostic and treatment services reported in studies included in the review by region and country¹

Country/ Region	Diagnostics								Medical						Surgical				
	Endo- scopy	Path- ology	Radio- logy	Blood Test- ing	Stool Test- ing	Trial of ATT	Surgical/ autopsy diagnosis	Clinical diagnosis only	TB Test- ing	Cortico- steroids	Amino- salicyclates	Immuno- modulators	Biologic agents	Nutritional therapy	Colect- omy	Ost- omy	Small bowel resection	Ileoanal pouch	Stricture- plasty
Overall (<i>n</i> = 216)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
South Asia (<i>n</i> = 129)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
India (<i>n</i> = 107)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X
Sri Lanka (<i>n</i> = 11)	X	X	X	X	X	X			X	X	X	X	X		X	X	X		X
Pakistan (<i>n</i> = 9)	X	X	X	X	X			X	X	X	X	X	X		X	X			
Nepal (<i>n</i> = 3)	X	X	X	X			X	X		X		X	X		X		X		
Bangladesh (<i>n</i> = 2)		X	X		X			X	X										
Middle East and North Africa (<i>n</i> = 67)	X	X	X	X	X		X		X	X	X	X	X	X	X		X	X	X
Tunisia (<i>n</i> = 41)	X	X	X	X	X		X		X	X	X	X	X		X		X	X	X
Egypt (<i>n</i> = 18)	X	X	X	X	X		X		X	X	X	X	X	X	X		X		
Morocco (<i>n</i> = 7)	X	X	X	X	X														
sub-Saharan Africa (<i>n</i> = 16)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
Nigeria (<i>n</i> = 5)	X	X	X	X	X			X		X	X						X		
Sudan (<i>n</i> = 3)	X	X	X	X	X		X		X	X	X								
Ethiopia (<i>n</i> = 2)	X	X	X	X	X	X	X			X	X				X		X		
Kenya (<i>n</i> = 2)	X	X																	
Uganda (<i>n</i> = 1)		X		X	X		X										X		

[illegible]

"X" indicates countries with at least one study describing the use of each service – see [Supplementary Table 4](#) for number of studies reporting each service. ¹Studies from Bolivia and Syria did not report on diagnostic or treatment services availability.

Tunisia, and Sudan (see [Supplementary Table 5](#)). Four of the five Indian studies^[49,51,56,57], as well as the one from Sudan^[41], reported that most individuals with Crohn's disease belong to the middle or upper class and have relatively high level of education. In contrast, one study from India reported that the majority of individuals with Crohn's are non-graduates (62.6%), are unemployed or unskilled workers (62.3%), and have an annual family income of less than 1000000 INR (approximately \$14K) (83.8%)^[52,58]. One Tunisian study from 2014 discussing socioeconomic characteristics reported that over half of the patients had a university education^[59], whereas another in 2017 reported that 22.2% had a university education^[60]. This study also reported that 34.3% of patients had "bad" socioeconomic conditions, 49% had "good" socioeconomic conditions, and 16.7 had "well" socioeconomic conditions^[60].

Three included studies from India and two from Nigeria describe out of pocket costs and insurance coverage of individuals with Crohn's disease (see [Supplementary Table 5](#)). Both of the studies from Nigeria reported that national health insurance programs are available but that coverage is limited, so all treatments were paid for out of pocket by patients^[61,62]. Studies from India were more variable, with one from 2009 explaining that cost of medications was not a factor^[56], another in 2017 reporting that 60% of patients were covered by private insurance^[63], and most recently in 2019 where 14.3% of patients discontinued Adalimumab due to high cost.

