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ABOUT COVER

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OPINION REVIEW

Alteration of the esophageal microbiota in Barrett's esophagus and esophageal adenocarcinoma

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Abstract

The incidence of esophageal adenocarcinoma (EAC) has increased in recent decades, and its 5-year survival rate is less than 20%. As a well-established precursor, patients with Barrett's esophagus (BE) have a persistent risk of progression to EAC. Many researchers have already identified some factors that may contribute to the development of BE and EAC, and the identified risks include gastroesophageal reflux (GER), male sex, older age, central obesity, tobacco smoking, *Helicobacter pylori* (*H. pylori*) eradication, and the administration of proton pump inhibitors (PPIs) and antibiotics. The human gut harbors trillions of microorganisms, the majority of which are bacteria. These microorganisms benefit the human host in many ways, such as helping in digestion, assisting in the synthesis of certain vitamins, promoting the development of the gastrointestinal immune system, regulating metabolism and preventing invasion by specific pathogens. In contrast, microbial dysbiosis may play important roles in various diseases, such as inflammation and cancers. The composition of the microbiota located in the normal esophagus is relatively conserved without distinct microbial preferences in the upper, middle and lower esophagus. Six major phyla constitute the esophageal microbiota, including *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *TM7*, similar to the oral microbiota. *Streptococcus* dominates the esophageal microbiota. However, the microbiota varies in different esophageal diseases compared to that in the healthy esophagus. The type I microbiota, which is primarily composed of gram-positive bacteria, is closely associated with the normal esophagus, while type II microbiota has enriched gram-negative bacteria and is mainly associated with the abnormal esophagus. These increased gram-negative anaerobes/microaerophiles

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include *Veillonella*, *Prevotella*, *Haemophilus*, *Neisseria*, *Granulicatella* and *Fusobacterium*, many of which are associated with BE. The microbial diversity in the esophagus is decreased in EAC patients, and *Lactobacillus fermentum* is enriched compared to that in controls and BE patients. Furthermore, the microbiota may be associated with BE and EAC by interacting with their risk factors, including central obesity, GER, *H. pylori*, administration of PPIs and antibiotics. Therefore, a large gap in research must be bridged to elucidate the associations among these factors. Some studies have already proposed several potential mechanisms by which the microbiota participates in human carcinogenesis by complicated interactions with the human host immune system and signaling pathways. The activation of the LPS-TLR4-NF-κB pathway may contribute to inflammation and malignant transformation. This exciting field of gastrointestinal microbiota allows us to unravel the mystery of carcinogenesis from another perspective. Further studies are needed to explore whether the microbiota changes before or after disease onset, to improve our understanding of the pathogenesis, and to find novel targets for prevention, diagnosis and therapy, which could offer more cost-effective and relatively safe choices.

Key words: Barrett's esophagus; esophageal adenocarcinoma; microorganisms; esophageal microbiota; alteration; dysbiosis

Core tip: Esophageal adenocarcinoma (EAC) is a malignancy with poor prognosis, and Barrett's esophagus (BE) is the only recognized precursor. As part of the human gut, the esophagus harbors distinctive microbiota, and dysbiosis may be related to BE/EAC. Many studies have attempted to characterize the esophageal microbiota in the normal esophagus and in different diseases, but more data are required. Studies on the esophageal microbiota in BE/EAC have mainly concentrated on these associations, and the underlying mechanisms remain blurred. This review focuses on the features and associations of esophageal microbiota and BE/EAC, which might provide some evidence of their relationships.

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INTRODUCTION

Esophageal cancer is one of the most common malignancies in the world, and the incidence of esophageal adenocarcinoma (EAC) has markedly increased in recent decades, as it accounts for almost half of all esophageal cancers[1,2]. The 5-year survival rate is less than 20%[3] because most EAC patients are first diagnosed in the advanced stages, which are not curable[4]. As a well-established risk factor for EAC, Barrett's esophagus (BE) confers a persistent risk of progression to EAC[5], increasing a patient's risk more than 30 times than the general population[6,7]. In the first global definition, namely, the Montreal definition, BE is defined as the replacement of normal squamous epithelial lining with metaplastic columnar epithelium[8]. Notably, the incidence of EAC progression from BE varies among studies. Some studies have indicated that more than 85% of newly diagnosed EAC patients have no history of BE[9,10], whereas other investigations[6,7] have reported that almost half of EAC patients have progressed from BE. Nevertheless, BE is the only well-recognized precursor of EAC, and the underlying mechanisms of pathogenesis and carcinogenesis need to be elucidated[11]. Many researchers have already shown that some factors may contribute to the development of BE and EAC, and the identified risks include, but are not limited to, gastroesophageal reflux (GER), male sex, older age, central obesity, tobacco smoking, *Helicobacter pylori* (*H. pylori*) eradication with antibiotics and acid suppression therapy[3,12-14]. However, preventive strategies are lacking.

The human gut harbors trillions of microorganisms[15-18], and the composition of the microbial communities that inhabit the mouth, esophagus, stomach and intestine are diverse and host-specific. The majority of microorganisms are bacteria and are estimated to comprise ~10^{14} bacterial cells, which is ten times more than the total number of human cells[19]. These microorganisms benefit the human host in many ways, such as helping in digestion, assisting in the synthesis of certain vitamins, promoting the development of the gastrointestinal immune system, regulating metabolism and preventing invasion by specific pathogens[15,16,19,20]. On the other hand, microbial dysbiosis may lead to tissue damage and play significant roles in various diseases, including inflammatory disorders and cancers[21-25]. Dysbiosis refers to an abnormal condition of the microbial ecosystem in a host[26]. Therefore, equilibrium must be achieved and maintained to support the interactions of the human host and microbiota. Before the 1990s, researchers mainly focused on the role of certain microorganisms by using protocols largely limited to culture-dependent methods, but cultivation is not suitable for defining a complicated microbial community and may induce bias as well[19]. Since the development of next-generation sequencing, which is independent of cultivation, the sensitivity of research techniques has been dramatically improved, and microbiota exploration has begun[20].

However, little is known about the relationship between the microbiota and the pathogenesis of BE and EAC. Here, we review the features of microbial communities in BE and EAC patients, which may provide some evidence of the relationships between altered esophageal microbiota and BE/EAC.

ESOPHAGEAL MICROBIOTA

The colon has the largest microbiota in the body[20], whereas the esophagus has far fewer microbes. Although bacterial communities have been observed with high inter- and intra-individual variations, an overlapping community has been identified between sites[27,28]. Previous studies[20,29-31] have suggested that *Firmicutes* (*Clostridium*, *Ruminococcus*, *Eubacterium*, *Peptostreptococcus*, *Peptococcus*, *Lactobacillus-L*), *Bacteroidetes*, *Proteobacteria* (*Enterobacteriaceae*) and *Actinobacteria* (*Bifidobacterium-BF*) phyla constitute the majority of the human gut microbiota. The composition of the microbiota located in the normal esophagus is relatively conserved, and the estimated resident microbes are mainly composed of more than 100 species, most of which have already been identified[32,33]. The dominant microbes that colonize the normal esophagus are *Streptococcus*[32,33], and Dong L *et al*.[28] reported that no distinct microbial preference exists in the upper, middle and lower esophagus. However, the microbiota varies in different esophageal diseases compared to the healthy esophagus[34].

In 2004, Pei Z *et al*.[32] found that six major phyla constituted the esophageal microbiota, namely, *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *TM7*, which are comparable to the oral microbiota. The genus *Streptococcus* dominates the esophageal microbiota[32]. Since then, more studies have emerged, and a classification for the esophageal microbiota was proposed[33]. In 2009, Yang L *et al*.[33] reported that type I microbiota, which is mainly composed of gram-positive bacteria, is closely associated with the normal esophagus and is dominated by the *Firmicutes* phylum. Consistent with previous studies, *Streptococcus* was the most dominant genus, and its relative abundance was higher. The type II microbiota is enriched in gram-negative bacteria (more than 50%) and is mainly associated with the abnormal esophagus. The relative abundances of 24 other genera are increased in the type II microbiota, many of which are relevant to BE. These increased gram-negative anaerobes/microaerophiles include *Veillonella*, *Prevotella*, *Haemophilus*, *Neisseria*, *Granulicatella* and *Fusobacterium*. Moreover, one study[19] conducted in Japan showed that the numbers of bacteria were similar in control, esophagitis and BE groups, despite the changes in the relative abundance of taxa. In patients with esophagitis or BE, the microbial diversity changed, and the abundance of *Streptococcus* species was reduced[33]. Gram-negative anaerobes/microaerophiles occupied greater proportions[33], such as *Veillonella*, *Prevotella*, *Fusobacterium* and *Neisseria*[19,33]. This shift from a gram-positive aerobic microbiota to a gram-negative anaerobic microbiota may be influenced by microenvironmental changes and related to abnormal disease states[33]. These consistent observations suggested that the altered microbiota is reliable in BE and could be further studied. Of note, Macfarlane S *et al*.[35] found that *Campylobacter* colonized the esophagus of the majority of BE patients and could not be identified in the control group. Moreover, Amir I *et al*.[36] strongly suggested that the family *Enterobacteriaceae* (mainly the genus *Escherichia*) is associated with esophageal abnormalities, such as esophagitis and BE, and may have a possible role in the pathogenesis of inflammation and metaplasia. Therefore, a large-scale joint multi-

center, multi-region, multi-race study about the alteration of the esophageal microbiota in BE/EAC is needed to provide more evidence.

Damage of the esophageal epithelium could affect the normal barrier and induce the translocation of other bacteria, thus influencing the microenvironment and immune homeostasis[11]. Although studies of specific bacteria in the development of EAC in addition to *H. pylori*[3] are rare, more attention has been paid to the local microbiota changes. The microbial diversity in the esophagus is decreased in EAC patients[12], regardless of the exact sampling locations. The decreased genera included some gram-negative and gram-positive taxa, such as *Veillonella* and *Granulicatella*. In contrast, *Lactobacillus fermentum* was found to be enriched in EAC patients compared to controls and BE patients. Notably, lactic acid bacteria could be dominant and affect the microenvironment[12]. A low microenvironmental pH may facilitate the growth of *Lactobacillus* spp. and *Streptococcus* spp. in the tumor niche[12]. Fermentation could produce more factors to inhibit the proliferation of other competitor microbes as well. Then, *Lactobacillus* may dominate the environment of the lower esophagus. Moreover, some specific species were demonstrated to have higher abundance. At the phylum level, the proportional abundance of *Tenericutes* was higher. At the genus level, the proportional abundances of *Fusobacterium*, *Megasphaera*, *Campylobacter*, *Capnocytophaga*, and *Dialister* were greater[12]. However, Blackett KL *et al*.[37] did not identify any specific taxa with significant differences, and Zaidi AH *et al*.[38] reported that *Streptococcus pneumonia* was present at a relatively higher abundance in control and BE groups compared to EAC in rat BE and EAC models. Interestingly, Peters BA *et al*.[39] found that the oral microbiota composition could reflect the prospective risk for EAC, and the genus *Neisseria* and the species *Streptococcus pneumoniae* were associated with EAC risk, which is consistent with the findings in the studies above. It was reasonable to conclude that the esophageal microbiota is largely influenced by the oral microbiota and that the oral microbiota composition could provide some evidence of EAC progression[17].

Furthermore, the microbiota may be associated with BE and EAC by interacting with their risk factors. One notable example is the case of obesity. As a chronic systemic disease and a proposed risk factor, obesity, particularly central obesity, is closely related to BE and EAC[20,40-42]. The linear pattern between increasing body mass index (BMI) and increasing risks of BE and EAC has been verified in several studies[43-46], which partially accounts for the increasing prevalence of EAC. Central obesity is closely related to EAC, even after adjustment for BMI[47,48], whereas the association between BMI and EAC risk disappeared after adjustment for central obesity. Moreover, the relationship between central obesity and BE has a similar pattern. Therefore, adiposity distribution may play an important role in BE and EAC pathogenesis. However, it is unclear whether weight loss could contribute to a reduced risk of BE and EAC. The possible mechanisms by which central obesity contributes to BE and EAC have been explored and discussed in several aspects. First, the increased abdominal adipose tissue might increase intra-abdominal pressure and gastric compression, disrupting the normal function of the gastroesophageal junction and promoting GER, which is also a well-recognized risk factor for BE and EAC[3]. Second, excess adipose tissue could secrete pro-inflammatory cytokines and adipokines[20], and these active factors could provoke inflammatory and metabolic changes in the body[40], such as stimulation of cell proliferation, apoptosis inhibition and neoplastic transformation. Third, the gut microbiota is altered in obese patients and has been associated with the activation of inflammation, which may play an important role in the development of BE and EAC[49]. *Streptococcus* and *Prevotella* species are the dominant bacteria in the upper gastrointestinal tract, and their ratio may be associated with central obesity and hiatal hernia length[27], which are two known risks of BE and EAC. In addition, the gut microbiota may be adjusted concomitantly along with dietary changes that humans experience and that are the main cause of central obesity, but some 'lost' taxa may be difficult to regain over generations[50]. Therefore, a large gap in research must be bridged to elucidate the associations among central obesity, microbiota and EAC[51].

Ultimately, the optimal method for esophageal microbiota sampling is fundamental and needs to be explored. Many studies have used invasive endoscopy to obtain focal tissues that are similar to those obtained by biopsy or endoscopic brushing to examine the microbiota in a larger area. Gall A *et al*.[27] showed that mucosal brush samples could enhance the detection of bacterial diversity in the esophagus and stomach and the microbiota compositions were similar after replicate sampling. Fillon SA *et al*.[52] proposed another minimally invasive sampling method, namely the overnight esophageal string test, and suggested that the compositions of the esophageal microbiota were similar between the traditional biopsy and the string test. Due to the lack of standard sampling methods, Elliott DRF *et al*.[12] studied the values of the Cytosponge prototype as a non-endoscopic sampling device to seek minimally

invasive methods for sampling the esophageal microbiota. For comparison, endoscopic biopsies, brushes and throat swabs were also included. Nevertheless, most of the microbial species overlapped among different sampling methods. However, the sampling area was larger with the Cytosponge, and the microbial DNA yield and total microbial abundance were higher. Moreover, the Cytosponge also provided clues for histological data, thus making it a valuable sampling method.

The important findings discussed above are summarized in Table 1. Nevertheless, some of these data were drawn from a specific population, and the sample sizes were not large. Consequently, these findings still need to be verified before any application to the general population, and more effort should be made to reveal the exact alteration of the microbiota in different diseases.

OTHER POSSIBLE FACTORS RELATED TO THE ESOPHAGEAL MICROBIOTA

Helicobacter pylori

It has been reported that more than 15% of human malignant cancers are related to infection or infection-associated inflammation[53], and many relevant studies are mainly focused on single microorganisms. There are two main theories to explain bacterial diseases[33]. Koch proposed the classic pathogen theory, which requires the presence of specific pathogens[54]. The other theory of microecological diseases is a new concept in which the whole microbiota contributes to pathogenicity[26]. As the only bacteria considered a class I human carcinogen, *H. pylori* is closely related to the progression of gastritis, gastric ulcer, gastric atrophy and gastric adenocarcinoma[55,56]. The bacterium has infected more than 50% of the world's population[57] and continues to spread. Although *H. pylori* is primarily localized to the gastric mucosa, its colonization could affect the gastric and esophageal microbiota[11,58], and the microbial composition in the esophagus and stomach overlaps to a certain extent[27].

The increasing incidence of BE and EAC may be inversely associated with *H. pylori* infection[59,60], and the inverse correlation between *H. pylori* and EAC risk has been well documented[10,27]. Meta-analyses[61-63] based on epidemiological and observational studies showed that EAC coincides with *H. pylori* eradication[49]. That is, *H. pylori* might affect carcinogenesis in the lower esophagus[27]. However, the inner mechanisms have only been partially revealed[10,27]. *H. pylori* harbors some factors that lead to chronic inflammation and cancer, such as cytotoxin-associated gene A (CagA), vacuolating cytotoxin (VAC) and adhesins. The bacterium could promote inflammatory responses by activating nuclear factor kappa B (commonly known as NF-κB)[56] and may induce the production of certain cytokines such as IL-1β, IL-2, IL-8 and tumor necrosis factor-α (TNF-α), which trigger inflammatory responses in the gastric epithelium. *H. pylori* may also directly damage host DNA, dysregulate DNA transcription factors such as caudal type homeobox 2 (Cdx2), and induce epithelial injury and acid secretory functions[55,56,58]. Another possible mechanism is that *H. pylori*-induced gastric atrophy causes a reduction in gastric acid, which is the main source of GER substances[20]. Some studies suggest that *H. pylori* eradication could increase the serum level of ghrelin, which may lead to obesity and affect gastric emptying[61], subsequently initiating the risks of BE and EAC. Moreover, *H. pylori* may stimulate apoptosis of EAC cells via the Fas-caspase cascade, which may account for another protective mechanism[61].

MEDICATIONS

Acid suppression therapies have been highly effective in acid-induced diseases, such as gastritis, esophagitis, BE and EAC. Proton pump inhibitors (PPIs) are considered benign and are commonly used in clinical practice[64]. The suppression of acid secretion could affect gastric acidity, volume and GER and even the bacterial composition in the stomach and esophagus, with potential consequences for human health and diseases[36,64]. The administration of PPIs could change the microbial composition in the esophagus and stomach in BE patients[34,36], which may contribute to the pathogenesis of BE, though this has not been well established. Studies have suggested that PPIs may directly target certain bacteria that contain P-type ATPase enzymes as part of their proton pumps, such as *Streptococcus pneumoniae* and *H. pylori*[65]. Moreover, the microenvironment could also be affected by the increased pH in the stomach and esophagus after PPI therapy. PPIs could reduce the number of gram-negative bacteria and decrease the risk for neoplasia in the esophagus[64]. To detail the changes, Amir I *et al*.[36] collected esophageal samples before and after 8 wk

Table 1 Esophageal microbiota studies on Barrett's esophagus and esophageal adenocarcinoma

Publication year	Sample population	Sequencing approach	Related notable findings	Ref
2004	Four patients with normal esophagus	16S rDNA	1 Members of six phyla, *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *TM7*, were represented. 2 *Streptococcus* (39%), *Prevotella* (17%), and *Veilonella* (14%) were most prevalent.	[32]
2007	Seven subjects without BE and seven patients with BE	16S rRNA	*Campylobacter* colonized the esophagus in the majority of BE patients and could not be identified in the control group.	[35]
2009	Thirty-four patients with normal, esophagitis, or Barrett's esophagus	16S rRNA	1 Esophageal microbiomes can be classified into two types. 2 The type I microbiome was mainly composed of gram-positive bacteria, dominated by the genus *Streptococcus* and concentrated in the phenotypically normal esophagus. 3 The type II microbiome contained a greater proportion of gram-negative anaerobes/microaerophiles and primarily correlated with esophagitis (Odds Ratio: 15.4) and BE (Odds Ratio: 16.5).	[33]
2012	Fifteen subjects	16S rRNA	The compositions of the esophageal microbiota were similar between the traditional biopsy and the overnight esophageal string test.	[52]
2014	Thirteen patients with esophagitis, six patients with BE, fifteen normal controls	16S rRNA	The *Enterobacteriaceae* family (mainly the genus *Escherichia*) is associated with esophageal abnormalities, such as esophagitis and BE.	[36]
2015	Twelve participants enrolled in the Seattle Barrett's Esophagus Research Program	16S rRNA	1 *Streptococcus* and *Prevotella* species dominate the upper GI and the ratio of these two species is associated with waist-to-hip ratio and hiatal hernia length, two known EAC risk factors in Barrett's esophagus. 2 Mucosal brush samples enhanced the detection of bacterial diversity in the esophagus and stomach, and the microbiota compositions were similar after replicate sampling.	[27]
2017	Twenty normal controls, twenty-four non- dysplastic BE twenty-three dysplastic BE	16S rRNA	1 The microbial diversity in the esophagus is decreased in EAC patients, regardless of the exact sampling locations. 2 *Lactobacillus fermentum* was enriched in EAC patients, and lactic acid bacteria dominated and affected the microenvironment.	[12]

2018	Twenty-seven dental and esophageal disease-free individuals	16S rRNA	1 The phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, and *TM7* were most abundant in both the oral cavity and the esophagus. 2 The genera *Streptococcus*, *Neisseria*, *Prevotella*, *Actinobacillus*, and *Veillonella* were most abundant in both oral cavity and esophagus, and *Streptococcus* in the esophagus. 3 No site-specific bacteria were found for three different segments (upper, middle, and lower) of the esophagus.	[28]

These studies displayed are sorted by the publication year. BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; GI: Gastrointestinal.

of PPI treatment in BE patients and found that PPI usage changed the esophageal microbiota. At the family level, *Comamonadaceae* was decreased, whereas other families, such as *Clostridiaceae* and *Lachnospiraceae*, were increased. However, long-term PPI therapy induced hypergastrinemia, which may upregulate cyclooxygenase-2 (COX-2) expression, cell proliferation and esophageal carcinogenesis[66]. PPI administration plays an important role in *H. pylori* eradication therapy. A study[67] conducted by Fischbach LA *et al*. showed that PPIs augment anti-*H. pylori* activity, and *H. pylori* appears to exert a protective role in esophageal neoplasia. The possible reason might be that PPIs have some direct protective effects in BE, which extend far beyond other effects[64]. At present, there is no direct evidence of a relationship between PPIs and esophageal carcinogenesis, and long-term preclinical and clinical studies with larger samples are needed to reveal the precise associations among PPIs, *H. pylori* and BE/EAC.

The introduction and worldwide application of antibiotics might also have contributed to the increasing incidence of BE and EAC[20]. The discovery and wide usage of antibiotics have cured many infectious diseases, but several unexpected effects have appeared, some of which may influence the progression of BE and EAC. Antibiotics may definitively change the gastrointestinal microbiota[3], and alteration of the microbial abundance and/or diversity might contribute to disease pathology[68]. Cho I *et al*.[69] found that the sub-therapeutic administration of antibiotics increased the abundance of *Firmicutes* and decreased the abundance of *Bacteroidetes*, which are the two main phyla in the colonic microbiota. Moreover, the overweight population harbored a higher ratio of *Firmicutes* to *Bacteroidetes* than the controls[26], and weight loss increased the abundance of *Bacteroidetes*. These data[26,69] indicated that long-term exposure to antibiotics changes the colonic microbiota, which may induce obesity and GER. In the same way, the use of antibiotics can change the microbiota in the esophagus. Tian ZY *et al*.[70] have suggested that *H. pylori* infection and antibiotic treatment changes the microbiota composition in the esophagus in a mouse model. In addition, an altered esophageal microbiota might play a more direct role than *H. pylori* or obesity in inflammation and in BE and EAC carcinogenesis[20]. On the other hand, antibiotics may help restore the normal esophageal microbiota to type I from type II by increasing the relative abundance of *Streptococcus*. Interestingly, antibiotics are another important part of *H. pylori* eradication therapy. The complicated associations among these factors need further investigation and validation[20].

MECHANISMS

Some studies have already proposed several potential mechanisms by which the microbiota participates in human carcinogenesis[20] by complicated interactions with the human host immune system and signaling pathways[56] (Figure 1). First, alteration of the microbiota may result in inflammation, and persistent chronic inflammation may promote carcinogenesis[71]. The disequilibrium between human immunity and microbiota may change the compositions of essential bacterial molecules at certain organs or sites and then form microorganism-associated molecular patterns (MAMPs)[71], such as toll-like receptors (TLRs) and nucleotide-binding-oligomerization-domain (NOD)-like receptors[72,73]. Then, the subsequent activation of related pathways may lead to the production and release of some target genes

involved in inflammation[73], such as cytokines, chemokines and other inflammatory factors. For example, specific bacterial invasion could promote the production of IL-17 and IL-23 and then induce an inflammatory response[74].

Bacterial products, or even the microbes themselves, could be sensed by some receptors on the epithelial membranes. The esophageal type II microbiota could produce larger amounts of gram-negative bacterial components, involving lipopolysaccharides (LPS)[19,75]. LPS could delay gastric emptying *via* COX1/2[76] and contribute to the development of GER by increasing the intra-gastric pressure. Notably, LPS could also affect the function of the lower esophageal sphincter, which may promote GER and carcinogenesis[75].

TLRs recognize known molecules from microbes[77,78] and have a well-recognized role in carcinogenesis[38]. As part of the pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), TLRs serve as receptors of various ligands, such as bacterial cell wall components and DNA and viral double-stranded RNA[77,78]. Therefore, TLRs mediate the interaction of the immune system with the microbiota[79]. Due to the connective roles between innate and adaptive immune responses, TLRs represent an important linking factor between inflammation and cancers[38,77,78,80]. As the natural ligand of LPS, TLR4 is expressed in the human esophageal epithelium, and its expression increases in BE and EAC[75]. TLR4 activation triggers the NF-κB pathway, which is related to inflammation-associated carcinogenesis[81] and mediates the initial metaplastic BE changes[82]. COX-2 is also upregulated as one of the downstream genes, which is related to gastric emptying[75] and occurs along the progression of BE and EAC[83]. Therefore, the activation of the LPS-TLR4-NF-κB pathway may contribute to inflammation and malignant transformation[20,75]. Similarly, activation of the Wnt signaling pathway could induce defects in cellular tight junction proteins and decrease the production of some mucins, which may have positive roles in the protection from carcinogenesis[84].

Second, bacteria may generate genotoxins that could cause genomic damage. For instance, the cytolethal distending toxin, which may be produced by gram-negative bacteria, can induce DNA damage and genomic instability[23,85]. Some bacterial products have tumor-promoting effects[86], such as CagA and VacA, and some bacteria may also activate procarcinogens to provoke inflammation and cancer development[87].

CONCLUSION

The number of studies on how microbial communities contribute to the pathogenesis of BE and EAC is increasing, and greater attention has been paid to the etiology and molecular mechanisms[49]. Although some observations are promising, the sample sizes of the related studies are not large enough, and the existing data are too limited to draw any convincing conclusion. The most appropriate methods and controls need to be proven as well. The mechanisms by which the microbiota affects the pathogenesis of BE and EAC are still not clear, and further studies are required. Whether the direct interactions between microbes and epithelia or the released products from microbes regulate local inflammation and immunity are under investigation[88]. Furthermore, it is important to identify when changes occur in the microbial composition during disease progression.

Without a doubt, exploring BE pathogenesis is a good way to study the carcinogenesis of EAC[11]. However, the alterations of microbial diversity in BE and EAC are modest[36], and the particular species of bacteria that can discriminate BE and EAC from controls have not yet been identified. Moreover, some low-abundance genera might be difficult to detect. Amir I *et al*.[36] reported that they could not identify any biomarker taxa for distinguishing BE from controls. Yang L *et al*.[33] showed that some gram-negative bacteria are enriched in BE and might thus be related to BE. Moreover, some genera might contribute to the etiology and pathogenesis of BE and EAC, or result from BE and EAC[11]. We still need to search for distinct microbial species that could be biomarkers for BE and EAC in individuals at higher risk, and the subtypes of EAC should be taken into consideration.

Nevertheless, finding the true causal mechanisms of dysbiosis is complicated, and the identification of a single species or a collection of species responsible for a particular disorder is sophisticated. The introduction of new techniques, such as next-generation sequencing, will definitely assist in revealing the mechanisms of the gastrointestinal microbiome in BE and EAC development, which might provide some evidence of their relations and the pathogenesis of BE and EAC. The microbiota in BE and EAC patients remains to be explored, particularly with the adjustment of other risks, such as sex and central obesity. It is crucial to improve our understanding of the

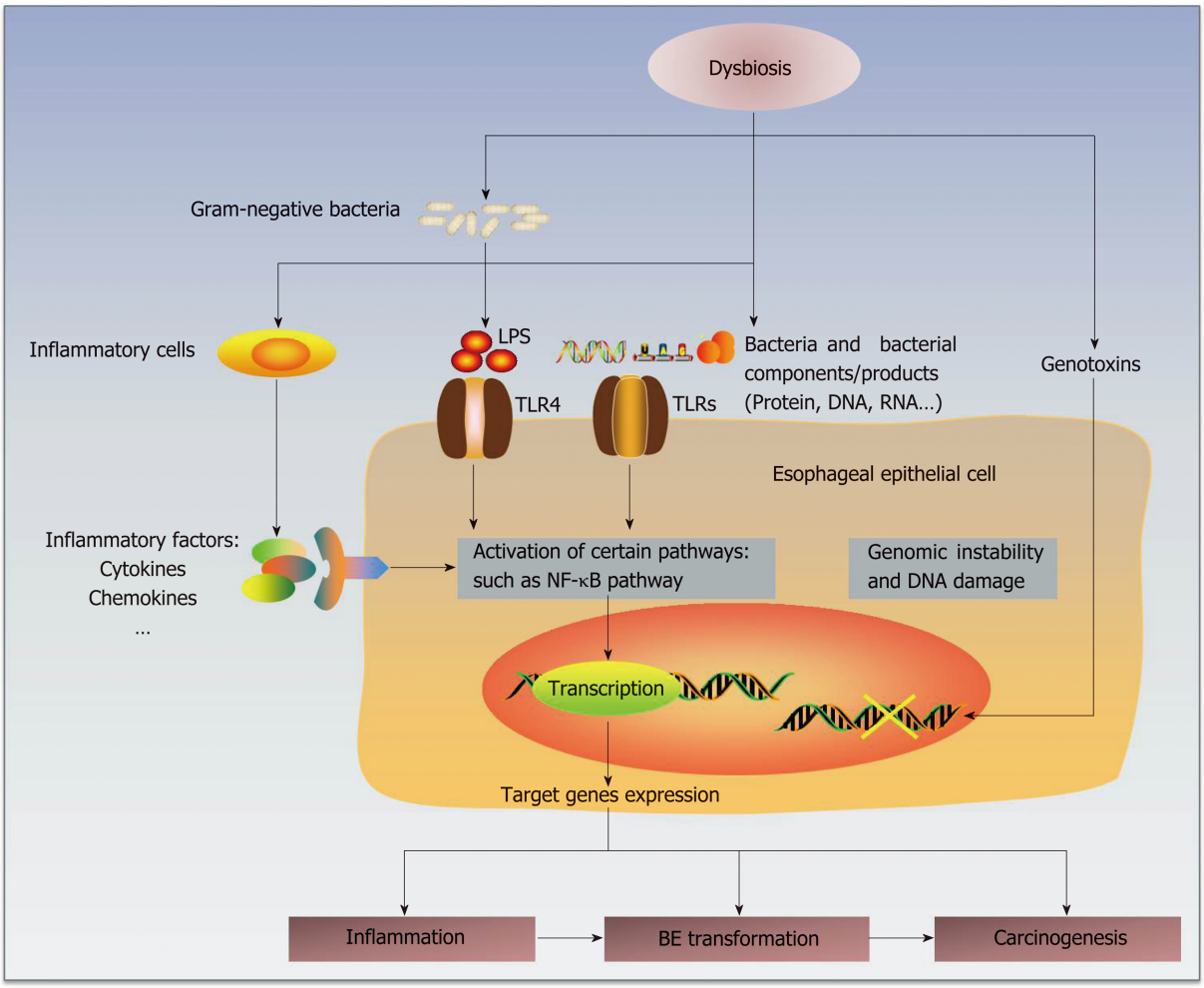

Figure 1 Hypothetical mechanisms by which the esophageal microbiota participates in the pathogenesis of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC). The microbial dysbiosis in the esophagus is associated with abnormal esophagus. Normal esophagus harbors a larger proportion of gram-positive bacteria, whereas the microbiota in abnormal esophagus is dominated by gram-negative bacteria. This shift from a gram-positive aerobic microbiota to a gram-negative anaerobic microbiota may interact with inflammatory cells and promote the production and secretion of inflammatory factors, such as cytokines and chemokines. In addition, the increase in gram-negative bacteria and their components/products, including LPS, DNA and RNA, may stimulate TLRs (mainly TLR4). TLR4 expression in the esophageal epithelium of BE/EAC is upregulated. As the natural ligands of LPS, TLR4 may play an important role in pathogenesis, whose activation could trigger the NF-κB pathway. These interactions mentioned above may stimulate activation of certain intercellular signaling pathways, such as NF-κB. This activation may upregulate the expression of target genes. Moreover, genotoxins generated by some bacteria may cause genomic instability and DNA damage. The end effects might be the induction of inflammation, BE transformation and carcinogenesis. However, whether the microbiota plays a causative role in BE/EAC progression is still unclear. LPS: lipopolysaccharides; NF-κB: nuclear factor kappa B; TLRs: toll-like receptors; BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma.

process by which the microbial composition may promote disorder progression[20].

The prognosis of EAC is poor, and the most pivotal factor is the tumor stage at diagnosis[3]. Given this situation in EAC patients, screening certain individuals with higher risks could be useful for early diagnosis. The cost-effectiveness and feasibility of endoscopic usage urge us to seek a less invasive and effective way of screening and early detection for BE and EAC[89]. Early detection of EAC will improve the survival rate and quality of life. Identification of BE patients who have higher risks of developing EAC could provide diagnostic clues and avoid unnecessary procedures, and the medical resources could be re-distributed to those who truly need attention. Understanding the pathogenesis and exploring biomarkers could also lead to early detection and prevention and improve the survival of some EAC patients. The therapeutic manipulation of the microbiota, such as prebiotics, probiotics or microbiota transplants, could be a potent approach in the management of inflammation and cancers[56].

The convincing associations between the microbiota and BE/EAC have indicated the importance of these studies. This exciting field of gastrointestinal microbiota allows us to unravel the mystery of carcinogenesis from another perspective, and the integration of different biomarkers may lead us to rapid advances. The introduction of animal models could be the proverbial icing on the cake. Future perspective studies

with sophisticated techniques are needed to explore whether the microbiota changes before or after disease onset, to improve our understanding of the pathogenesis, and to find novel targets for prevention, diagnosis and therapy, which could offer more cost-effective and relatively safe choices[19,56,90].

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REVIEW

Inflammatory bowel diseases and spondyloarthropathies: From pathogenesis to treatment

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Abstract

Spondyloarthropathies (SpA) include many different forms of inflammatory arthritis and can affect the spine (axial SpA) and/or peripheral joints (peripheral SpA) with Ankylosing spondylitis (AS) being the prototype of the former. Extra-articular manifestations, like uveitis, psoriasis and inflammatory bowel disease (IBD) are frequently observed in the setting of SpA and are, in fact, part of the SpA classification criteria. Bowel involvement seems to be the most common of these manifestations. Clinically evident IBD is observed in 6%-14% of AS patients, which is significantly more frequent compared to the general population. Besides, it seems that silent microscopic gut inflammation, is evident in around 60% in AS patients. Interestingly, occurrence of IBD has been associated with AS disease activity. For peripheral SpA, two different forms have been proposed with diverse characteristics. Of note, SpA (axial or peripheral) is more commonly observed in Crohn's disease than in ulcerative colitis. The common pathogenetic mechanisms that explain the link between IBD and SpA are still ill-defined. The role of dysregulated microbiome along with migration of T lymphocytes and other cells from gut to the joint ("gut-joint" axis) has been recognized, in the context of a genetic background including associations with alleles inside or outside the human leukocyte antigen system. Various therapeutic modalities are available with monoclonal antibodies against tumour necrosis

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factor, interleukin-23 and interleukin-17, being the most effective. Both gastroenterologists and rheumatologists should be alert to identify the co-existence of these conditions and ideally follow-up these patients in combined clinics.

Key words: Spondyloarthropathies; Axial spondyloarthropathies; Peripheral spondyloarthropathies; Ankylosing spondylitis; Inflammatory bowel disease

Core tip: Spondyloarthropathies (SpA) are subdivided to axial and peripheral SpA with ankylosing spondylitis (AS) being the prototype disease of the former. They have many extra-articular manifestations the most common of which is bowel involvement. Inflammatory bowel disease (IBD) (silent or clinically evident) occurs much more frequently in AS compared to the general population and associates with AS disease activity. Both axial and peripheral SpA occur more frequently in Crohn's disease than ulcerative colitis. Pathogenetic mechanisms that have been proposed to explain the link between SpA and IBD include dysregulated microbiome and migration of T lymphocytes and other cells from gut to the joint.

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INTRODUCTION

Under the term spondyloarthropathy (SpA) are classified many inflammatory arthropathies with similar clinical and imaging features. However, diagnostic laboratory or pathological findings with high specificity are lacking. SpA affects mainly the spine, but symptomatology from the peripheral joints as well as from entheses and other tissues might occur. SpA include: psoriatic arthritis (PsA), peripheral SpA, enteropathic [also known as inflammatory bowel disease (IBD)-related] arthritis, reactive arthritis, undifferentiated spondyloarthropathy and axial spondyloarthropathy (axSpA) which includes non-radiographic axial spondyloarthropathy (nraxSpA), and Ankylosing spondylitis (AS)[1]. The latter is considered the prototype of these diseases[2]. Enthesitis (inflammation of the entheses which are the insertions of the ligaments and tendons into the bone) is thought to be one of the key manifestations in SpA, helping to distinguish from other inflammatory arthropathies[1]. Apart from the skeletal disease, extra-articular manifestations like uveitis, psoriasis and inflammatory bowel disease often occur[2], offering significant help in the diagnosis of these diseases and being part of their classification criteria[3,4]. Of note, IBD contributes to the diagnosis of axSpA, as it has a positive likelihood ratio of 4.3 for axSpA diagnosis in patients with chronic low back pain[5,6].

According to the latest Assessment of SpondyloArthritis Society (ASAS) criteria, SpA can be classified to axSpA or peripheral SpA. The former pertains to patients with low back pain for ≥ 3 mo and age of onset < 45 years old and requires either sacroiliitis on imaging and at least one other SpA feature (*e.g.*, dactylitis, enthesitis) or positivity for HLA-B27 and at least two other SpA features (Table 1)[4]. Difference between nraxSpA and axSpA is the lack of radiographically confirmed sacroiliitis in the former. Under the term "peripheral SpA" are classified patients without current low back pain, with peripheral arthritis, or enthesitis or dactylitis plus at least one or two of the following SpA features: (uveitis, psoriasis, crohn´s disease/ulcerative colitis, preceding infection, positive HLA-B27, sacroiliitis on imaging) or (arthritis, enthesitis, dactylitis, inflammatory back pain in the past, family history of SpA), respectively[3].

It is well known that there is a close association between IBD and SpA[7]. Purpose of our review was to present in detail the existing epidemiological data and treatment approaches to these patients and to delineate the current diagnostic challenges. Also, we aimed to describe the underlying pathogenetic mechanisms that have been

Table 1 Assessment of Spondyloarthritis International Society Classification criteria for axial spondyloarthropathy and peripheral spondyloarthropathy

Axial Spondyloarthropathy		
Patients with back pain ≥ 3 months and age at onset <45 years		
Sacroiliitis on imaging1 plus ≥ *1*		*HLA-B27 plus* ≥ *2 other*
Spondyloarthropahty feature	OR	**Spondyloarthropahty features**
(Imaging arm)		(Clinical arm)
	Spondyloarthropahty features	
	Inflammatory back pain	
	Arthritis	
	Enthesitis (heel)	
	Uveitis	
	Dactylitis	
	Psoriasis	
	Crohn's Disease/Ulcerative colitis	
	Good response to NSAIDs	
	Family history of spondyloarthropahty	
	HLA-B27	
	Elevated CRP	
	Peripheral Spondyloarthropathy	
	Arthritis OR Dactylitis OR Enthesitis[2]	
	PLUS	
≥ 1		≥ 2
Uveitis		Arthritis
Psoriasis		Enthesitis
Inflammatory bowel disease	OR	Dactylitis
Preceding infection		IBP (past)
HLA-B27		Family history of Spondyloarthropahty
Sacroiliitis on imaging		

ASAS (ASAS, Assessment of Spondyloarthritis international Society) classification criteria for axial Spondyloarthropathy (axSpA) and peripheral Spondyloarthropathy (peripheral SpA).
[1]Definite radiographic sacroiliitis according to the modified New York criteria or positive sacroiliac magnetic resonance imaging.
[2]Without current back pain. NSAIDS: Non-steroidal anti-inflammatories; HLA: Human leukocyte antigen; CRP: C-reactive protein; IBP: Inflammatory back pain (3,4).

suggested to link these two entities.