Challenges and barriers

Of the 21 LLMICs included in this review, 14 hypothesized at least one specific diagnostic, management, access, or financial challenge or barrier to individuals with Crohn's disease and providers (see [Supplementary Table 6](#) for a summary of

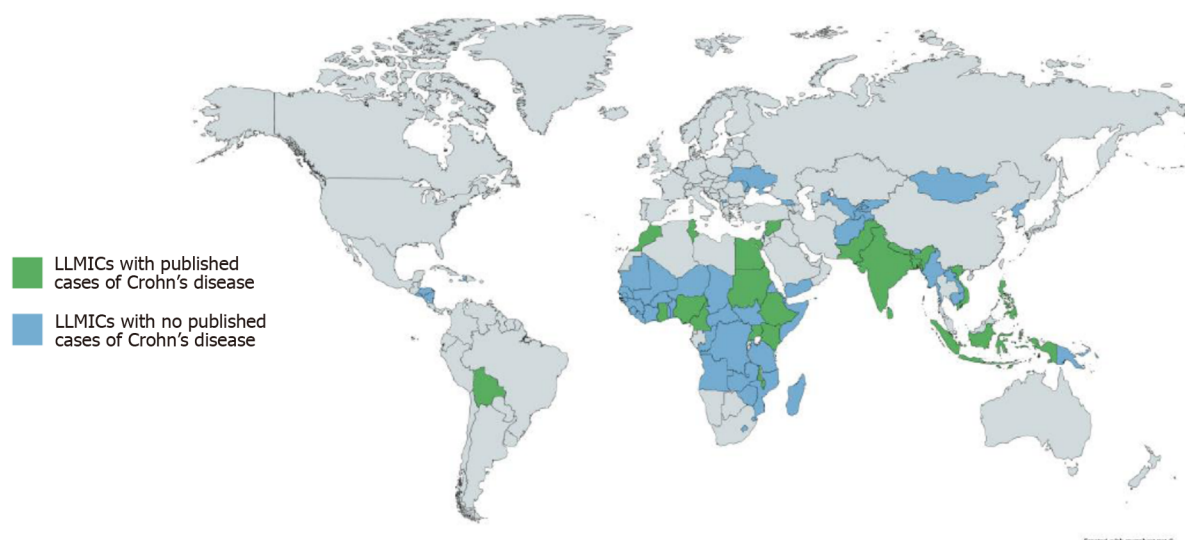
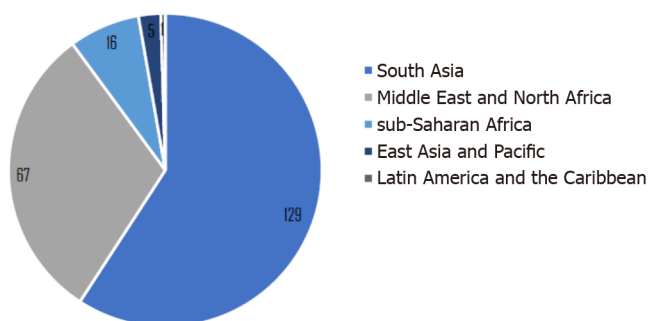
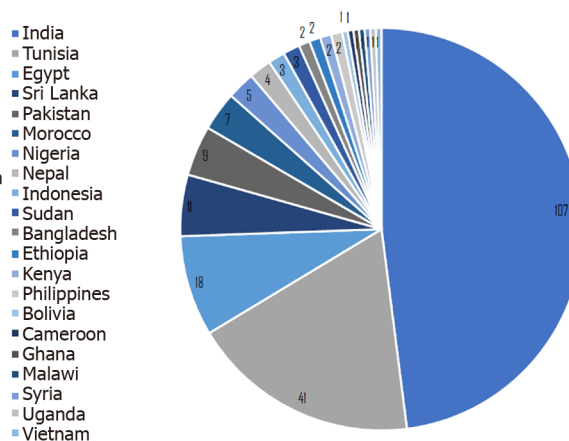
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Figure 2 Studies with Crohn's disease. Low and lower-middle income countries with and without published Crohn's disease studies (A); summary of studies describing Crohn's disease cases by world region (B) and low and lower-middle income countries (C).

diagnostic, management, access, and financial challenges and barriers to individuals with Crohn's and providers in LLMICs). The most commonly reported provider challenge is differentiating between Crohn's and intestinal tuberculosis (ITB), due to the high prevalence of TB in LLMICs and its overlap of symptoms and endoscopic features. This can result in long delays in disease diagnosis and thus appropriate treatment. A total of 36 studies in 10 countries in the review included distinguishing between Crohn's and ITB as a diagnostic challenge to providers (Table 4). This was followed by diagnostic delays due to perceived rarity of IBD and lack of clinical awareness among providers, which was mentioned in 17 studies from eight countries, and lack of quality diagnostic facilities, which was mentioned in 14 studies from eight countries. Management challenges to Crohn's providers were discussed less frequently than diagnostic challenges, with three studies reporting limited use of biologics due to cost, and one reporting risk of TB reactivation on biologics.

The most frequently reported patient barrier was cost of Crohn's surgeries and medications, particularly biologics. Patients' inability to afford the costs of their treatment in general was mentioned in nine studies, and high cost of biologics specifically in three studies (Table 4). Lack of access to high quality health care facilities was another common patient barrier, with nine studies describing access to care as a patient barrier.

Table 4 Most frequently reported diagnostic, management, access, and financial challenges and barriers to Crohn's patients and providers in low and lower-middle income countries

Provider diagnostic challenges	Number of countries	Number of studies
Difficulty differentiating between Crohn's and ITB	10	36
Low disease index of suspicion/clinical awareness due to perceived rarity of Crohn's leads to underdiagnosis	8	17
Lack of quality diagnostic facilities and investigational modalities	8	14
Difficulty differentiating between Crohn's and other infectious diseases	7	16
Difficulty differentiating between Crohn's and UC	5	7
Diagnosis of Crohn's made on histological exam of resected colon	2	3
Lack of reliable TB testing modalities	2	2
Provider Management Challenges		
Use of biologics is limited due to cost	1	3
High risk of TB infection reactivation in patients treated with biologics	1	1
Patient Access Barriers		
Lack of access to high quality health care services	4	9
Lack of education/knowledge about disease	3	3
Lack of access to Crohn's medications	1	1
Patient Financial Barriers		
Patients unable to afford treatment in general (medications and surgeries)	6	9
High cost of diagnostic testing	3	4
Lack of insurance coverage	2	4
Patients unable to afford biologics	1	3

ITB: Intestinal tuberculosis; TB: Tuberculosis; UC: Ulcerative colitis.

DISCUSSION

Summary of findings

The majority of publications describing Crohn's disease in low and lower-middle income countries are from South Asia, with nearly half of the studies included in this review taking place in India. However, 73% of LLMICs worldwide don't have any published studies on Crohn's, highlighting a major lack of published data. There is an even more severe lack of population-based epidemiologic data about Crohn's in LLMICs, with only four LLMICs reporting incidence or prevalence data-- India, Indonesia, Sri Lanka, and the Philippines, all of which are in Asia. Given the numerous diagnostic challenges facing gastroenterologists in diagnosing Crohn's in LLMICs, this gap in knowledge might be reflective of providers' inability to diagnose the disease due to these challenges, or simply a lack of resources to publish the data, rather than a true absence of IBD in these populations. It is quite telling that a majority (211) of the studies included in this review are from large tertiary facilities in capitol cities, which have the capacity to diagnose Crohn's disease (see [Supplementary Table 7](#) for a summary of all facilities included in the review).

The primary diagnostic challenges facing Crohn's disease providers in LLMIC are: difficulty differentiating between Crohn's and ITB, limited clinical awareness and low index of suspicion, and a lack of quality diagnostic facilities and investigational modalities. Distinguishing between Crohn's and ITB is a serious issue for gastroenterologists in countries where TB is prevalent. Crohn's and ITB overlap substantially in terms of their clinical symptoms, as well as their endoscopic, pathologic, and radiologic findings, but have entirely different treatment methods. Many providers in LLMICs treat TB empirically before even considering Crohn's disease, which can delay diagnosis by months or years. Algorithms for accurately diagnosing Crohn's and TB is an important area of gastroenterology research in LLMICs.