IBD AND SPA: EPIDEMIOLOGY, AND ASSOCIATION WITH DISEASE CHARACTERISTICS

IBD in the context of SPA

IBD [including Crohn's disease (CD) and ulcerative colitis (UC)] is not rare in AS, with its prevalence ranging from 6%-14%[2,6,8,9]. In detail, in a large population, control-matched study, including 4101 patients with AS, Stolwijk *et al*[6] found that at the time of AS diagnosis, the cumulative incidence was 4%. Additionally, in a French large, prospective study for early inflammatory back pain, IBD occurred in 7.2% of patients with newly diagnosed AS[10]. Furthermore, in an early axSpA cohort frequency of IBD was calculated to be 2.6% (1.7% for AS and 0.9% for nraxSpA, difference was not significant)[11]. In fact, it has been suggested that the risk for IBD is more pronounced in the first years of AS diagnosis and falls at baseline levels approximately 10 years after[6]. However, this has not been confirmed by a SLR and meta-analysis[2]. In that, Stolwijk *et al*[6] found that prevalence of IBD in AS was 6.8% (95%CI: 6.1%-7.7%), which is much higher than the percentages observed in the general population (0.01% to 0.5%). Likewise, in a large population study was shown that the incidence rate of IBD was 5.3-fold increased to the AS patients compared to healthy controls. For nraxSpA the results seem to be largely similar. In a meta-analysis addressing the

prevalence of extra-articular disease in nraxSpA versus AS, it was found that IBD was almost equally frequent (pooled prevalence difference of 1.4% in favour of AS) between these two entities[8].

The question remains open whether we can predict which SpA patients suffer from or will develop IBD. Stolwijk *et al*[6] 2014 found that IBD was in general more common in males and that its frequency decreases with age, in AS. In a multi-centre AS study with a long follow-up, no differences were recorded between patients who had a history of IBD at baseline and those who did not[9]. On the other hand, development of IBD was associated with disease activity and spinal pain scores at baseline and worse physical function and patient well-being, at the time of IBD diagnosis[9]. Additionally, in a case control study[12] it was found that anterior uveitis was less frequent in patients with IBD-related spondyloarthropathy compared to those with SpA without bowel involvement[12]. Interestingly, in a sub-analysis of the GIANT cohort, it was shown that in patients with axSpA, there is a link between bone marrow edema of the sacroiliac joints and the gut inflammation. For this, SPARCC (Spondyloarthritis Research Consortium of Canada) scores which is a tool to measure MRI-defined sacroiliitis and ileocolonoscopy were used, respectively. It was found that SPARCC scores were higher in patients with chronic gut inflammation, compared to those without gut lesions[13].

Clinically silent IBD in SPA

Despite clinically evident IBD in the context of AS is observed in less than 15%, it has been suggested that clinically silent macroscopic and microscopic gut inflammation occurs in about 60% of AS patients[14-16]. From them, 5%-20% will develop CD within 5 years[17,18]. Microscopic gut inflammation, in axSpA, has been associated with younger age, male gender, progressive disease, early disease onset, radiologic sacroiliitis, high disease activity as assessed by the BASDAI and restricted spinal mobility measured by the Bath Ankylosing Spondylitis Metrology Index[9,16]. No association was identified with other extra-articular features or with the status of HLA-B27. Results were comparable between nraxSpA and AS[16].

SPA occurring in patients with IBD

Seeing the opposite flip of the coin, SpA is encountered in about 10-39% of patients with IBD, being the most frequent extra-intestinal manifestation in these individuals[16,19-25]. SpA is more commonly observed in patients with CD compared to those with UC[26-28]. Axial/arthritis symptomatology usually follows IBD diagnosis, but in about 20% the opposite is the case[19,23] especially for axial disease[20]. In general, AS and sacroiliitis (symptomatic or not) is estimated to occur in about 2%-16% and 12%-46% of IBD patients, respectively[19,20,22,23,27,29], both being more common in CD than in UC[19,30]. In a recent meta-analysis, it was shown that prevalence of AS and sacroiliitis in IBD were 3% (95%CI: 2%-4%) and 10% (95%CI: 8%-12%), respectively.

Comparing CD patients with and without AS, in a small single centre study, Liu *et al* did not observe any differences between these two groups[31]. Of note, they demonstrated that there was a significant correlation between disease activities of these two entities. These were measured by CD activity index for CD and with BASDAI for AS. They also showed that activity of CD significantly correlated with functional disability in AS, as assessed by Bath AS functional index - BASFI. All these possibly imply that there is a tight connection in the pathogenetic mechanisms of these conditions.

On the other hand, in a study examining possible associations between clinical and other characteristics with the occurrence of AS or SI in patients with CD, it was found that there was an association between SI and peripheral arthritis as well as between AS and uveitis, in these patients[32]. Besides, it has been suggested that in CD patients, colitis is more commonly associated with arthritic involvement compared with patients suffering from ileitis, while regarding UC, it seems that isolated proctitis is rarely combined with rheumatic manifestations[20,23].

Finally, patients with IBD-related ankylosing spondylitis and IBD-related isolated sacroiliitis are HLA-B27 positive in about 25%-78% and 7%-15%, respectively[20,22,32,33], possibly suggesting that isolated sacroiliitis is of different nature compared to AS in the setting of IBD[23]. These percentages are also lower compared to the prevalence of HLA-B27 observed in patients with AS which range from 80%-90%[1,20,34-36].

Peripheral SpA is also common in IBD with its prevalence ranging from 0.4% to 34.6%[19,28,37]. A recent systematic review and meta-analysis found that the pooled prevalence of peripheral arthritis, in the context of IBD was 13% (95%CI: 12%-15%) with its prevalence being much higher in the younger ages: 25% (95%CI: 19%-32%) and 2% (95%CI: 1%-5%) for age groups between 20-30 and 50-60 years old, respectively[30]. As observed for axial disease, peripheral SpA is more common in CD compared to UC[28,30,38]. A large retrospective study in the IBD Oxford clinics, had

shown that peripheral arthritis occurred in 10% and 6% of patients with CD and UC, respectively[38,39]. This study, led to identification of two major groups of peripheral arthritis in the context of IBD, namely: oligoarticular (< 5 joints are affected) and polyarticular (≥ 5 joints are affected)[20]. Some authors suggest that in the first group, which is more frequent than the second[39], arthritis is usually asymmetrical, non-erosive, affects lower limbs[20] and is associated with IBD activity and positivity for HLA-B27[23,39]. Patients belonging in the second group tend to have a more chronic course and be destructive and unrelated with IBD activity and HLA-B27 status[38]. Furthermore, Yüksel *et al*[28], examining the characteristics of peripheral arthritis in patients with IBD, they found that erythema nodosum and pyoderma gangrenosum were more commonly observed in IBD patients who also had peripheral arthritis, compared to those without. Various risk factors have been reported for peripheral arthritis in the context of IBD including: family history of IBD, appendicectomy, smoking and presence of other extra-intestinal manifestations[19,40,41].

Finally, some IBD patients might exhibit clinical features of SpA (*e.g.*, dactylitis) without fulfilling diagnostic criteria for SpA[20,42]. The frequency of dactylitis in patients with SpA in the context of IBD varies from 0% to 15.5%, but it seems to be around 5%[12,30] and therefore less common than in patients with SpA without IBD[12]. Incidence of enthesitis also varies largely, among different studies, in these patients[12,30]. A case control study[12] found that enthesitis was also less frequent in IBD-SpA patients compared to SpA individuals without IBD. For both dactylitis and enthesitis, no differences in their frequency were detected between CD and UC patients, while they occurred more frequently in patients with IBD and psoriasis compared to the IBD patients without skin disease[12].

PATHOPHYSIOLOGY

The pathophysiology of spondylathropathies associated with IBD involves the so-called ''gut-synovial axis'' hypothesis, which implicates environmental and host factors. Many of them act as triggers leading to initiation of inflammation in genetically predisposed individuals (Figure 1). Several studies have confirmed the link between joint and gut inflammation. It seems likely that both bacterial antigens and reactive T-cell clones, activated into the gut home the joint. However, the exact immunological mechanisms linking gut and joint inflammation are not fully understood[43,44].

Genetic predisposition

Genetic predisposition seems to carry a significant role in linking these conditions. In a large genotyping study, investigating risk variants for AS, it was shown that many of these were also linked with CD and UC[45]. Additionally, in a genealogic study in Iceland it was shown that first and second-degree relatives of patients with AS had increased risk (3.0 and 2.1, respectively) for IBD and vice versa[46].

Genetic factors play an important role, through alterations in both the adaptive and innate immune pathways[43,44]. Certain human leukocyte antigen (HLA) alleles have been recognized in patients with IBD who are at higher risk for having SpA. As mentioned, 25%-78% of patients with AS and IBD are positive for HLA class I molecule B27 (HLA-B27)[23,43,44]. Furthermore, MHC class II allele DRB1 0103 along with HLA-B35 and HLA-B27 are frequently associated with type I peripheral arthritis[23,47,48], while approximately 38% of patients with active UC or CD have been identified as carrying the allele DRB1 0103. On the other hand, type II peripheral arthritis is associated with HLA-B44[44,49].

Genetic factors outside the HLA system, have also been described. Variations of CARD15 gene (which encodes the protein product NOD2) increase the risk of CD about 4-40 times and has been linked to the development of sacroiliitis in IBD patients[50-52]. In addition, patients with AS and CARD15 mutations are at higher risk for subclinical intestinal inflammation[44,51,53]. NOD2 is an intracellular receptor for bacterial molecules and is expressed in the surface of macrophages, lymphocytes, paneth cells and intestinal epithelial cells. This receptor plays a role in the innate immune response by activating nuclear factor-κB (NFκB) which is a transcriptional regulator of a large variety of genes encoding pro-inflammatory cytokines, adhesion molecules, cytokines, growth factors and enzymes)[43,44,48,51,54]. As a result, NOD2 protein is responsible for positive regulation of immune defense in the gut and induction of a pro-inflammatory state[54]. However, though NOD2 gene mutations are associated with the clinical expression of CD in 20%-30% of patients there is no established association between presence of NOD2 mutations and development of SpA in IBD patients[44,51,55].

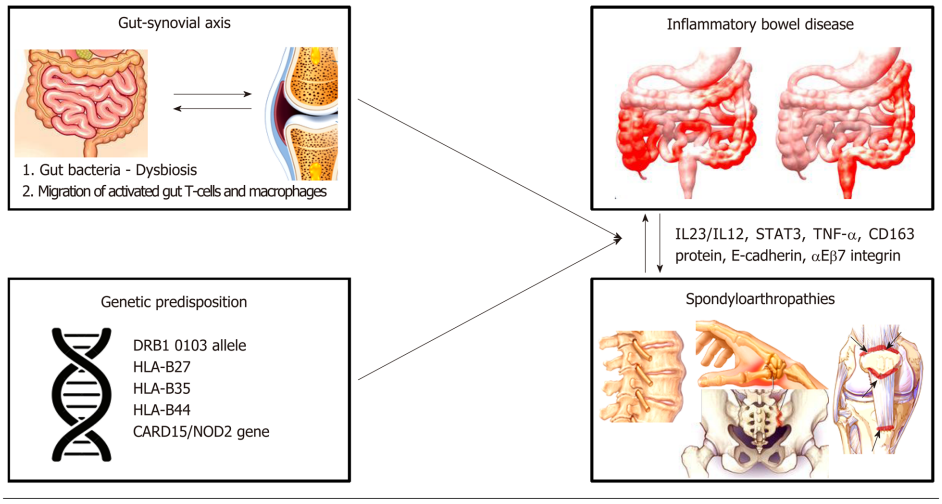

Figure 1 Pathogenic mechanisms linking gut and joint inflammation. The pathogenic link between spondyloarthropathies (SpAs) and inflammatory bowel disease (IBD) involves the so-called "gut-synovial axis" hypothesis. Various environ mental (gut bacteria-dysbiosis) and host factors (migration of activated gut-T cells and macrophages) leading to initiation of inflammation in genetically predisposed individuals may act as triggers of inflammatory responses against gut and joints components. IBD patients carrying specific human leukocyte antigens (HLA) alleles (such as DRB1 0103 allele, HLA-B27, HLA-B35, HLA-B44) and mutations of the CARD15/NOD2 gene are at higher risk of developing SpAs. Recently, up-regulation of adhesion molecules (E-cadherin, αEβ7 integrin), increased levels of pro-inflammatory cytokines (tumor necrosis factor-α), macrophages expressing CD163 protein, interleukin (IL)-12/IL-23 signaling pathway and signal transducer and activator of transcription 3 protein have also been implicated in the pathophysiology of SpAs in IBD patients. IL: Interleukin; STAT: Signal transducer and activator of transcription; TNF: Tumor necrosis factor; HLA: Human leukocyte antigen; CARD15: Caspase recruitment domain-containing protein 15; NOD: Nucleotide-binding oligomerization domain-containing protein 2.

Furthermore, CD, AS and PsA have been associated with polymorphisms in some common genes like IL-23R, IL-12B, STAT3, and CARD9, all of them implicated in the anti-IL-23/IL-17 axis[56-60].

Having said that, IL-23/-17 axis seems to play an important role in both axSpA and IBD (regarding the latter, the evidence mainly pertains to CD rather than UC)[61-63]. This axis is mainly regulated by IL-23, resulting in the production of IL-17, IL-22 and to a lesser extent of tumor necrosis factor (TNF)[1] by the so-called Th17 cells, which are a subgroup of T-helper cells. These cytokines are also produced from other cells like innate lymphoid cells[64]. In the gut of patients with CD or patients with SpA, it has been observed increased expression of IL-23[65]. Similarly, in the peripheral blood of AS patients there is increased number of Tγδ cells expressing IL-23R and producing IL-17; additionally, increased expression of IL-23 is noticed in patients' facets. Interestingly, it seems that there are some cells able to produce IL-17 irrespective of the presence of IL-23. This, as discussed below, might has some implications in the therapeutic approach of these patients[66].

Links between the gut and the joints

Several other findings also highlight the common underlying pathogenetic mechanisms between IBD and SpA. aEβ7 integrin which is expressed by intraepithelial T cells in the intestinal mucosa and binds to the glycoprotein E-cadherin expressed by gut epithelial cells, has been found to be upregulated on colonic T cells from AS patients and also from lymphocytes obtained from synovial tissue of SpA patients[67,68]. The E-cadherin molecules have been also observed to be up-regulated in the gut of patients with IBD and SpA individuals with subclinical gut inflammation[51,67-69].

In another study increased levels of macrophages expressing the protein CD163 have been reported in both gut mucosa of IBD patients with and without SpA and in the synovial tissue and gut from SpA patients[51,70,71]. Finally, animal models have shown that prolonged exposure to the pro-inflammatory cytokine TNF-α might lead to a phenotype resembling IBD-SpA[72]. In the last decade, it was recognized that a common target of this cytokine could be the synovial fibroblasts and the intestinal myofibroblasts[68,73].

The Gut-synovial axis

Two - probably complementary- theories have been formulated to explain the development of SpA in patients with IBD. These theories include both alterations in gut bacteria and migration of gut lymphocytes to the joint[43,44]. Changes in the gut

microbiome, which is also known as dysbiosis, have been associated with SpA. In detail, *Faecalibacterium prausnitzii* has been found to be in reduced numbers, in stools of SpA patients[18,74,75]. Also, in AS patients, increased numbers of *Dialister* microbes in ileal and colon biopsies have been correlated with Ankylosing Spondylitis Disease Activity Score (ASDAS)[18,75] and of *Ruminococcus gnavus* in the stools with Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)[18,76,77]. Furthermore, other studies have detected in the inflamed joints of these patients certain bacteria like *Yersinia enterocolitica*, *Salmonella enteritidis* and *typhimurium*, or antigens related to them[26,78,79]. Role of microbiome in the pathogenesis of SpA is also supported by data from animal models. For example, the arthritis and inflammatory colitis features developed in HLA-B27 transgenic rats are ameliorated when they are raised in germ-free conditions[18,80]. These observations suggest that gut and joint inflammation process depends on the presence of bacteria into the gastrointestinal tract, which emphasizes the role of autoimmunity and antigen mimicry[26,44].

The second hypothesis is based on experimental studies which showed that gut T-cells activated by antigens migrate to the joints and induce inflammation[43,44,51]. In state of inflammation alterations occur in the mucosal vasculature, such as vasodilation, hyperemia and increased vascular permeability which are induced by various inflammatory cytokines, resulting in enhanced extravasation of leukocytes. Furthermore, the migration pathways of lymphocytes are altered by aberrant expression patterns of adhesion molecules, inflammatory cytokines and receptors[51]. It is known that integrins α4β7 and αEβ7 and MadCAM-1 mucosal vascular receptor play an important role in the lymphocytes' gut homing. It has been shown that leukocytes populations from inflamed gut can bind to synovial vessels and home into the joint, using multiple adhesion molecules[81], such as αEβ7 integrins, vascular adhesion protein-1 (VAP-1) and intracellular adhesion molecule-1 (ICAM-1/CD54)[26] . The increased number of T cells expressing αEβ7 integrins in the synovial membrane is in favor of the mucosal origin of these cells, however this hypothesis remains to be proven[68].

Additionally, It has been shown that macrophages from the gut of IBD patients are able to adhere in endothelial cells of synovial tissue[81], further enhancing the activation of T cells locally[26]. Collectively, one could argue that gut T-cells are activated in the Peyer's patches and mesenteric lymph nodes, express a pattern of adhesion molecules that under specific conditions leads to migration of these activated T-cells into the joint causing inflammation[43,44,51,82]. Further studies are needed to fully understand the pathogenic pathways linking IBD and SpA.

TREATMENT

Given the common pathogenetic mechanisms underlying SpA and IBD, therapeutic approach to these entities is largely similar. However, there are some differences in the safety and efficacy of the various treatment modalities used.

Non-steroidal anti-inflammatory drugs (NSAIDs) although commonly used in SpA, should be generally avoided in IBD especially in the active ones, but short courses (*e.g.*, 2 wk) do not seem to cause exacerbations[20]. On the other hand, short courses of systemic steroids, although demonstrate some efficacy for CD or UC are not effective for axSpA[20,83]. Local steroids injections and low doses of systemic steroids have been used for peripheral SpA[20].

The most common conventional Disease modifying antirheumatic drugs (DMARDs) used for treatment of SpA are methotrexate and sulfasalazine, both demonstrating some efficacy for peripheral but not for axial SpA[84,85]. Furthermore, methotrexate has proved to be helpful in inducing and maintaining remission in CD patients[86,87] but is not recommended as first line treatment of UC. Similarly, sulfasalazine has some efficacy in CD (but not for ileal CD)[20] and UC[88,89].

Anti-TNF regimes are the gold-standard treatment for the patients with co-existing IBD with SpA who are not controlled with conventional DMARDs. All of them are approved for the treatment of axSpA[90] while infliximab and adalimumab are the most well studied for patients with IBD, both having indication for CD and UC[87]. Etanercept, was not effective for IBD[91]. Many hypotheses have been made to explain its lack of efficacy, including that this might be related to its insufficiency to induce apoptosis in the T cells of the lamina propria[92]. Also, etanercept blocks both TNFa and TNFb. The latter seems to regulate, in the lamina propria, T-cell dependent IgA production, which in turn controls the intestinal microbiota composition[93,94].

Interestingly, new onset IBD in the context of AS, has been observed in patients who started treatment with anti-TNF reagents[95,96]. These cases were more frequently resembling CD rather than UC and have been associated with commencement of

etanercept[97-99]. Of note, a large multicentre AS study examining the presence and development of extra-articular manifestations did not find any correlation between biologics use and development of IBD[9]. Whether there is a true association between treatment with anti-TNF drugs in AS patients and new-onset of IBD, remains to be defined.

The role of the IL-23/-IL-17 axis in the pathogenesis of SpA and IBD is supported by many studies of basic and clinical research[61]. Despite, monoclonal antibodies targeting the key cytokines of the axis (*i.e.*, IL-23 and IL-17) were thought to be very effective, data from clinical trials did not fully support this notion. Ustekinumab, a monoclonal antibody against the common p40 subunit of IL-12 and IL-23, has proved to be effective for CD but does not appear to work for AS. Although data derived from post hoc analyses of phase 3 trials in patients with psoriatic spondylitis were promising, ustekinumab did not achieve the primary endpoint in phase 3 trials for AS and non-radiographic axSpA[100]. Similarly, risankizumab, which is an antibody specifically targeting the p19 subunit of IL-23 failed to show clinical and radiological efficacy in a phase 2 trial for AS[101]. Data from clinical trial about other antibodies against p19 subunit of IL-23 are eagerly awaited. To explain the differences observed in the efficacy of anti-IL-23 between psoriatic spondylitis and AS, it is not irrational to speculate that the pathogenetic mechanisms underlying AS are somewhat different to those of spondylitis in the context of PsA.

Secukinumab, which is a monoclonal antibody against IL-17 recently received approval for AS and therefore is another therapeutic option in patients with axSpA. Phase 3 trials are now underway for secukinumab in nraxSpA. One could expect, based on the underlying pathogenetic mechanisms that secukinumab would have good results in CD. However, results in phase 2 trials were negative with the drug being numerically worse than placebo. Many hypotheses have been formed to explain the failure of secukinumab in CD. *Candida albicans* proliferation has been proposed as a plausible explanation for the CD exacerbation given the role of IL-17 in fighting fungal infections[61,102]. Although new cases of IBD in axSpA patients treated with secukinumab have been described[103], a recently published study analysing data from 21 clinical trials from patients with psoriasis, psoriatic arthritis and ankylosing spondylitis, has shown that exposure adjusted incidence rates for IBD did not increase over time with secukinumab treatment[104]. Interestingly, a recent study provided some evidence supporting that suppression of IL-17F but not IL-17A was indeed protective for colitis by inducing T regulatory cells via modifications in colonic microbiota[105].

An obvious question is how ustekinumab, which blocks IL-23 and subsequently IL-17 works for CD but secukinumab does not? There is accumulated evidence that IL-17 can be produced also -to a lesser extent possibly- in an IL-23 independent manner from innate lymphoid, T γδ or other types of cells[64,106]. Therefore, blocking IL-23 leaves some "basal" levels of IL-17. Lee *et al*[107] have shown, that T γδ cells in the lamina propria are the producers of gut-protective IL-17, in an IL-23 independent way. Its effect is possibly mediated through regulation of the tight-junction protein "occludin" which maintain barriers integrity.

Vedolizumab, a gut selective α4β7 integrin antagonist, has shown to be effective in patients with CD[108,109] and for inducing or maintaining therapy in UC patients[110]. Results of this drug in articular symptoms are somewhat conflicting. Whether this drug is linked with exacerbation or new-onset arthralgias or inflammatory arthritis remains to be answered[87,111-113]. Of note, a recent post hoc analysis of the "Gemini" trials showed that vedolizumab was associated with decreased likelihood of new or worsening arthritis/arthralgia in CD patients while in UC the incidence was similar between patients treated with the active drug or with placebo[114].

JAK inhibitors are a new drug class category with promising results in various immune mediated diseases. Genome wide association studies have shown that there is association between CD and single nucleotide polymorphisms in the JAK-STAT pathway[115]. Results in a phase 2 trial for CD has shown that tofacitinib was not effective[116]. However, newer and more selective JAK-inhibitors, like filgotinib and upadacitinib have favorable results in achieving clinical remission in phase 2 trials for CD[117,118]. For UC, tofacitinib after the promising results with patients achieving higher rates of clinical remission and clinical response compared to placebo[119] received Food and Drug Administration (FDA) approval for patients with moderate to severely active UC.

As regards to the efficacy of JAK-inhibitors in SpA, tofacitinib has shown favorable results in phase 2 trials of AS with 80.8% of the patients treated with tofacitinib achieving ASAS20 improvement at week 8, compared to 41.2% of placebo-treated patients[120]. Recently published results from a phase 2 clinical trials showed also that filgotinib was effective for AS with patients experiencing significant clinical improvement, compared to placebo, at week 12. A phase 2b/3a clinical trial assessing

the efficacy and safety of upadacitinib in patients with AS is currently underway (NCT03178487). Whether JAK-inhibitors could be another potential therapeutic option in patients with IBD and SpA remains to be defined from future studies.

DIAGNOSIS - THE ROLE OF CALPROTECTIN

Although colonoscopy is being considered as the gold-standard for IBD diagnosis, a recent study has shown that capsule endoscopy was superior to classical colonoscopy in diagnosing CD in the context of SpA. It was shown that small bowel inflammation was present in 42.2% and 10.9% of the patients who underwent capsule endoscopy and classical colonoscopy, respectively. Interestingly, positive findings were not associated with symptomatology from the gastrointestinal system but with elevated faecal calprotectin levels, confirming that many SpA patients have "silent" IBD[121]. Calprotectin measured in the serum or in the stools has been used to identify subclinical bowel inflammation in patients with SpA. Cypers *et al*[14] have found that elevated serum calprotectin levels have been associated with subclinical microscopic colitis in SpA patients. In detail, individuals who had both CRP and calprotectin elevated had a frequency of bowel inflammation of 64% compared to 25% in patients who had low levels of these proteins. Additionally, in patients who had high levels of either serum calprotectin or CRP, frequency of bowel inflammation was significantly higher in SpA patients with high faecal calprotectin compared to those with low[14]. In a recent study, Ostgard *et al*[122] confirmed that faecal calprotectin could serve as a biomarker to identify patients with subclinical bowel inflammation. It has to be noted however that faecal calprotectin levels can be influenced by NSAIDs use, which is quite common in SpA patients[123]. Interestingly, patients with elevated faecal calprotectin levels had more inflammation in the sacroiliac joints compared to those with low levels. Also, the former had better response to adalimumab as assessed by ASDAS. It has to be said however, that these patients received an extra loading dose of 80 mg adalimumab, at baseline[122]. The concept of calprotectin as biomarker of treatment response has been suggested also previously: In proof of concept trials for SpA, serum calprotectin has been found to be decreased after treatment of axSpA and peripheral SpA with infliximab and etanercept, respectively[124].

DISCUSSION - POINTS TO CONSIDER

It is increasingly being recognized that there is a very close link between IBD and SpA. As outlined in this review, there are several hints for that: epidemiological, clinical, laboratory (*i.e.*, positivity for HLA-B27) histopathologic and pathogenetic. Regarding the latter, it is very intriguing to define to which extent these are common between these entities and identify the diversities that lead to different clinical expressions. However, many limitations impede this venture. Firstly, over the last years, many different criteria have been used for the classification of SpA, which comprise a group of relatively heterogenous diseases. Besides, classification criteria in SpA do not mean necessarily a certain diagnosis and *vice versa*[125]. Secondly, these patients, depending on the cardinal manifestation, are followed up by a gastroenterologist or a rheumatologist that might overlook the articular or bowel manifestations of the disease, respectively. To that end, the effective communication between different professions and the interdisciplinary approach, through combined clinics for example, in imperative.

Treatment of this entities has progressed significantly over the last years. To the successful anti-TNF reagents, drugs targeting IL-23 and IL-17 as well as the JAK-inhibitors have been added to the clinician's arsenal. However, treating patients with co-existing SpA and IBD, should not only include these manifestations but also considerate other extra-articular and extra-intestinal manifestations like skin disease or uveitis. Comprehensive algorithms, designed by clinicians of many disciplines are urgently needed, in light of the numerous emerging therapeutic modalities.

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MINIREVIEWS

Harnessing the potential of gene editing technology using CRISPR in inflammatory bowel disease

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Abstract

The molecular scalpel of clustered regularly interspersed short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) technology may be sharp enough to begin cutting the genes implicated in inflammatory bowel disease (IBD) and consequently decrease the 6.3 billion dollar annual financial healthcare burden in the treatment of IBD. For the past few years CRISPR technology has drastically revolutionized DNA engineering and biomedical research field. We are beginning to see its application in gene manipulation of sickle cell disease, human immunodeficiency virus resistant embryologic twin gene modification and IBD genes such as Gatm (Glycine amidinotransferase, mitochondrial), nucleotide-binding oligomerization domain-containing protein 2, KRT12 and other genes implicated in adaptive immune convergence pathways have been subjected to gene editing, however there are very few publications. Furthermore, since Crohn's disease and ulcerative colitis have shared disease susceptibility and share genetic gene profile, it is paramount and is more advantageous to use CRISPR technology to maximize impact. Although, currently CRISPR does have its limitations due to limited number of specific Cas enzymes, off-target activity, protospacer adjacent motifs and crossfire between different target sites. However, these limitations have given researchers further insight on how to augment and manipulate enzymes to enable precise gene excision and limit crossfire between target sites.

Key words: Clustered regularly interspersed short palindromic repeats; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Gene excision; Gene editing; Gene therapy; Financial impact of inflammatory bowel disease on healthcare; Clustered regularly interspersed short palindromic repeats crossfire

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Core tip: Using clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) to harness the potential of gene editing technology implicated in inflammatory bowel disease. This revolutionary way of editing genes and its application of gene manipulation is only gaining momentum. Genes such as Gatm (Glycine amidinotransferase, mitochondrial) and nucleotide-binding oligomerization domain-containing protein 2 have been utilized to show CRISPR is able to manipulate these genes precisely, and this is just the beginning.

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INTRODUCTION

Clustered regularly interspersed short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology has drastically revolutionized DNA engineering and biomedical research in the past few years. It has enabled researchers to model disease and advance therapeutic intervention techniques in a pragmatic and clinically efficacious way. The Figure 1 below demonstrates the exponential growth in publications and interest in CRISPR technology over the past few years. Abreast is Figure 2 demonstrating use of CRISPR technology in inflammatory bowel disease (IBD). Strong interest in CRISPR has driven gene therapy publications down in the past few years as seen in Figure 3. Application of CRISPR is gaining momentum and is seen in sickle cell disease gene modification of hematopoietic stem cells, embryonic stem cells, gene-based therapy for HIV-1 infected individuals and other disease gene modification[1-6]. It is a new era in biomedical research for use of CRISPR technology in IBD (Figure 4). These advancements in biomedical research with the use of CRISPR will enable researchers to begin treating IBD and other gene modifying diseases, thereby relieving the healthcare financial burden of IBD.

IBD affects an estimated 1.5 million people in North America[7,8]. These patients have considerable morbidity and the associated financial burden on healthcare system is estimated to be 6.3 billion dollars annually with two thirds of that due to hospitalizations and pharmaceutical therapy alone[9-11]. This does not take into account the financial implications of work and disability of patients and impact on daily life. Even though gene therapy remains expensive in any disease treatment due to pharmaceutical patents, considerable thought should be taken to propel CRISPR use in IBD because the use of this novel technology is less taxing than previously used epigenetic modification[12].

Prior to the discovery of CRISPR/Cas9 enzyme, RNA sequencing[7,8], modular protein recognition DNA such as zinc finger nuclease or TAL effector nuclease were used in epigenetic modification and disease modeling, which are complex and require very specific protein engineering and programming[13]. However, with the revolutionary CRISPR technology, researchers now may edit genomes in a much simpler and precise way as modeled by bacteria[14,15].

CRISPR/Cas9 was found in Streptococcus pyogenes, which uses this mechanism as defense against invading viruses[14]. Upon infection by a virus the bacteria stores the viral DNA sequence in between sections of regularly interspaced palindromic repeated segments that are closely associated with genes that encode CRISPR-associated proteins. In order for the system to confer underlying efficacy and specificity, the CRISPR and the viral DNA is converted to two RNA moieties: TracrRNA and crRNA, the tracrRNA confers enzymatic activity and the crRNA determines substrate specificity[13,16]. Once the moieties bind together, they traverse the cell seeking any genetic material that matches the crRNA. Upon sequence matching, the tracrRNA part of Cas enzyme clips the target DNA in specific nucleotide bases disabling its replication. This incredibly precise system can be utilized in therapeutic advances and programed to target any sequence in the cell. This novel mechanism will be assessed for potential application and targeting of genes implicated in IBD.

CRISPR DNA engineering has advanced quickly and is evolving rapidly. It has

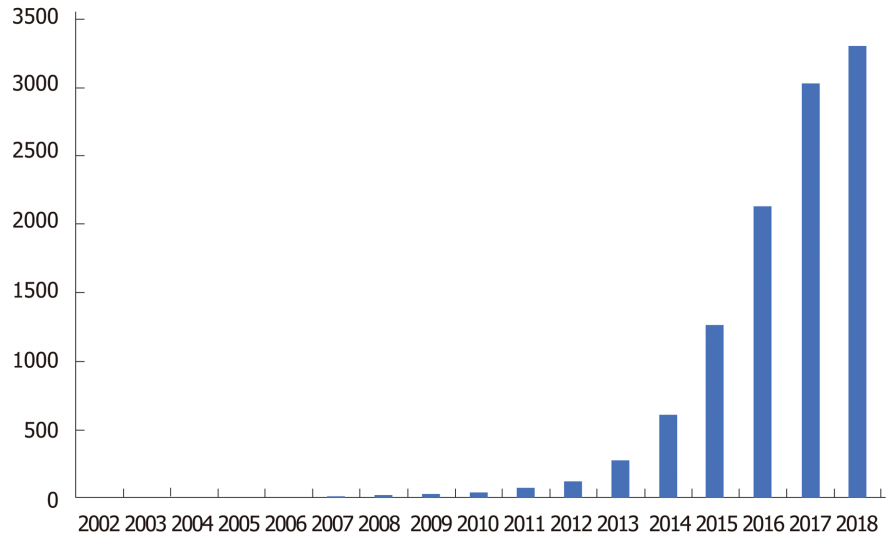

Figure 1 Number of CRISPR publications by year.

already been used in diseases such as sickle cell, beta-thalassemia or HIV resistant human embryos[1,2,6]. Given its exponential application in disease variants, it may suggest that it's an optimal time to further our understanding of IBD pathogenesis and treatment. Primarily IBD refers to two major categories of chronic relapsing inflammatory intestinal disorders: Ulcerative colitis (UC) and Crohn's disease (CD). With the advent of genetic research both diseases have seen notable success culminating in the discovery of over 160 susceptible genes, among which, many potentially may be targeted by the CRISPR technology[17,18]. Around one-third of these loci/genes described, confer susceptibility to both CD and UC, which may make targeting and gene editing a viable option[18,19]. The genetic architecture of IBD has shed much light on central themes in IBD and the level of cellular process that the pathogenesis emerges. A putative CD-susceptibility locus has been mapped to chromosome 16 around locus D16S409 and D16S419 which may shed more light etiology of IBD[20]. In CD a common genetic theme is seen in defective processing of intracellular bacteria, autophagy and innate immunity. In UC, genetic evidence demonstrates genes that are responsible for proper barrier function are important in preventing UC. However, when analyzing genetic data in more detail, CD and UC have shared disease susceptibility and shared genetic gene profile that may be targeted[18]. Table 1 demonstrates common genes implicated and their strength of role in IBD[18].

Recent research has identified and linked a gene, Gatm, to IBD using CRISPR/Cas9 gene-editing technology. When the Gatm gene is activated, it induces synthesis of creatine which helps in intestinal mucosal barrier, which helps protect the intestinal wall against inflammation that's caused by bacteria[21]. When inducing a frame-shift mutation in the Gatm gene *via* CRISPR/Cas9 there was signs of inflammatory response in the intestinal wall. This demonstrated its important role in mucosal barrier protection and potential manipulation of this gene using CRISPR. Protection from inflammation by an intact barrier is vital to decrease immune response, which is suppressed or increased by enhancers in the immune activation cascade. In the inflammatory response pathway, IL2RA plays a role in signaling T cells to hamper or increase the response. If the enhancers that switch on IL2RA are defective the T cells won't suppress inflammation and chronic inflammation is associated with 15%-20% of all human malignancies[8,22]. Inflammation also results in autoimmune disorders such as IBD and inflammatory-induced colon cancer mediated by NF-kB pathway[22-24]. Previously it has been demonstrated that single nucleotide polymorphisms -SNPs' mutation of IL2RA leads to improper activation of T cells and subsequently resulting in autoimmune disorders. These SNP's may be targeted by CRISPR/Cas9 and repaired with non-homologous end-joining repair. This has been demonstrated in KRT12 mutations-specific targeting of SNP's as well[25]. Recent advancements in CRISPR/Cas9 specificity and potency of targeted genes demonstrate that SNP's or genes that have point mutations may be targeted and editing may be attempted.

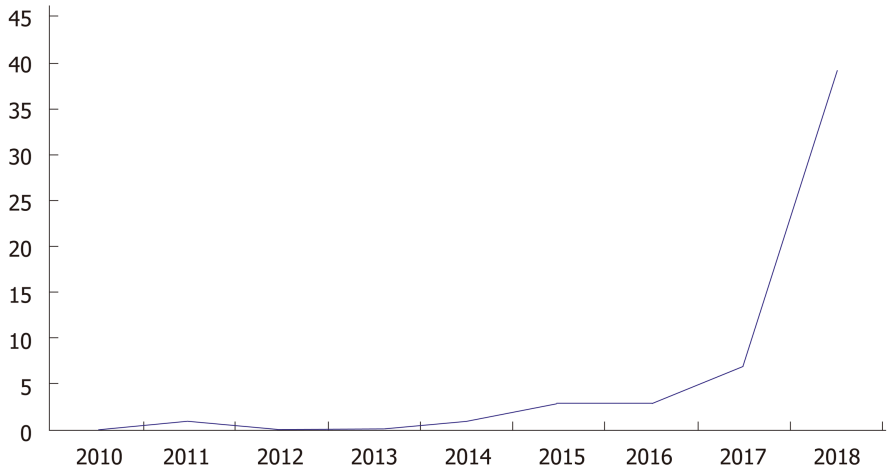

Figure 2 Publications on inflammatory bowel disease using CRISPR.

POTENTIAL THERAPEUTIC TIMING

CD and UC in general have three stages of disease progression, mild-moderate; moderate-severe; and severe. Currently there are no studies indicating potential therapeutic timing when to target affected genes using CRISPR in IBD, however several multicenter trials conducted administering human recombinant IL-10 during active mild/moderate stage of CD or during refractory CD as well as patients undergoing curative ileal or ileocolonic resection[26,27]. However, results did not show significant clinical improvement or higher remission rates secondary to too low IL-10 dose and adverse effects of medication. In addition, IL-10 alone failed to effectively suppress variety of dysregulated pro-inflammatory cytokines[26,27]. In later stages of disease process, significant dysregulation of pro-inflammatory cytokines and redundant pathways occur, such as NF-kB receiving activation from different pathways[28] thus single target impact is futile. Given that CRISPR can simultaneously multiplex several genes, it will aid researchers to devise appropriate intervention timing[29]. We also suggest early intervention is optimal to prevent progression of disease and reduce complications. It is imperative to conduct studies to best identify role of CRISPR in various stages of disease.

CURRENT STATUS

Currently, CRISPR is applied in many fields of scientific study. In biotechnology it is used to modify Maize genome in protoplasts. In drug development, it is used to understand modes of drug resistance and drug-target interactions. In epigenetics, it has taken the place of zinc finger nuclease and TALEN in epigenetic modification because the indel frequency is more superior[6]. Since the CRISPR debut, researchers are improving and enhancing the specificity and accuracy of the Cas9. Currently the Cas9 not only cuts the DNA, but can be altered to perform desired functions. The Cas9 protein has a deaminase region that may be altered to increase highly specific alternation of genome sequence, which will allow for broader specific DNA bases manipulation[13]. It can also promote gene transcription using enzyme by deactivating the endonuclease activity and add transcriptional activator to increase transcription. The Cas9 can silence domains that recruit factors so that genes are blocked and they are not transcribed. In general targeting studies, Cas9 can be tagged with fluorescent dye so genes can be followed. Furthermore, Cas9 can be multiplexed with multiple guide RNAs to generate multiple breaks in order to cut out large sequences of DNA in one experiment[29]. This limits time and repetitiveness of experiments conducted and time is of an essence in this race to invent even-more versatile or efficient variations of this powerful enzyme, which greatly simplifies the editing of DNA. Furthermore, very recently successful attempts were made to edit CCR5 gene in human embryos to enable resistance to HIV[30]. Although, ethics and implications of such studies are currently widely debated. Also, recently KRAS oncogenic alleles were modified leading to decreased cancer cell growth without disturbing wild type alleles[31]. Since major oncogenic mutations occur on codon-12 of KRAS exon-2, direct targeting of oncogenic KRAS single-nucleotide missense substitution c.35G>T mutation using

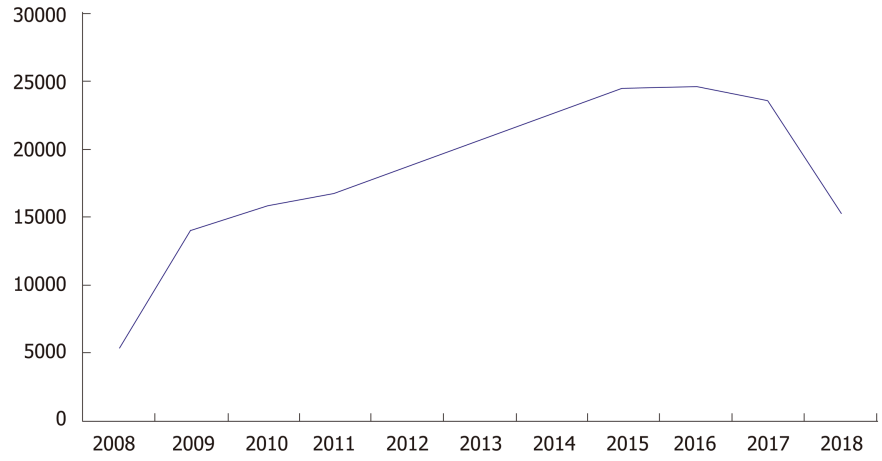

Figure 3 Total publications using gene therapy.