Comparison to prior reviews

This review is unique from prior global studies of IBD because it is the first to be focused specifically on Crohn's disease in LLMICs. Its scoping nature has also allowed us to adapt our research questions and inclusion criteria throughout the review process, thus identifying a larger number of LLMICs reporting individuals with Crohn's disease compared to prior reviews. These countries are: Bangladesh, Bolivia, Cameroon, Egypt, Ethiopia, Ghana, India, Indonesia, Kenya, Malawi, Morocco, Nepal, Nigeria, Pakistan, the Philippines, Sri Lanka, Sudan, Syria, Tunisia, Uganda, and Vietnam. This review also documents diagnostic barriers that suggest that current incidence and prevalence estimates for these countries are likely too low.

Research implications/future directions

Crohn's disease research in general is very important in LLMICs, especially in those countries where there is no or very little data. What little data is published from LLMICs is generated primarily from large tertiary hospital and likely does not reflect the status of Crohn's in the rest of the country. This lack of published data has contributed to a perceived rarity of Crohn's by gastroenterologists in LLMICs, reducing their awareness of the disease and thus likelihood of accurately diagnosing it. It is critical to study and publish data on Crohn's in LLMICs, even if they are single facility-based or case-studies, and to set up clinical data registries so that population-based epidemiologic research can shed light on the true burden of Crohn's in these settings. A survey of gastroenterology providers in LLMICs, including in-depth interviews, would also be useful to more comprehensively capture the nuances of diagnosing and treating Crohn's disease in low resource settings. Future studies should also collect and report data on the geography of where patients' reside, as well as socioeconomic information such as income-level or employment.

Practice implications

Crohn's disease is a time-consuming and expensive disease to diagnose and treat, and thus cost is the most significant barrier faced by Crohn's patients in LLMICs. Biologics are especially expensive, and in some countries such as Nigeria, are only available through a special application to the government. This is further exacerbated by a general lack of insurance coverage among these patients. Although Crohn's has historically been documented primarily among wealthier populations living in urban centers, this review does give some indication of Crohn's in patients living in rural areas in India, Sri Lanka, Egypt, Tunisia, Ethiopia, Kenya, Indonesia, the Philippines, and Bolivia. Education of both gastroenterologists and other providers in LLMICs about the symptoms and findings of Crohn's, especially at rural lower-level facilities, is also essential to improving disease awareness among providers and thus accurate diagnosis. Diagnosis is further delayed by a lack of reliable diagnostic facilities in remote areas of LLMICs, particularly endoscopy and pathology. Decentralizing Crohn's care by training providers at lower-level care facilities in endoscopy and colonoscopy can potentially reduce underdiagnosis of Crohn's in these areas. This may also be an opportunity to utilize telemedicine and telepathology technologies.

CONCLUSION

This scoping review has shown that Crohn's disease does indeed exist in LLMICs in South Asia, the Middle East and North Africa, sub-Saharan Africa, East Asia and Pacific, and Latin America and the Caribbean, sometimes in large numbers. There is a pressing need to study the population epidemiology of Crohn's disease in LLMICs to fully understand the burden of Crohn's disease among the world's poorest people. This is particularly important in those countries where it has not been studied at all, and in rural areas, where there is likely the most significant underdiagnosis and lack of access to medical care.

ARTICLE HIGHLIGHTS**Research background**

Crohn's disease is a chronic gastrointestinal disease that has been recognized in the rapidly developing upper-middle income countries of East Asia, and South America.

In contrast, previous systematic reviews of published literature have found few studies on either the prevalence or incidence of Crohn's coming from the low- and lower-middle income countries (LLMICs). In the absence of primary population data regarding Crohn's in these countries, there has been a perception that the burden of Crohn's remains low among the global poor. As part of an effort to understand the non-communicable burden of disease among the world's poorest, we have conducted a scoping review with broad inclusion criteria focused on the experience with Crohn's in LLMICs.

Research motivation

The correct diagnosis of Crohn's requires a complex chain of events beginning with patients seeking care and ending with colonoscopy and histology. A break in any part of this chain resulting from gaps in financing, education, equipment, or supplies on the part of the patient or provider can result in a missed diagnosis. Crohn's may also have an impact on patients living in extreme poverty that is out of proportion with the disease prevalence. Follow-up care for Crohn's, like many other chronic diseases, requires frequent visits to health facilities, a steady supply of medications (including biologics), and often surgery. The absence of the services could result in a high rates of disability and death among the poor affected by Crohn's in LLMICs.