CRISPR/Cas9 system inhibited cancer cell growth and is dependent on efficient target cell transduction[31].

LIMITATIONS

The excitement generated by the new CRISPR technology in the science community is reflected by exponential publications and application in various fields of study, including agriculture, drug development and epigenetic control but its revolutionary genome editing method is far from perfect. The CRISPR is an RNA based DNA recognition system. It is dependent on a guide molecule composed of RNA to recognize a sequence in the DNA that has specific molecular features. The problem is that the original Cas9 can only land on genome segments that have a trio of 'NGG' (N being any nucleotide) nucleotide base pairs and cut only limited fraction of the genome. The human genome contains 3.2 billion-bases and only one-sixteenth of the genome where Cas9 can land. This limits the specific target genes of interest and leads to "off-target" mutations however to limit these effects a highly specific guide RNA sequence is selected[15,32]. Also, recently researchers modified the enzyme and developed an xCas9s that has a broader range of three-base landing pads, referred to as protospacer adjacent motifs or PAMs. It works best with NGN sequence, which occurs in one-fourth of the genome. It allows for researches to perform gene knockout studies, which help them, determine what gene is implicated in disease. However, since majority of diseases are associated with "point mutations", it is difficult to target and repair a mutated gene. Not only that, once the enzyme clips the desired DNA gene, the repair mechanism of the cell is wobbly and during repairs it tends to insert or delete DNA bases. Furthermore, Cas9 enzyme only cuts DNA and only 2% of the genome codes directly from DNA to protein and 98% of genome is regulatory gene sequences. This poses a challenge for precisely modifying RNA. Although, very recently a new enzyme was characterized called Cpf1, which was found in Staphylococcus aureus, and is capable of cleaving both DNA and RNA[33]. This will allow targeting of RNA gain-of-function mutations such as NOD2 or other mutations that can be edited. Furthermore, Cpf1 is also smaller which makes transfection much easier[33].

Although some of these limitations of the Cas9 are quickly becoming insightful opportunities and researchers are drastically improving it by altering its capabilities. The new enzymes are more precise than the original Cas9 and now there is xCas9, dCas9 and dCas13 which are capable of editing specific base pairs. For example, dCas13 can convert base A to I in RNA and I is a universal base[34]. Such manipulation of bases is a very appealing target for therapies, particularly inflammation. Furthermore, because RNA is around in the cell for a short period of time before it is degraded repeated administration of RNA base editors would need to be given[34]. Also, sequencing RNA is problematic and laborious[35]. On the other hand this may seem disadvantageous but working with RNA may limit some off target genome mutations. In addition to off target genome mutations, crossfire between different cells may occur. Intended target may be either gain of function mutation or loss of function mutation, either way, altering alleles may have their own detrimental effect.

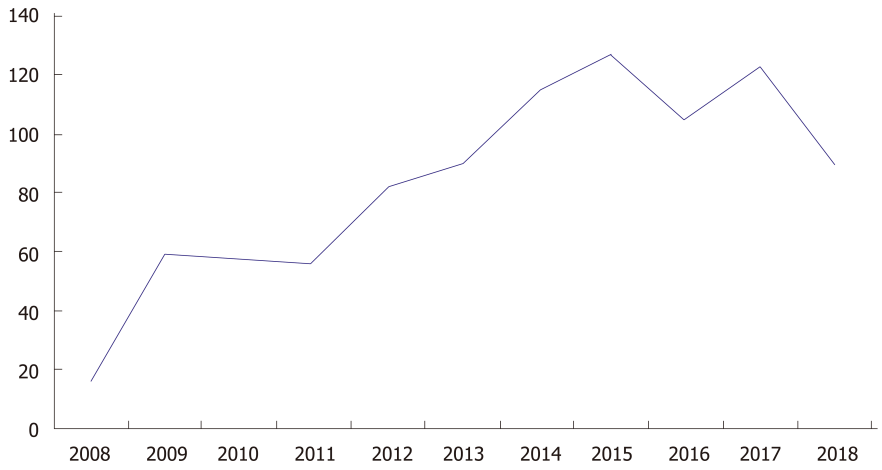

Figure 4 Publications on inflammatory bowel disease using gene therapy.

For example, as indicated in Figure 5, NOD2 loss of function leads to Crohn's and gain of function causes endothelial to mesenchymal transition of glomerular endothelial cells causing diabetic nephropathy[36]. However, some of these cross reactions may not be as detrimental. In Figure 6, altering ATG16L1 allele to T300A ATG16L1 only incases risk for CD type, but not disease onset[37]. Figures 5-7 below demonstrate positive and negative effects of altering main genes implicated in IBD; NOD2, STAT3 ATG16L1, IL23R genes using CRISPR technology[37-41].

CONCLUSION

Despite CRISPR/Cas technology limitations, as new innovative techniques such as anti-CRISPR proteins and new Cas proteins are developed to advance precise DNA editing, application of this revolutionary mechanism is at its prime time to hone in on genes implicated in IBD. Implementation of CRISPR in IBD research will lead to better outcomes and may decrease financial burden on the health care system.

Table 1 Genes to target for optimal impact using CRISPR

Gene	Strength of role in inflammatory bowel disease	Pathway	Function
NOD2[42]	+++++	Cellular Innate Immunity	Intermediate in MDP to NFkB and IL1B production
IRGM	++		
LRRK2	+		
ATG5	+	Autophagy	Phagophore to autophagosome to autophagolysosme creation
ATG16L1[42]	++++		
IRGM	++		
Gatm	+++	Defective Barrier	Leaky lamina propria allowing antigen entry and T cell response
ECM1	+		
CDH1	+		
LAMB1	+		
HNF4A	+		
GNA12	+		
IL10	+++++	Adaptive Immune	Convergence of STAT3, IL10RB, NOD2, ATG16L1 pathway
CARD9/15	++		
CCR6	++		
IL2RA	+		
MST1	+		
TNFSF15	+++		
REL	+		
STAT3	+++++	Th17 Mediated	Activation of Th17 adaptive immunity
IL23R[42]	+++++		
IL12B	++		
FUT2	+		

Genes in bold have publications using CRISPR technology. Strength of role is based on + of publications and relationship to inflammatory bowel disease: > 5 +; > 10 ++; > 20 +++; > 40 ++++; > 80 +++++. NOD2: Nucleotide-binding oligomerization domain-containing protein 2; IRGM: Immunity related GTPase M; LRRK2- leucine-rich repeat kinase 2; ATG5: Autophagy related 5; ATG16L1: Autophagy-related protein 16-1; Gatm: Glycine amidinotransferase, mitochondrial; ECM1: Extracellular matrix protein 1; CDH1: Cadherin 1; LAMB1: Laminin subunit beta 1; HNF4A: Hepatocyte nuclear factor 4 alpha; GNA12: G protein subunit alpha 12; IL-10: Interleukin 10; CARD9/15: Caspase-associated recruitment domain; CCR6: Chemokine receptor 6; IL2RA: Interleukin 2 receptor subunit alpha; MST1: Macrophage-stimulating protein; TNFSF15: TNF superfamily member 15; REL: Rel protein; STAT3: Signal transducer and activator of transcription 3; IL23R: Interleukin 23 receptor; IL12B: Interleukin 12; FUT2: Fucosyltransferase 2.

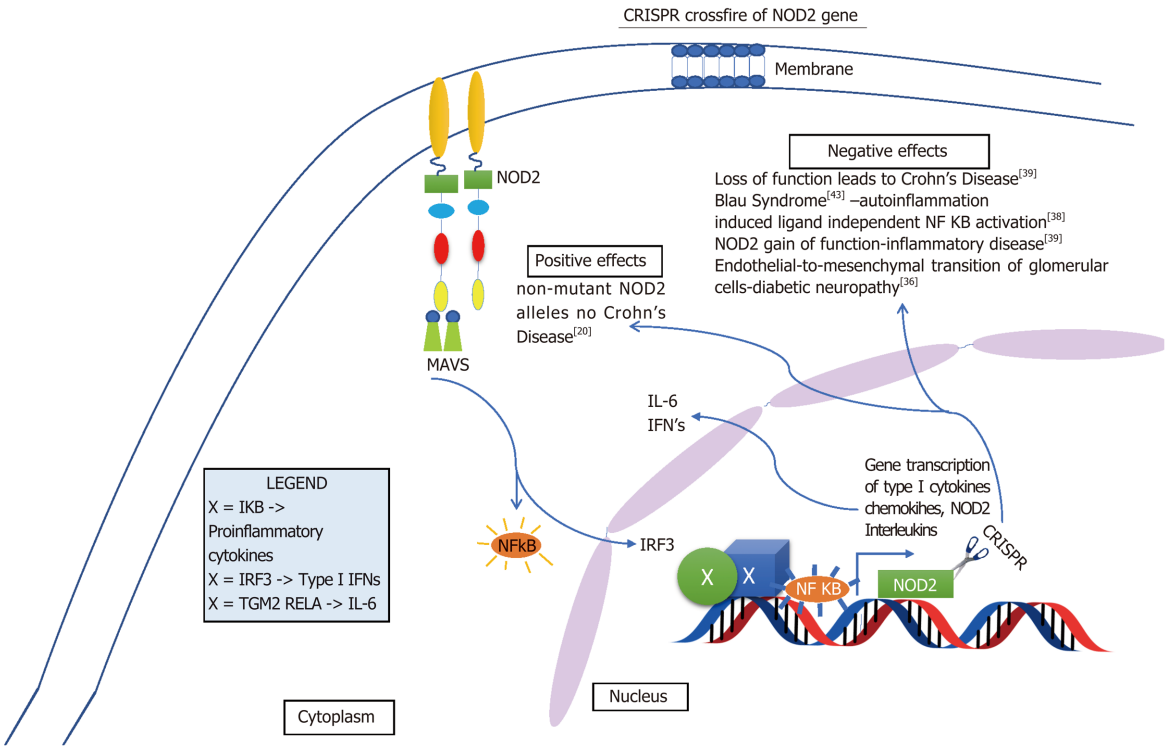

Figure 5 Positive and negative effects of CRISPR when targeting NOD2 gene.

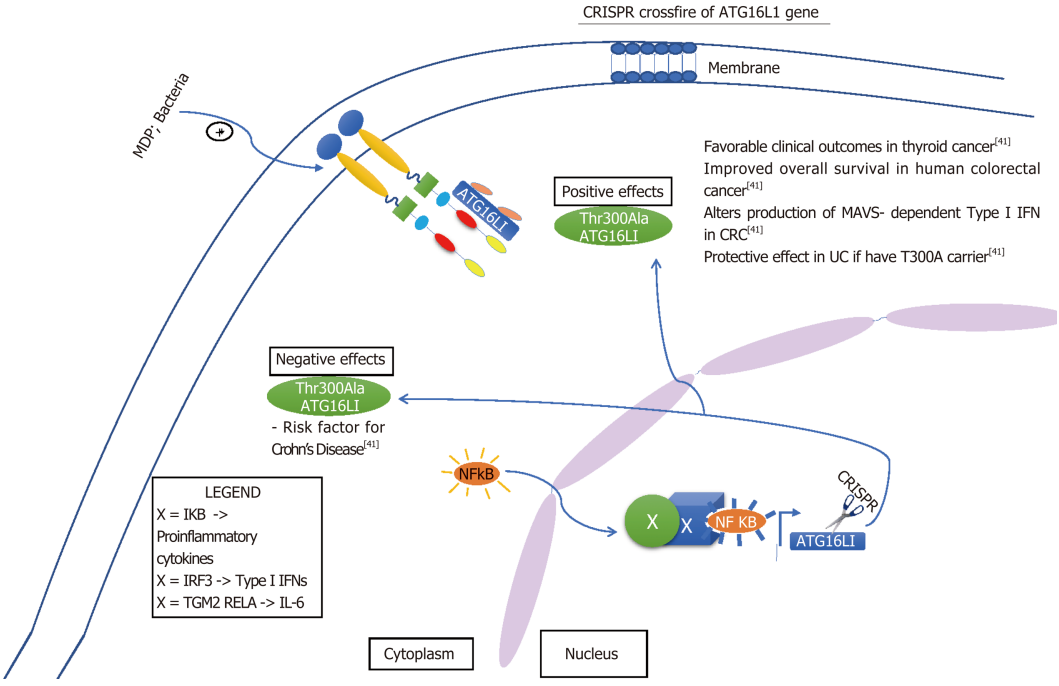

Figure 6 Positive and negative effects of CRISPR when targeting gene ATG16L1. UC: Ulcerative colitis; CRC: Colorectal cancer.

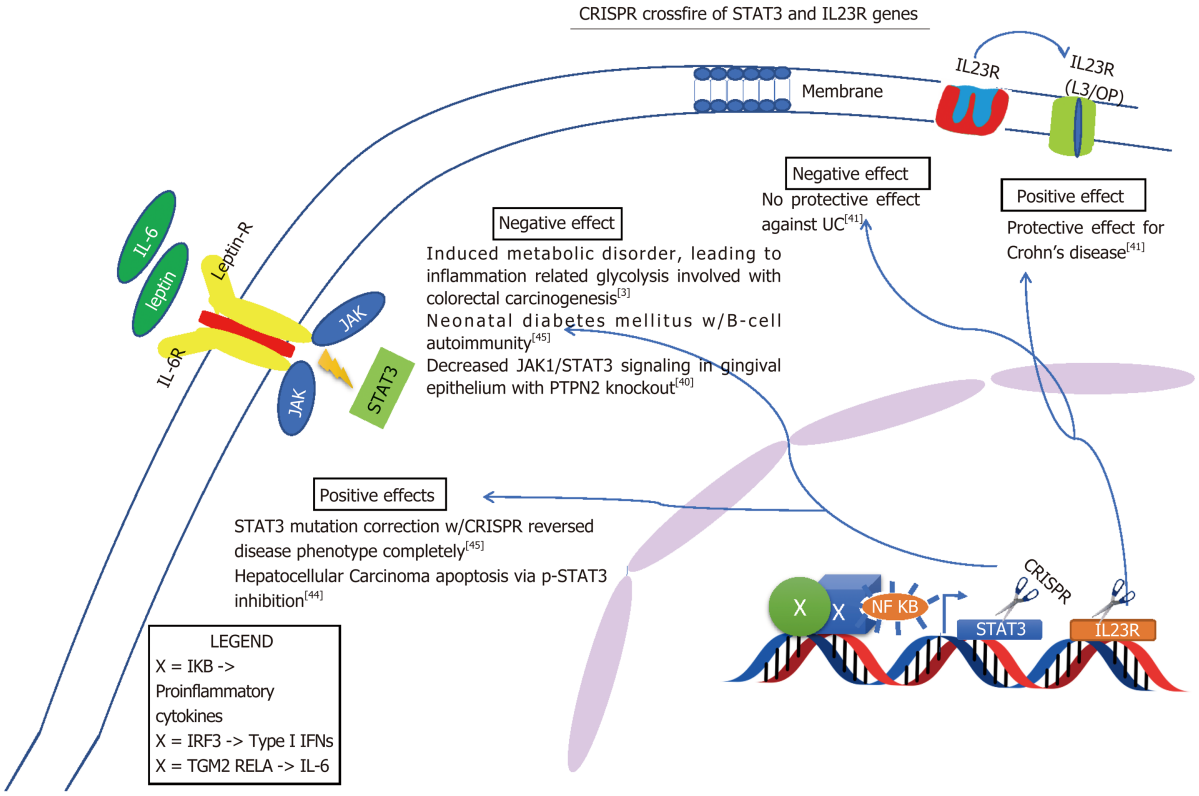

Figure 7 Positive and negative effects of CRISPR when targeting STAT3 and IL23R genes. UC: Ulcerative colitis.

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MINIREVIEWS

Diversity of *Saccharomyces boulardii* CNCM I-745 mechanisms of action against intestinal infections

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Abstract

The yeast *Saccharomyces boulardii* CNCM I-745 is one of the probiotics recommended for the prevention of antibiotic-associated diarrhea. Studies conducted *in vivo* and *in vitro* demonstrated that in the case of infectious diseases there are two potential sites of action of *Saccharomyces boulardii* CNCM I-745: (1) An action on enteropathogenic microorganisms (adhesion of bacteria and their elimination or an effect on their virulence factors: Toxins, lipopolysaccharide, *etc*.); and (2) a direct action on the intestinal mucosa (trophic effects, effects on epithelial reconstitution, anti-secretory effects, anti-inflammatory, immunomodulators). Oral administration of *Saccharomyces boulardii* CNCM I-745 to healthy subjects does not alter their microbiota. However, in the case of diseases associated with the use of antibiotics or chronic diarrhea, *Saccharomyces boulardii* CNCM I-745 can restore the intestinal microbiota faster. The interaction of *Saccharomyces boulardii* CNCM I-745 with the innate immune system have been recently demonstrated thus opening up a new therapeutic potential of this yeast in the case of diseases associated with intestinal infections but also other pathologies associated with dysbiosis such as inflammatory diseases.

Key words:*Saccharomyces boulardii* CNCM I-745; Probiotics; Yeast; Intestinal infection; Mechanism

Core tip: The efficacy of lyophilized probiotic yeast *Saccharomyces boulardii* CNCM I-745 was clinically demonstrated with controlled studies of intestinal infections associated with antibiotics and acute diarrhea in children. This review summerizes scientific data describing the mechanism of *Saccharomyces boulardii* protection against infection and emphazises the diversity of potential mechanism of action that this

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probiotic yeast can have against pathogenic microorganisms. More recently, effects on the recovery of the intestinal microbiota as well as on the immune system have been demonstrated, thus opening up a new therapeutic potential of this yeast.

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INTRODUCTION

Probiotics are living microorganisms that when administered in adequate amounts have a beneficial effect on the host. Most are bacteria, the best known of which are strains of *Lactobacillus* spp., and *Bifidobacterium* spp. However, there is a non-bacterial microorganism classified as a probiotic agent: this is the yeast *Saccharomyces boulardii* (*S. boulardii*) CNCM I-745 (Figure 1). *S. boulardii* was discovered by the French microbiologist Henri Boulard in 1920 during a visit to Indochina. The microbiologist noted that people who drank a decoction prepared from the outer skin of the lychee and mangosteen fruits did not develop diarrhea. He isolated the causative agent and named it *S. boulardii*. The initial strain was deposited at the National Collection of Microorganism Culture (CNCM) at the Institut Pasteur under the reference CNCM I-745. As a yeast, *S. boulardii* CNCM I-745 differs from bacterial probiotics in size, cell wall composition, antibiotic resistance and metabolic properties[1,2]. *S. boulardii* CNCM I-745 is the first identified yeast that has been studied for use as a probiotic in human medicine. This strain responds to a manufacturing process and a mode of conditioning that preserves the characteristics of the initial strain and its viability. The probiotic actions demonstrated by this strain of yeast are not extrapolatable to other strains. The aim of this review is to synthesize the experimental studies that have been carried out with the yeast *S. boulardii* strain CNCM I-745 in order to understand its mechanism of action against pathogenic microorganisms.

CLINICAL DATA IN THE CASE OF INFECTIOUS DIARRHEA

S. boulardii strain CNCM I-745 is prescribed in adults and children for the prevention and treatment of diarrhea, including antibiotic and post-antibiotic diarrhea associated with *Clostridium difficile* (*C. difficile*). Three prospective, randomized, double-blind, placebo-controlled clinical trials demonstrated the efficacy of *S. boulardii* CNCM I-745 in preventing diarrhea associated with antibiotic therapy in adults[3-5]. In children, two studies showed that *S. boulardii* CNCM I-745 has a preventive effect in diarrhea associated with antibiotics[6,7]; the second study also demonstrated a curative effect. A recently published meta-analysis[8] analyzed data from 21 randomized studies comprising a total of 4780 adult or child patients that received antibiotics. The results of this study support those of the first meta-analysis conducted in 2005[9] and show an efficacy of *S. boulardii* CNCM I-745 in the pediatric and adult population regardless of the type of antibiotic prescribed. When considering studies that reveal a significant benefit of the administration of *S. boulardii* strain CNCM I-745, an early treatment with the yeast during antibiotic therapy appears to be an essential factor with the administration of *S. boulardii* strain CNCM I-745 for the entire duration of antibiotic treatment. Several clinical studies show the effectiveness of *S. boulardii* CNCM I-745 in relapsing colitis and *C. difficile* diarrhea[10-12]. There are several studies evaluating *S. boulardii* CNCM I-745 in patients with gastroenteritis that may be due to a viral, bacterial, or parasitic infection. A 2012 meta-analysis of 11 randomized controlled trials found that *S. boulardii* decreased the duration of diarrhea and hospitalization due to gastroenteritis in all countries examined[13-18].

Tourists traveling to countries with warm climates, particularly in tropical or subtropical regions, are at high risk of diarrhea. In 80% to 85% of cases, diarrhea is due to pathogenic bacteria [*Escherichia coli* (*E. coli*), enteropathogenic, *Campylobacter jejuni, Shigella*, *Salmonella*, *Yersinia enterocolitica*]. Viruses [Norwalk or Rotavirus (RV)] and parasites [*Entamoeba histolytica* (*E. histolytica*), *Giardia lamblia*, *Cyclospora*, *Cryptosporidium*] are less frequently the causative pathogen. In many cases, the cause

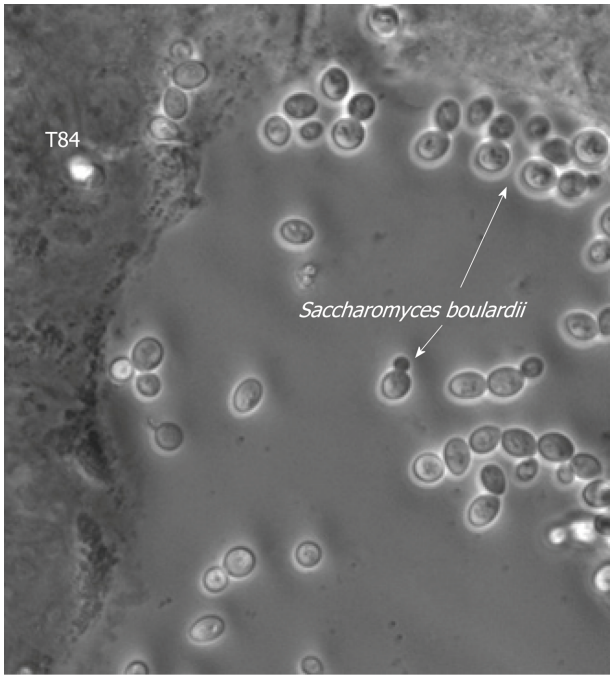

Figure 1 Transmission microscopy image showing *Saccharomyces boulardii* CNCM I-745 on a culture of human epithelial cells (T84 lineages). (Source: Pontier-Bres R and Czerucka D). The arrows indicate budding yeast located either in the spaces between the cells or near the cell walls and the formation of a protective barrier.

can not be determined. Two randomized controlled studies were conducted with *S. boulardii* CNCM I-745 with a total of four treatment arms with different doses. Kollaritsch *et al*[19] analyzed the results from 1231 Austrian travelers that were randomized into 2 arms, receiving *S. boulardii* CNCM I-745 at two different doses (250 and 500 mg daily) or a placebo for 3 wk. This treatment was started 5 d prior to the trip and continued throughout the trip. In the placebo group, the rate of diarrhea was 43% while it was 34% for the traveler receiving 250 mg of *S. boulardii* CNCM I-745 and 32% for those receiving the highest dose. No side effects have been reported[19]. In a second study by Kollaritsch *et al*[20] conducted with 3000 Austrian tourists, travelers received either a dose of *S. boulardii* CNCM I-745 (250 mg or 1 g per day) or a placebo. The treatment was initiated 5 days prior to departure and continued throughout the trip (for a mean duration of 3 wk). Patients that received a placebo had a higher frequency of diarrhea (39% *vs* 34% for the low dose and 29% for the high dose, $P < 0.05$).

PRECLINICAL-STUDIES: EFFECTS ON ENTEROPATHOGENIC MICROORGANISMS

Several studies using animal or cellular models have shown that *S. boulardii* CNCM I-745 has a beneficial effect against infections by various pathogenic bacteria such as *C. difficile*, *Vibrio cholerae*, *Salmonella*, *Shigella*, *E. coli*, viruses (RV) and finally, pathogenic yeasts, for example *C. albicans*. *S. boulardii* CNCM I-745 acts either directly on bacterial toxins or on the pathogen, and can also act directly on the intestinal mucosa of the host and modulate the response to infection (Figure 2). The protective action of *S. boulardii* CNCM I-745 with respect to an infection often results from the complementary effect of several mechanisms.

Anti-toxin activity

Vibrio cholerae: The first studies on the mechanism of action of *S. boulardii* CNCM I-745 with respect to pathogenic bacteria focused on the *Vibrio cholerae*. In 1986, Vidon *et al*[21] demonstrated, *in vivo*, that the administration of *S. boulardii* CNCM I-745 to ligated jejunal loop in rats that had been innoculated with cholera toxin (CT) significantly reduced the fluid and sodium secretion induced by CT. This effect has been confirmed in models of intestinal epithelial cells[22,23]. The action of *S. boulardii* CNCM I-745 (Figure 3A) is associated with a decrease in CT-induced cAMP. It requires live yeasts and it is associated to a protein factor present in the culture

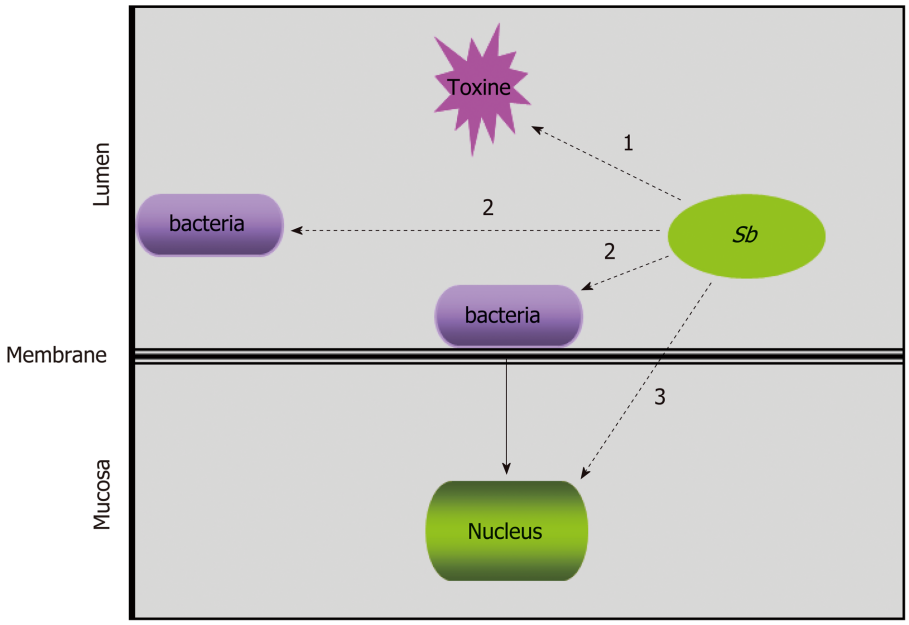

Figure 2 The hypothesized targets of *Saccharomyces boulardii* CNCM I-745 during bacterial infections: *Saccharomyces boulardii* CNCM I-745 may act directly on toxins "1", on pathogenic bacteria "2" or on host cells "3". Sb: *Saccharomyces boulardii* CNCM I-745.

supernatant of *S. boulardii* CNCM I-745. This protein factor, at 120 kDa, acts directly on enterocytes by inhibiting the cAMP-induced secretion of chloride (Cl^-) triggered by CT, and also by other cAMP-agonists like Vaso-intestinal peptide (VIP) or the Labile toxin (LT) of *E. coli*[22,23]. Another study shows that *S. boulardii* CNCM I-745 can attach CT to its cell wall, which is another mechanism of action against this toxin[24].

***Clostridium difficile*:** The mechanism of action of *S. boulardii* CNCM I-745 with respect to *C. difficile* infections has been the most studied. The administration of *S. boulardii* CNCM I-745 significantly reduces the mortality induced by *C. difficile* colitis in clindamycin-treated hamsters[25] and mice inoculated either by *C. difficile*[26] or by toxins A and B produced by the bacteria[27]. A recent study demonstrated the protective effect of *S. boulardii* CNCM I-745 against different ribotypes of *C. difficile* that are associated with outbreaks[28]. Among the various mechanisms of action proposed (action on the bacterium or its toxins see Figure 3B), the direct action of *S. boulardii* CNCM I-745 on toxins A and B of *C. difficile* and their receptors is the most supported. In fact, the injection of toxin A into the ileal loop does not induce inflammatory diarrhea if the animal has been previously treated with *S. boulardii* CNCM I-745 or its supernatant[29]. *In vitro* studies have shown that the culture supernatant of *S. boulardii* CNCM I-745 inhibits the adhesion of toxin A to its receptor[30]. A 54kDa protease identified in this supernatant can degrade *C. difficile* toxins A and B and their receptors[29,30]. The inflammation associated with *C. difficile* colitis is due to the activation of pro-inflammatory pathways by toxins A and B: nuclear translocation of NF-κB factor and activation by phosphorylation of MAP kinases that induce cytokine synthesis. The culture supernatant of *S. boulardii* CNCM I-745 inhibits interleukin 8 (IL-8) synthesis as well as nuclear translocation of NF-κB and inhibits toxin A-induced phosphorylation of ERK1/ 2 and JNK in epithelial cells[31]. In addition to acting on toxins and their receptors, the mechanism of action of *S. boulardii* CNCM I-745 with respect to *C. difficile* infections appears to involve modulation of the immune system. In fact, *S. boulardii* CNCM I-745 increases the level of circulating anti-toxin A IgA in mice that have been stimulated with inactivated toxin A[32].

Bacillus anthracis*:Bacillus anthracis*, the etiological agent of anthrax, infects the host by three routes of entry: cutaneous (most common), digestive and inhalation. The virulence factors are the capsule responsible for sepsis and two toxins responsible for toxemia. The toxins are composed of three peptides: The protective antigen (PA) and two enzymatic factors. The PA binds to cell-surface receptors and, after endocytosis, injects into the cytosol the two enzymatic factors: The lethal factor (LF) and the edematogenic factor (EF). The lethal toxin (LT) formed by the combination of PA with LF is a 90 kDa protein with metalloprotease activity that specifically cleaves MEK-2

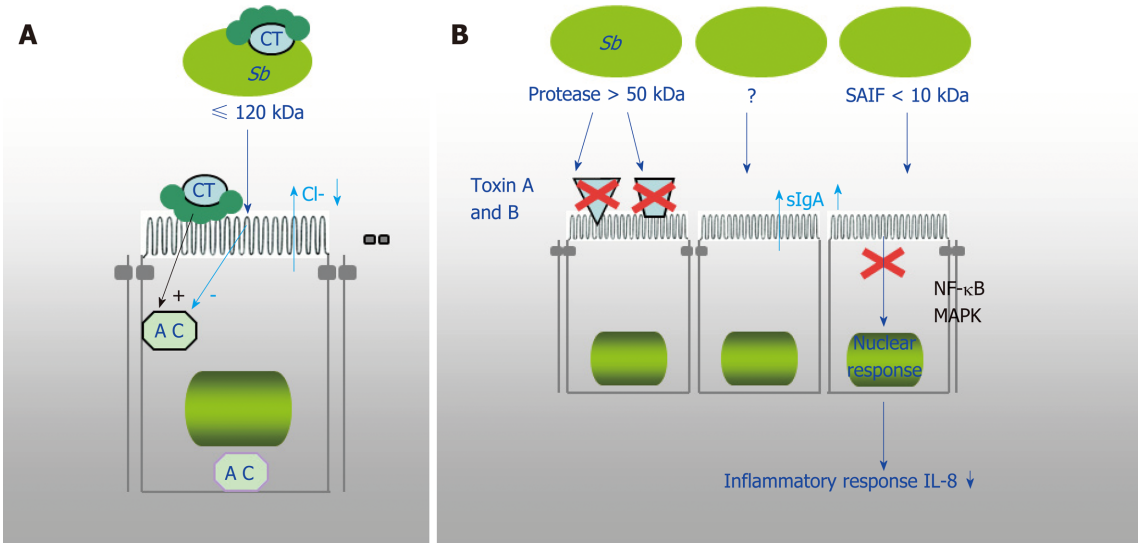

Figure 3 Demonstrated mechanism of *Saccharomyces boulardii* CNCM I-745 action against bacterial toxins. A: *Saccharomyces boulardii* CNCM I-745 (*S. b*) produces a 120 kDa protein that inhibits adenylate cyclase and cholera toxin (CT)-induced chloride secretion. *S. boulardii* CNCM I-745 can also bind to CT; B: *S. boulardii* CNCM I-745 secretes a protease (> 54kDa) that lyses *C. difficile* toxins A and B and their receptors and a protein (< 10 kDa) that inhibits the signaling pathways involved in interleukin 8 synthesis. AC: Adenylate cyclase; CT: Cholera toxin; Sb: *Saccharomyces boulardii* CNCM I-745; IL-8: Interleukin 8.

protein kinase. This toxin affects actin filaments in endothelial and epithelial cells and triggers morphological changes that result in the opening of tight junctions. Incubating cells with *S. boulardii* CNCM I-745 prior to exposure to LT toxin maintains the structure of the actin fibers and junctions[33]. Furthermore, the cleavage of MEK-2 is delayed in the presence of *S. boulardii* CNCM I-745. The yeast acts directly on the LT subunits by cleaving the PA antigen and attaching the antigen and LF to its surface. These results suggest the use of *S. boulardii* CNCM I-745 as a preventive agent for *B. anthracis* infection.

Action on pathogenic bacteria

Enteropathogenic *E. coli* and enterohaemorrhagic *E. coli*: Among pathogenic *E. coli*, enteropathogenic *coli* (EPEC) cause infectious diarrhea in developing countries, while enterohaemorrhagic *coli* (EHEC), that produce the shiga-like toxin, are involved in foodborne infections in industrialized countries. The adhesion of these bacteria to the intestinal mucosa is a crucial step in their pathogenicity. After contact with the mucosa, these bacteria inject effector proteins into the host through the type III secretion system. These effector proteins induce changes in the structure of tight junctions, stimulate the activation of MAP kinases and the factor NF-κB and, consequently, activate the synthesis of IL-8. Studies on monolayers of polarized human epithelial cells (Figure 4) showed that incubation with *S. boulardii* CNCM I-745 prevents the increase in intestinal permeability induced by EPEC and EHEC infection[34,35]. In addition, the structure of the tight junctions is maintained in these infected cells after exposure to the yeast. In EHEC-infected cells, *S. boulardii* CNCM I-745 inhibits phosphorylation of the myosin light chain that is directly involved in maintaining the integrity of tight junctions. *S. boulardii* CNCM I-745 also has an anti-inflammatory effect that inhibits the activation of mitogen-activated protein kinases, the nuclear translocation of NF-κB, and, consequently, the synthesis of IL-8[34,35]. In a mouse model infected with *Citrobacter rodentium* (the equivalent of EPEC in rodents) *S. boulardii* CNCM I-745 decreases the flux of mannitol and improves the histological score of infected animals[36]. *In vivo* the yeast acts directly on the expression of the virulence factors of the bacterium: Tir (Translocated receptor intimin), a factor directly involved in the adhesion of bacteria to the surface of enterocytes, and EspB, an effector protein injected into the host cell by the type III secretion system. Another study shows that *S. boulardii* CNCM I-745 can act on bacterial factors, in this case lipopolysaccharide (LPS) of *E. coli*. Buts *et al*[37] reported that *S. boulardii* CNCM I-745-synthesized phosphatase could de-phosphorylate *E. coli* O55B5 LPS. Injection to rats of LPS exposed to phosphatase purified from *S. boulardii* CNCM I-745 resulted in a reduction in circulating TNF-compared to the level induced by untreated LPS.

***Salmonella enterica* Typhimurium and *Shigella flexneri*:** Ingestion of food contaminated with *Salmonella enterica* Serovar Typhimurium (hereafter referred to as *Salmonella typhimurium*) results in diarrhea. *Salmonella typhimurium* are invasive

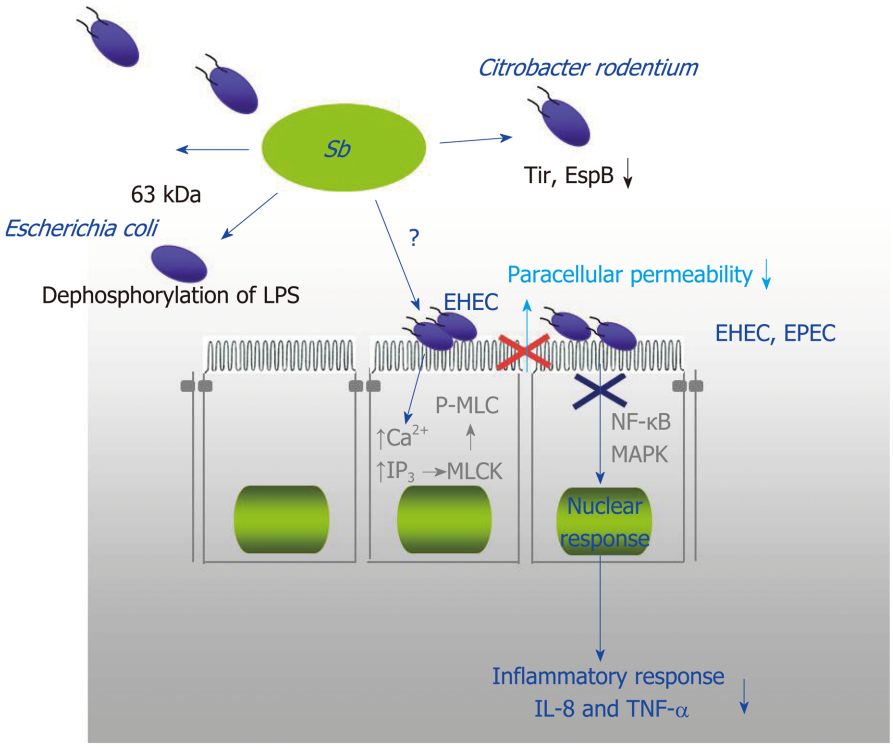

Figure 4 The protective mechanisms against enteropathogenic *Escherichia coli* (enteropathogenic *coli*, enterohaemorrhagic *coli* and *Citrobacter rodentium*). They include an effect on the mucosa with *Saccharomyces boulardii* CNCM I-745 inhibiting the pathways involved in opening tight junctions (phosphorylation of myosin light chain kinase), inhibition of activation pathways of mitogen-activated protein kinases and NF-κB that are involved in the synthesis of Interleukin 8, and finally a direct effect of *S. boulardii* CNCM I-745 on bacteria (dephosphorylation of lipopolysaccharide of *Escherichia coli* and modification of the expression of pathogenicity factors of *Citrobacter rodentium*). EHEC: Enterohaemorrhagic *coli*; EPEC: Enteropathogenic *coli*; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MLCK: Myosin light chain kinase; P-MLC: Phosphorylation of myosin light chain; IL-8: Interleukin 8; TNF-α: Tumor necrosis factor α Sb: *Saccharomyces boulardii* CNCM I-745.

bacteria that use several entry routes into the mucosa: microfold cells, enterocytes and a specific population of dendritic cells (DC). These bacteria are also able to induce a pro-inflammatory response by activating the MAP kinase and NF-κB pathways. Inoculation of *S. boulardii* CNCM I-745 to gnotoxenic or conventional mice infected with *Salmonella typhimurium* or *Shigella flexneri* protects against mortality (*Shigella flexeneri*) or reduces the severity of intestinal lesions (*Salmonella typhimurium*)[38]. This protective effect is not related to a reduction in the level of intestinal population of these bacteria. In the case of Salmonella infection, the protective effect of *S. boulardii* CNCM I-745 has been confirmed in a conventional mouse model and a mouse model with intestinal flora impaired by antibiotic treatment[39,40]. In treated animals, mortality and translocation of *S. typhimurium* to the liver and spleen were reduced. *In vitro*, *S. boulardii* CNCM I-745 decreases enterocyte invasion by *S. typhimurium*, which is correlated with decreased activation of the Rac pathway, a pathway directly used duirng invasion by these bacteria (Figure 5)[39]. *S. typhimurium*, like *E. coli*, are peritrichous bacteria with flagella that give them the ability to swim. Mutants devoid of flagellum are immobile and not invasive. A recent study showed that *S. boulardii* CNCM I-745 modifies the motility of bacteria by a steric effect and also by chemotaxis, decreasing the invasiveness of *S. typhimurium*[41]. In polarized T84 cells, *S. boulardii* CNCM I-745 maintains the structure of tight junctions in infected monolayers. In addition, in this model, the yeast prevents the activation of NF-κB and MAP kinase pathways and the synthesis of IL-8, which are associated with cell infection. The maintenance of tight junctions and the anti-inflammatory effect of *S. boulardii* CNCM I-745 were also confirmed in the case of *Shigella* infection[42]. The adhesion of *E. coli* and Salmonella to *S. boulardii* CNCM I-745 cell walls was reported in an earlier study[43] and has recently been confirmed *in vitro* and *in vivo* for *S. typhimurium* by scanning and confocal microscopy[39,40]. Salmonella adherent to the yeast wall was visualized by confocal microscopy on sections of caecum (Figure 6). The adhesion constitutes one of the mechanisms of action of *S. boulardii* CNCM I-745. By imaging

bioluminescent *S. typhimurium*, it was shown that *in vivo S. boulardii* CNCM I-745 modifies bacterial propagation in the gut of living mice during the first hours of infection. The yeast accelerates the spread of Salmonella along the digestive tract with the bacteria being detected in the feces as early as 6 h after the onset of infection. In addition, the administration of *S. boulardii* CNCM I-745 modifies the site-dependent (ileum *vs* caecum) pro and anti-inflammatory responses at the early stages of infection[40].