Research objectives

As part of an effort to understand the non-communicable burden of disease among the world's poorest, we have conducted a scoping review with broad inclusion criteria focused on the experience with Crohn's in LLMICs. Specifically, this review seeks to answer the following research questions: (1) What is the published evidence regarding the burden of Crohn's disease in communities and health facilities in LLMICs? Is underdiagnosis a problem? (2) What is the diagnostic and treatment capacity for Crohn's disease in LLMICs? What services, equipment, and medications are used to diagnose and manage Crohn's in LLMICs? (3) What challenges and barriers are there to providers and patients with Crohn's disease in LLMICs in terms of diagnosis, treatment, and long-term management? And (4) What is known from the published literature regarding the social and demographic characteristics of patients with Crohn's disease in LLMICs?

Research methods

The authors conducted a scoping review utilizing a full search strategy of studies that describe cases of individuals with Crohn's disease in an LLMIC as defined by The World Bank that was developed and conducted in PubMed, Embase and World Health Organization Global Index Medicus. To capture possible undiagnosed or misdiagnosed cases of Crohn's in LLMICs that do not have any published Crohn's data, as well as to understand their diagnostic and treatment capacity for Crohn's, studies that mention the use of diagnostics (*i.e.* colonoscopy, small bowel follow-through, stool calprotectin), findings (*i.e.* skip lesions, cobblestone, small bowel obstruction), and treatments (*i.e.* colectomy, small bowel resection, infliximab) utilized in managing Crohn's disease were also included. Two independent reviewers screened the titles and abstracts of all of the publications found in this search, reviewed selected publications, and extracted relevant data, which underwent descriptive review and was analyzed in Excel.

Research results

The database search yielded 4486 publications, 216 of which were determined to be relevant to the research questions. Of all 79 LLMICs, only 21 (26.6%) have publications describing individuals with Crohn's. The majority of Crohn's studies identified are from India (49.5%), followed by Tunisia (19.0%), Egypt (8.3%), and Sri Lanka (5.1%). The mean number of Crohn's patients reported per study is 56.84 and the median is 22, with a wide range from one to 980. Of the 21 LLMICs included in the review, only two countries (India and Sri Lanka) had studies estimating prevalence and only four countries (India, Indonesia, Sri Lanka and Philippines) had studies reporting incidence of Crohn's disease with most of the data coming from one multi-country study.

Of the 216 studies included in the review, 112 discussed the utilization of diagnostic and treatment services, all of which include cases that were confirmed *via* both colonoscopy and histology. Of the 21 LLMICs included, all but Bolivia and Syria had at least one study discussing the utilization of Crohn's diagnostic services: Blood testing, stool testing, TB testing, radiology/imaging, endoscopy, and pathology services. Corticosteroids, aminosalicylates, and immunomodulators are the most

frequently reported medications overall, while biologic agents are the least available. The most frequently discussed Crohn's surgery overall is colectomy, followed closely by small bowel resection. Ileoanal pouches were not specifically described in any of the included studies, but several studies described other or unspecified anal surgery for Crohn's disease. Of the 216 studies in 21 countries included in this review, only 29 studies in 11 countries discussed patient geographic, socioeconomic, or cost information.

Of the 21 LLMICs included in this review, 14 hypothesized at least one specific diagnostic, management, access, or financial challenge or barrier to individuals with Crohn's disease and providers. The most commonly reported provider challenge is differentiating between Crohn's and intestinal tuberculosis (ITB), due to the high prevalence of TB in LLMICs and its overlap of symptoms and endoscopic features. This can result in long delays in disease diagnosis and thus appropriate treatment. This was followed by diagnostic delays due to perceived rarity of IBD and lack of clinical awareness among providers, which was mentioned in 17 studies from eight countries, and lack of quality diagnostic facilities, which was mentioned in 14 studies from eight countries. The most frequently reported patient barrier was the cost of Crohn's surgeries and medications, particularly biologics. Lack of access to high quality health care facilities was another common patient barrier, with nine studies describing access to care as a patient barrier.