***Helicobacter pylori*:** *Helicobacter pylori* (*H. pylori*) is major causative agent of gastritis, peptidic ulcer disease and is strongly associated with gastric cancer and lymphoma of the gastric mucosa-associated lymphoid tissue. Currently recommended treatments for the eradication of *H. pylori* is standart triple therapy combining two antibiotics with a proton pump inhibitor. This therapy produces excellent cure rates but present side effects such as (diarrhoe, nausea/vomoting and even *C. difficile* infection) and emergence of antibiotic-resistant strains. Introduction of probiotics, especially *S. boulardii* as adjuvent treatment show promising results in reducing side effect[44,45]. Investigation on *S. boulardii*'s mechanism of action demonstrated that this yeast prevents binding of *H. pylori* on duodenal cells, whereas bacterial probiotical strains do not. It may be linked with *S. boulardii* neuramidase activity that modifies *H. pylori* binding site on the duodenal cells [46]. In a murine model of Helicobacter infection using a close species of *H. pylori* (*H. suis*), *S. boulardii* decreased the Helicobacter bacterial load, inhibited the formation of lymphoid follicles and reduced expression levels of inflammatory cytokines and chemokines in the stomach. It also increased the production of anti-helicobacter specific IgA and sIgA and beta-defensin in the small intestine after the infection [47].

Action on other pathogenic microorganims

***Candida albicans*:** *Candida albicans* (*C. albicans*) is the most commonly isolated opportunistic pathogenic fungus in humans, responsible for localized and systemic infections. The prevention of *C. albicans* infections is one of the first effects attributed to *S. boulardii* CNCM I-745[48]. In the gnotoxenic mouse, continued administration of *S. boulardii* CNCM I-745 precludes the implantation of a strain of *C. albicans* in the gastrointestinal tract[49]. Two studies in immunocompromised mice and rats indicate that *S. boulardii* CNCM I-745 also reduces the translocation of *C. albicans* to mesenteric lymph nodes (MLN)[50,51]. In a mouse model of dextran sulfate sodium- induced colitis, *S. boulardii* CNCM I-745 reduced both inflammation and intestinal colonization by *C. albicans*[52]. The antagonistic effects of *S. boulardii* CNCM I-745 on *C. albicans* have recently been demonstrated *in vitro*[53]. *S. boulardii* CNCM I-745 has an inhibitory effect on the filamentous growth of *C. albicans*, as well as its adhesion and the formation of biofilm on different surfaces. The same authors identified the active compound as capric acid, an AGCC synthesized by *S. boulardii* CNCM I-745, which reduces the virulence of *C. albicans*[54]. *In vitro*, *S. boulardii* CNCM I-745 decreases the adhesion of *C. albicans* to Caco-2 and Intestin 407 cells, and inhibits IL-8 expression[55]. *S. boulardii* CNCM I-745 also shows an anti-inflammatory effect in intraepithelial lymphocytes, of a mouse model, that were exposed to *C. albicans*[56]. In the presense of the yeast, the authors observed an increase in the anti-inflammatory cytokines IL-4 and IL-10, and a decrease in the pro-inflammatory cytokine IL-1.

Rotavirus: Rotavirus (RV) infection is the most frequent and severe form of acute gastroenteritis in infants and children worldwide, and frequently requires hospitalization. RV infects mature enterocytes of the small intestinal villi, inducing broad functional and structural damage. In humans, RV causes watery diarrhea that results from a combination of osmotic and secretory effects. The non-structural protein 4 (NSP4) produced by RV plays a key role in secretory diarrhea by inducing a redox imbalance, resulting in the secretion of chloride by intestinal epithelial cells. In Caco-2 cells infected with the viral strain SA11, the secretion of chloride is induced in association with an increase in reactive oxygen species[57]. The supernatant of *S. boulardii* CNCM I-745 reduces oxidative stress in these cells and strongly inhibits RV-induced chloride secretion. These results were confirmed on human intestinal biopsies exposed to NSP4. *S. boulardii* CNCM I-745, via a soluble metabolite, prevents oxidative stress and inhibits NSP4-induced chloride secretion[57].

***Entamoeba histolytica*:** Amoebiasis ranks third among the most deadly parasitic diseases in the world. About 10% of the world's population is infected with amoeba parasites of the genus Entamoeba, the most pathogenic of which is *E. histolytica*. It is a protozoan that can surround itself with a thin shell to form a cyst a few microns in diameter. When these cysts are ingested, they germinate in the small intestine to give rise to the vegetative form, the trophozoites, which enter the large intestine, proliferate and re-encysted. It is in this form that *E. histolytica* is expeled in feces and is

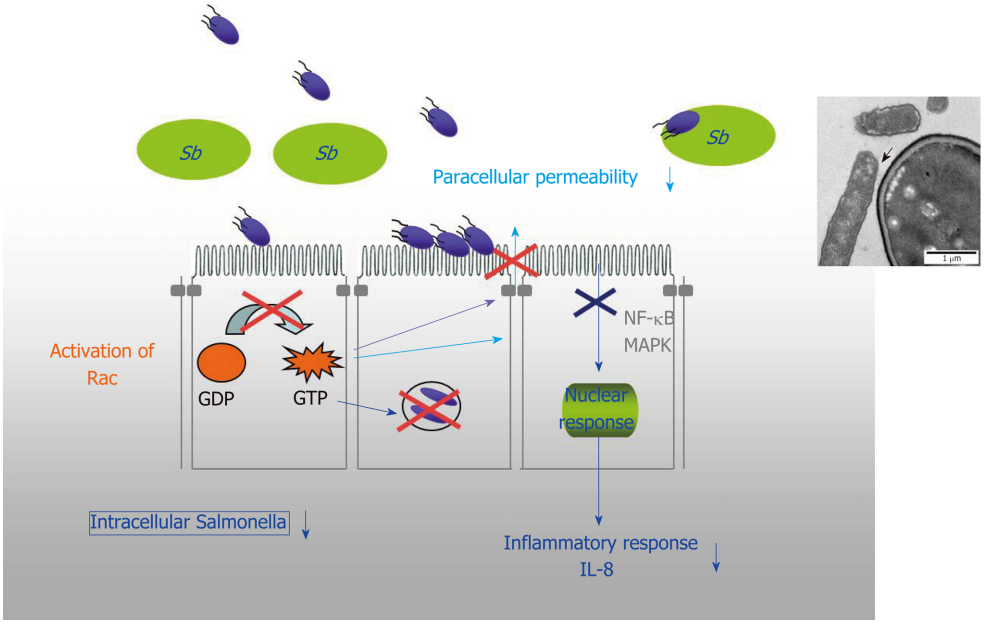

Figure 5 The mechanisms of protection against enteroinvasive bacteria (Salmonella and *Shigella*). In the case of Salmonella, there is a decrease in the activation of the small GTPase pathway and consequently a decrease in the number of intracellular bacteria and the maintence of tight junctions. *Saccharomyces boulardii* CNCM I-745 also induces inhibition of mitogen-activated protein kinases and NF-κB activation pathways that are involved in interleukin 8 synthesis. And finally, a direct effect of *S. boulardii* CNCM I-745 on bacteria with the modification of their motility and the adhesion of Salmonella to *S. boulardii* CNCM I-745. IL-8: Interleukin 8; MAPK: Mitogen-activated protein kinase; Sb: *Saccharomyces boulardii* CNCM I-745.

likely to contaminate other people. If the infection remains generally asymptomatic, the parasite can, however, cause painful and bloody diarrhea (amoebic dysentery), ulcers, and, in the more severe forms, lead to abscesses in the liver, lungs and brain. In young rats, infection with this species can produce lesions similar to those observed in humans. A model of caecal amoebiasis in rat was used to study the effect of *S. boulardii* CNCM I-745 on the development of lesions[58]. In this model, young rats infected with *E. histolytica* were treated with *S. boulardii* CNCM I-745. In the yeast treated group the number of sick pups was significantly lower and the macroscopic appearance of the lesions on the cecum, as well as the presence of amoebae, was decreased. The lesions were similar to those of the control animals, but their healing process was accelerated. In addition, *S. boulardii* CNCM I-745 showed no amoebicidal activity. The antagonistic effect of the yeast has been explained *in vitro* by competition between the yeast and amoebae at binding sites on erythrocytes[59].

EFFECT ON MUCOSA THAT CAN BE IMPLICATED IN THE ANTI-PATHOGENIC EFFECT ANTI-SECRETORY EFFECTS

Intestinal fluid secretion is driven by active Cl^- secretion creating the electro-chemical gradient for paracellular Na^+ secretion and the osmotic driving force for transcellular water secretion. Cl^- is transported into the cell at the basolateral membrane by a $Na^+/K^+/Cl^-$ cotransporter which is driven by Na^+ and Cl^- concentration gradients produced by Na^+K^+-ATPase and basolateral K^+ channels. The electrochemical gradient drives Cl^- secretion across the luminal membrane by two Cl^- channels: (1) The cAMP-activated channel CFTR; and (2) the Ca^{2+}-activated channel. These channels are an attractive target for potentially antidiarrheal therapeutics. The effect of *S. boulardii* CNCM I-745 on these channels has been the subject of several studies using pharmacological agents. The first study conducted in the jejunum of pigs or in tissue from animals treated with *S. boulardii* CNCM I-745, shows a decrease in Cl^- secretion induced by a phosphodiesterase inhibitor: Theophylline[60]. Another study performed *in vitro* reports that *S. boulardii* CNCM I-745 inhibits the secretion induced by receptor (vasointestinal peptide or prostaglandin E2) or non-receptor (forskolin) c-AMP mediated Cl^- secretion, thus demonstrating that *S. boulardii* CNCM I-745 can directcly affect adenylate cyclase activity[22]. In the same study *S. boulardii* CNCM I-745 showed decreased Ca^{2+}-stimulated Cl^- secretion induced by carbachol. These inhibitory effects on Cl^- secretion have been reproduced with bacterial toxin: For exemple in the case of CT or LT-toxin produce by Enterotoxigenic *E. coli*, which induces secretory diarrhea

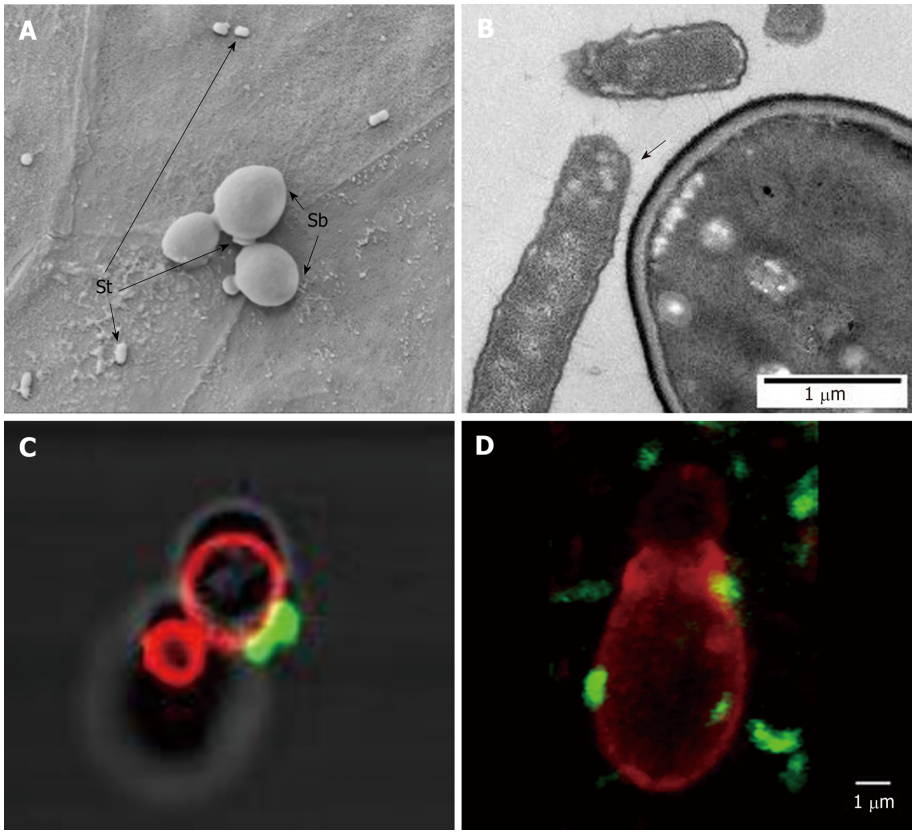

Figure 6 Scanning electron microscopy image showing *Saccharomyces boulardii* CNCM I-745 and *Saccharomyces typhimurium* on a monolayer of T84 polarized cells. A and B: Electron microscopy image showing *Saccharomyces typhimurium* adhesion to *Saccharomyces boulardii* CNCM I-745; C and D: Confocal microscopy images showing *S. typhimurium* (*Fluorescein IsoThioCyanate* labelling), which adheres to *S. boulardii* CNCM I-745 (rhodamine labeling) *in vitro* (C) and *in vivo* on mouse cecum sections (D). Photos A and B: D. Czerucka[1], P. Gounon[2], P. Rampal[1]; C and D: D. Czerucka[1], R. Pontier-Bres[1], P. Rampal[1] ([1]CSM, Monaco, microscopy Platform Cote d'Azur, MICA; [2]University of Nice-Sophia-Antipolis). Sb: *Saccharomyces boulardii* CNCM I-745; St: *Salmonella typhimurium*.

primarely by activating cAMP-activated Cl^- secretion. The authors demonstrate that the culture supernatant of *S. boulardii* CNCM I-745 exerts an anti-secretory effect, suggesting the existence of a factor secreted by the yeast that could act on the cAMP dependent Cl^- secertion[23]. The anti-secretory effect of *S. boulardii* CNCM I-745 on the secretion of Cl^- was also suggested in a rat model exposed to prostaglandin-2[61]. However in this model, *S. boulardii* CNCM I-745 stimulates the absorption of Cl^- in the jejunum and colon. *S. boulardii* CNCM I-745 exerted an antisecretory effect in other models that did not involve Cl^- channels. In a rat study, prophylactic administration of *S. boulardii* CNCM I-745 showed a potent effect on castor oil-induced secretory diarrhea in a dose-dependent manner[62]. This effect is significantly inhibited by L-arginine, suggesting the involvement of the nitric oxide pathway. Finally, *S. boulardii* CNCM I-745 also has an effect on short chain fatty acid (SCFA) synthesis: Butyrate, acetate and propionate, which play a role in the absorption of water and electrolytes. Treatment with clindamycin decreases the daily production of acetate, propionate and butyrate in pigs[63]. Simultaneous administration of *S. boulardii* CNCM I-745 and the antibiotic maintains acetate and propionate at their initial levels. In another study performed on patients receiving exclusive enteral nutrition, treatment with *S. boulardii* CNCM I-745 reduced fecal SCFA concentrations to normal, particularly for butyrate[64]. This property may account in part to the anti-diarrheal and anti-inflammatory effects of *S. boulardii* CNCM I-745.

One of the roles of the intestinal epithelium is to prevent microorganisms present in the intestinal lumen from accessing the tissues. Immune exclusion is the process by which microorganisms are prevented from crossing the intestinal barrier. This is done through tight junctions that provide a mechanical barrier and the combination of mucus-containing fluid and secreted IgA that allows the sequestration of microorganisms. Finally, the intestinal immune system also plays a crucial role in eliminating pathogenic bacteria that evade immune exclusion. In previous sections of this review we saw that *S. boulardii* CNCM I-745 was able to maintain the structure of

tight junctions in monolayers of infected cells. The effect of *S. boulardii* CNCM I-745 on IgA is the subject of one of the first studies on the mechanism of action of this yeast. This study showed that *S. boulardii* CNCM I-745 induces an increase in the concentration of secretory IgA in the intestinal fluid and an increase in the secretory component of the polymeric immunoglobulin receptor in cryptic cells of the intestinal mucosa in young rats 14 d after weaning[65]. This effect appears transient, as another study done on adult rats shows that after 21 d of administration of *S. boulardii* CNCM I-745 the yeast has no effect on IgA secretion, intestinal mucosal or circulating lymphocyte populations[66]. The effect of *S. boulardii* CNCM I-745 on IgA was confirmed in *C. difficile* infections, and furthermore, administration of the yeast to mice exposed to inactivated toxin A increases the level of circulating anti-toxin A IgA[32]. More recently, *in vitro* and *in vivo* studies showed a direct action of *S. boulardii* CNCM I-745 on cells of the innate immune system (Figure 7). DC plays a key role in the balance between immunity and tolerance, and participate in the activation of lymphocytes. A recent study found that *S. boulardii* CNCM I-745 modulates the properties of DC after treatment with amoxicillin[67]. Membrane markers of antigen activity (MHC-II and CD86) are upregulated in DC of female rats treated with the antibiotic. In the presence of the yeast, these antigens are negatively regulated and the intestinal flora regains its equilibrium more quickly after stopping antibiotic treatment. This study suggests that *S. boulardii* CNCM I-745 exerts a modulatory effect on the specific immune response to microbial antigens. This has been confirmed on human DC isolated from the blood of healthy subjects exposed to LPS, which induces DC activation[68]. The authors show that a < 3 kD fraction of the culture supernatant of *S. boulardii* CNCM I-745 decreases the expression of co-stimulatory CD40 and CD80 molecules and the chemokine receptor CCR7 (a receptor that causes chemotactic migration to secondary lymphoid organs) induced by LPS. This fraction also decreases the secretion of pro-inflammatory cytokines (TNF and IL-6) and stimulates the secretion of IL-10 in DC. In addition, *S. boulardii* CNCM I-745 decreases DC-induced T cell activation[68]. In another study, the same group demonstrated that supernatant of *S. boulardii* CNCM I-745 may modify the profile of DC (CD40 and CD80 co-factors, CCR7 and cytokine profile) in the blood of patients with Crohn's Disease or ulcerative colitis [69]. In addition, this supernatant inhibits the Th1 polarization of lymphocytes and induces the secretion of IL-8 and TGF, two cytokines involved in the reconstitution of the epithelium. Recently our group demonstrated that in a model of Salmonella-infected mice, *S. boulardii* CNCM I-745 decreases the DC population expressing $CD103^+$ that migrate to the MLN and are responsible for the bacterial translocation to MLN. The yeast also acts on another population of macrophage which express the fractalkine receptor CX3CR1 ($CX3CR1^{hi}$ MΦs) and are present in the Lamina Propria (LP). Thanks to extensions between epithelial junctions, this population of highly phagocytic cells recognize intra-luminal antigens, including pathogenic bacteria. They phagocyte them, and unlike $CD103^+$DCs do not migrate into the MLN but are locally induced in the LP activated T lymphocytes. The population of these $CX3CR1^{hi}$ MΦs in *S. boulardii* CNCM I-745-treated mice infected with Salmonella. The increase in this population comes from the expansion of circulating pro-inflammatory monocytes (mono $Ly6C^{hi}$, $CX3CR1^{int}$) that are recruited in the bone marrow. These results could be confirmed *in vitro* on cells isolated from the bone marrow of mice treated or not with S. *boulardii* CNCM I-745 and then infected. In the presence of the yeast, monocytes differentiate to $CX3CR1^{hi}$ MΦs and their ability to phagocyte Salmonella increases significantly. These results demonstrate that *S. boulardii* CNCM I-745 can act on the immune response during the early phase of infection[70]. The $CX3CR1^{hi}$ MΦs has been involved in tolerance to the microbiota. In mice with induced dysbiosis and treated with antibiotics, the population of $CX3CR1^{hi}$ MΦs decreases. Administration of *S. boulardii* CNCM I-745 during antibiotic therapy increases the population of $CX3CR1^{hi}$ MΦs. The impact of this increase on the reconstitution of intestinal flora remains to be demonstrated.

EFFECTS ON THE INTESTINAL MICROBIOTA

Several studies conducted in humans and mice show that *S. boulardii* CNCM I-745 has no effect on the intestinal microbiota of healthy subjects, however, in some diseases there is an effect on intestinal dysbiosis (Table 1). In a model of amoxicillin-treated mice, antibiotic treatment increased the Enterobacteriaceae and *Bacteroides* populations and decreased *Clostridium coccoides* and *Eubacterium rectale*. Treatment with *S. boulardii* CNCM I-745 did not influence changes in the gut microbiota during antibiotic treatment but accelerated the return to normal that occurred after 10 days in these mice versus 22 d in untreated mice[67,71]. Swidsinski *et al*[72] developed an

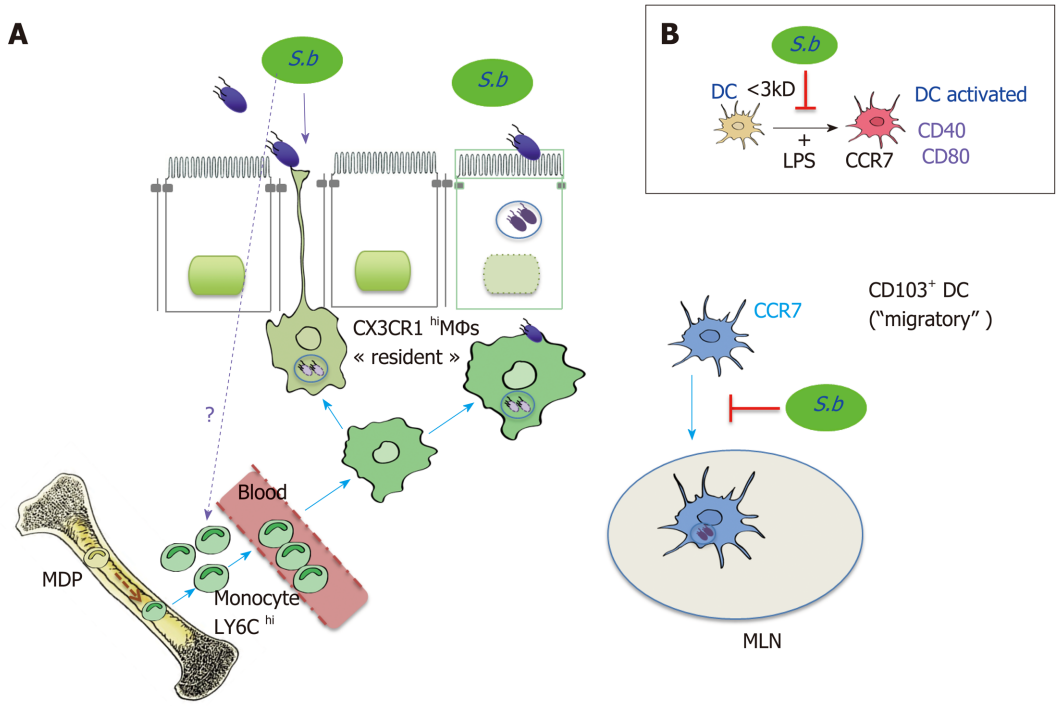

Figure 7 Effect of *Saccharomyces boulardii* CNCM I-745 on intestinal mononuclear phagocytes: Dendritic cells expressing CD103 (CD103$^+$DC) and macropahge expressing the fractalkine receptor (CX3CR1MΦs). A.CD103$^+$DC which expresses the CCR7 on their surface phagocytes the Salmonella (ST) and migrate to the mesenteric lymph nodes (MLN). CX3CR1MΦs which have a high phagocytosis capacity, include bacteria, do not migrate, but remain in the LP where they stimulate T lymphocytes. These MΦs are able to form extensions that pass between the epithelial cells and capture the antigens in the intestinal lumen, among other pathogenic bacteria such as ST. *Saccharomyces boulardii* CNCM I-745 induces the recruitment of CX3CR1MΦs and promotes phagocytosis of ST by these cells. *S. boulardii* CNCM I-745 effects the expansion of Ly6C inflammatory monocytes, which are the precursors of CX3CR1 DCs in the bone marrow. In addition, *S. boulardii* CNCM I-745 reduces the number of ST that migrate to MLN by decreasing the number of migratory DCs. B.*In vitro* studies have shown that *S. boulardii* CNCM I-745 can modify lipopolysaccharide activation of migratory DCs. This effect would be due to a molecule of low molecular weight (< 3 kDa) present in *S. boulardii* CNCM I-745 conditioned medium[64]. Sb: *Saccharomyces boulardii*CNCM I-745; ST: Salmonella; MLN: Mesenteric lymph nodes; DCs: Dendritic cells; LPS: Lipopolysaccharide.

innovative technique based on *in situ* hybridization (FISH) of stool samples collected by coring, which enables quantitatively assessing microbiota in the mucus, in the germinal reserve area and in the central fermentation area. This technique was used to compare and localize bacterial populations in healthy subjects and patients with idiopathic chronic diarrhea treated or not with *S. boulardii* CNCM I-745. Stools from healthy subjects are characterized by, a mucus layer of 5-60 m with homogeneous fluorescence, high concentrations of three usual bacterial groups (*Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii*) and low concentrations of occasional bacterial groups. In patients with diarrhea, the authors observed a thickening of the protective mucous layer, mucous layer incorporation in the stool, a reduced concentration of usual bacteria, an inhibition of the metabolism with appearance of areas devoid of hybridization signal and a stratification with increased levels of the occasional bacteria. Treatment with *S. boulardii* CNCM I-745 has no effect on the microbiota of healthy subjects, but the microbiological and clinical symptoms of diarrhea are reversible after treatment with the yeast. *S. boulardii* CNCM I-745 reduces the thickness of the mucus layer and increases the concentration of two usual bacterial groups: *Bacteroides* and *Roseburia*. Yeast treatment also decreases the abundance of the occasional bacterium *Akkermansia muciniphila*.

In a recent study using the same technique (corning and FISH), Swidsinski *et al*[73] investigated the effect of *S. boulardii* CNCM I-745 on the reconstitution of intestinal flora after antibiotic treatment in patients with vaginal infection. In this study a group of patients was treated with metronidazole and ciprofloxacin, a second group received yeast during antibiotic treatment and a third group was treated with yeast after antibiotic therapy. Antibiotic treatment significantly decreased the number of bacteria in the dominant group (*Clostridium cocoides*, *Eubacterium rectum*, *Faecalibacterium prausnitzii*, *etc*.) mainly located in the fermentation zone. Treatment with *S. boulardii* CNCM I-745 during antibiotic therapy increased these populations and post-antibiotic treatment allowed these populations to return to normal. Identification of bacteria in the stool by bacterial 16S RNA sequencing confirmed that

Table 1 Effets of *Saccharomyces boulardii* CNCM I-745 on gut microbiota in various diseases

Disease	Technique	Alteration of the microbiota	Effet of *S.boulardii* CNCM I-745	Ref.
Antibiotic treatment (mice)	FISH and cytometry	Increase in Enterobacteriaceae and *Bacteroides* Drastic decrease in *Clostridium cocoides* and *Eubacterium rectale*	Rapid return to normal for: *Bacteroides*, *Clostridium cocoidesEubacterium rectale*, *PrevotellaPorphyromonas*	[67,71]
Chronic diarrhea (humans)	Coring and FISH	Increase in *BifidobacteriumEubacterium cylindrodes*, *Clostridium histolyticus* et Decrease in *Bacteroides* et Roseburia	Decrease in *Bacteroides* and *Roseburia*	[72]
Antibiotic Treatment (humans)	Sequencing	Increase in *Parabacteroides* and *Escherichia/Shigella* Decrease in *Ralstonia*	Reduces microbiota variations due to antibiotic treatment	[74]
Antibiotic Treatment (women[1])	Corning and FISH	Decrease in dominant microbiota:*Clostridium cocoides*, *Eubacterium rectale*, *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii*	Group A/*Sb*: Increase in *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii* Groupe *Sb*-A: Rapid return to normal	[73]

FISH: Fluorescence in situ hybridation; A/*Sb*: Group of patients concomitantly treated with antibiotics and *Saccharomyces boulardii* CNCM I-745; *Sb*-A: Group of patientstreated with *Saccharomyces boulardii* CNCM I-745 after antibiotic treatment; *S.boulardii*; *Saccharomyces boulardii*.
[1]Patients with vaginal infections.

S. boulardii CNCM I-745 did not alter the microbiota composition of healthy subjects[73]. In patients treated with amoxicillin, there was a decrease in *Ralstonia* and an increase in *Parabacteroides* and *Escherichia/Shigella* in the fecal microbiota. In the group treated with *S. boulardii* CNCM I-745 and antibiotic, changes in the composition of the microbiota are significantly attenuated. A summary of the effect of *S. boulardii* CNCM I-745 on antibiotic-associated dysbiosis is presented in Figure 8. These results suggest that an optimal use of *S. boulardii* CNCM I-745 would be to administer it during and after antibiotic therapy. More and Swidsinski[75] recently published a review that summarizes all preclinical and clinical data on the effect of *S. boulardii* CNCM I-745 on intestinal microbiota associated and not associated with mucus.

CONCLUSION

S. boulardii strain CNCM I-745 is a probiotic yeast that by virtue of being a eukaryote differs from other probiotic strains, which are of bacterial origin (prokaryote). The research shows a great diversity in its mode of action and types of targets: pathogens, pathogenic toxins, gut microbiota and intestinal epithelium. Two main mechanisms were demonstrated: the first one is a large capacity of the wall to fix bacteria and toxins which facilitates their elimination during intestinal transit and the second one is the synthesis by this yeast of several active factors. These factors include high molecular weight proteins, some of which have antisecretory effects, others act as proteases that degrade toxins or their receptors. Factors of small size and protein or non-protein nature that exhibit anti-secretory or anti-inflammatory activities are also involved in its action. Finally, *S. boulardii* CNCM I-745 acts on different components that maintain the intestinal barrier: Tight junctions that regulate permeability; reconstitution of the microbiota after antibiotic therapy; and, activation of innate immunity which stimulates innate defenses of the host during infection. The optimization of the use of this probiotic in infections requires a better knowledge of the different mechanisms of action.

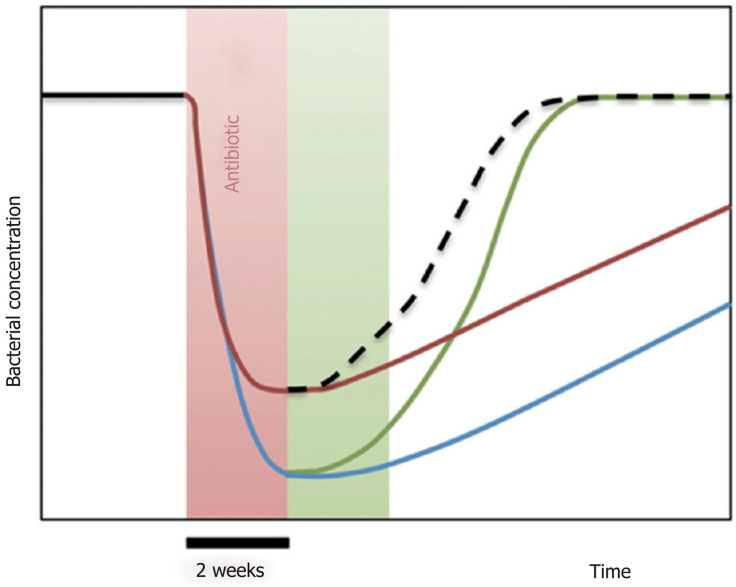

Figure 8 Diagram illustrating the impact of *Saccharomyces boulardii* CNCM I-745 on dysbiosis during antibiotic therapy. A 2-wk treatment with antibiotics (red zone of the graph) induces a sudden decrease in the dominant bacterial populations of the microbiota (blue curve). Treatment with *Saccharomyces boulardii* CNCM I-745 during antibiotic therapy (red curve) reduces the sudden decrease in bacterial populations. When *S. boulardii* CNCM I-745 is administered after antibiotic therapy (green zone of the graph, green curve) the yeast accelerates the restoration of the intestinal flora to its initial level. An optimal use of yeast would be administration during and after antibiotic therapy, which is presented by the hatched curve resulting from the red and green curve (from reference[75]).

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ORIGINAL ARTICLE

Basic Study

Characteristics of mucosa-associated gut microbiota during treatment in Crohn's disease

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Author contributions: He C, Wang H and Liao WD contributed to equally to this work; He C and Zhu ZH designed the research; Wang H, Liao WD, Peng C, Shu X and Zhu X enrolled the qualified patients and collected the mucosal samples; Wang H analyzed the information of the patients during treatment; He C performed the bioinformatic analysis and wrote the paper; all authors have read and approved the final version to be published.

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Abstract

BACKGROUND

The dysbiosis of the gut microbiome is evident in Crohn's disease (CD) compared with healthy controls (HC), although the alterations from active CD to remission after treatment are unclear.

AIM

To characterize the mucosa-associated gut microbiota in patients with CD before and after the induction therapy.

METHODS

The basic information was collected from the subjects and the CD activity index (CDAI) was calculated in patients. A 16S rRNA sequencing approach was applied to determine the structures of microbial communities in mucosal samples including the terminal ileal, ascending colon, descending colon and rectum. The composition and function of mucosa-associated gut microbiota were compared between samples from the same cohort of patients before and after treatment. Differential taxa were identified to calculate the microbial dysbiosis index (MDI) and the correlation between MDI and CDAI was analyzed using Pearson correlation test. Predictive functional profiling of microbial communities was obtained with PICRUSt.

RESULTS

There were no significant differences in microbial richness among the four anatomical sites in individuals. Compared to active disease, the alpha diversity of CD in remission was increased towards the level of HC compared to the active stage. The principal coordinate analysis revealed that samples of active CD were clearly separated from those in remission, which clustered close to HC. Sixty-five genera were identified as differentially abundant between active and quiescent CD, with a loss of *Fusobacterium* and a gain of potential beneficial bacteria

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including *Lactobacillus*, *Akkermansia*, *Roseburia*, *Ruminococcus* and *Lachnospira* after the induction of remission. The combination of these taxa into a MDI showed a positive correlation with clinical disease severity and a negative correlation with species richness. The increased capacity for the inferred pathways including Lipopolysaccharide biosynthesis and Lipopolysaccharide biosynthesis proteins in patients before treatment negatively correlated with the abundance of *Roseburia*, *Ruminococcus* and *Lachnospira*.

CONCLUSION
The dysbiosis of mucosa-associated microbiota was associated with the disease phenotype and may become a potential diagnostic tool for the recurrence of disease.

Key words: Crohn's disease; Mucosa-associated gut microbiota; Active; Remission; 16S rRNA sequence

Core tip: The dysbiosis of gut microbiome is associated with the development of Crohn's disease (CD), although the alteration from active CD to remission after treatment is unclear. This study illustrated that the composition of mucosa-associated gut microbiota in active CD significantly changed after the induction of remission regardless of drugs used, which got close to healthy subjects. We speculate that the maintenance of gut microbiota balance may be potential therapeutic target for reducing the risk of disease relapse.

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INTRODUCTION

Crohn's disease (CD), a subtype of inflammatory bowel disease (IBD), has become a global disease with accelerating incidence over the last few decades[1]. CD is characterized by multiple episodes of exacerbation and remission, with clinical manifestations of diarrhea, abdominal pain, fistulas and perianal lesions, which may affect the whole digestive tract and cause systemic symptoms[2]. Current therapies for IBD include anti-inflammatory and immunomodulatory treatments such as 5-aminosalicylates, corticosteroids, thiopurines, thalidomide and anti-tumor necrosis factor alpha, all of which aim to achieve clinical remission and mucosal healing[3,4]. The pathogenesis of CD is multifactorial and involves the interplay of host genetics, immune dysregulation and environmental factors resulting in an aberrant immune response and subsequent intestinal inflammation[5].

Recent progress in understanding the composition and function of human microbiota has revealed the important role of microbiota in immune homeostasis[6]. Accumulating studies using culture-independent techniques have shown the dysbiosis of gut microbiota in patients with CD, including decreased bacterial diversity, with an expansion of putative aggressive groups (such as *Enterobacteriaceae*, *Fusobacterium*) combined with decreases in protective groups (such as *Faecalibacterium*, *Roseburia*)[7,8]. In addition to observing the characteristics of gut microbiota in CD, a study by Wang *et al*[9] evaluated their dynamic changes after infliximab (IFX) therapy and found that the dysbiosis could be corrected in patients with a sustained therapeutic response. Furthermore, a prospective study assessed the stool metagenomes of IBD patients starting biologic therapy and demonstrated a higher abundance of butyrate producers at baseline in therapy-responsive CD patients, indicating the predicative effect of the gut microbiome in treatment response[10]. Due to the dysregulated microbiota in the pathogenesis of IBD, several studies have reported the potential effect of restoring dysbiotic gut microbiota, including the use of probiotics and unprocessed donor feces, in the management of IBD[11]. It is necessary

to clarify the key bacteria that play a role in disease remission and relapse, and then, precise manipulation of these bacteria may become a therapeutic target in the future.

To date, most studies investigating the gut microbiota of CD have typically used fecal samples since they are readily obtained[7-9]. However, the composition of fecal microbiota has been shown to be significantly different from mucosal microbiota; this difference is believed to directly affect epithelial and mucosal function and to be more deeply involved in the pathophysiology of CD[12,13]. To collect sufficient mucosal samples, we processed endoscopically uninflamed mucosa, which was thicker than the inflamed mucosa and probably more appropriate for microbial analysis[14]. In addition, only limited differences in microbiota composition were observed between inflamed and uninflamed mucosa in patients[15]. The previous cross-sectional study of the alterations between CD patients and healthy controls (HC) could be misread based on the interindividual differences, which make it difficult to characterize the critical bacteria in CD. Thus, we investigated the mucosal-associated microbiome in paired samples from CD patients before and after clinical treatment by 16S rRNA gene sequencing to determine the association between gut microbiota and disease activity.

MATERIALS AND METHODS

Study cohort

A prospective study was performed in nine CD patients who were enrolled in flare at baseline and then induced remission after clinical therapy. Inclusion criteria were a diagnosis of CD confirmed by endoscopy and histology and the activity of the disease was measured by the CD activity index (CDAI). Six HC without previous history of chronic disease were also recruited in the study from the First Affiliated Hospital of Nanchang University, China. Exclusion criteria for the two groups included severe concomitant disease involving the liver, heart, lung or kidney, pregnancy or breast-feeding, and treatment with antibiotics and prebiotics during the previous 4 wk. The mucosal samples were collected during the colonoscopy and both the patients and healthy subjects underwent intestinal washing before the examination. We did not collect both inflamed and noninflamed tissues since a previous study showed that the mucosal microbiota of inflamed and noninflamed regions of the gastrointestinal tract in CD or ulcerative colitis (UC) were indistinguishable, with virtually no taxa demonstrating disproportional abundances at a significant threshold nor any significant diversity differences observed[15]. Written informed consent was obtained from all the subjects and this study was approved by the Medical Ethics Committee of Nanfang Hospital.

Sample collection and DNA extraction

A total of 74 mucosal biopsies were collected from 15 participants, including 9 patients with CD and 6 healthy individuals. Specimens of terminal ileum, ascending colon, descending colon and rectum in noninflamed mucosa were taken during colonoscopic examination. Sampling included both active and remission stages for each patient who underwent clinical treatment. All the samples were immediately put in liquid nitrogen and stored at - 80 °C before processing.

Microbial DNA was extracted from the mucosal biopsies using the E.Z.N.A. stool DNA kit (Omega Biotek, Norcross, GA, United States) according to the manufacturer's protocols. The 16S rDNA V3-V4 region of the Eukaryotic ribosomal RNA gene was amplified by PCR (95 °C for 2 min, followed by 27 cycles at 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 30 s and a final extension at 68 °C for 10 min) using primers 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT, where the barcode is an eight-base sequence unique to each sample. PCRs were performed in triplicate 50 μL mixture containing 5 μL of 10 × KOD Buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (5 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA.

16S rRNA gene sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and qualified using QuantiFluor-ST (Promega, United States). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) using Illumina Hiseq 2500 following standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive database (Accession Number: SRP157001).

Sequencing data analysis

The raw data were filtered to obtain clean reads by eliminating the adapter pollution and low-quality sequences. Paired end clean reads were merged as raw tags using FLASH (Fast Length Adjustment of Short reads, v 1.2.11) with a minimum overlap of 10 bp and mismatch error rates of 2%[16]. Noisy sequences of raw tags were filtered by QIIME (v1.9.1) pipeline under specific filtering conditions to obtain high-quality clean tags[17]. The tags were then clustered as Operational Taxonomic Unit (OTU) by scripts of USEARCH (v 7.0.1090) software with a 97% similarity threshold[18]. The representative OTU sequences were taxonomically classified using Ribosomal Database Project classifier v.2.2 trained on the Greengenes database[19,20]. Finally, an OTU table and a phylogenetic tree were generated for diversity analysis. To estimate the diversity of the microbial community of the sample, we calculated the within-sample (alpha) diversity by Wilcoxon rank test for two groups and multiple group comparisons were made using Kruskal-Wallis test. Beta diversity was estimated by computing weighted Unifrac distance and was visualized with principal coordinate analysis (PCoA). Statistical differences ($P < 0.05$) between the two groups in the relative abundance of bacterial phyla and genera were evaluated using Metastats (Kruskal-Wallis test for more than two groups).

According to the genera average abundance in patients before and after treatment, genera were divided into before-enriched and after-enriched. The correlation network of genera differentially enriched in before and after group was constructed by Pearson correlation test based on the abundance. The correlation network was visualized using Cytoscape (version 3.3.0). Pearson correlation test was also performed for investigating microbial dysbiosis index (MDI) and CDAI, as well as differential genera and predicted pathways.

Functional annotation

The metagenomes of the gut microbiome were imputed from 16S rRNA sequences with PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). This method predicts the gene family abundance from the phylogenetic information with an estimated accuracy of 0.8. The closed OTU table was used as the input for metagenome imputation and was first rarefied to an even sequencing depth prior to the PICRUSt analysis. Next, the resulting OTU table was normalized by 16S rRNA gene copy number. The gene content was predicted for each individual. Then, the predicted functional composition profiles were collapsed into level 3 of the KEGG database pathways. The output file was further analyzed using Statistical Analysis of Metagenomic Profiles (STAMP) software package[21].

RESULTS

Bacterial microbiota diversity

To determine the effect of disease activity on microbial composition, we collected four parts of mucosal samples including ileum, ascending colon, descending colon and rectum from nine patients in active and remission stages. Patients characteristics are described in Supplement Table 1. The CDAI was significantly decreased in the After group compared to the Before group, indicating that the remission had been induced after clinical treatments (Figure S1). The Before group included samples from three anatomical sites (ileum, ascending colon, descending colon) and the After group supplemented with rectum mucosa, except for three samples that were unqualified for sequencing. Given the ethical issue, we did not collect mucosa from the four sites in healthy subjects, but the samples in each site were relatively uniform (3 from ileum, 4 from ascending colon, 3 from descending colon and 4 from rectum). After filtering and bioinformatic processing, a median yield of 275041 high-quality reads were obtained per sample.

The gut microbiota richness, measured by observed species and Shannon index, was not significantly different among ileum, ascending colon, descending colon and rectum in both CD and HC (Figure S2, numbers of observed species, $P = 0.12$ for CD and $P = 0.49$ for HC; Shannon index, $P = 0.78$ for CD and $P = 0.91$ for HC, Kruskal-Wallis test). The analysis of beta diversity based on the unweighted UniFrac distances showed that there was no significant difference among the four regions of intestinal tract in both CD and HC (Adonis analysis, $P = 0.85$ for CD, $P = 0.94$ for HC). Interestingly, analysis of alpha diversity as calculated by number of observed species and Chao1 index revealed that the microbial biodiversity of the patients in remission was significantly increased towards the HC compared to their active stage before treatment (Figure 1A, numbers of observed species, $P < 0.0001$; Chao1 index, $P < 0.0001$; Kruskal-Wallis test). Beta diversity represented by PCoA analysis clearly showed that the samples from patients after treatment, which clustered separately

from those before treatment, tended to approach that of HC (Figure 1B, Adonis analysis, $P = 0.001$).

Bacterial microbiota composition and correlations

To investigate the specific changes of microbiota in patients with active and remission CD, we assessed the relative abundance of taxa before and after treatment. At the phylum level, the *Fusobacteria* was lower in the After group than the Before group (Figure 2A). In addition, the *Firmicutes* was significantly decreased in CD whereas the *Proteobacteria* was overrepresented in CD relative to HC. At the genus level, we observed 65 bacterial taxa that displayed different abundance between Before and After group. Compared with the After group, 14 bacterial taxa were enriched in the Before group, while 51 bacterial taxa were depleted in the Before group. The Before-enriched bacterial taxa included *Fusobacterium*, *Streptococcus*, *Bacillaceae*, etc. Bacterial taxa that were depleted in the Before group included *Anaerostipes*, *Roseburia*, *Ruminococcus*, *Lactobacillus*, *Akkermansia*, *Lachnospira*, etc, which were significantly more abundant in HC than CD (Figure 2B). Then, we used these taxa to calculate the MDI, which is the log of (total abundance in organisms increased in Before group) over (total abundance of organisms decreased in Before group) for the samples. The MDI showed a positive correlation with clinical disease severity (CDAI), and a negative correlation with species richness (Figure 2C), suggesting the close association between gut microbiota disorder and disease activity.

We further compared the effect of different medications including thalidomide, azathioprine (AZA), AZA plus prednisolone and IFX on gut microbiota. First, the observed species and Chao1 index, which represents alpha diversity, tended to increase after treatment in four groups, although without significance in IFX and AZA (Figure S3, numbers of observed species, $P < 0.05$ for thalidomide, $P < 0.01$ for AZA plus prednisolone, $P > 0.05$ for AZA and IFX; Chao1 index, $P < 0.05$ for thalidomide, $P < 0.01$ for AZA plus prednisolone, $P > 0.05$ for AZA and IFX, t test). The analysis of beta diversity based on the unweighted UniFrac distances showed that the overall microbial composition of the After group was significantly different from the Before group regardless of treatment drugs (Adonis analysis, $P = 0.005$ for thalidomide, $P = 0.019$ for AZA, $P = 0.004$ for AZA plus prednisolone, $P = 0.018$ for IFX). Furthermore, the differential taxa between After and Before groups were identified according to each treatment. The relative abundance of *Roseburia*, *Ruminococcus* and *Anaerostipes* was significantly increased after IFX treatment while the number of *Fusobacterium* and *Streptococcus* was lower. The relative abundance of *Roseburia*, *Ruminococcaceae* and *Lachnospira* was significantly increased while *Fusobacteriaceae* was decreased after patients were treated with AZA. In the AZA plus prednisolone group, the relative abundance of *Lactobacillus*, *Ruminococcus* and *Lachnospiraceae* was increased while that of *Fusobacterium* and *Bacillaceae* was decreased after treatment. In the thalidomide group, the relative abundance of *Lactobacillus* and *Roseburia* was also increased after treatment (Supplement Table 2). Collectively, these alterations in microbiota composition between Before and After group were similar among the four medications.

Spearman correlation test was performed to evaluate the relationships among the genera identified in MDI. Significant positive correlations were found in the genera depleted in the Before group including *Lachnospira*, *Roseburia*, *Anaerostipes*, *Ruminococcus*, which were abundant in HC, suggesting their synergy as commensal bacteria in maintaining gut microbiota homeostasis and promoting the mucosal healing process (Figure 3). On the other hand, the genera enriched in the Before group such as *Fusobacterium* and *Bacillaceae* showed negative correlations with those depleted in the Before group, indicating an antagonistic relationship between harmful bacteria in the active stage and beneficial bacteria in the remission stage.

Microbial functions altered during CD treatment

To infer the metagenome functional content based on the microbial community profiles obtained from the 16S rRNA gene sequences, we used PICRUSt[22]. The pathways including Lipopolysaccharide (LPS) biosynthesis proteins and LPS biosynthesis, which were enriched in patients before treatment compared to HC, tended to decrease after the induction of remission (Figures 4A and S4). On the other hand, the pathways including Folate biosynthesis, Starch and sucrose metabolism as well as Glycolysis/Gluconeogenesis which were deficient in active CD patients tended to increase after treatment and approached that of HC. Intriguingly, the abundance of LPS biosynthesis proteins and LPS biosynthesis were negatively correlated with three genera including *Roseburia*, *Ruminococcus* and *Lachnospira*, which were more abundant in HC and patients in remission, suggesting their potential role in anti-inflammation in CD (Figure 4B-D).

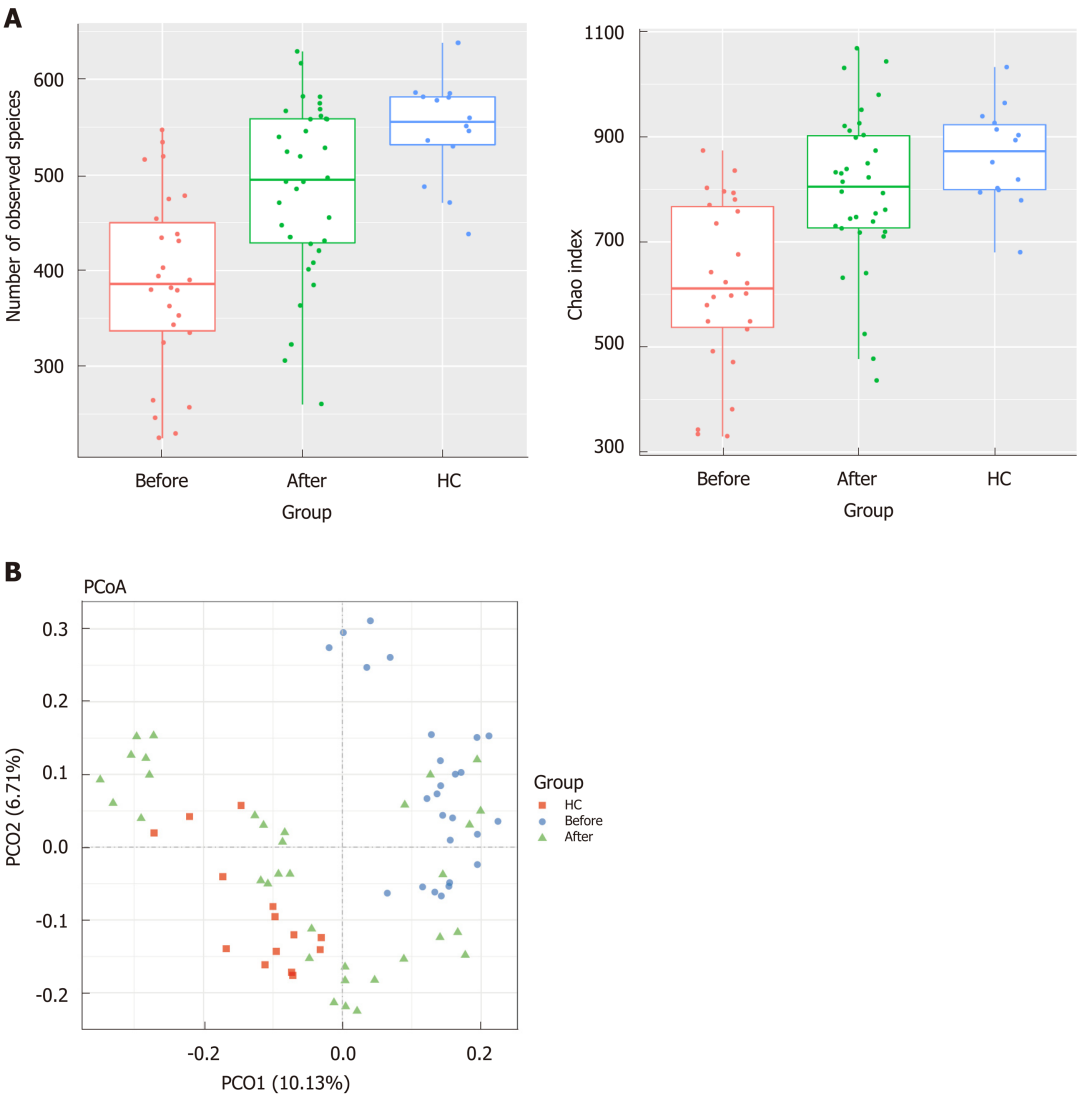

Figure 1 Richness and diversity in the mucosa-associated microbiota of the patients with Crohn's disease and healthy controls before and after induction of remission. A: The number of observed species was 388.26 ± 94.22 in the Before group, 475.03 ± 96.01 in the After group and 547.85 ± 52.85 in the healthy controls (HC) group. The Chao1 index was 617.78 ± 161.04 in the Before group, 771.40 ± 146.62 in the After group and 864.08 ± 91.59 in the HC group; B: Plots shown were generated using the weighted version of the UniFrac-based Principal coordinate analysis. Samples from After group (green triangle) clustered separately from Before group (blue circle) while got close to the HC group (red square). HC: Healthy controls.

DISCUSSION

In the current study, we analyzed, using a 16S rRNA sequencing approach, the alterations of the gut mucosal microbiota in CD patients during their treatment. The comparison from the same cohort showed that the composition of mucosa-associated microbiota changed significantly after the induction of remission, with increased diversity as well as a restoration of potential beneficial bacteria. The MDI that was identified using differential microbiota between patients before and after treatment showed the correlation of microbial dysbiosis with disease activity.

Accumulating evidence has demonstrated the disorder of gut microbiota in CD, and this disorder is considered as an essential factor in driving inflammation[7,23]. The gut microbial community of CD patients is characterized by reduced diversity as well as compositional changes in phylum level, including a decreased abundance of Firmicutes and an increased abundance of Proteobacteria when compared to HC[24]. However, it remains unclear whether these alterations of gut microbiota are associated with disease activity. Consistent with previous studies, we found the dysbiosis of mucosa-associated microbiota in patients with CD and then analyzed its composition before and after the induction of remission. Due to the distinct microbiome signatures in different sub-phenotypes of CD, we enrolled the patients with the same behavioral phenotype to exclude bias[25]. Both the alpha and beta diversity showed that the structure of gut microbiota in patients before treatment was

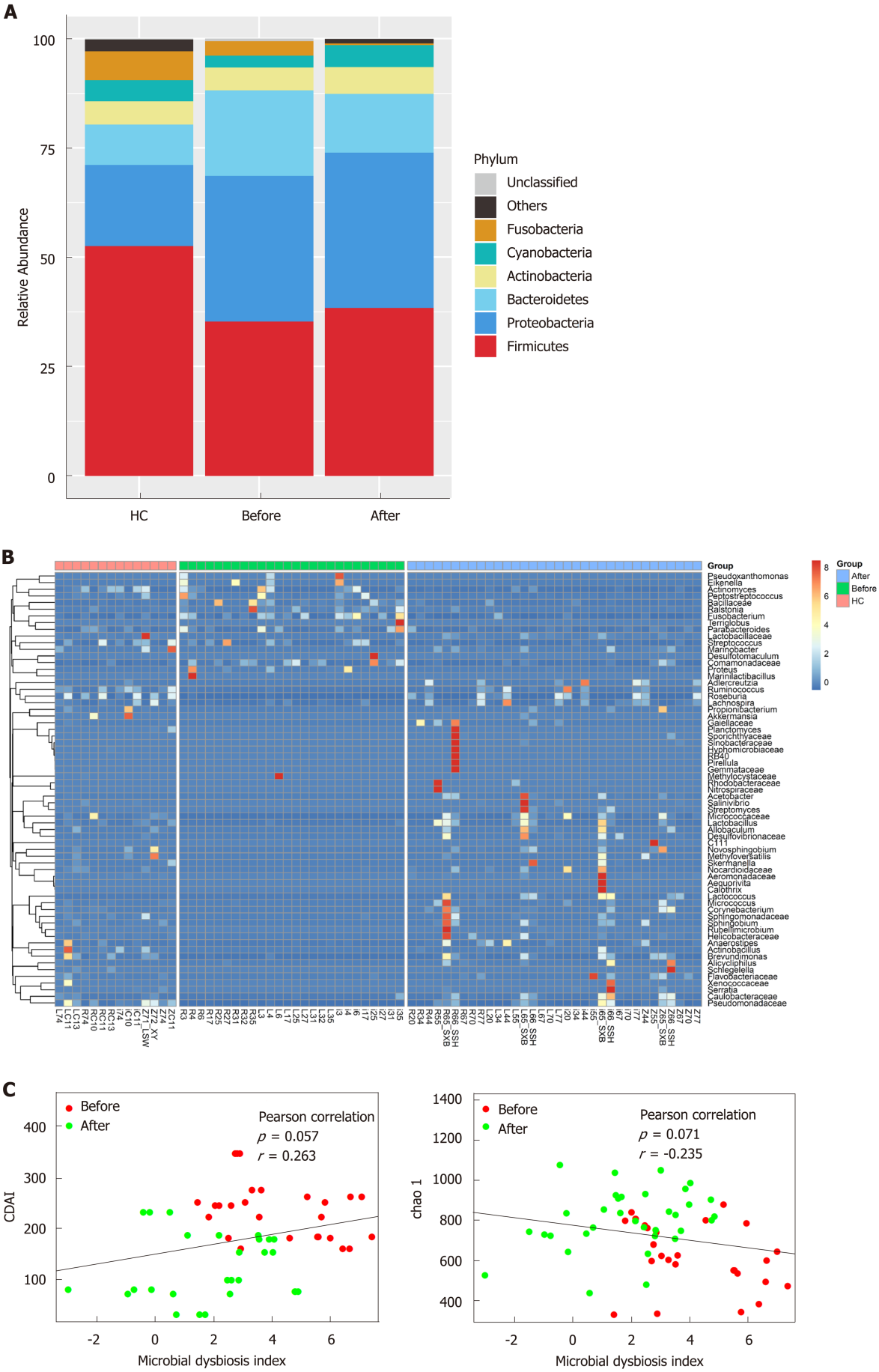

Figure 2 The microbial dysbiosis index characterizes the activity of Crohn's disease. A: The composition of mucosa-associated microbiota at phylum level; B:

The heatmap of 65 differentially abundant genera between patients before and after treatment. Each column represented a mucosal sample from patients before and after treatment as well as healthy controls. The first letter means the site of the colon (L: Ascending colon; R: Descending colon; i: Terminal ileum; Z: Rectum); C: The identified 65 genera were used to calculate the microbial dysbiosis index (MDI). The Pearson correlation analysis was constructed between MDI and the Crohn's disease activity index as well as MDI and Chao index. MDI: Microbial dysbiosis index; HC: Healthy controls; CDAI: Crohn's disease activity index.

significantly different from those after treatment, approaching that of HC and indicating the partial restoration of microbial balance after treatment. A previous study by Wang *et al*[9] reported the dynamic changes of fecal microbiota during IFX therapy in pediatric CD, and IFX has been demonstrated to diminish CD-associated gut microbial dysbiosis. Another earlier study using a polymerase chain reaction-denaturing gradient gel electrophoresis also found that treatment with Adalimumab induced short-term changes in the microbiota composition, and these changes seem to parallel the partial recovery of the gut bacterial ecology[26]. AZA is the most commonly employed 6-mercaptopurines drug, and these drugs have been found to exert anti-inflammatory effects by targeting not only human macrophages but also the gut microbiota linked to CD[27]. Consistent with these previous findings, the present study showed that the mucosa-associated gut microbiota changed after treatment, regardless of the drug used, and thus we speculate that the alterations of gut microbiota may be associated with the change in disease status from active to remission. The causal relationship between disease activity and microbiota, however, needs further investigation using a germ-free animal model.

To further clarify the critical taxa that may be associated with the activity of CD, we identified 65 genera showing significant difference before and after treatment, and these genera were used to calculate the MDI. Interestingly, the positive correlation between MDI and CDAI indicates that the activity of CD may be associated with the dysbiosis of gut microbiota. A previous study in treatment-naïve children with CD also described the MDI using the differential taxa between CD and healthy subjects, and then demonstrated that the MDI characterized CD severity[28]. Some MDI-associated taxa were common to both studies including *Fusobacterium*, *Lachnospira*, *Ruminococcus* and *Parabacteroides*. The relative abundance of *Lachnospira*, *Ruminococcus* and *Roseburia*, which are known to produce short chain fatty acids (SCFAs) was significantly increased in patients with clinical remission compared to those with active CD. SCFAs, which are mainly composed of acetate, propionate and butyrate, are the end products of the fermentation of dietary fiber by the gut microbiota and have been shown to exert multiple beneficial effects on mammalian energy metabolism[29]. Recently, numerous studies have shown the loss of SCFA-producing taxa in CD and that IFX treatment was able to restore their levels in responsive patients, indicating their association with disease severity[9,24,30]. The beneficial effect of SCFAs on CD is probably due to their anti-inflammation capacity which is documented both *in vitro* and *in vivo*[31]. Chronic inflammation is a hallmark of CD and results from the recruitment and activation of immune cells from the circulation. Butyrate elicits anti-inflammatory effects *via* the inhibition of IL-12 and the upregulation of IL-10 production in human monocytes, repressing production of pro-inflammatory molecules TNF-α, IL-1β, nitric oxide, and reducing NF-κB activation[32,33]. A clinical study explored the therapeutic effect of butyrate on patients with CD and found that the administration of butyrate induced clinical improvement and remission in 53% of patients, in whom butyrate successfully downregulated mucosal levels of NF-κB and IL-1β[34]. Thus, it is conceivable to modulate the dysbiotic gut microbiota using probiotics, prebiotics and fecal microbiota transplantation (FMT) for the management of CD. Several studies have reported the potential of FMT for CD treatment, and we speculate that it may helpful to select donors with a high abundance of the beneficial bacteria, which are deficient in patients to improve the therapeutic efficacy[35].

Analysis of the inferred metagenome in our study showed that the abundance of LPS biosynthesis proteins and the LPS biosynthesis pathway in patients with CD were significantly decreased after treatment while the abundance of Folate biosynthesis, glycolysis/gluconeogenesis, starch and sucrose metabolism were increased, suggesting that the induction of remission could partially rectify the dysbiosis of gut microbiota and restore the homeostasis of metabolic function. LPS, one important component in the outer membrane of gram-negative bacteria, plays a critical role in triggering inflammatory responses that could further result in various diseases such as CD[36]. The serum levels of LPS were demonstrated to increased markedly in active CD patients compared with patients in remission and HC and were positively correlated with the severity of the disease[37]. Moreover, the blockade of intestinal mucosal inflammation with IFX could reduce the levels of LPS and IFX has been reported to diminish the CD-associated gut microbial dysbiosis[9,37]. Analysis of

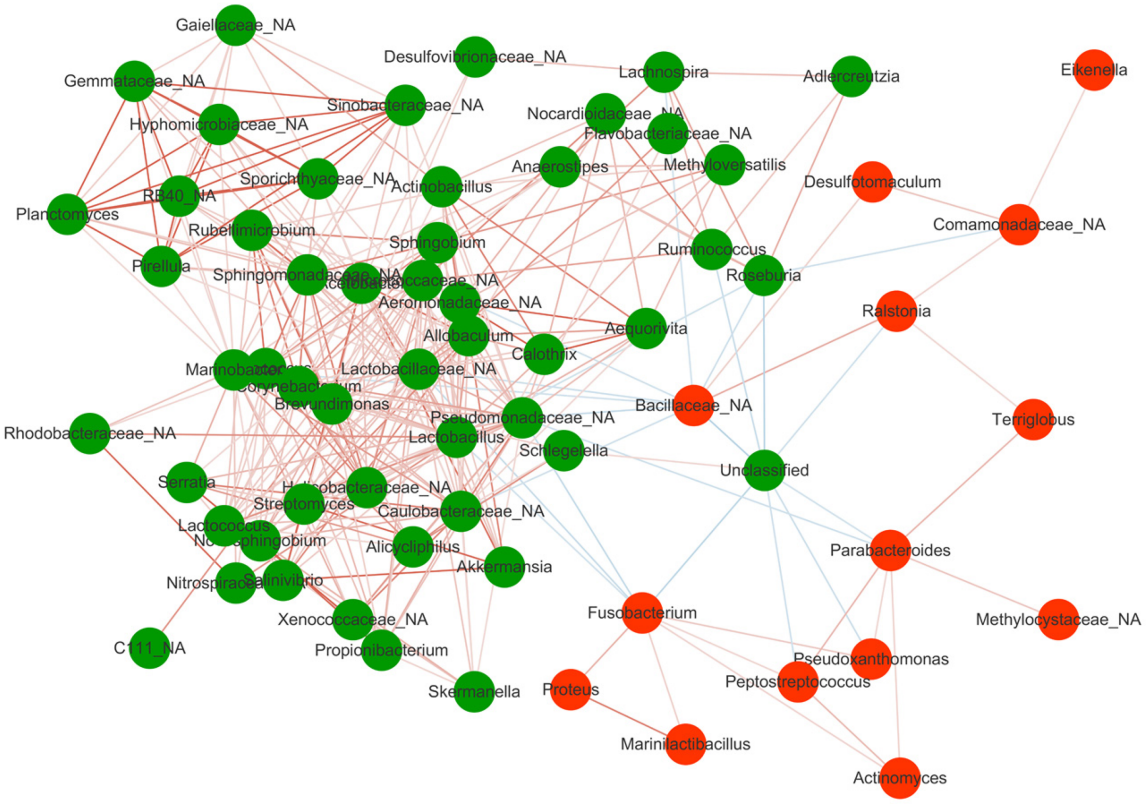

Figure 3 Spearman correlations among the 65 Crohn's disease-associated genera in gut mucosal samples. The green circle represented taxa enriched in patients after treatment while the red circle represented taxa enriched in patients before treatment. Lines between nodes denoted Pearson correlation ($r > 0.2$ and $P < 0.05$). The red and blue lines represented the positive and negative correlation, respectively.

microbiota genes in this study showed a prominent upregulation of LPS-related pathways in patients before treatment, and this upregulation may be due to the overgrowth of LPS-producing bacteria such as *Fusobacterium* and *Eikenella*. Folate deficiency is common in patients with IBD, mostly in CD patients. Up to 80% of patients with CD present with low levels of serum folate, and this is more common in active disease than at times of remission[38,39]. Folate is produced in large quantities by the colonic microbiota, mainly probiotics such as *Lactobacillus* and *Bifidobacterium*[40]. The enrichment of *Lactobacillus* in CD patients after treatment as shown in our study may promote the biosynthesis of folate whose deficiency is associated with disease severity.

This study has some limitations. First, the number of patients was somewhat small. Therefore, the characteristics of mucosa-associated gut microbiota during CD treatment and the predictive value of MDI in disease activity warrant verification in large populations. Second, the patients in this study received different drugs for inducing remission, which may cause some bias. Our results showed that the composition of gut microbiota in patients before treatment was significantly different from those after treatment regardless of the drug used. Due to the small sample size in each treatment group, the effect of drugs on gut microbiota needs further investigation.

In conclusion, the results in this study presented that both the structure and function of mucosa-associated bacterial microbiota in patients with CD changed significantly during treatment, approaching healthy status. The dysbiosis of gut microbiota, which is associated with disease activity, is partially restored after the induction of remission with characteristics including increased biodiversity and an increase in SCFA-producing bacteria. Therefore, the disturbance of gut microbiota, especially the overgrowth of pathogenic bacteria and the depletion of beneficial bacteria may act as a potential therapeutic target for CD treatment.

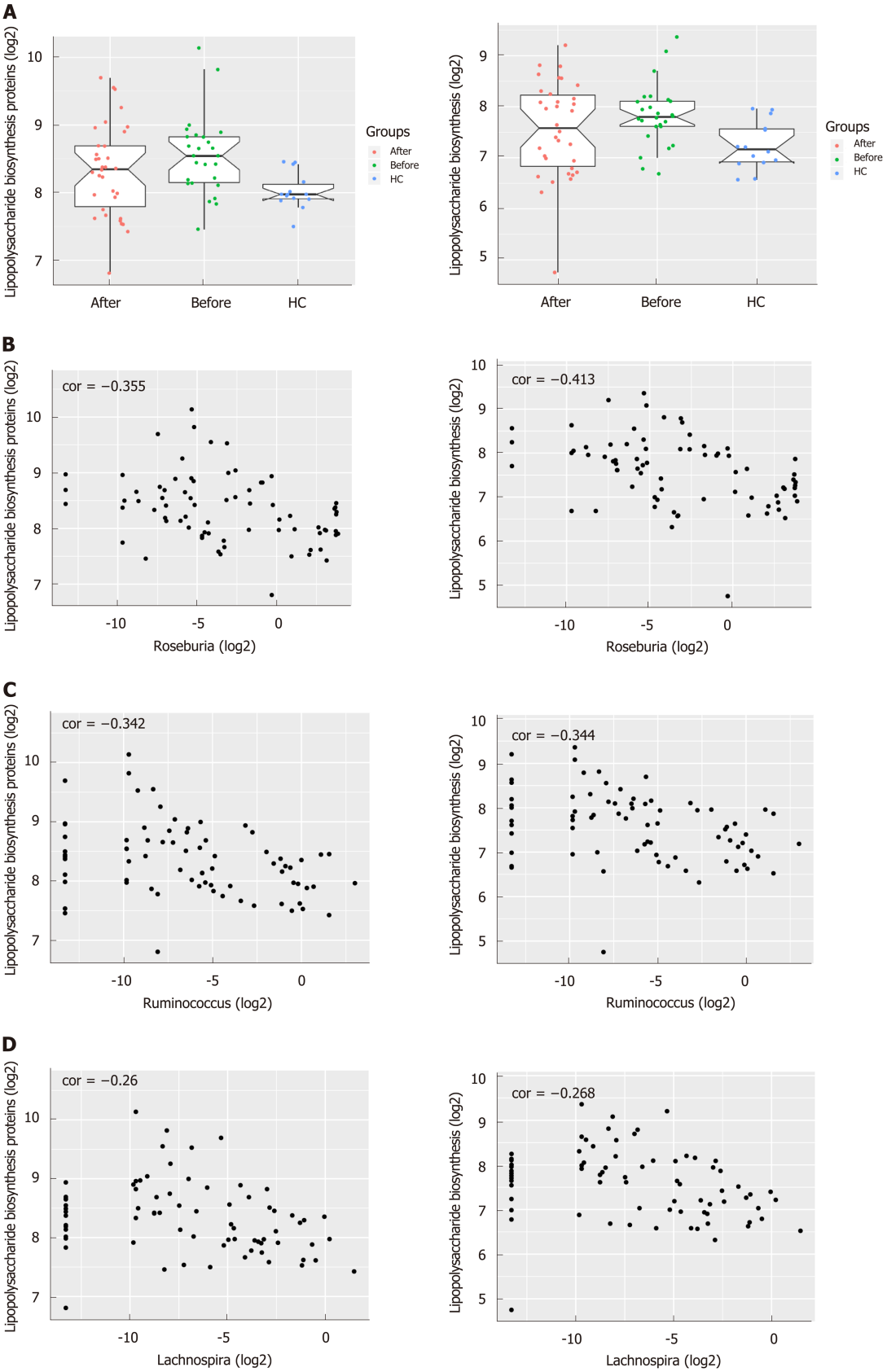

Figure 4 The predicted functional module involving pro-inflammatory pathways altered in Crohn's disease compared to healthy controls. A: Pathways including Lipopolysaccharide biosynthesis proteins and Lipopolysaccharide biosynthesis predicted to show significant different abundances among before, after and healthy controls group according to Kyoto Encyclopedia of Genes and Genome pathway analysis. The Crohn's disease-depleted genera including *Roseburia*, *Ruminococcus* and *Lachnospira* were negatively correlated with Lipopolysaccharide biosynthesis proteins (P = 0.001 for *Roseburia*, P = 0.002 for *Ruminococcus* and P = 0.025 for *Lachnospira*) and Lipopolysaccharide biosynthesis (P = 0.0002 for *Roseburia*, P = 0.002 for *Ruminococcus* and P = 0.021 for *Lachnospira*). B: *Roseburia*; C: *Ruminococcus;* D: *Lachnospira*.

ARTICLE HIGHLIGHTS

Research background

Accumulating evidence demonstrated the alterations of gut microbiota in patients with Crohn's disease (CD) compared to healthy subjects. However, comparative analysis of mucosal microbiota in the same cohort of patients before and after treatment remains limited. The different characteristics of mucosa-associated gut microbiota between active and quiescent CD may provide as a predictive tool for disease relapse as well as a potential therapeutic target for treatment.

Research motivation

Most studies investigating the gut microbiota of CD have used fecal samples while only few studies have investigated the mucosal microbiota, which is believed to directly affect epithelial function and may be more deeply involved in the pathogenesis of CD. Although the dysbiosis of gut microbiota have been reported in patients with CD as compared with healthy controls, the microbial changes during treatment and their association with disease activity are largely unknown.

Research objectives

To illustrate the global alterations of mucosa-associated microbiota in patients with active CD before and after treatment.

Research methods

A total of 74 mucosal biopsies were collected from 15 participants including 9 patients with CD and 6 healthy individuals. Sampling included both active and remission stages for each patient who underwent clinical treatment. The gut microbiota was sequenced by 16S rRNA analysis.

Research results

Our results showed that the structure of gut microbiota in patients with active CD changed significantly after the induction of remission, including the decreased abundance of pathogenic bacteria and increased abundance of beneficial bacteria.

Research conclusions

The dysbiosis of mucosa-associated gut microbiota in active CD was partially restored after treatment, indicating the association of microbiota and disease activity.

Research perspectives

The variations of gut microbiota may act as a tool to supervise and predict the recurrence of CD, and the maintenance of microbial homeostasis could become a potential therapeutic target for the disease.

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ORIGINAL ARTICLE

Retrospective Study

Role of abdominal ultrasound for the surveillance follow-up of pancreatic cystic neoplasms: a cost-effective safe alternative to the routine use of magnetic resonance imaging

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Abstract

BACKGROUND

Patients with pancreatic cystic neoplasms (PCN), without surgical indication at the time of diagnosis according to current guidelines, require lifetime image-based surveillance follow-up. In these patients, the current European evidenced-based guidelines advise magnetic resonance imaging (MRI) scanning every 6 mo in the first year, then annually for the next five years, without reference to any role for trans-abdominal ultrasound (US). In this study, we report on our clinical experience of a follow-up strategy of image-based surveillance with US, and restricted use of MRI every two years and for urgent evaluation whenever suspicious changes are detected by US.

AIM

To report the results and cost-efficacy of a US-based surveillance follow-up for

(CEAVNO)".

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known PCNs, with restricted use of MRI.

METHODS
We retrospectively evaluated the records of all the patients treated in our institution with non-surgical PCN who received follow-up abdominal US and restricted MRI from the time of diagnosis, between January 2012 and January 2017. After US diagnosis and MRI confirmation, all patients underwent US surveillance every 6 mo for the first year, and then annually. A MRI scan was routinely performed every 2 years, or at any stage for all suspicious US findings. In this communication, we reported the clinical results of this alternative follow-up, and the results of a comparative cost-analysis between our surveillance protocol (abdominal US and restricted MRI) and the same patient cohort that has been followed-up in strict accordance with the European guidelines recommended for an exclusive MRI-based surveillance protocol.

RESULTS
In the 5-year period, 200 patients entered the prescribed US-restricted MRI surveillance follow-up. Mean follow-up period was 25.1 ± 18.2 mo. Surgery was required in two patients (1%) because of the appearance of suspicious features at imaging (with complete concordance between the US scan and the on-demand MRI). During the follow-up, US revealed changes in PCN appearance in 28 patients (14%). These comprised main pancreatic duct dilatation (n = 1), increased size of the main cyst (n = 14) and increased number of PNC (n = 13). In all of these patients, MRI confirmed US findings, without adding more information. The bi-annual MRI identified evolution of the lesions not identified by US in only 11 patients with intraductal papillary mucinous neoplasms (5.5%), largely consisting of an increased number of very small PCN (P = 0.14). The overall mean cost of surveillance, based on a theoretical use of the European evidenced-based exclusive MRI surveillance in the same group of patients, would have been 1158.9 ± 798.6 € per patient, in contrast with a significantly lower cost of 366.4 ± 348.7 € (P < 0.0001) incurred by the US-restricted MRI surveillance used at our institution.

CONCLUSION
In patients with non-surgical PCN at the time of diagnosis, US surveillance could be a safe complementary approach to MRI, delaying and reducing the numbers of second level examinations and therefore reducing the costs.

Key words: Ultrasound; Pancreatic cystic neoplasms; Magnetic resonance imaging; Surveillance

Core tip: Considering the high incidence of pancreatic cystic neoplasms (PCN) in the general population and the low risk of malignant progression in these patients, health care providers need to consider cost-effective follow-up programs. Current guidelines advise only magnetic resonance imaging (MRI) surveillance for the routine follow-up of these patients. This image-based surveillance carries issues concerning accessibility and high costs. The present retrospective analysis enrolling 200 patients has demonstrated that a modified surveillance based on ultrasound and restricted use of MRI is both safe and significantly more cost-effective. However, this retrospective study requires confirmation by a prospective randomized controlled clinical trial comparing the two follow-up regimens in patients with non-surgical PCN.

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INTRODUCTION

The widespread use of high resolution imaging has resulted in a marked increase in the incidental detection of pancreatic cystic lesions, such that these lesions are encountered in 3% of abdominal computed tomography (CT) examinations and in up to 13%-19.6% of magnetic resonance imaging (MRI) scans[1-3]. The MRI prevalence data are in agreement with the previously reported autopsy studies demonstrating pancreatic cystic lesions in up to a quarter of cases[4]. Cystic pancreatic lesions are a heterogeneous group. The classification of pancreatic cyst neoplasms (PCNs) is based either on their neoplastic potential or their epithelial or mesenchymal origin.

The two most common PCN lesions are the intraductal papillary mucinous neoplasms (IPMN) and the mucinous cystic neoplasms (MCN). Both are benign, but with an established risk of malignant progression; whereas others are always benign, without any risk for malignant transformation, *e.g.*, serous cystic neoplasm (SCN). More rarely, PCN are overtly malignant at the time of diagnosis (cystadenocarcinomas). PCN not only have diverse histological and imaging appearances, but also differ in their clinical presentation, biological behavior, growth pattern, and risk of malignancy[5]. Accurate risk stratification and decisions regarding treatment and follow-up strategies necessitate precise lesion characterization and diagnosis. Several recommendations have been published on their pathology and management. The most relevant are the International Consensus Guidelines (2006[6], 2012[7] and 2017[8]), the guidelines of the American Gastroenterological Association (2015[9]), and the European study group on cystic tumors of the pancreas guidelines (2012[10] and 2018[11]). All these guidelines consider repeated MRI scans with similar frequency as the preferred surveillance tool in the follow-up strategy of non-surgical PCN. The European evidence-based guidelines on pancreatic cystic neoplasms consider the presence of jaundice, mural nodes (> 5 mm) and main duct dilatation ≥ 10 mm as "absolute indications" for surgery. The presence of main duct dilatation 5-9.9 mm, cyst growth rate > 5 mm/year, mural nodes < 5 mm, cystic diameter ≥ 40 mm, increased serum markers and new onset of diabetes or acute pancreatitis are relative indications for surgical intervention. In the presence of one or more of these features, surgical indication has to be balanced by the patients' general condition, including co-morbid disorders.