A lack of published data has contributed to a perceived rarity of Crohn's by gastroenterologists in LLMICs, reducing their awareness of the disease and thus likelihood of accurately diagnosing it. It is critical to study and publish data on Crohn's in LLMICs, even if they are single facility-based or case-studies, and to set up clinical data registries so that population-based epidemiologic research can shed light on the true burden of Crohn's in these settings.

Research conclusions

This scoping review has shown that Crohn's disease does indeed exist in LLMICs in South Asia, the Middle East and North Africa, sub-Saharan Africa, East Asia and Pacific, and Latin America and the Caribbean, sometimes in large numbers.

This scoping review demonstrates that although there is a lack of rigorous epidemiologic data on Crohn's disease in LLMICs, it actually does exist, sometimes in large numbers, in these settings and presents unique challenges that need to be addressed to advance non-communicable diseases care in low resource settings.

There is a pressing need to study the population epidemiology of Crohn's disease in LLMICs to fully understand the burden of Crohn's disease among the world's poorest people. This is particularly important in those countries where it has not been studied at all, and in rural areas, where there is likely the most significant underdiagnosis and lack of access to medical care.

Patients with Crohn's disease do indeed exist in low resource settings and there are existing models of service delivery that could be learned from and adapted to meet the unique challenges of management. For example, this study has found that one of the primary diagnostic challenges facing Crohn's disease providers in LLMIC are: difficulty differentiating between Crohn's and ITB. Crohn's and ITB overlap substantially in terms of their clinical symptoms, as well as their endoscopic, pathologic, and radiologic findings, but have entirely different treatment methods. Many providers in LLMICs treat TB empirically before even considering Crohn's disease, which can delay diagnosis by months or years.

Decentralizing Crohn's care by training providers at lower-level care facilities in endoscopy and colonoscopy can potentially reduce underdiagnosis of Crohn's in these areas. This may also be an opportunity to utilize telemedicine and telepathology technologies.

This study utilized broad inclusion criteria to map the landscape of studies showing where and how services for the management of Crohn's disease are provided and organized.

If there are existing models of service delivery for patients with Crohn's disease in low resource settings, there can also be valuable lessons in how these models can be adapted and translated to similar areas still in need of services.

This review seeks to answer the following research questions: (1) What is the published evidence regarding the burden of Crohn's disease in communities and health facilities in LLMICs? Is underdiagnosis a problem? (2) What is the diagnostic and treatment capacity for Crohn's disease in LLMICs? What services, equipment, and medications are used to diagnose and manage Crohn's in LLMICs? (3) What challenges and barriers are there to providers and patients with Crohn's disease in LLMICs in terms of diagnosis, treatment, and long-term management? And (4) What

is known from the published literature regarding the social and demographic characteristics of patients with Crohn's disease in LLMICs?

Education of both gastroenterologists and other providers in LLMICs about the symptoms and findings of Crohn's, especially at rural lower-level facilities, is essential to improving disease awareness among providers and thus accurate diagnosis. Decentralizing Crohn's care by training providers at lower-level care facilities in endoscopy and colonoscopy can potentially reduce underdiagnosis of Crohn's in these areas. This may also be an opportunity to utilize telemedicine and telepathology technologies.

Research perspectives

Crohn's disease research in general is very important in LLMICs, especially in those countries where there is no or very little data. What little data is published from LLMICs is generated primarily from large tertiary hospitals and likely does not reflect the status of Crohn's in the rest of the country.

It is critical to study and publish data on Crohn's in LLMICs, even if they are single facility-based or case-studies, and to set up clinical data registries so that population-based epidemiologic research can shed light on the true burden of Crohn's in these settings.

A survey of gastroenterology providers in LLMICs, including in-depth interviews, would also be useful to more comprehensively capture the nuances of diagnosing and treating Crohn's disease in low resource settings. Future studies should also collect and report data on the geography of where patients' reside, as well as socioeconomic information such as income-level or employment. In addition, algorithms for accurately diagnosing Crohn's and TB is an important area of gastroenterology research in LLMICs.

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