Surgical intervention in the remaining "low risk" PCN (Wirsung diameter < 5 mm, cyst size < 40 mm, growth rate < 5 mm/years and no mural nodes) is not indicated at the time of diagnosis. Instead, accurate surveillance follow-up is recommended in the first instance. This consists of a MRI every 6 mo during the first year and then annually for the next 5 years, in order to detect the appearance of progressive changes, *e.g.*, an increase in the size of the major lesion, main pancreatic duct dilatation or mural nodules. Although MRI is considered the gold standard imaging technique[12-14] to follow-up on these lesions, it has some issues, including limited access to this imaging modality and high costs[15,16]. In addition, MRI examinations are lengthy and can be uncomfortable for patients, particularly those who suffer from claustrophobia. Additionally, there are patients in whom MRI is contraindicated. Other imaging modalities used include endoscopic ultrasound (EUS) with or without fine needle aspiration (FNA), transabdominal ultrasound (US), contrast enhanced US (CEUS) and contrast enhanced-EUS (CH-EUS). EUS is recommended in the current guidelines as an adjunct to the other imaging modalities in the assessment of patients harboring PNC with features identified during the initial investigation or follow-up, which may indicate the need for surgical resection. Despite its accuracy, EUS-FNA is invasive and thus should be performed only when the results are expected to change clinical management. Although US and CEUS are included in the Italian consensus guidelines for the diagnostic work-up and follow-up of cystic pancreatic neoplasms[17], this recommendation is not included in the European Evidence Based Guidelines; although CH-EUS is considered for evaluation of mural nodules[11].

A pragmatic approach is needed, especially in public healthcare hospitals, in the clinical management of patients harboring relatively common benign lesions but a varying risk of malignant transformation. Although these PCN do not require surgery at the time of diagnosis, there is an evidence-based absolute need for expensive image-based long-term surveillance follow-up. Hence, cost considerations, with the emphasis on cost-efficacy and utility of long-term surveillance, is particularly essential in public healthcare systems. However, in the quest for cost containment, an alternative cheaper follow-up system for non-surgical PCN is only acceptable if it is safe and proven to be fit for purpose, *i.e.* if it does not miss malignant evolution of PCN. Recently, a few publications[18-20] have evaluated the role of US in monitoring PCN. Nevertheless, to date there has not been any reports of a safe alternative follow-up strategy based on US with limited MRI use outlined by the present study.

MATERIALS AND METHODS

Patients selection and data acquisition

Records from all patients with PCN diagnosis without indications for surgery (absolute or relative), who were enrolled in our modified surveillance protocol between January 2012 and January 2017, were reviewed retrospectively. The patient cohort for this retrospective study was obtained from our institutional, prospectively-collected database and selected as patients with confirmed diagnosis of PCN without absolute or relative indications for surgery according to the current European evidence-based guidelines[11]. The diagnostic US criteria for suspect PNC were the identification of one or more partial or completely anechoic areas within the pancreatic parenchyma and/or dilation of Wirsung duct > 2 mm, in the absence of identifiable causes of obstruction. In the protocol, the US diagnosis was always confirmed with an MRI scan. Exclusion criteria were suspected or proven malignancy at the time of diagnosis (presence of solid vascularized tissue in the cyst, presence of nodal or distant metastasis at imaging, or positive histopathological findings)[18], PCN with absolute or relative surgical criteria[11], clear diagnosis of SCN, absence of diagnostic MRI scan, and follow-up period less than 10 mo. The last date of entrance into the US follow-up for the group of patients included in the study was January 1, 2017, with an end-point date of January 1, 2018 for the surveillance follow-up period.

After US diagnosis and MRI confirmation, all scheduled patients were followed-up with a non-conventional surveillance protocol that was used in our Unit since 2012. It consisted of a US scan every 6 mo for the first year and then, in patients with stable disease, annually from the second to the fifth year. A planned MRI was performed routinely every two years for stable disease, or at any time when suspicious changes were observed on US. Abdominal US was always performed just before the planned routine MRI in the second year of follow-up (Figure 1). The reasons for reducing the imaging intervals and advancing the MRI were dilatation of the main duct > 50%, increased size of the cyst ≥ 2 mm from previous examinations, or development of new lesions (Figure 2). The development of new PCN is diagnosed by conventional US as a new anechoic area into the pancreatic parenchyma. Stable disease was defined as PNC without detectable changes between two subsequent follow-up images.

Retrieved data included baseline patient characteristics, Wirsung caliber (the widest portion regardless of location), PCN size (largest diameter), connection of the cyst to the ductal system, and numbers and locations at the time of diagnosis. The duration of the follow-up period, Wirsung caliber, PCN modifications discovered during surveillance with subsequent performance of US as a follow-up examination, and MRI were also evaluated. Finally, the number and cost of US and MRI performed for each patient were obtained.

Costs of a single examination were expressed in Euros and obtained from our regional rates as follows: 60 € for US and 480 € for MRI, with an extra 254 € for a contrast-enhanced study. The total number of US and MRI scans (both routine and urgent) for each patient included in the study, together with the total number of examinations, were obtained. In the same way, the overall number of MRI alone, which would have been performed if the same patient group had undergone the standard guidance-approved MRI only surveillance[11], were calculated. According to the guidelines, a short MRI protocol without the administration of contrast provides equivalent information to a longer contrast enhanced MRI protocol for the surveillance of PCN. As a consequence, the MRI exam in the 'virtual' control group was estimated as 480 € and scheduled, as suggested, every 6 mo. This enabled the calculation of the theoretical overall cost of the control group.

These data were used in the cost-analysis comparison between the US-MRI restricted surveillance-based follow-up strategy and surveillance had the same group of patients been subjected to the evidenced-based existing guidelines surveillance with MRI alone. Sensitivity, negative predictive value, and the accuracy of US with respect to MRI in the follow-up were evaluated. Specifically, the sensitivity, negative predictive value and accuracy refer to the ability of US to detect changes in PNC, with respect to the gold standard MRI at two years. The diagnostic criteria evaluated for this analysis are the same for both US and MRI, which include a detected increase in the number of PCN (detection of new anechoic areas not identified in previous examination; increased of size PCN > 2 mm; increase of Wirsung caliber > 50%). In this series, no patients developed mural nodules or PCN wall thickness. Hence, these were not included in the analysis.

Imaging protocol

B-mode US was performed in all patients at our department by surgeons with expertise in US examinations and with over 25 years of experience in pancreatic surgery and imaging. All patients signed an informed consent to authorize the

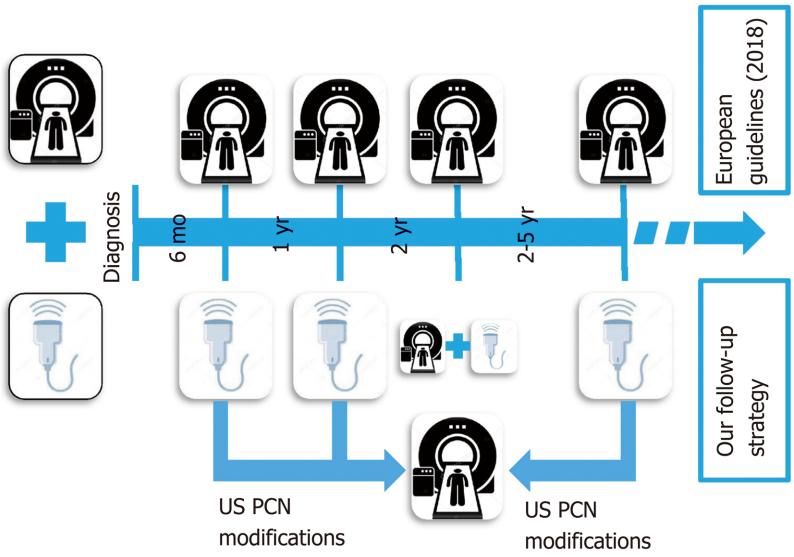

Figure 1 The two follow-up strategies comprising the standard magnetic resonance imaging *vs* non-conventional abdominal US surveillance. US: Ultrasound; PCN: Pancreatic cyst neoplasms.

scientific use of the collected data. A GE Logiq 9 (GE Healthcare, Milwaukee, WI, United States) with a probe frequency of 3.5-5.0 MHz was utilized. Patients were scanned supine or in other positions with grey scale imaging and Color-Doppler. Harmonic imaging was routinely used to reduce artefact and increase the signal-to-noise ratio. The position, size, boundaries, and contents of all PCN, as well as the diameter of the pancreatic duct, if visible, were recorded. During the examinations, previous US or MRI images, including those performed at the time of diagnosis, were available using Resolution and then Suit Estensa PACS (Esaote Spa, Genova, Italy). They were used as correlative images to identify known PNC and evaluate their modifications. CEUS was only used occasionally in a few selected patients with relative surgical indications, which had shown at the time of the diagnosis septa or cystic wall thickness by B-mode US. Hence, it was not considered in the present analysis.

Patients underwent MRI on a superconductive 1.5T system (Signa HDx; GE Healthcare) using a twelve-channel phased-array body coil for both excitation and signal reception. Immediately before starting the examination, scopolamine methyl-bromide (20 mg; Buscopan®, Boehringer Ingelheim, Italy) was administered intramuscularly to avoid peristaltic artefacts. The standard imaging protocol included, first, T1-weighted breath-hold SPGR in-phase and out-of-phase axial sequences (with and/or without fat suppression), and T2-weighted axial sequences (both breath-hold, single-shot fast spin-echo and respiratory-triggered, fat-suppressed fast spin-echo) of the upper abdomen. Next, MRCP was performed by respiratory-triggered, three-dimensional, heavy T2-weighted fast spin-echo (3D FRFSE) sequence and breath-hold, thick-slab, single-shot FSE T2-weighted sequences performed in the coronal and oblique-coronal projections. Diffusion-weighted MR imaging of the pancreatic region was performed using an axial respiratory-triggered spin-echo echo-planar sequence with multiple *b* values (300, 500, 700, 1000 s/mm^2) in all diffusion directions. If there were some doubts on the pre-contrast study, a three-dimensional fat-suppressed Liver Acquisition with Volumetric Acceleration (LAVA) sequence was obtained in the axial and sometimes coronal plane before and after intravenous injection of Gadolium-based contrast agents. Post-contrast graphic images were obtained in the arterial, portal-venous and delayed (between 3 and 5 min) phases. Acquisition time for the whole examination ranges from 30 to 35 min. The size of the voxels and therefore the spatial resolution depends on matrix size, the field-of-view (FOV), and slice thickness. Moreover, higher magnetic field allows improved resolution. In our study, the MR cholangiography matrix was 256 × 160, the slice thickness/spacing was 2.4/-1.2 mm, and the FOV was about 40. By applying these parameters, the 1.5 T MR device allowed evaluation of variation in the dimension of 2 mm. The spatial resolution of the 1.5 T MR commercial device has been reported in the literature by Arizono *et al*[21] as 1.1 × 1.0 mm (inplane resolution) and 0.84 mm (minimum slice resolution).

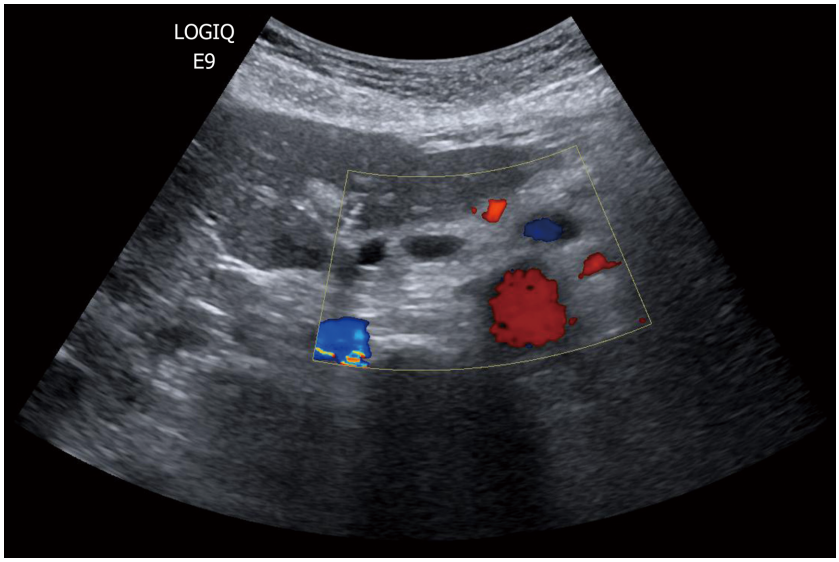

Figure 2 Development of new pancreatic cyst neoplasms as new anechoic areas into the pancreatic parenchyma detected by conventional ultrasound.

Statistical analysis

SPSS version 21.0 (IBM Corp., Armonk, NY, United States) and STATA version 13 (STATA Corp., College Station, TX, United States) software were employed for statistical evaluation. Continuous variables are reported as mean ± standard deviation (SD) and compared using Student's *t*-test. Variables with a non-normal distribution are expressed as median and compared using the Wilcoxon Test. To determine the competency of US policy in the surveillance period relative to the gold standard MRI, a receiver operating characteristic curve (ROC) test was performed with a calculation of sensitivity, negative predictive value, accuracy and area under the curve (AUC). P values less than 0.05 were considered statistically significant.

RESULTS

Two hundred patients harboring 261 PCN were followed-up with the US-Restricted MRI surveillance program described above. At diagnosis, 140 patients (74.5%) had a single PNC and 51 patients had multiple PNC (25.5%), the multiple PNC being referred to as IMPNs. The median number of cysts was two (range 2-5). The study group comprised 138 (69%) females and 62 (31%) males with a mean age of 67 ± 14 years. At the time of diagnosis, the median Wirsung diameter was 2.6 (range 1.8-4.5 mm) and the mean cystic diameter was 16 ± 13 mm, with 97 (37%) measuring less than 10 mm. Most lesions were located in the pancreatic head (106 PCN, 40% of total). Connections to the ductal system and MRI diagnosis of IPMN was documented in 148 (74% of total patients). The mean follow-up period was 25.1 ± 18.2 mo. Surgery was required in two patients (1%) because of the appearance of suspicious features on the surveillance scans (with complete concordance between the US and the on-demand MRI scans). In the first patient, a 35 mm lesion located in the tail was detected by the 1-year US scan, which confirmed a rapid increase in cyst diameter reaching 42 mm. After MRI, EUS was also performed. However, due to the distal localization of the lesion and poor acoustic window, needle aspiration was not performed. This female patient was treated by laparoscopic distal pancreatectomy. Pathological examination of the excised specimen confirmed a cystic neuroendocrine tumor. For the second patient with a PCN located in the pancreatic head, follow-up imaging showed a progressive Wirsung dilatation that was accompanied by a rising serum Ca 19.9 level and, hence, had clear-cut indications for surgery. The final histologic diagnosis of the excised lesion was confirmed as a mucinous carcinoma arising from IPMN and staged T2N0M0. Data summarized in Table 1.

In 28 patients (14%), US showed non-surgical changes in PCN during surveillance, consisting of pancreatic duct dilatation in one case (from 2 to 3 mm) and an increase in diameter of the main cyst in 14 cases (median increased = 2.5 mm; range 2-5 mm). One female 88-year-old patient with PCN in the head of the pancreas that enlarged by 5 mm in one year was treated conservatively because of significant ischemic heart disease and diminished cardiac function. The lesion remained stable during a follow-

Table 1 Patient characteristics

Characteristics	
Number of patients	200
Baseline total PCN	261
Multiple PNC patients, *n* (%)	51 (25)
Sex ratio, M:F	62:138
Age in yr, mean ± SD	67 ± 14
Main duct diameter in mm, median (range)	2.6 (1.8-4.0)
Cystic size in mm, mean ± SD	16 ± 13
PNC < 10 mm, *n* (%)	97 (37)
Location, uncinate process, *n* (%)	73 (28)
Location, pancreatic head, *n* (%)	106 (40)
Location, pancreatic body, *n* (%)	54 (21)
Location, pancreatic tail, *n* (%)	28 (11)
Radiologically suspected IPMN, *n* (%)	148 (74)
Mean follow-up, mo (± SD)	25.1 (± 18.2)
Surgery during follow-up, *n* (%)	2 (1)

PNC: Pancreatic cystic neoplasm; IPMN: Intraductal papillary mucinous neoplasm.

up of 28 mo. In the remaining 13 patients, US discovered new cystic lesions during image-based surveillance. All these patients underwent MRI, which confirmed the US findings.

In 11 patients (5.5% of total), the routine 2 years MRI identified evolution of the lesions not detected at the same time US ($P = 0.14$), but mainly related to an increased number of PCN (6 cases; 54%). In all these cases, the new PCN detected by MRI were located in the uncinate and tail of the pancreas. In five cases (46%), the routine MRI demonstrated a median PCN enlargement of 3 mm (range 3-4 mm) not detected by US. However, all these patients had PCN diameters < 15 mm and an MRI every 6 mo would not have altered the clinical management of these patients. In the present study, the follow-up surveillance program did not identify the development of mural nodules or thickness of the wall either by US or MRI.

Considering the MRI as the gold standard, US used in PCN surveillance showed a sensitivity of 72%, negative predictive value of 94%, accuracy of 95% and AUC of a ROC curve of 86% (confidence interval 77%-94%, $P < 0.001$) (Figure 3).

The mean cost of surveillance for each patient according to the proposed US-restricted MRI surveillance follow-up strategy was 366.4 ± 348.7 €. Had we used the surveillance recommended by the European evidenced-based guidelines with MRI in the same group of patients, the costs incurred would have been 1158.9 ± 798.6 € ($P < 0.0001$), *i.e*. nearly trebled (Figure 4).

However, the cost of our proposed US-restricted MRI-based surveillance could be higher than the above cost of 366 ± 349 €, since it would be influenced by the number of patients with PCN requiring urgent MRI. This is because of changes documented by surveillance imaging (total = 30 in present cohort) and/or the need for a contrast-based MRI scanning contrast phase ($n = 5$ in present study). The overall costs of our proposed follow-up strategy still remain significantly lower than the exclusive MRI-based surveillance, *i.e*. 907.2 ± 382.9 € *vs* 1511.6 ± 790.4 € respectively, $P < 0.05$).

DISCUSSION

The crucial objective in the clinical management of patients harboring PCN is the early identification of those at high risk of malignant degeneration[22]. To this effect, MRI is considered the gold standard imaging modality[13] in all the published guidelines[8,11], both in the diagnostic workup and in the subsequent follow-up. MRI is useful for establishing the diagnosis and presence of any connection between PCN and the ductal system, the baseline cystic diameter and other features. Likewise, the follow-up imaging-based surveillance used has to be capable of the risk features/changes predictive of neoplastic evolution[23-25]. Despite its proven efficacy, MRI has certain issues, including contrast-related side effects, claustrophobia, limited accessibility and high costs. EUS is helpful in resolving PCN with suspicious features,

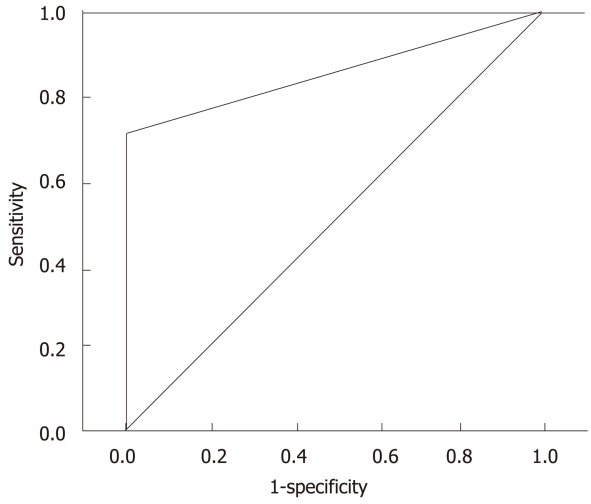

Figure 3 Receiver operating characteristic analysis showing the accuracy of ultrasound in the follow-up respect to gold standard magnetic resonance imaging performed 2 year after diagnosis *vs* those performed "on demand" during the follow-up.

but on its own exhibits modest useful diagnostic performance for these lesions. However, when combined with fine-needle aspiration (FNA), the diagnostic yield and accuracy of EUS are increased significantly. Nevertheless, because of the invasive nature of FNA, this combination should be reserved in selected PNC cases with suspicious features on the MRI that suggest a need for surgery. Otherwise, because of its invasive nature, EUS with FNA is not suitable or recommended as a surveillance follow-up modality[11].

In the management of patients with PCN who are largely asymptomatic and often young, within the context of increasing costs of secondary and tertiary healthcare in both public and private hospitals, cost considerations cannot be ignored. The clinical management of patients is essentially based on image-based surveillance follow-up, and we need an imaging protocol that is both fit for purpose and affordable. In this context, US is an imaging modality worthy of consideration as an alternative imaging modality in the follow-up of PCN. US can be used to evaluate most patients by assessing pancreatic duct caliber, diameter of the wall, and the internal aspect of pancreatic cysts[19]. US scanning for the detection and evaluation of PCN has been analyzed in a few reports, with the most important being the report by Sun *et al*[19]. This seminal publication is based on a study involving 57 patients who underwent blinded US on the same day that each had an MRI. The authors demonstrate that an abdominal US for established PNC provides visualization and accurate measurement of many PCN of the cyst size, location, and other lesion characteristics. The conclusion from this study was that US was a valid adjunct of MRI in monitoring patients harboring PCN diagnosed by MRI. In another recent report, Jeon *et al*[20] reported that the detection rate and utility of US is significantly improved by repeat imaging if the initial diagnostic US image is available for use as a reference map; thereby confirming the increased usefulness of US in PCN surveillance. Apart from the patients' stature, the cyst location, availability of initial (baseline) images that affect US successful evaluation, and different PCN changes are not detected equally by US. In line with these studies, we have shown that during follow-up, Wirsung duct caliber and cyst diameter are the factors that are well-visualized with US scan, whereas the development of new cysts and small mural nodes are detected less successfully. When detectable, the US features indicative of the development of new cysts and small mural nodes include the appearance of new anechoic areas in the pancreatic parenchyma and the appearance of a solid iso/iper-echoic component inside the anechoic cystic area[26].

Although dilatation of pancreatic duct caliber was clearly detected by US, the growth rate of PCN was missed in five cases and detected by MRI. However, all these missed lesions that measured smaller than 15 mm did not affect management. The development of new cyst(s) in other part of the pancreas was the main limitation of US surveillance, largely due to the inability for complete US exploration of the gland. However, the clinical importance of detecting small new PCN remains debatable. Since the clinical records of the patients included in the present study contained no data on mural nodes, the ability of US to detect these changes was not evaluated. Nevertheless, our view is that with conventional US, the difficulty of distinguishing

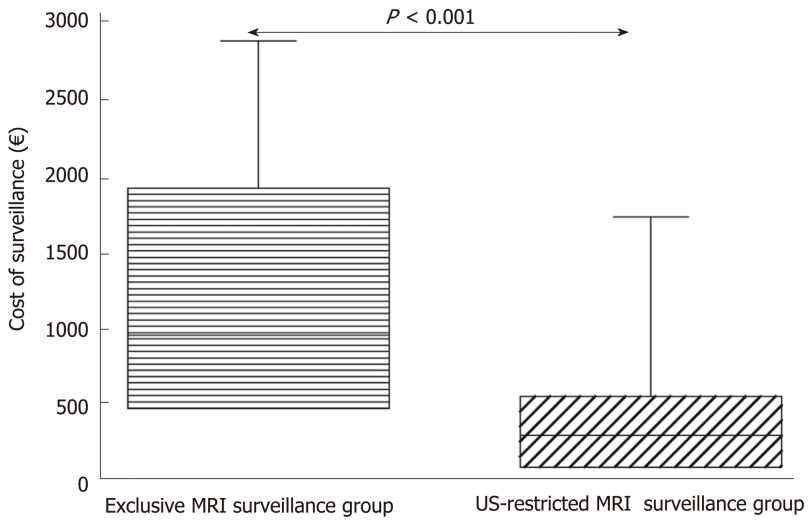

Figure 4 The box-plot graph showing the statistical difference between the overall cost for MRI alone and US-restricted MRI surveillance follow-up. US: Ultrasound; MRI: Magnetic resonance imaging.

genuine mural nodes from mucin plugs is substantial. CEUS can be useful to enhance the ability of conventional US to image and detect changes in the inner wall of pancreatic cysts[27]. In some reports[20], CEUS alone has not been found to be useful or reliable in documenting suspicious abnormalities that warrant change in management. In addition, these studies consider MRI to be better for detecting malignant transformation of IPMN, such that a detected intra-cystic solid mural node inevitably requires a second level MRI exam, thus limiting the utility of CEUS. The use of US as part of the surveillance protocol in the follow-up of patients with PCN has certain advantages: ease of performance by fully trained ultrasonographists, fast, widespread availability, and low cost.

The main potential risk of delaying the MRI imaging routine could be to miss early detection of worrisome features, however this has never been found in this study. Furthermore, even in this instance, the additional risk appears to be very low. In fact, relative indications for surgery according to European evidence-based guidelines[11] are not an expression of degeneration but only of increased risk, which is estimated in about 5.7% in patients with one relative indication for surgery[28].

Although CEUS has been proven to be more sensitive than US, it is a more complex procedure to be performed, requires venous access and the availability of the contras media, and is more time-consuming and costly (about double with respect to a conventional US). Furthermore, CEUS is not as panoramic as the MRI, because the various phases must be focused only on a precise target instead of on the whole gland.

For these reasons, we think that CEUS is a good diagnostic technique in selected cases, particularly when we have to study a precise finding of a B mode US, as an alternative or complimentary study to MRI. However, it is not a good technique for a routine follow-up.

The results of the present study indicate the potential benefit of including US scanning with restricted MRI, as outlined in our hospital protocol, for surveillance of patients with PCN. The study has confirmed that restricting MRI imaging to patients with progressive PCN modifications identified by abdominal US can reduce hospital costs without incurring missing patients who develop changes that require prompt surgical intervention or overt malignant transformation. Several studies[29-31] have confirmed the utility and importance of a cheaper imaging alternative to the MRI protocol. In our institution, the MRI protocol includes diffusion-weighted imaging for a more reliable definition of suspicious PCN elements, together with the routine use of contrast-enhanced sequences. The routine MRI sequences differ substantially from those reported by Pozzi-Mucelli *et al*[30], and our costs seem lower despite the comprehensive protocol. In Italy, several factors influence the imaging workflow, and these include examination time. US is the most acceptable imaging modality in this setting because it is quick, widely accessible and low cost. This is confirmed by the Italian Consensus guideline for diagnostic work-up and follow-up of PCN[17]. This may be different in other countries where a short MRI is preferred.

The primary goal of any surveillance program is to reduce the frequency of high-

level tests exemplified by MRI without compromising patient safety. The shortcomings of US are related to the patients' acoustic window and the undeniable fact that US is operator-dependent. Hence, the successful outcome of the present study may be related to the expertise of the surgeon US operators involved. For this reason, we believe that this program should be followed only in tertiary care hospitals by a dedicated team with specialist expertise in managing pancreatico-biliary disorders. Even so, there can be no doubt that patients/PNC locations with poor acoustic window cannot be safely followed by US. In our opinion, the standard exclusive MRI surveillance is needed for the follow-up of these patients.

We acknowledge that the study has some limitations. The first is its retrospective nature, which prevented the inclusion of patients with PCN who could not be assessed by US because of a poor acoustic window, thereby increasing the risk of selection bias. However, in the literature, cases with poor acoustic windows precluding US assessment are not common and range from 2%-12% of cases[32]. The retrospective nature of the study may also influence extrapolated cost estimations based on the same cohort of patients undergoing surveillance by exclusive MRI surveillance. Another limitation of the study is the short follow-up, as this may inflate the performance of US since many PCNs remained unchanged during the study period. Clearly, the two surveillance regimens for patients with PCN (MRI-based surveillance (current gold standard) *vs* our proposed US-restricted MRI surveillance protocols) need to be evaluated and confirmed by a prospective RCT with both clinical and health economic endpoints.

In conclusion, in patients with good US window, and with PCN without absolute or relative surgical criteria, abdominal US performed by an expert physician could be a safe complementary approach to MRI. This would delay and reduce the numbers of second-level examinations and therefore reduce the cost of surveillance. Considering the growing pressure for the allocation of healthcare resources, US is an inexpensive option for follow-up of a large number of PCN patients in a protracted period. However, the proposed abdominal US-restricted MRI surveillance protocol needs to be evaluated and confirmed by a prospective RCT against the currently recommended MRI-based surveillance, with the RCT having both clinical and health economic endpoints.

ARTICLE HIGHLIGHTS

Research background

The current international guidelines only consider magnetic resonance imaging (MRI) for the follow-up of patients with pancreatic cystic neoplasms (PCN). Given the great number of patients with PCN that have to be followed-up due to the inherent risk of malignant progression, the use of abdominal ultrasound (US) might be a quick, easily accessible and cost-saving imaging modality. Recent publications have evaluated the role of US in monitoring PCN, but none have proposed a safe alternative follow-up surveillance based on US with restricted MRI use.

Research motivation

We performed this study in order to evaluate the safety and cost-efficacy of US as a diagnostic tool to simplify the follow-up of selected patients with low risk pancreatic cystic neoplasms.

Research objectives

The objectives of this study were: (1) to evaluate the safety of the use of US in the surveillance of patients with good acoustic window and low-risk pancreatic cystic neoplasms; and (2) to propose an alternative follow-up protocol that reduces the cost with respect to the cost incurred by current international guidelines.

Research methods

We retrospectively evaluated the safety and costs of a follow-up surveillance for patients with low-risk PCN, performed with 6 monthly abdominal US for the first year, and then annually and with recourse to MRI scans performed every 2 years, or for confirmation of suspicious US findings.

Research results

Between January 2012 and January 2017, we followed 200 patients with a specific protocol that included abdominal US scans for pancreatic cystic neoplasms. During a follow-up period of 25.1 ± 18.2 mo, MRI identified evolution of the lesions not detected by US in only 11 patients (5.5%). However, MRI every 6 mo would not have changed patient management in any case. The mean cost of surveillance for each patient based on theoretical application MRI surveillance (recommended by international guidelines) within the group of patients included in the study would have incurred costs of 1158.9 ± 798.6 €, compared to the surveillance costs incurred by the proposed US-restricted MRI protocol of 366.4 ± 348.7 € ($P < 0.0001$).

Research conclusion

Abdominal US seems to provide a cost-effective surveillance that reduces the frequency of MRI scans without affecting patient outcome. This is important in reducing the financial burden on hospital healthcare, aside from reducing the examination time and MRI-related issues and side effects. For patients with PCN, we have proposed a follow-up surveillance that includes abdominal US, and demonstrated that it is safe and complementary to MRI. In addition, it effectively delays and reduces the number of MRI scans, thereby reducing the cost of surveillance.

Research perspectives

The results of the present study need to be confirmed by a comparative prospective randomized trial with both clinical (long-term patient outcome safety) and health economic primary endpoints.

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ORIGINAL ARTICLE

Clinical Trials Study

Ombitasvir/paritaprevir/ritonavir + dasabuvir +/- ribavirin in real world hepatitis C patients

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Abstract

BACKGROUND

The hepatitis C virus (HCV) NS5A inhibitor ABT-267 (ombitasvir, OBV), the HCV NS4/4A protease inhibitor ABT-450 (paritaprevir, PTV), the CYP3A inhibitor ritonavir (r) and the non-nucleoside NS5B polymerase inhibitor ABT-333 (dasabuvir, DSV) (OBV/PTV/r + DSV) with or without ribavirin (RBV) is a direct-acting antiviral regimen approved in the United States and other major countries for the treatment of HCV in genotype 1 (GT1) infected patients. Patients with HCV who are considered "hard-to-cure" have generally been excluded from registration trials due to rigorous study inclusion criteria, presence of comorbidities and previous treatment failures.

AIM

To investigate the efficacy of this regimen in HCV G1-infected patients historically excluded from clinical trials.

METHODS

Patients were ≥ 18 years old and chronically infected with HCV GT1 (GT1a, GT1b or GT1a/1b). Patients were treatment-naïve or previously failed a regimen including pegylated interferon/RBV +/- telaprevir, boceprevir, or simeprevir. One hundred patients were treated with the study drug regimen, which was administered for 12 or 24 wk +/- RBV according to GT1 subtype and presence/absence of cirrhosis. Patients were evaluated every 4 wk from treatment day 1 and at 4 and 12 wk after end-of-treatment.

RESULTS

Many of the patients studied had comorbidities (44.2% hypertensive, 33.7% obese, 20.2% cirrhotic) and 16% previously failed HCV treatment. Ninety-six patients completed study follow-up and 99% achieved 12-wk sustained virologic

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response. The majority (88.4%) of patients had undetectable HCV RNA by week 4. The most common adverse events were fatigue (12%), headache (10%), insomnia (9%) and diarrhea (8%); none led to treatment discontinuation. Physical and mental patient reported outcomes scores significantly improved after treatment. Almost all (98%) patients were treatment compliant.

CONCLUSION
In an all-comers HCV GT1 population, 12 or 24-wk of OBV/PTV/r + DSV +/- RBV is highly effective and tolerable and results in better mental and physical health following treatment.

Key words: Hepatitis C; Ombitasvir; Paritaprevir; Ritonavir; Dasabuvir; Genotype 1

Core tip: The hepatitis C virus (HCV) NS5A inhibitor ABT-267 (ombitasvir, OBV), HCV NS4/4A protease inhibitor ABT-450 (paritaprevir, PTV), CYP3A inhibitor ritonavir (r) and non-nucleoside NS5B polymerase inhibitor ABT-333 (dasabuvir, DSV) (OBV/PTV/r + DSV) with or without ribavirin (RBV) is an approved direct-acting antiviral regimen less frequently studied in an all-comers population. This study included 96 all-comers; many had comorbidities (44.2% hypertensive, 33.7% obese, 20.2% cirrhotic) and 16% previously failed HCV treatment. In these patients, 12 or 24-wk of OBV/PTV/r + DSV +/- RBV was highly effective (99% sustained virologic response for 12 wk treatment), tolerable and resulted in better mental and physical health.

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INTRODUCTION

The National Health and Nutrition Examination Survey (NHANES) approximates that 3.2 million hepatitis C virus (HCV) patients are living in the United States[1]. Among these patients, most will develop chronic infection that can ultimately lead to hepatic fibrosis and cirrhosis[2]. Cirrhotic HCV patients have an increased risk of developing decompensated cirrhosis, hepatocellular carcinoma (HCC)[3,4]. and commonly require liver transplantation[5].

The recent era of improved treatment regimens for HCV has contributed to much better outcomes in these patients[6], with the goal of treatment being to eradicate the virus and prevent the development of cirrhosis and its complications. Successful treatment of HCV is defined in terms of sustained virologic response (SVR), which is undetectable levels of HCV viral RNA in the blood 12 wk after completion of therapy[7]. Currently, treatment for HCV involves the use of direct-acting antiviral (DAA) regimens, which are molecules that target specific nonstructural proteins of the virus and disrupt viral replication and infection. One such DAA regimen is the HCV NS5A inhibitor ombitasvir (OBV), HCV NS4/4A protease inhibitor paritaprevir (PTV), the CYP3A inhibitor ritonavir and the non-nucleoside NS5B polymerase inhibitor dasabuvir (DSV) (OBV/PTV/r + DSV) with or without ribavirin (RBV). In registration trials for this regimen, SVR rates reached as high as 99% in HCV genotype 1 (GT1) patients[8].

Patients with HCV who are considered "hard-to-cure" have generally been excluded from registration trials due to rigorous study inclusion criteria, presence of comorbidities and previous treatment failures. Here we report the results of a phase 4, open label study that evaluated the safety and efficacy of OBV/PTV/r + DSV +/- RBV in a real-world clinical setting in patients who have historically been excluded from clinical trials. We also report on the patient quality of life, dosing adherence and whether resistance-associated substitutions (RASs) impact achievement of SVR.

MATERIALS AND METHODS

All patients provided written informed consent. The study was conducted in accordance with the International Conference on Harmonisation, applicable regulations, and the principles of the Declaration of Helsinki. Upon signing the informed consent, patients were considered enrolled and screening and baseline assessments were initiated. Patients were screened from April 21, 2016 through December 5, 2016. Screening procedures at baseline included a medical history, physical exam, blood chemistries, HCV RNA PCR and HCV genotype assessments.

Eligible patients were ≥ 18 years old and chronically infected with HCV GT1 (GT1a, GT1b or GT1a/1b). Patients were either treatment naïve or previously failed a regimen including pegylated interferon (pegIFN)/RBV with or without telaprevir, boceprevir, or simeprevir. Patients were excluded from the study if they were currently taking or planning on taking any medications contraindicated in the OBV/PTV/r + DSV package insert, had evidence of decompensated liver disease (Child-Pugh B or C, including the presence of clinical ascites, bleeding varices, or hepatic encephalopathy), hemoglobin < 8 g/dL, platelets < 25000 cells/mm^3, all nucleated cells < 500 cells/mm^3, bilirubin > 3, international normalized ratio ≥ 2.3, serum albumin < 2.8 and glomerular filtration rate < 30 mL, alanine aminotransferase or aspartate aminotransferase > 10 × ULN, consume > 3 alcoholic drinks daily or have uncontrolled HIV or hepatitis B virus coinfection. Patients with cirrhosis were required to have serum alpha fetoprotein < 100 ng/mL and imaging ruling out HCC within 3 mo of screening visit.

Treatment was initiated when screening and baseline procedures were completed, and patient eligibility was confirmed. Patients received the US package insert recommended dose: Two OBV/PTV/r 12.5/75/50 mg tablets once daily (in the morning) and one DSV 250 mg tablet twice daily (morning and evening) with a meal without regard to fat or calorie content. RBV was given to some patients based on approved US package insert and was dosed at 1000 mg/d (< 75 kg) or 1200 mg/d (≥ 75 kg), divided and administered twice-daily with food. Duration of treatment was determined based on GT1 subtype and the presence or absence of cirrhosis. GT1a patients with compensated cirrhosis were treated for 24 wk including RBV, GT1a patients without cirrhosis and GT1b patients with compensated cirrhosis were treated for 12 wk including RBV, and GT1b patients without compensated cirrhosis were treated for 12 wk without RBV.

For patients treated for 12 wk, study visits took place at day 1 (baseline) and weeks 4, 8 and 12 (end-of-treatment, EOT). For patients treated for 24 wk, study visits took place at day 1 (baseline) and weeks 4, 8, 12, 16, 20 and 24 (EOT). All patients had follow-up visits 4 and 12 wk after their last dose of study medication. Patients were considered to be off study if they discontinued early or did not complete protocol defined visits. The study protocol was approved by the Integ Review Institutional Review Board. The study was registered on ClinicalTrials.gov.

Definitions and outcome measures

The primary endpoint was SVR 12 wk after the last treatment dose for the all treated population. The all treated population was defined as all patients that were consented and received at least 1 dose of study medication. SVR was defined as HCV RNA below the lower limit of quantification (LLOQ) 12 wk after the end of treatment (SVR12). Plasma HCV RNA levels were measured using the COBAS TaqMan HCV test (version 2.0), for use with the High Pure System, which has an LLOQ of 25 IU per milliliter.

Outcomes for patients not achieving an SVR12 were recorded as on-treatment virologic failure (VF), post-treatment virologic relapse through post-treatment week 12 or failure due to other non-virologic reasons (*e.g.*, premature discontinuation, adverse event, lost to follow-up, consent withdrawn). HCV RNA was assessed at each study visit and post treatment weeks 4 and 12 (or early post treatment discontinuation).

Key safety parameters that were recorded included dose discontinuations/modifications due to adverse events (AEs), treatment related serious AEs (SAEs), and laboratory test abnormalities. The onset and end dates, severity and relationship to study drug were recorded for each AE. Patients were questioned and/or examined by the investigator or a qualified designee for evidence of AEs at each treatment visit. The determination of AE severity rested on medical judgment and was made with the appropriate involvement of the investigator or a designated sub-investigator. The severity of AEs, with the exception of laboratory values, was graded according to the WHO grading system. Investigators relied on clinical judgment in assigning severity to abnormal laboratory AEs. Data on all treatment emergent AEs were collected from the start of study drug until 30 d after receipt of the last dose. Clinical laboratory

testing was performed at all visits during the treatment period.

The first pre-defined secondary endpoint evaluated the effect of baseline RASs on SVR12. Baseline sampling for RASs was obtained on day 1 for all patients and thereafter only in patients with detectable HCV RNA after previously testing negative (breakthrough) or patients who were undetectable at the end of treatment but had detectable HCV RNA during the follow up period (relapse). Resistance testing was performed by Quest Diagnostics. The patient subgroups evaluated included all RASs and different classes of RASs.

Another pre-defined secondary endpoint evaluated patient reported outcomes, examined *via* the Short Form–36 version 2 Health Survey (SF36v2), at baseline compared to end of treatment. Patients completed this self-administered questionnaire, which assessed functional health and well-being at baseline and at 12 wk. The results consisted of eight scaled scores, which are the weighted sums of the questions in their section. Scores were aggregated into a mental component summary (MCS) and a physical component summary (PCS); higher scores were indicative of better health.

The final secondary endpoint was to evaluate adherence in patients receiving this treatment regimen. Pills were counted by study personnel at each treatment visit. Treatment compliance was defined as the subject having a total missed pill count of ≤ 20% of the total dispensed pill count over the course of their treatment duration. For patients on RBV, the total dispensed pill count was 840 over 12 wk and 1680 over 24 wk. For patients not on RBV, the total dispensed pill count was 336 over 12 wk and 672 over 24 wk. Patient adherence was reported according to treatment arm.

Statistical analysis

All patients who consented and received at least one dose of study medication were included in the primary analysis for both efficacy and safety (all-treated population). Descriptive summaries consisted of frequencies and percentages for categorical measures and of the number of patients, mean, standard deviation, median, minimum, and maximum values for continuous measures. Descriptive summaries were presented for select subgroups. Tabular summaries presented included age, sex, and race and other parameters measured at baseline. Since this was a single arm study design, no power statement was calculated.

RESULTS

Patients

A total of 104 patients were screened and 100 patients were treated with the study drug regimens. The majority of patients (n = 89, 89%) were treated with OBV/PTV/r + DSV + RBV, with 75 (75%) undergoing 12 wk of treatment and 25 (25%) undergoing 24 wk of treatment. The vast majority of patients (86%) were infected with GT1a (Figure 1). Patient characteristics at baseline, including current comorbidities and history of previous HCV treatments are shown in Table 1.

Efficacy

The final date for collection of data was October 3, 2017. One hundred (100) patients received at least one dose of study drug, 96 patients completed follow-up and 95 (95%) achieved SVR12. The HCV RNA results for patients completing treatment with SVR are depicted in Figure 2. The vast majority (88.4%) were undetectable by week 4. Of the 4 patients who exited the study early, 3 were lost to follow-up and 1 was terminated early due to noncompliance. The one patient who completed treatment but did not achieve SVR was a 48-year-old Black male infected with GT1a HCV. He was treatment-naïve, FibroScan® showed no fibrosis (F0) and no baseline RASs were detected. His baseline viral load was 2694796 IU/mL and he was prescribed 12 wk of OBV/PTV/r + DSV + RBV. He had undetectable HCV RNA at treatment week 4 and treatment week 8; however, he missed 6 doses of RBV 8 wk into treatment. He was counseled on medication compliance and didn't miss any other doses. At end of treatment (week 12), HCV RNA was detected (742 IU/mL) and he was considered a treatment-failure. The patient did not return for his 12-wk follow-up visit.

Safety

The number of AEs reported totaled 123. The most common AEs were fatigue (12%), headache (10%), insomnia (9%) and diarrhea (8%) (Table 2). There were no serious AEs or AEs leading to discontinuation reported in this study.

Baseline variants

Sampling for RASs was performed on 99 patients at baseline. There were no

Table 1 Demographic and clinical characteristics of patients *n* (%)

Characteristic	Patients
Age	
n	104
Mean (yr ± SD)	49.9 ± 10.5
Min, max (yr)	29.0, 78.0
Median (yr) (IQR)	51.5 (41.0, 58.2)
Gender	
n	104
Female	46 (44.2)
Male	58 (55.8)
Ethnicity	
n	104
Hispanic or Latino	48 (46.2)
Not Hispanic or Latino	56 (53.8)
Race	
n	104
White	91 (87.5)
Black	11 (10.6)
Unknown	2 (2)
Current comorbidities	
n	104
Hypertension	46 (44.2)
Obesity	35 (33.7)
Cirrhosis, Child-Pugh A	21 (20.2)
Hepatic steatosis	18 (17.3)
Diabetes mellitus, type II	17(16.3)
Hyperlipidemia	17 (16.3)
Chronic kidney disease	3 (3)
Coronary artery disease	3 (3)
Atherosclerosis	1 (1)
Congestive heart failure	1 (1)
HIV	1 (1)
Genotype	
n	100
GT1a	86 (86)
GT1b	14 (14)
Prior HCV treatment	
n	100
IFN	2 (2)
IFN+RBV	3 (3)
PegIFN	1 (1)
PegIFN+RBV	10 (10)

SD: Standard deviation; IQR: Interquartile range; GT1: Genotype 1; IFN: Interferon; PegIFN: Pegylated interferon; RBV: Ribavirin.

detectable mutations in the NS5a or NS5b genes in 88 (88%) and 98 (98%) of these patients, respectively. A probable resistance to OBV was found in one patient (1%) with an NS5a mutation, but no resistance to daclatasvir, elbasvir, ledipasvir or velpatasvir was noted. In addition, there were 4 (4%) cases of possible resistance to OBV, daclatasvir, elbasvir, ledipasvir and velpatasvir. With regard to NS5b RASs, one patient (1%) had a possible resistance to DSV.

Patient reported outcomes

Patient reported outcomes were analyzed in patients with both baseline and end of

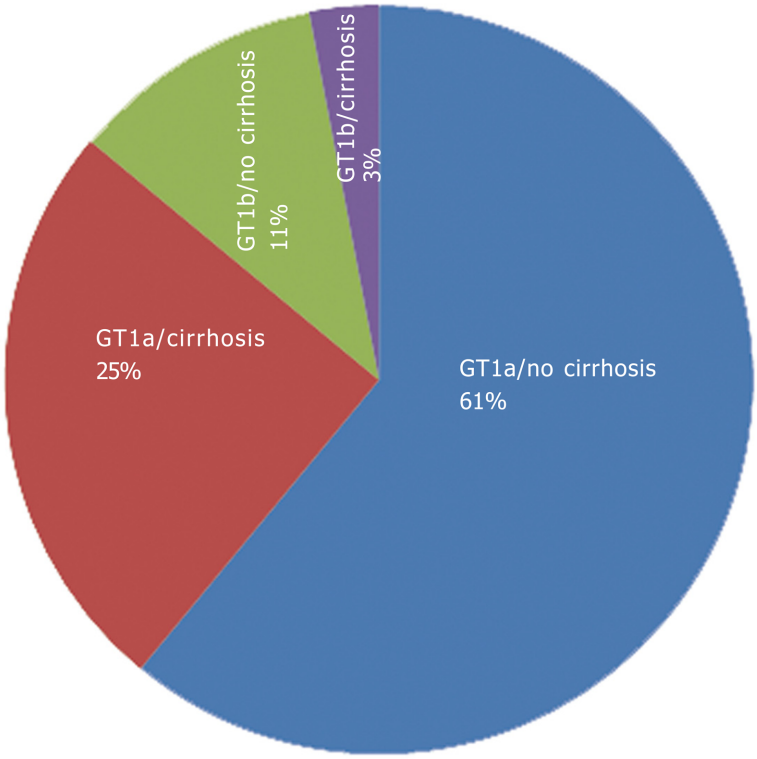

Figure 1 Hepatitis C genotype and presence/absence of cirrhosis for patients. GT1: Genotype 1.

treatment (Week 12 or Week 24) scores (n = 67). These results are portrayed in Table 3. Overall, PCS scores and MCS scores were significantly higher following treatment compared to baseline (P = 0.04 and P = 0.011, respectively). Of the 8 scaled scores, all end of treatment scores were higher compared to baseline, with statistically significant improvement observed for 5 sub-scores (physical, general health, vitality, emotional and mental health).

Adherence

Treatment compliance was assessed in all 100 patients and 98 (98%; 95% confidence interval, 93 to 99.4) of these patients were considered compliant. The two noncompliant patients were in the OBV/PTV/r + DSV + RBV 12-wk treatment arm. All patients in the OBV/PTV/r + DSV 12-wk arm and the OBV/PTV/r + DSV + RBV 24-wk treatment arm achieved 100% compliance. Four patients terminated the study early (prior to 12 wk) and are included in these data; all 4 met the criteria for treatment compliance while on treatment.

DISCUSSION

In a real world, all-comers population of HCV GT1 patients, a 12 or 24-wk multi-targeted DAA regimen of OBV/PTV/r + DSV +/- RBV was highly effective with a 95% SVR12 rate. Of the 96 patients who completed follow up, 99% (95/96) achieved SVR12. The presence of baseline RASs had no impact on the ability to achieve SVR12. Furthermore, treatment was associated with a low rate of treatment discontinuation unrelated to AEs. No AEs were considered serious. These efficacy and safety results are comparable to those seen in controlled clinical trials of OBV/PTV/r + DSV +/- RBV[8-11]. Although newer DAA regimens with shorter durations have been recently approved, our data on this older regimen remains important; this regimen is an approved treatment option and is still used in some developing countries.

Our study population was made up of slightly more males than females, which is consistent with the distribution of HCV in the United States[1]. Although there were no Asian patients and a small percentage of Black patients, this study represented an even distribution of Hispanics and non-Hispanics with HCV. HCV data are important in this population. Hispanics are a large and fast-growing minority group in the United States[12] and, according to one study[13], Hispanics infected with HCV are at a significantly higher risk of developing cirrhosis and HCC than non-Hispanic Whites.

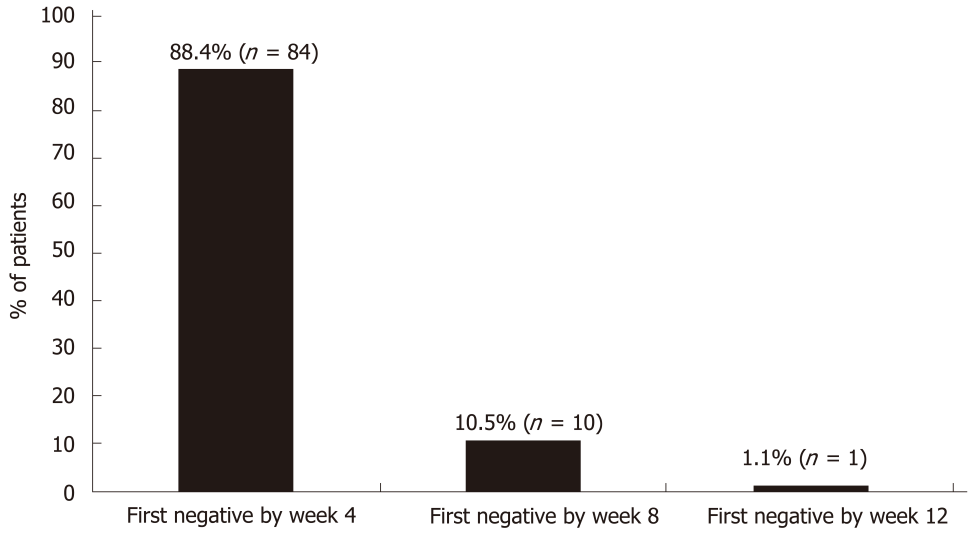

Figure 2 Hepatitis C virus RNA results for patients completing treatment with sustained virologic response.

Traditionally, individuals with HCV who have comorbidities are considered "hard-to-cure" and are often excluded from controlled clinical trials. The HEARTLAND study sought to enroll these individuals; many of the individuals studied had hypertension (44.2%), obesity (33.7%), Child-Pugh A cirrhosis (20.2%), steatosis (17.3%), type II diabetes mellitus (16.3%) and hyperlipidemia (16.3%). Including these populations in HCV treatment studies is essential. Data has demonstrated that, among patients with HCV, > 99% have at least one comorbidity, with hypertension being one of the most common[14]. In addition, NHANEs investigators predict that the incidence of cirrhosis will peak by the year 2020, affecting 1.76 million HCV-infected individuals in the United States[1]. Finally, metabolic conditions such as steatosis, obesity and diabetes are emerging as independent co-factors of fibrogenesis[15].

Individuals who previously failed HCV treatment are also often disqualified from registration studies or evaluated in separate retreatment studies. Sixteen percent of patients in HEARTLAND were HCV treatment experienced, with the majority (62.5%) previously treated with PegIFN + RBV. The inclusion of these patients did not affect overall SVR rates. This is consistent with what other studies of OBV/PTV/r + DSV +/- RBV have demonstrated: combining antiviral drugs with multiple mechanisms of action is effective regardless of the prior response to PegIFN + RBV[9,16].

In addition to including underrepresented HCV patients, data were collected in the "real world" setting, further demonstrating its applicability in clinical practice. Moreover, results are similar to other published real-world data on this regimen. For example, in a study of 5168 HCV GT1 patients, who were included irrespective of cirrhosis status or prior HCV treatment experience, real world SVR12 rates were consistently high (94 to 99%) for the OBV/PTV/r + DSV +/- RBV regimen[17]. Another study performed in Taiwan showed SVR12 rates of 98% and good tolerability in patients administered this regimen[18].

The data generated from the secondary analyses performed in this study are also consistent with published reports. With regard to resistance testing, according to the American Association for the Study of Liver Diseases (AASLD) HCV Guidance, Recommendations for Testing, Managing, and Treating Hepatitis C, a subset of patients with HCV will have viral variants harboring substitutions associated with resistance to DAAs, especially with NS5A inhibitor-containing regimens. This may negatively impact treatment response[19]. In HEARTLAND, SVR rates approached 100% despite the presence of baseline NS5A and NS5B RASs in 12% and 2% of patients, respectively. This reinforces what is stated in the AASLD HCV guidance: the magnitude of the negative impact of RASs varies according to many factors and RAS testing alone will not dictate optimal DAA regimen selection[19].

Another secondary analysis was the effect of OBV/PTV/r + DSV +/- RBV on patient reported outcomes. Such outcome assessments provide patients' perspective on the impact of treatment on daily life and work. HEARTLAND demonstrated, *via* data collected from the SF36v2 short form, that OBV/PTV/r + DSV +/- RBV significantly improved patient reported outcomes for total physical and mental components. The MALACHITE-I and MALACHITE-II controlled clinical studies in HCV G1 patients evaluated the same secondary endpoints using a similar method

Table 2 Adverse events reported during the HEARTLAND study

Most common adverse events	Patients reporting AE (*n* = 100)
Fatigue	12 (12%)
Headache	10 (10%)
Insomnia	9 (9%)
Diarrhea	8 (8%)
Anemia	6 (6%)
Nausea	6 (6%)
Pruritus	5 (5%)
Rash	4 (4%)
Upper respiratory infection	3 (3%)
Urinary tract infection	3 (3%)

AE: Adverse event.

and demonstrated matching trends. Overall, patients treated with OBV/PTV/r + DSV +/- RBV have better mental and physical health following HCV treatment[20].

Finally, patient adherence was addressed in HEARTLAND. Adherence is important when successfully treating HCV and becomes even more critical in the real-world setting. Often, efficacy rates reported from clinical trials are substantially reduced when drugs are approved and used in clinical practice. The reasons for this are multifactorial and can be due to side effects, complexity of the regimen and/or other patient-related factors[21]. In our study, we demonstrated excellent adherence rates (98% treatment compliance) in the real-world setting using a complex regimen. This study was designed to enroll approximately 100 patients with baseline factors that may have limited their ability to enroll in registration trials including 28% with cirrhosis. The safety and efficacy results reported in this study are impressive and comparable to those reported in registration trials.

In conclusion, the all oral, DAA regimen containing OBV/PTV/r + DSV +/- RBV was associated with a 99% SVR at post-treatment week 12 in GT1-infected patients, with and without compensated cirrhosis.

Table 3 Mean short form 36 version 2 scores using the normative based scores

	End of treatment	Baseline	*P* value
	(*n* = 67)	(*n* = 67)	(Paired)
Physical functioning	45.6 ± 11.3	44.4 ± 11.0	0.3389
Physical	45.3 ± 10.8	42.2 ± 10.4	0.0152
Bodily pain	46.3 ± 12.0	43.9 ± 11.2	0.1103
General health	49.0 ± 10.8	44.6 ± 10.5	0.0004
Vitality	50.4 ± 11.9	46.0 ± 10.6	0.0066
Social functioning	45.4 ± 11.9	42.7 ± 12.1	0.0530
Emotional	45.2 ± 12.1	41.8 ± 11.4	0.0272
Mental Health	46.6 ± 12.6	44.1 ± 10.4	0.0287
Physical component summary	46.8 ± 10.7	44.4 ± 9.1	0.0404
Mental component summary	47.1 ± 13.7	43.6 ± 11.6	0.0112

ARTICLE HIGHLIGHTS

Research background

The hepatitis C virus (HCV) is a prevalent virus that, if left untreated, leads to chronic liver disease and, ultimately, death. The new era of direct acting antiviral (DAA) treatment regimens has the potential to cure the virus [*i.e.*, achieve sustained virologic response (SVR)] in the majority of patients. The HCV NS5A inhibitor ombitasvir (OBV), HCV NS4/4A protease inhibitor paritaprevir (PTV), the CYP3A inhibitor ritonavir and the non-nucleoside NS5B polymerase inhibitor dasabuvir (DSV) (OBV/PTV/r + DSV) with or without ribavirin (RBV) is a DAA regimen that achieves SVR rates as high as 99% in HCV genotype 1 (GT1) patients in controlled clinical studies. However, there are patients who are considered "hard to cure" that are traditionally excluded from registration trials due to rigorous study inclusion criteria, presence of comorbidities and previous treatment failures. This phase 4, open label study evaluated the safety and efficacy of OBV/PTV/r + DSV +/- RBV in a real-world clinical setting in patients who have historically been excluded from clinical trials. This study is completed.

Research motivation

Controlled clinical studies demonstrate 99% SVR rates in patients with HCV GT1, however, many patients in these studies do not meet the inclusion criteria for these studies. In a real world population of HCV patients, many have comorbidities or history of previous HCV treatment failures. We sought to examine the efficacy and safety of OBV/PTV/r + DSV +/- RBV in real world HCV patients who are generally underrepresented in clinical trials. This study also examined patient quality of life, dosing adherence and whether resistance-associated substitutions (RASs) impact achievement of SVR, which are all real world issues encountered in HCV patients. The results of this study will determine if controlled clinical trial results can be expected in everyday HCV patients seen in clinical practice.

Research objectives

The primary objective of this study was to examine the efficacy and safety of OBV/PTV/r + DSV +/- RBV in real world HCV patients generally underrepresented in clinical trials. This study found that this treatment regimen was highly effective and no adverse events were considered serious; these results are comparable to those seen in controlled clinical trials with this treatment regimen. Therefore, including patients with comorbidities or a history of previous HCV treatment(s) did not affect the results. According to this one study, the results demonstrated in controlled clinical trials involving OBV/PTV/r + DSV +/- RBV can be applied to everyday HCV patients seen in clinical practice.

Research methods

Patients were ≥ 18 years old and chronically infected with HCV GT1 (GT1a, GT1b or GT1a/1b). Patients were treatment-naïve or previously failed a regimen including pegylated interferon/RBV +/- telaprevir, boceprevir, or simeprevir. One hundred patients were treated with the study drug regimen, which was administered for 12 or 24 wk +/- RBV according to GT1 subtype and presence/absence of cirrhosis. Patients were evaluated every 4 wk from treatment day 1 and at 4 and 12 wk after end-of-treatment.

Research results

Many of the patients studied had comorbidities (44.2% hypertensive, 33.7% obese, 20.2% cirrhotic) and 16% previously failed HCV treatment. Ninety-six patients completed study follow-up and 99% achieved 12-wk sustained virologic response. The majority (88.4%) of patients had undetectable HCV RNA by week 4. The most common adverse events were fatigue (12%), headache (10%), insomnia (9%) and diarrhea (8%); none led to treatment discontinuation.

Physical and mental patient reported outcomes scores significantly improved after treatment. Almost all (98%) patients were treatment compliant.

Research conclusions

In an all-comers HCV GT1 population, 12 or 24-wk of OBV/PTV/r + DSV +/- RBV is highly effective and tolerable and results in better mental and physical health following treatment.

Research perspectives

Results of he approved use of the OBV/PTV/r + DSV +/- RBV regimen in HCV GT1 patients in clinical practice can potential mirror results obtained in controlled clinical trials. The availability of real world data on approved HCV treatment regimens is extremely useful in clinical practice. Newer DAA regimens with shorter treatment durations have been recently approved. These regimens should also be evaluated in the real world population of HCV patients. Future clinical studies need to evaluate the efficacy and safety of these newer DAA regimens in real world patients. Patients with comorbidities and those who have had previous HCV treatment failures should be included in these studies. In addition, secondary measures should include physical and mental outcomes, the affects of RASs and adherence to the newer regimens.

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ORIGINAL ARTICLE

Observational Study

Nested case-control study on risk factors for opportunistic infections in patients with inflammatory bowel disease

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Abstract

BACKGROUND

When opportunistic infections occur, patients with inflammatory bowel disease (IBD) commonly display a significantly increased rate of morbidity and mortality. With increasing use of immunosuppressive agents and biological agents, opportunistic infections are becoming a hot topic in the perspective of drug safety in IBD patients. Despite the well-established role of opportunistic infections in the prognosis of IBD patients, there are few epidemiological data investigating the incidence of opportunis-tic infections in IBD patients in China. Besides, the risk factors for opportunistic infection in Chinese IBD patients remain unclear.

AIM

To predict the incidence of opportunistic infections related to IBD in China, and explore the risk factors for opportunistic infections.

METHODS

A single-center, prospective study of IBD patients was conducted. The patients were followed for up to 12 mo to calculate the incidence of infections. For each infected IBD patient, two non-infected IBD patients were selected as controls. A conditional logistic regression analysis was used to assess associations between putative risk factors and opportunistic infections, which are represented as odds ratios (OR) and 95% confidence intervals (CIs).

RESULTS

Seventy (28.11%) out of 249 IBD patients developed opportunistic infections. *Clostridium difficile* infections and respiratory syncytial virus infections were found in 24 and 16 patients, respectively. In a univariate analysis, factors such as the severity of IBD, use of an immunosuppressant or immunosuppressants, high levels of fecal calprotectin, and C-reactive protein or erythrocyte sedimentation

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rate were individually related to a significantly increased risk of opportunistic infection. Multivariate analysis indicated that the use of any immunosuppressant yielded an OR of 3.247 (95%CI: 1.128-9.341), whereas the use of any two immunosuppressants yielded an OR of 6.457 (95%CI: 1.726-24.152) for opportunistic infection. Interestingly, when immunosuppressants were used in combination with infliximab (IFX) or 5-aminosalicylic acid, a significantly increased risk of opportunistic infection was also observed. The relative risk of opportunistic infection was greatest in IBD patients with severe disease activity (OR = 9.090; 95%CI: 1.532-53.941, relative to the remission stage). However, the use of IFX alone did not increase the risk of opportunistic infection.

CONCLUSION
Factors such as severe IBD, elevated levels of fecal calprotectin, and the use of immunosuppressive medications, especially when used in combination, are major risk factors for opportunistic infections in IBD patients. The use of IFX alone does not increase the risk of opportunistic infection.

Key words: Nested case-control study; Opportunistic infections; Inflammatory bowel disease

Core tip: When opportunistic infections occur, patients with inflammatory bowel disease (IBD) commonly display a significantly increased rate of morbidity and mortality. With increasing use of immunosuppressive agents and biological agents, opportunistic infections are becoming a hot topic in the perspective of drug safety in IBD patients. Despite the well-established role of opportunistic infections in the prognosis of IBD patients, there are few epidemiological data investigating the incidence of opportunistic infections in IBD patients in China. Besides, the risk factors for opportunistic infections in Chinese IBD patients remain unclear.

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INTRODUCTION

Opportunistic infection refers to any infection caused by a weakened immune system and typically does not occur in people with normal immune function[1]. When they occur, patients presenting with opportunistic infections commonly display a significantly increased rate of morbidity and mortality. With increasing use of immunosuppressive agents and biological agents, opportunistic infections are becoming a hot topic in the perspective of drug safety in patients with inflammatory bowel disease (IBD). Studies have shown that opportunistic infections are closely related to the recurrence of IBD[2,3]. Severe opportunistic infection was observed in 3% of IBD patients, significantly increasing their mortality[4]. According to the consensus of The European Crohn's and Colitis Organization on opportunistic infections in IBD, IBD patients taking glucocorticoids, immunomodulators, and biologic therapies are all at an increased risk of opportunistic infection. Malnutrition and old age are also key risk factors for opportunistic infections[5]. A multicenter prospective study conducted in Japan followed 570 patients with IBD for one year and observed 52 (9.1%) cases of opportunistic infection. Further analysis found that being over 50 years of age and the use of immunosuppressive agents are contributing risk factors for opportunistic infections in patients with IBD[6]. A study conducted by Tourner *et al*[7] also concluded that immunosuppressive medications and increased patient age are related with an increased risk of opportunistic infections in IBD patients. On the other hand, positive prevention, early diagnosis, and timely control of opportunistic infections are the current focuses for improving the prognosis of IBD patients.

Despite the well-established role of opportunistic infections in the prognosis of IBD

patients, there are few epidemiological data investigating the incidence of opportunistic infections in IBD patients in China. The level of fecal calprotectin (FC) is a commonly used diagnostic measure for patients with IBD as its levels generally increase in patients with IBD. It is generally accepted that FC levels are a stable and reliable measure of IBD severity with a high sensitivity and specificity. However, the relationship between FC levels and the incidence of opportunistic infections in IBD has never been studied.

Therefore, this study sought to investigate the relationship between IBD and opportunistic infections from a cohort of patients with IBD using a nested case-control method. Specifically, we sought to predict the incidence of oppor-tunistic infections in patients with IBD in China, and determine the relationship between FC and opportunistic infection, with an aim to provide a scientific basis for the effective prevention and control of opportunistic infections in patients with IBD.

MATERIALS AND METHODS

Research design and patient groups

This study involving 301 IBD patients [139 with ulcerative colitis (UC) and 162 with Crohn's disease (CD)] enrolled from January to December 2017. The probability of an opportunistic infection was calculated during a one-year follow-up period. Common opportunistic infections in patients with IBD include viral infections (herpes viruses, human papillomavirus, influenza virus, and JC virus), bacterial infections (tuberculosis, nocardia, *Clostridium difficile*, pneumococcus, legionella, and listeriosis), fungal infections (histoplasmosis, cryptococcosis, *Pneumocystis jirovecii* infection, aspergillosis, and candidiasis), and parasite infections (*Strongyloides stercoralis*)[8]. Patients were screened for opportunistic infection before enrollment to exclude those currently infected. Infection was based on laboratory results, in which viral IgM positivity and DNA copy were diagnosed as viral infection. The diagnosis of tuberculosis was based on the detection of *Tubercle bacillus*. *Clostridium difficile* was detected by PCR. Positive fecal cultures of mold and candida were diagnosed as fungal infections. Clinical visits were conducted once a month. Clinical follow-up and laboratory examinations [including routine blood examination, C-reactive protein (CRP), hepatic and renal function, FC, infection indicators, *etc*.] were conducted once a month. In addition, disease activity was assessed at each follow-up. Truelove and Witts disease severity classification criteria[9] and CD activity index (CDAI)[10] were used to evaluate the disease activity of patients with UC and CD, respectively. These diagnoses and disease activity index were admitted by the attending physician.

This study was approved by the ethics committee of First Affiliated Hospital of Zhejiang Chinese Medical University, and patients under the age of 16 were admitted to our study with the consent from their parents or guardians.

Case-control study

IBD patients with opportunistic infections were selected as the case group. For each case, two uninfected patients were chosen as controls and were matched according to age (at an interval of 10 years; ≤ 19, 20-29, 30-39,40-49, and ≥ 50 years).

Data collection

The demographic characteristics of patients were collected, including age, gender, course of the disease, current smoking habits, previous bowel surgery, comorbidity, disease activity, the type of IBD, and current medications. All patients were diagnosed with UC or CD based on the previously criteria[11].

Statistical analysis

Demographic characteristics were analyzed by the *t*-test. The univariate and multivariate analyses were performed with SPSS version 23 (SPSS, Tokyo, Japan).

RESULTS

IBD patients' demographic and clinical features

A total of 301 IBD patients were enrolled in this study, 52 of whom were lost to follow-up. Of the remaining 249 patients, 119 had UC and 130 had CD. The patients' ages ranged from 12 to 78 years (mean age, 45.24 years). During one year of follow-up, we found 70 IBD patients who developed an opportunistic infection (Table 1). The incidence of opportunistic infections was 28.11%. *Clostridium difficile* infection was found to be the most common opportunistic infection in patients with IBD (9.64%),

followed by respiratory syncytial virus infection (6.43%). Fifteen (6.02%) and eleven (4.42%) patients were infected with Epstein-Barr virus and Fungal, respectively. One patient developed active tuberculosis.

There were 70 patients in the case group, including 33 UC patients and 37 CD patients. In contrast, the control group consisted of 140 patients, including 66 UC patients and 74 CD patients. Selected demographic and clinical characteristics of cases and controls are provided in Table 2. There were no statistically significant differences between the two groups in age, gender, type of IBD, duration of IBD, duration of medication use, prior surgery or smoking history. The data of the two groups were well balanced and comparable.

Severe disease activity is a risk factor for opportunistic infections in IBD patients

Patients' gender, age, type of IBD, duration of medication, course of the disease, history of prior surgery, and smoking habits were not related to increased risk of opportunistic infections, according to the univariate analysis. However, severe disease activity in IBD patients was related to an increased rate of opportunistic infections (P < 0.001, OR = 18.404; 95%CI: 3.833-88.375). Multivariate analysis also indicated that severe IBD was the greatest risk factor for opportunistic infections (P < 0.001, OR = 18.404; 95%CI: 3.833-88.375).

Elevated level of FC is a risk factor for opportunistic infections in IBD patients

In our study, FC higher than normal levels (>200 μg/g) was related to an increased risk of opportunistic infections both in our univariate analysis (P < 0.001, OR = 4.431; 95%CI: 2.265-8.667) and multivariate analysis (P = 0.023, OR = 2.467; 95%CI: 1.133-5.373).

Immunosuppressive medications, especially when used in combination, are risk factors for opportunistic infections in IBD patients

Compared with the use of 5-aminosalicylic acid (5-ASA), the use of infliximab (IFX) or 5-ASA+ IFX was not related to the incidence of opportunistic infections in our univariate analysis (Table 3) and multivariate analysis. On the contrary, the use of any immunosuppressant (steroids, azathioprine, tacrolimus, thalidomide, or methotrexate) was related to a significantly increased odd for opportunistic infection, compared with the use of 5-ASA both in univariate analysis (P < 0.047, OR = 2.668; 95%CI: 1.012-7.033) (Table 3) and multivariate analysis (P = 0.029, OR = 3.235; 95%CI: 1.125-9.306) (Figure 1). Especially when two or more immunosuppressants were used in combination, it represented the second largest risk factor for opportunistic infections in both univariate analysis (P < 0.001, OR = 10.375; 95%CI: 2.948-36.508) (Table 3) and multivariate analysis (P = 0.006, OR = 6.462; 95%CI: 1.727-24.172) (Figure 1). In addition, the use of 5-ASA and any immunosuppressant(s) or IFX with any immunosuppressant(s) was related to significantly increased infection rates both in univariate analysis (P < 0.01) (Table 3) and multivariate analysis (P < 0.05) (Figure 1).

High levels of CRP and erythrocyte sedimentation rate (ESR) are not related to a significantly increased risk of opportunistic infection

Our univariate analysis showed that high levels of CRP (OR = 3.98; 95%CI: 1.994-7.944) and ESR (OR = 3.744; 95%CI: 1.87-7.494) were related to an increased risk of opportunistic infections (Table 3). However, the results of multivariate analysis did not confirm this finding (Figure 1).

DISCUSSION

In our study, we prospectively predicted the occurrence of opportunistic infections in IBD patients. A total of 70 (28.11%) of the 249 patients developed opportunistic infections. Currently, there are few epidemiological data on the rate of opportunistic infection in IBD patients in China. In a study performed at Ruijin Hospital affiliated to Shanghai Jiao Tong University, of 130 patients with IBD, 12.3% developed CD infection[12]. In another study, positive serum IgG for cytomegalovirus (CMV) was found in 73% of UC patients in Wuhan, China and 89% of CD patients, and the rate in the healthy population was 50.69%[13]. Data from Peking Union Medical College Hospital indicated that the CMV infection rate in UC patients undergoing surgical operations was 46.2%[14], compared to 36.7% among refractory UC patients in Tianjin[15]. A multicenter prospective study conducted in Japan included 570 IBD patients who were followed for one year, and the authors observed 52 cases (9.1%) of opportunistic infection[7]. Separately, a national study in France found that the

Table 1 Opportunistic infections in inflammatory bowel disease patients evaluated between January 1, 2017 and December 31, 2018

Organism	Number of UC patients	Number of CD patients
Mycobacterium tuberculosis	1	0
Epstein-Barr virus	10	5
Cytomegalovirus	3	1
Hepatotropic virus	0	1
Clostridium difficile	11	13
Adenovirus	0	3
Syncytial virus	5	11
Coxsaccie virus	1	2
Herpes simplex virus	3	2
Herpes zoster	0	1
Mold	6	3
Candida albicans	2	0
Streptococcus	1	2

Three patients were co-infected with Epstein-Barr virus (EBV) and respiratory syncytial virus (S virus). Two patients were co-infected with EBV and mold. One patient was co-infected with EBV and *Clostridium difficile* (CDI). One patient was co-infected with EBV and cytomegalovirus (CMV). One patient was co-infected with EBV and herpes simplex virus (HSV). One patient was co-infected with EBV, S virus, and Coxsaccie virus (C virus). One patient was co-infected with mold and CDI. One patient was co-infected with adenovirus and S virus. One patient was co-infected with adenovirus and C virus. One patient was co-infected with CDI and C virus. One patient was co-infected with CDI and S virus. One patient was co-infected with CDI, mold, and Streptococcus. UC: Ulcerative colitis; CD: Crohn's disease.

incidence of opportunistic infections in IBD patients was 7.9% after five years of follow-up[16]. The high incidence of opportunistic infection in IBD patients in China may be related to the level of economic development and the abuse of antibiotics. In most regions of China, expensive biological agents are not covered by medical insurance, which limits their extensive use in China. However, most immunosuppressants, such as steroids and azathioprine, are cheap and are accepted by the majority of IBD patients. China is known to be one of the countries with the worst rates of antibiotic abuse in the world. It is estimated that China produced 248000 tons of antibiotics in 2013[17], and the per capita consumption of antibiotics was 10 times that of the United States[18]. The direct consequence of abuse of antibiotics is widespread bacterial drug resistance, which results in an increased rate and severity of opportunistic infections.

Infection with *Clostridium difficile*, an anaerobic, gram-positive bacillus, is the most common nosocomial infection which was most commonly observed in the IBD patients in our study (9.64%). IBD patients are at an increased risk for infection with *Clostridium difficile*[19-23]. Compared with those with IBD alone, patients with *Clostridium difficile* and IBD had longer hospital stay, requiring colon surgery intervention[24,25]. The use of antibiotics is closely related to *Clostridium difficile* associated diarrhea. Antimicrobials may disrupt the normal gastrointestinal flora, leading decreased colonization resistance and allowing toxigenic strains of *Clostridium difficile* to cause diseases. The abuse of antibiotics in China is serious. In addition, IBD patients are often treated with antibiotics for IBD deterioration or immunosuppressive complications, so *Clostridium difficile* infection can easily occur. *Clostridium difficile* is an increasing problem in immunocompromised patients, which can lead to higher rates of colectomy and mortality[26]. *Clostridium difficile* infection initiates with disruption of normal colonic microbiota, for which IBD patients are at high risk, conferring them an additional risk of *Clostridium difficile* infection[27,28]. In our pooled analysis, *Clostridium difficile* has the highest infection rate among opportunistic infections in IBD patients due to the extensive use of antibiotics and immunosuppressive agents.

Our study found that disease severity was also a risk factor for opportunistic infections in IBD patients. Previously, the relationship between disease activity and the incidence of opportunistic infections was not clear. We speculate that when IBD patients progress to late-stage disease, a nonspecific inflammatory reaction is initiated in the gut mucosa, promoting pathogen invasion. The invasion of pathogens subsequently aggravates intestinal inflammation, creating a positive feedback loop. Furthermore, the increased energy consumption and low immunity state induced by

Table 2 Demographic and clinical features of inflammatory bowel disease patients with (cases) and without (controls) opportunistic infection

	Cases (*n* = 70)	Controls (*n* = 140)	*P*-value
Median age	45.67 ± 15.79	44.13 ± 14.21	0.45
Gender			0.11
Male	39	89	
Female	31	51	
Type of IBD			0.82
UC	33	66	
Proctitis	4	24	
Left-sided	7	15	
Extensive	22	27	
CD	37	74	
Ileitis (L1)	9	23	
Colitis (L2)	6	3	
Ileocolitis (L3)	17	26	
Upper gastrointestinal tract (L4)	1	4	
L1 + L4	0	5	
L2 + L4	0	2	
L3 + L4	4	11	
Duration of IBD (yr)	6.42 ± 6.01	5.12 ± 4.04	0.28
Duration of medication (yr)	2.73 ± 2.02	2.54 ± 2.11	0.71
Prior surgery	25	41	0.31
Smoking	2	13	0.11

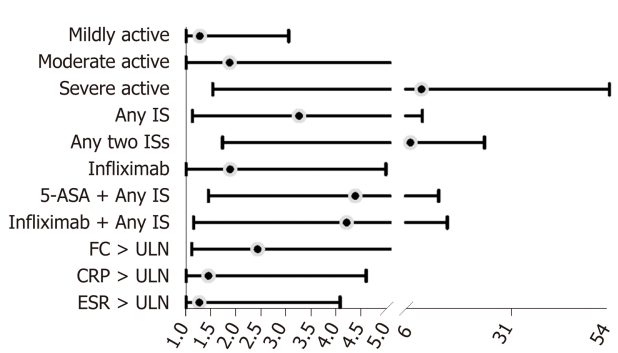
L1; Ileitis; L2: Colitis; L3: Ileocolitis; L4: Upper gastrointestinal tract; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

inflammation diminish IBD patients' ability to resist external pathogens, leading to further opportunistic infection.

A reliable measure of long-term outcome is paramount in predicting the course of the disease, responsiveness to treatment, potential for complications, and need for hospitalization and/or surgery[29]. Many studies have shown that mucosal healing is the best predictor of positive long-term outcomes[30-33]. Endoscopy is currently regarded as the gold standard test for the assessment of mucosal healing[34]. However, its invasive nature and high cost make it an unfeasible modality for frequent monitoring. Calprotectin is an abundant calcium-binding protein, which is derived mainly from neutrophils and a lesser extent monocytes and reactive macrophages. FC is a sensitive marker of intestinal inflammation in IBD, as its concentration reflects the migration of neutrophils into the intestinal cavity[35]. Thus, FC has emerged as a novel diagnostic tool to detect and monitor intestinal inflammation and reflect disease activity in IBD. Measurements for FC are simple, rapid, specific, sensitive, and inexpensive compared to its counterparts[36,37]. Along with CRP, ESR, and FC, our study discovered that elevated FC was a risk factor for opportunistic infections in IBD patients. One explanation for this finding may be that the level of FC serves as an indicator for intestinal inflammation and therefore, disease activity. This makes FC level a more sensitive and specific marker than CRP and ESR. When the level of FC in IBD patients is elevated, it suggests that intestinal inflammation has occurred and the disease is in an active stage, conferring the patient to opportunistic infections.

In our study, immunosuppressive agents included methotrexate, 6-mercaptopurine, azathioprine, thalidomide, tacrolimus and steroids. From this study, we have found that the use of immunosuppressive medications alone increased the risk of an opportunistic infection about 3.247-fold. When any two immunosuppressive medications were used in conjunction, the risk was increased greatly to about 6.457-fold. This result from our study is consistent with recent results in the literature[7,8,38,39]. Additionally, it was determined that when immunosuppressive medications were combined with 5-ASA or IFX, the risk of opportunistic infection was increased greatly to about 4 to 5 folds. This conclusion is consistent with the findings reported by Tourner *et al*[7], Lawrance *et al*[40], and Kirchgesner *et al*[16].

The relationship between biologics and opportunistic infections has not been



	Odds ratio (95%CI)	*P* value
Mildly active	1.274 (0.533-3.041)	0.586
Moderate active	1.870 (0.600-5.825)	0.280
Severe active	9.090 (1.532-53.941)	0.015
Any IS	3.247 (1.128-9.341)	0.029
Any two ISs	6.457 (1.726-24.152)	0.006
Infliximab	1.878 (0.709-4.974)	0.205
5-ASA + Any IS	4.367 (1.446-13.192)	0.009
Infliximab + Any IS	4.193 (1.151-15.274)	0.030
FC > ULN	2.423 (1.113-5.273)	0.026
CRP > ULN	1.445 (0.456-4.583)	0.532
ESR > ULN	1.266 (0.395-4.064)	0.691

Figure 1 Risk factors for opportunistic infection in inflammatory bowel disease patients (multivariate analysis). 5-ASA: 5-aminosalicylic acid; IS: Immunosuppressant (steroids, thiopurine, thalidomide, tacrolimus, or methotrexate); FC: Fecal calprotectin; CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; ULN: Upper limit of normal; CI: Confidence interval. *P*-values and 95% confidence intervals (CIs) for mild, moderate, and severe disease were compared with remission. *P*-values and 95%CIs for any immunosuppressant (IS), any two IS, infliximab, 5-aminosalicylic acid (5-ASA) + any IS, and infliximab + any IS were compared with 5-ASA.

clearly established. Some studies[41-44] indicated that biologics increase the incidence of opportunistic infections, while others do not support this conclusion[45,46]. In our study, we found no association between the use of IFX alone and the increased risk of opportunistic infections. One possible explanation is that IFX may not cause opportunistic infections within the one-year period of the study (shorter than the previous five-year period)[7].

Despite the important findings, this study had several limitations. First, the sample size of our study was small. A larger sample size would have allowed for more accurate estimation of the incidence of opportunistic infections and increase in reliability of risk factors in Chinese IBD patients. Second, our study was a single-center clinical study, which cannot represent the situation of IBD patients in China as a whole.

In conclusion, severe disease activity, elevated levels of FC, and immunosuppressive medications, especially when used in combination, are risk factors for opportunistic infections in IBD patients. The use of IFX alone has nothing to do with opportunistic infections. With the increasing use of immunosuppressants in IBD and the advocacy of combination therapy, patients and physicians need to pay attention to and prevent opportunistic infections. As the genetic background, living environment, lifestyle, and diet of IBD patients in China are different from those in Western countries, our study of opportunistic infection could provide vital information for clinicians and IBD patients in China and other countries.

Table 3 Risk factors for opportunistic infection in inflammatory bowel disease patients (univariate analysis)

	Cases (*n* = 70)	Controls (*n* = 140)	*P*-value	Odds ratio	95%CI
Disease activity					
Remission	25	85		Reference	
Mildly active	20	40	0.145	1.673	0.837-3.345
Moderate	14	13	0.004	3.604	1.505-8.626
Severe	11	2	<0.001	18.404	3.833-88.375
Treatment					
5-ASA	20	83		Reference	
Any IS	8	12	0.047	2.668	1.012-7.033
Any two IS	10	4	<0.001	10.375	2.948-36.508
Infliximab	13	24	0.109	1.992	0.857-4.632
5-ASA + any IS	11	9	0.002	5.072	1.853-13.887
Infliximab + any IS	9	7	0.003	5.336	1.773-16.058
FC > ULN	57	69	<0.001	4.431	2.265-8.667
CRP > ULN	26	18	<0.001	3.98	1.994-7.944
ESR > ULN	25	18	<0.001	3.744	1.87-7.494

5-ASA: 5-aminosalicylic acid; IS: Immunosuppressant (steroids, thiopurine, thalidomide, tacrolimus, or methotrexate); FC: Fecal calprotectin; CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; ULN: Upper limit of normal; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Opportunistic infection refers to any infection caused by a weakened immune system and typically does not occur in people with normal immune function. When they occur, patients presenting with opportunistic infections commonly display a significantly increased rate of morbidity and mortality. A number of studies have been conducted in Western countries and Japan to investigate the incidence of and risk factors for opportunistic infection in inflammatory bowel disease (IBD) patients. Currently, there are few epidemiological data on the rate of opportunistic infection in IBD patients in China. The risk factors for opportunistic infection in Chinese IBD patients remain unclear.

Research motivation

The main topics in our study are to predict the incidence of opportunistic infections related to IBD in China, and explore the risk factors for opportunistic infections. The key problems to be solved is to ensure compliance of enrolled IBD patients. The significance of solving these problems for future research in this field is to more accurately predict the incidence of and risk factors for opportunistic infections.

Research objectives

The main objectives in our study were to predict the incidence of opportunistic infections related to IBD in China, and explore the risk factors for opportunistic infections. The realized objectives include that the incidence of opportunistic infection in IBD patients in China is higher than that in Western countries and factors such as severe IBD, elevated levels of fecal calprotectin, and the use of immunosuppressive medications, especially when used in combination, are major risk factors for opportunistic infections in IBD patients, according to our single-center study. The significance of realizing these objectives for future research in this field is to alert patients and physicians to pay attention to and prevent opportunistic infections. Meanwhile, our study can provide important information for clinicians and IBD patients in China and other countries.

Research methods

The research design that was adopted to realize the objectives is observational study and nested case-control study. Observational study is to observe and record the characteristics of research objects in a natural state, and describe and compare the results. In our study, the patients were followed for up to 12 mo to identify the incidence of infections. In nested case-control studies, cases and controls come from the same cohort, so the selection bias in effect estimation is reduced and comparability is good. For each infected IBD patient, two non-infected IBD patients were selected as controls in our study.

Research results

Our study found that the incidence of opportunistic infection in IBD patients in China is higher than that in Western countries and factors such as severe IBD, elevated levels of fecal calprotectin, and the use of immunosuppressive medications, especially when used in combination, are major risk factors for opportunistic infections in IBD patients. Meanwhile, the

use of infliximab alone does not increase the risk of opportunistic infection. Our findings remind patients and physicians to pay attention to and prevent opportunistic infections. As the genetic background, living environment, lifestyle, and diet of IBD patients in China are different from patients in Western countries, our study of opportunistic infection could provide important information for clinicians and IBD patients in China and other countries. The following problems remain to be solved: (1) Our study was a single-center clinical study, which cannot represent the situation of IBD patients in China as a whole; and (2) the sample size of this study for both ulcerative colitis and Crohn's disease patients was small. A larger sample size would have allowed for more precise estimation of the incidence of opportunistic infections and increase in reliability of risk factors in Chinese IBD patients.

Research conclusions

The new findings of this study are that the incidence of opportunistic infection in IBD patients in China is higher than that in Western countries, according to our single-center study, and factors such as severe IBD, elevated levels of fecal calprotectin, and the use of immunosuppressive medications, especially when used in combination, are major risk factors for opportunistic infections in IBD patients. Meanwhile, the use of infliximab alone does not increase the risk of opportunistic infection. The original insights into the current knowledge that this study offered is when opportunistic infection occurs, patients commonly display a significantly increased rate of morbidity and mortality. Therefore, prevention and identification of opportunistic infections are critical. The implications of this study for clinical practice in the future is patients and physicians need to pay attention to and prevent opportunistic infections.

Research perspectives

From this study, we can conclude that the incidence of opportunistic infection in IBD patients in China is higher than that in Western countries, and doctors should pay attention to and prevent the incidence of opportunistic infections. The future research direction is to conduct a multi-center study to evaluate the incidence of opportunistic infection in Chinese IBD patients, and more accurately screen out the risk factors leading to the occurrence of opportunistic infection in Chinese IBD patients, so as to effectively prevent the occurrence of opportunistic infection.

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META-ANALYSIS

Effect of prophylactic clip placement following endoscopic mucosal resection of large colorectal lesions on delayed polypectomy bleeding: A meta-analysis

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Abstract

BACKGROUND

The role of prophylactic clipping for the prevention of delayed polypectomy bleeding (DPB) remains unclear and conclusions from prior meta-analyses are limited due to the inclusion of variety of resection techniques and polyp sizes.

AIM

To conduct a meta-analysis on the effect of clipping on DPB following endoscopic mucosal resection (EMR) of colorectal lesions ≥ 20 mm.

METHODS

We performed a search of PubMed and the Cochrane library for studies comparing the effect of clipping *vs* no clipping on DPB following endoscopic resection. The Cochran *Q* test and I^2 were used to test for heterogeneity. Pooling was conducted using a random-effects model.

RESULTS

Thirteen studies with a total of 7794 polyps were identified, of which data was available on 1701 cases of EMR of lesions ≥ 20 mm. Prophylactic clipping was associated with a lower rate of DPB (1.4%) when compared to no clipping (5.2%) (pooled OR: 0.24, 95%CI: 0.12-0.50, $P < 0.001$) following EMR of lesions ≥ 20 mm. There was no significant heterogeneity among the studies ($I^2 = 0\%$, $P = 0.67$).

CONLUSION

Prophylactic clipping may reduce DPB following EMR of large colorectal lesions. Future trials are needed to further identify risk factors and stratify high risk cases in order to implement a cost-effective preventive strategy.

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Core tip: The role of prophylactic clipping for the prevention of delayed polypectomy bleeding (DPB) remains unclear and conclusions from prior meta-analyses are limited due to the inclusion of variety of resection techniques and polyp sizes. We conducted a meta-analysis that included 7794 polyps in 1701 cases of endoscopic mucosal resection (EMR) and found that prophylactic clipping may reduce DPB following EMR of large colorectal lesions. Future trials are needed to further identify risk factors and stratify high risk cases in order to implement a cost-effective preventive strategy.

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INTRODUCTION

Colonoscopy has been shown to decrease the risk of death from colorectal cancer through the early identification and removal of pre-malignant or early stage cancerous lesions[1]. Endoscopic resection (ER) is the preferred first-line treatment for most of these superficial neoplasms and is associated with lower costs, morbidity, and mortality when compared to surgery[2,3]. Most colonic polyps are less than 10 mm and can be safely and effectively resected with conventional snare polypectomy. Conversely, larger lateral spreading lesions (LSLs) or sessile polyps, particularly those ≥ 20 mm in size, are usually removed by endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD). While ESD continues to gain traction as an alternative for lesions with suspected superficial invasion or subtypes of non-granular LSLs[4,5], its definitive role in Western clinical practice is yet to be defined. Hence, wide-field EMR remains the preferred therapy for large non-cancerous colorectal lesions.

Bleeding is the most common adverse event following ER of colorectal lesions. Bleeding can be immediate (during the procedure) or delayed (post-operatively), and has been estimated to occur in 1%-6% of cases[6,7]. In the absence of coagulopathy, the risk of delayed polypectomy bleeding (DPB) is nearly negligible for the resection of small polyps < 10 mm. Conversely, the incidence of DPB increases with polyp size[8-10]. Several studies have evaluated the effect of prophylactic clipping on DPB following ER, with mixed results[11-13]. The inclusion of small polyps and different ER techniques (*i.e.*, conventional polypectomy, EMR, ESD) significantly limits the interpretability of the data. The primary aim of this study was to conduct a meta-analysis on the effect of prophylactic clipping on DPB following EMR of colorectal lesions ≥ 20 mm. A secondary aim was to evaluate the effect of clipping on the incidence of adverse events following colorectal ER.

MATERIALS AND METHODS

Search strategy and study selection

We identified studies through a literature search of two databases (MEDLINE through PubMed and the Cochrane Library) with the last search performed in January 2018. The PubMed search strategy was constructed by using the following string of search terms: ("clip" OR "clipping") AND ("colon" OR "colorectal" OR "colonic") AND ("endoscopic"). The search of the Cochrane library was conducted using similar search terms. A review of the reference list of included studies was performed to identify any relevant articles missed through the original search strategy. Titles and abstracts were screened by two investigators (F.A. and D.R.W) for relevance to the study. The full text of potentially eligible studies was subsequently reviewed by the two investigators (F.A and D.R.W). Disagreements were resolved by

consensus or by consulting with a third investigator (D.Y).

Inclusion and exclusion criteria

Studies eligible for inclusion were: (1) Prospective or retrospective, case-control, or cohort studies and clinical trials; (2) studies reporting incidence of DPB following ER; and (3) those that included outcomes on both patients with prophylactic clipping *vs* non-clipping after resection. Exclusion criteria were: (1) Case reports; (2) single arm retrospective or prospective case series; (3) studies not reporting incidence of DPB; (4) reviews, commentaries, surveys; and (5) duplicate studies.

Data extraction

Data from each eligible study were extracted using a standardized data extraction sheet. The extracted data included: (1) Study authors; (2) year of publication; (3) setting (location); (4) study period; (5) patient demographics (age, gender); (6) number of patients/lesions; (7) lesion characteristics (size, location, morphology); (8) type of ER (conventional polypectomy, EMR, ESD); (9) incidence of adverse events, including DPB and perforation; and (10) follow-up period.

Outcomes and definitions

The aim of this study was to conduct a meta-analysis studying the effect of prophylactic clipping on DPB following EMR of colorectal lesions ≥ 20 mm. A secondary aim was to evaluate the effect of prophylactic clipping on the incidence of adverse events following colorectal ER. Prophylactic clipping was defined as endoscopic clipping performed with the aim of reducing the risk of delayed (post-operative) adverse events. DPB was defined as bleeding occurring post-operatively (upon conclusion of the ER and after scope withdrawal from the patient). Conventional polypectomy was defined as removal of a colorectal lesion with a forceps or snare without prior submucosal injection. In contrast, EMR was defined as resection achieved by first lifting the target lesion with a submucosal injection followed by snare polypectomy. ESD was defined as any resection in which submucosal dissection was performed.

Assessment of methodologic quality

For prospective trials, the quality of each study was assessed using the risk-of-bias tool as outlined in the Cochrane Handbook for Systematic Reviews of Interventions (version 5.1.0). The methodologic quality of retrospective studies was assessed using the Newcastle-Ottawa scale[14]. The quality of all studies was assessed by 3 investigators (F.A, D.R.W, J.J.F). Funnel plots were generated to evaluate for any potential publication bias. Visual inspection of the funnel plot was used detect significant publication bias when less than 10 studies were available for meta-analysis as recommended by the Cochrane Handbook. Egger's regression test was used when more than 10 studies were included in the meta-analysis.

Statistical analysis

We obtained or calculated the proportions and 95%CI for each categorical variable and the mean or median for continuous data when possible. The pooled means and OR were calculated utilizing a random effects model. The random effects model was used regardless of underlying statistical testing of heterogeneity since it provides more conservative estimations of the pooled effects that are more likely to contain the true effect. The Cochran Q test and I^2 were used to assess heterogeneity of included studies. I^2 values of < 25%, 25%-50% and > 50% were considered to represent low, moderate and high heterogeneity, respectively. P values < 0.05 were considered significant and all tests were two tailed. The study was performed in accordance with the PRISMA recommendations for reporting systematic reviews and meta-analyses. Analysis was conducted using Stata, version 15 (Stata Corp, College Station, TX, United States) and RevMan 5.3 (The Cochrane Collaboration, Copenhagen).

RESULTS

Search results

Figure 1 depicts the study selection flow diagram. Overall, 255 studies were identified using our search strategy, of which 110 were duplicates. Of the remaining 145 studies, 120 were excluded after screening titles and abstracts. Full text review was then performed on 25 studies using the predefined inclusion and exclusion criteria, after which 13 studies were retained. Of the 13 studies, 7 were randomized control trials (RCTs)[13,15-21] and 6 were cohort studies (2 prospective, 4 retrospective)[11,12,22-25]. Studies were published between 2003 and 2017. Nine studies were conducted in Asia, 2 in

Europe, and 2 in the United States. These 13 studies were included in the meta-analysis evaluating the impact of prophylactic clipping on adverse events following colorectal ER. Of these, 4 studies with available data on specific parameters (lesion size, type of ER, clipping *vs* no clipping, incidence of DPB) were included in the analysis on the effects of prophylactic clipping on DPB after EMR of lesions ≥ 20 mm.

Study characteristics are summarized in Table 1. Colorectal ER was performed in 7794 polyps, of which 3567 (45.8%) underwent prophylactic clipping. Out of the 13 studies identified, 7 studies excluded all pedunculated polyps whereas 1 study did not report details on polyp morphology[23]. Of the remaining six studies, 3772 out of 5225 polyps (72%) were reported as pedunculated. Eleven studies specified that the lesion located in the right colon (2695 out of 6309; 42.7%). Overall, 7 studies included data on EMR only, 3 studies reported outcomes on both EMR and conventional polypectomy, 2 on ESD alone, and 1 on both ESD and EMR. Most lesions (82%; 6377) were removed by EMR, followed by conventional polypectomy (14%; 1118), and ESD (4%; 299). While several studies reported the number of patients in each group (clipping *vs* non-clipping), a few studies only described the number of lesions in each arm[11,12,15,18,22-25]; hence, the number of lesions was used in the analysis.

Quality assessment

The risk of bias in the 6 nonrandomized studies was evaluated according to the Newcastle-Ottawa assessment scale (Supplementary Table 1). The average quality score was 8 out of the highest possible score of 9. Five of the 6 included cohort studies were of high methodological quality (score 8-9/9), and 1 was of low quality (score 4-5/9). The risk of bias for the 7 RCTs is shown in Supplementary Table 2. Blinding of participants and personnel was not performed in any of the included RCTs. Methods for random sequence generation and allocation concealment were described by 5 studies. All RCTs were found to have adequate assessment of incomplete outcomes and avoided selective reporting.

Meta-analysis results

Effect of prophylactic clipping on DPB following EMR of colorectal lesions ≥ 20 mm: Of the 13 studies on colorectal ER, data from 4 studies were available to evaluate the incidence of DPB after EMR of lesions ≥ 20 mm[11,21-23]. In all, clipping was performed in 592 (34.8%) cases of the 1701 EMRs of lesions ≥ 20 mm. Clipping was associated with a lower incidence of DPB (8 out of 592; 1.4%) when compared to no clipping (58 out of 1109; 5.2%) (pooled OR: 0.24, 95%CI: 0.12-0.50, $P < 0.001$). There was little heterogeneity among the included studies ($I^2 = 0\%$, $P = 0.67$) (Figure 2A). There was no evidence of substantial publication bias based on visual inspection of the funnel plot (Figure 2B).

Effect of prophylactic clipping on the incidence of adverse events following colorectal ER: DPB, the incidence of DPB was reported in all 13 studies included in the meta-analysis. The overall pooled incidence of DPB was 2.1% (160 out of 7794 lesions) (Table 2). DPB was reported in 46 (1.3%) cases with prophylactic clipping as compared to 114 (2.7%) in the non-clipping arm (pooled OR: 0.50; 95%CI: 0.25-0.91, $P = 0.02$) (Figure 3A). A sensitivity analysis was performed by using patient instead of lesion numbers when available and this did not alter the overall pooled outcome (pooled OR 0.49; 95%CI: 0.27-0.89, $P = 0.02$). There was significant heterogeneity among the included studies ($I^2 = 50\%$, $P = 0.03$). When only RCTs were included in the analysis, compared with no clipping, the pooled OR for DPB with clipping was 0.77 (95%CI: 0.36-1.65, $P = 0.51$), suggesting no significant difference between the two groups (Figure 3A). However, there was moderate heterogeneity among these RCT results ($I^2 = 42\%$, $P = 0.12$). In all, there was no evidence of substantial publication bias based on the visual inspection of the funnel plot and Egger's regression test ($P = 0.57$) (Figure 3B).

Perforation following Colorectal ER. Eight studies evaluated the rate of perforation following ER. No cases of perforation were reported in six studies, whereas the remaining two observed a total of 2 cases of perforation in each group (clipping *vs* non-clipping). Hence, the overall pooled rate for perforation was 0.19% (4 out of 2031 lesions), with no significant difference between the two groups (pooled OR: 1.05; 95%CI: 0.15-7.48, $P = 0.96$).

Subgroup analyses

Lesion Size ≥ 20 mm: Eight studies with available data on outcomes for lesions ≥ 20 mm included 910 cases with clipping and 1445 without clipping following ER (EMR or ESD). The overall pooled rate of DPB was 3.8% for lesions ≥ 20 mm. Prophylactic clipping of lesions ≥ 20 mm was associated with a lower rate of DPB when compared to no clipping (1.8% *vs* 5.1%) (pooled OR: 0.33, 95%CI: 0.18-0.62, $P < 0.001$), with no

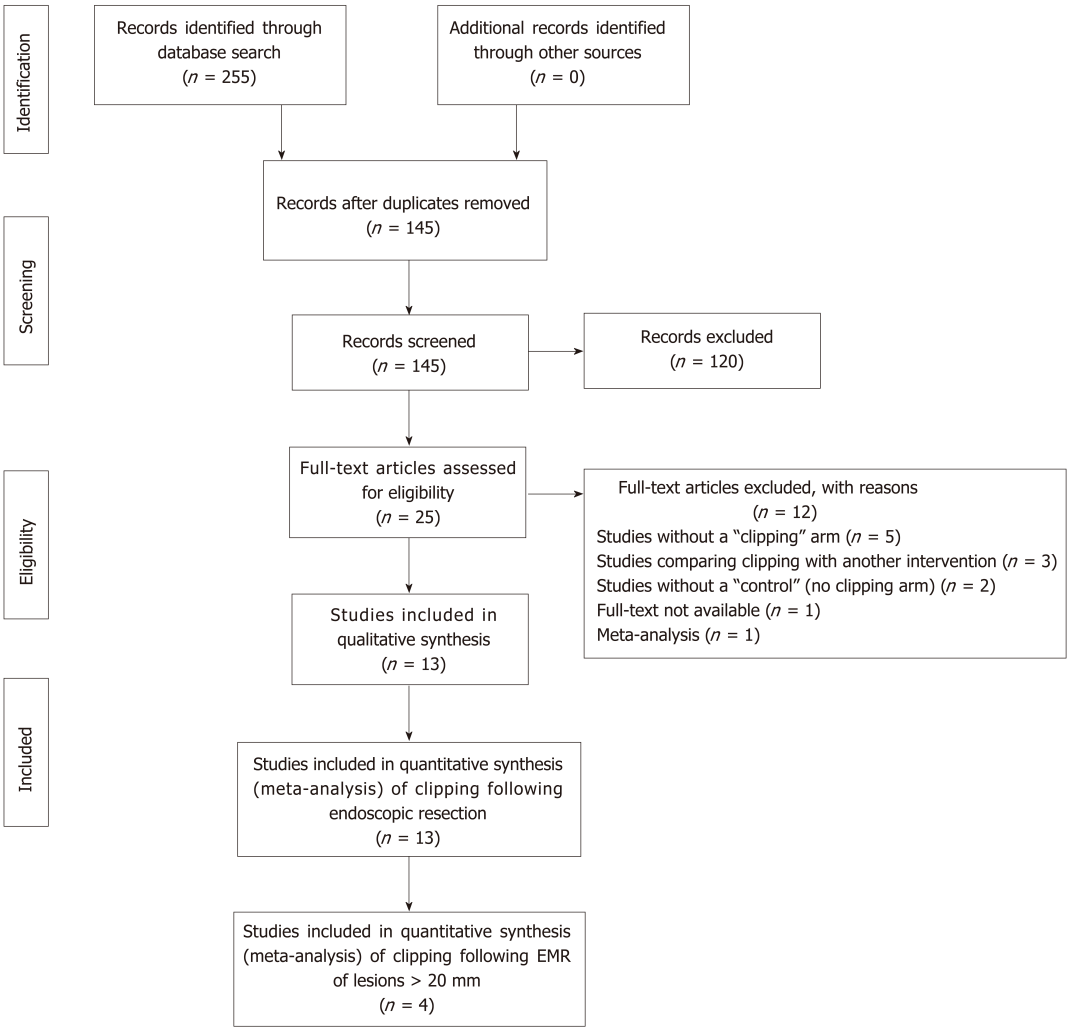

Figure 1 PRISMA flow diagram.

significant heterogeneity among the available studies (I^2 = 10%, P = 0.36) (Figure 4).

Polyp morphology (pedunculated) and right-colon location: Out of the 13 studies included in the meta-analysis, only two studies specified outcomes on DBP for pedunculated polyps[16,21]. The pooled incidence for DPB in pedunculated polyps from these two studies did not show a difference between clipping (1.1%) *vs* no clipping (1.1%) (pooled OR: 0.77, 95%CI: 0.17-3.46, P = 0.73). Only 1 study specified the incidence of DPB in right-sided colonic lesions. The authors did not report a significant difference in the rate of DPB between the two groups (1.3% with clipping *vs* 6% without clipping; OR: 2.28, 95%CI: 0.79-6.58, P = 0.13)[16].

DISCUSSION

DPB is the most common adverse event following ER of colorectal lesions. Prophylactic clipping has been suggested as a strategy for the prevention of DPB, although prior data has been marred by conflicting findings. The results from this meta-analysis suggests that endoscopic clipping may be associated with a lower occurrence of DPB after colorectal EMR of lesions ≥ 20 mm in size.

Nishizawa *et al*[26] recently reported the results of their meta-analysis on the effect of prophylactic clipping after colorectal ER. A total of 7 RCTs with 3059 cases were included. In their study, the rate of DPB was similar between cases with clipping (2.1%) *vs* no clipping (2.7%) (OR 0.76; 95%CI: 0.39-1.47; P = 0.414). Similarly, when only RCTs were included in our meta-analysis, clipping did not affect the rate of DPB when compared to no clipping after ER (OR 0.77; 95%CI: 0.36-1.65, P = 0.51). However, it is important to highlight that nearly all of the cases included in these

Table 1 Study characteristics

Study	Study design	Country	Endoscopic Resection (*n*)			Intervention	Age, mean ± SD	Gender (M/F)	Patients (*n*)	Lesions (*n*)	Lesion Size in mm, mean ± SD	Pedunculated (*n*)	Right colon (*n*)
			CP	EMR	ESD								
Shioji *et al*[20], 2003	RCT	Japan	-----	413	----	Clip	64 ± 9	118/38	156	205	7.8 ± 3.9	67	97
						Non-Clip	63 ± 12	130/37	167	208	7.8 ± 4.1	65	90
Kaltenbach *et al*[25], 2007	Cohort	United States	-----	125	----	Clip	68 ± 9	100/0	Not reported	49	16.7 ± 7	Excluded	49
						Non-clip				76			0
Dior *et al*[23], 2012	Cohort	France	------	139	----	Clip	66 (23-90)[1]	76/62	Not reported	75	Not reported	Not reported	63
						Non-clip				64			
Liaquat *et al*[11], 2012	Cohort	United States	------	472	----	Clip	67.1 ± 10.9	250/213	Not reported	225	31 (20-100)[1]	Excluded	273
						Non-clip				247			
Matsumoto *et al*[12], 2012	Cohort	Japan	403		----	Clip	63 ± 12	140/135	Not reported	174	27.1 ± 9.6	Excluded	Not reported
						Non-clip				229			
Mori *et al*[18], 2014	RCT	Japan	------	148	----	Clip	Not reported	Not reported	Not reported	73	15.3 ± 2.84	24	9
						Non-clip				75	15.5 ± 2.60	24	10
Tominaga *et al*[21], 2014	RCT	Japan	------	801	----	Clip	67 (22-88)[1]	151/60	211	385	7.7 (5-30)[1]	229	79
						Non-clip	66.6 (15-94)[1]	148/68	216	416	8.5 (5-35)[1]	245	114
Dokoshi *et al*[15], 2015	RCT	Japan	54	234	----	Clip	67.1 ± 8[2]	109/45	Not reported	154	< 10 mm: 98, 10-20 mm: 48, > 20 mm: 8	41	73
						Non-clip	67.8 ± 11[2]	99/35		134	< 10 mm: 86, 10-20mm: 48, > 20 mm: 6		
Zhang *et al*[13], 2015	RCT	China	----	286	62	Clip	67.9 ± 12.6	112/62	174	174	10-20 mm: 111, 20-40 mm: 63	Excluded	22
						Non-clip	64.2 ± 9.8	107/67	174	174	10-20 mm: 107, 20-40 mm: 67		27
Albéniz *et al*[22], 2016	Cohort	Spain	----	1056	----	Clip	67.9 ± 10.9	770/444	Not reported	281	30.5 ± 11.8	Excluded	Not reported
						Non-clip				775			
Matsumoto *et al*[16], 2016	RCT	Japan	1064	2300	----	Clip	65 (25-87)	534/218	752	1636	< 5 mm: 388, > 5 mm: 1248	1467	823
						Non-clip	66 (25-88)	513/234	747	1728	< 5 mm: 447, > 5 mm: 1281	1595	845
Osada *et al*[19], 2016	RCT	Japan	----	----	26	Clip	68.8 ± 8.7	9/4	13	13	677.2 ± 306[3]	Excluded	Not reported
						Non-clip	66.2 ± 10.4	7/6	13	13	790 ± 220[3]		
Harada *et al*[24], 2017	Cohort	Japan	----	----	211	Clip	70.7 ± 9.2	124/87	Not reported	123	< 30 mm: 65, 30-60 mm: 58, > 60 mm: 2	14	50

	Non-Clip	88	< 30 mm: 23, 30-60 mm: 53, > 60 mm: 12

[1]Range;
[2]Standard error;
[3]Area in mm^2. RCT: Randomized controlled trial; CP: Conventional polypectomy; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

RCTs (2847 out of 3059; 93%) involved polyps < 20 mm in size. DPB is a rare occurrence following ER of small colorectal lesions. Indeed, most if not all of these lesions can be safely and completely excised with conventional cold snare polypectomy with no risk for DPB[27,28]. Hence, it is not surprising that prophylactic clipping did not impact the rate of postoperative bleeding in patients included in those trials.

It is well known that the incidence of DPB is directly associated with lesion size, and has been more frequently reported after the resection of lesions ≥ 20 mm[8,29,30]. Nonetheless, the study by Nishizawa *et al*[26] did not report a difference in postoperative bleeding for lesions ≥ 20 mm with clipping *vs* no clipping (pooled OR 0.78; 95%CI: 0.23-2.68). The small number of cases with lesions ≥ 20 mm included in their study (97 with clipping and 115 without clipping) may have underpowered their analysis to detect any meaningful differences. In contrast, in effort to specifically evaluate the risk of DPB in lesions of clinically significant size, we included a total of 2355 polyps ≥ 20 mm in size. Our results demonstrated that clipping following the ER of lesions ≥ 20 mm was associated with a reduction in the risk of DPB when compared to no clipping (1.8% *vs* 5.1%, pooled OR: 0.33, 95%CI: 0.18-0.62, $P < 0.001$), with little heterogeneity among the studies ($I^2 = 10\%$, $P = 0.36$). Furthermore, given that colorectal lesions ≥ 20 mm are primarily removed with EMR, we specifically evaluated the risk of DPB in this group. Similarly, our meta-analysis demonstrated that clipping after EMR of lesions ≥ 20 mm significantly reduced the risk of bleeding when compared to no clipping (1.4% *vs* 5.2%; pooled OR: 0.24, 95%CI: 0.12-0.50, $P < 0.001$). When compared to conventional polypectomy, EMR, particularly when performed for the removal of larger lesions, inherently results in an extended residual mucosal defect[31]. Prophylactic clip closure of the defect reduces exposure of the submucosal tissue to the colonic luminal milieu, which may in turn reduce the risk of DPB and other adverse events, including abdominal pain and post-polypectomy syndrome[13].

Several issues remain to be addressed before this practice can be fully advocated. It is important to note that prophylactic clipping is not without its limitations. From a health economics standpoint, a prophylactic clipping strategy may not be cost effective and justifiable for all colorectal lesions removed by EMR[32]. Certainly, the added cost of clips and lengthier procedure should be weighed against the potential incremental expenditures associated with DPB (*i.e.*, emergency room visits, readmissions, need for transfusions, repeat therapeutic interventions). Given the above limitations, a strategy of clipping targeted to patient and/or lesion characteristics would likely prove most efficient. Patient characteristics that may warrant prophylactic clipping may include those requiring resumption of anti-coagulant or anti-thrombotic therapy following resection, those with a high comorbidity burden who may not hemodynamically tolerate significant hemorrhage or patients with low likelihood of post-procedural follow up and access to care[11,33]. Lesion characteristics that may benefit from clipping may include those that are larger than 20 mm, pedunculated, located in the right colon or a combination of the aforementioned factors. Future well-designed RCTs are needed to further define the role of prophylactic clipping in the prevention of DPB in select lesions, specifically after EMR of large colonic lesions.

This study has several strengths. Given that DPB often occurs following ER of larger lesions, we specifically evaluated the efficacy of prophylactic clipping with respect to lesion size. Furthermore, many studies on prophylactic closure for DPB do not differentiate between the types of endoscopic intervention (*i.e.*, EMR *vs* ESD), which significantly limits the interpretability of the results as both of these approaches are technically distinct and carry inherently different risks for post-procedural adverse events[34,35]. In this meta-analysis, we demonstrate that prophylactic clipping reduces the risk of DPB in arguably the most clinically significant group: lesions ≥ 20 mm removed with EMR. These observations have direct clinical implications as vast majority of these lesions in the West are approached with EMR.

Table 2 Incidence of delayed polypectomy bleeding and perforation following endoscopic resection

Author/Year	Endoscopic resection (*n*)			Intervention	Polyps	DPB	Perforation
	CP	EMR	ESD				
Shioji *et al*[20], 2003	-----	413	----	Clip	205	2	0
				No clip	208	2	0
Kaltenbach *et al*[25], 2007	-----	125	-----	Clip	49	0	0
				No clip	76	0	0
Dior *et al*[23], 2012	------	139	------	Clip	75	0	Not reported
				No clip	64	3	Not reported
Liaquat *et al*[11], 2012	------	472	-----	Clip	225	4	1
				No clip	247	24	1
Matsumoto *et al*[12], 2012	403		------	Clip	174	3	Not reported
				No clip	229	14	Not reported
Mori *et al*[18], 2014	------	148	-------	Clip	73	2	0
				No clip	75	0	0
Tominaga *et al*[21], 2014	-------	801	-------	Clip	385	4	Not reported
				No clip	416	9	Not reported
Dokoshi *et al*[15], 2015	54	234	-------	Clip	154	4	0
				No clip	134	3	0
Zhang *et al*[13], 2015	------	286	62	Clip	174	2	1
				No clip	174	12	1
Albéniz *et al*[22], 2016	------	1056	------	Clip	281	4	Not reported
				No clip	775	30	Not reported
Matsumoto *et al*[16], 2016	1064	2300	------	Clip	1636	18	Not reported
				No clip	1728	15	Not reported
Osada *et al*[19], 2016	------	------	26	Clip	13	0	0
				No clip	13	0	0
Harada *et al*[24], 2017	------	------	211	Clip	123	3	0
				No clip	88	2	0

CP: Conventional polypectomy; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

We also acknowledge the limitations of this study. All available studies reporting the effect of clipping on DPB were included in this meta-analysis in efforts to capture sufficient cases for subgroup analyses. The inclusion of cohort studies, in addition to RCTs, potentially introduces selection bias. Nonetheless, the overall quality of the included cohort studies was satisfactory based on the Newcastle-Ottawa scale and there was little heterogeneity among the studies. Furthermore, given that the main aim of the study was to evaluate DPB in lesions ≥ 20 mm following EMR, only a few studies were available, and thereby these results should be interpreted with caution and underscores the need of additional well-designed trials. Secondly, the lack of data on polyp morphology, location in the colon, and management of anti-coagulant/anti-thrombotic medications prior to ER in many of the included studies limited our ability to perform additional sub-analyses or draw any meaningful conclusions on these important subgroups.

In summary, this meta-analysis suggests that prophylactic clipping may reduce DPB after ER of colorectal lesions. Clip closure was associated with a significant reduction in the incidence of DPB in lesions ≥ 20 mm following EMR. Future trials are needed to further identify risk factors for DPB and help implement a cost-effective preventive strategy.

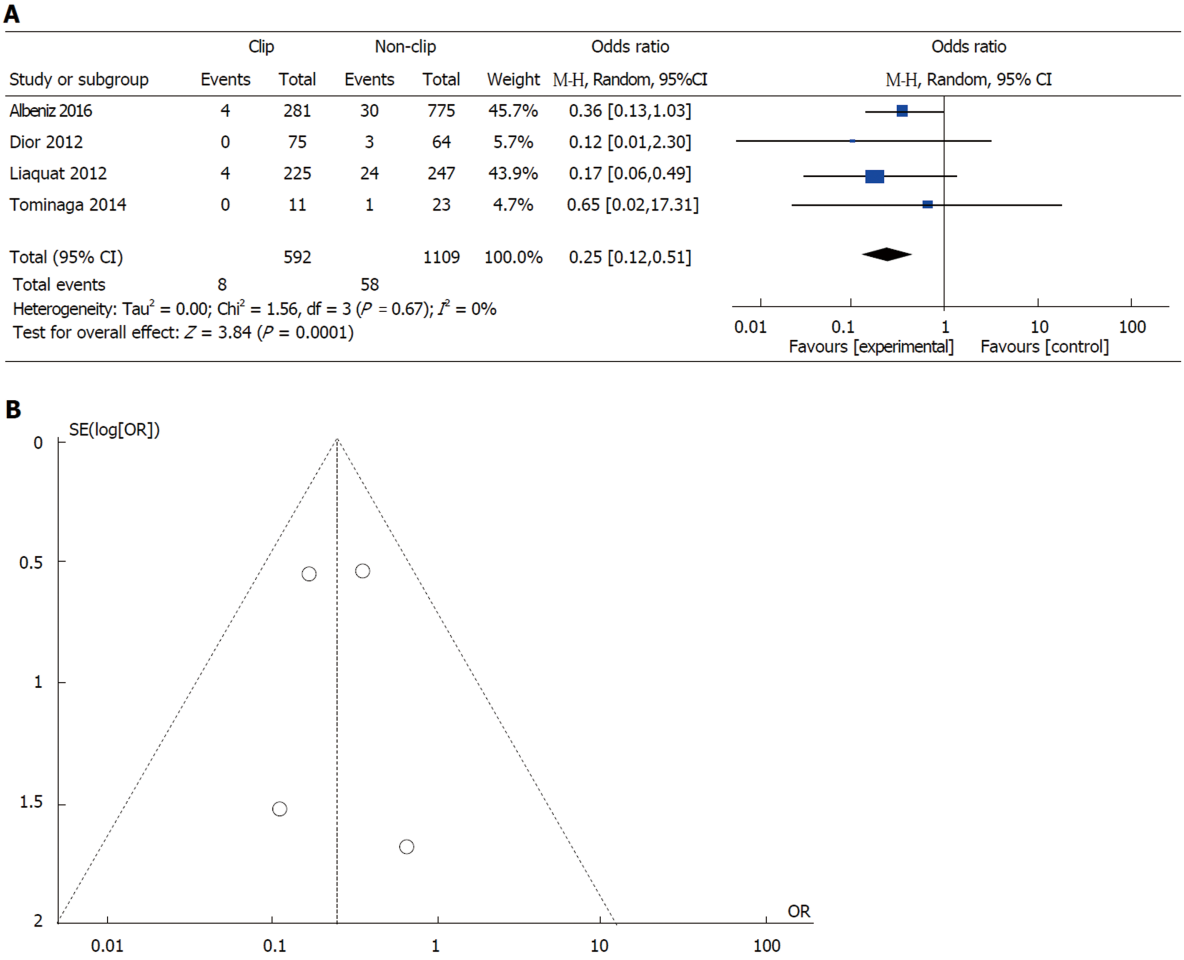

Figure 2 Forrest plot of the included studies evaluating the rate of delayed polypectomy bleeding after colorectal endoscopic mucosal resection of lesions ≥ 20 mm (A) and funnel plot of studies evaluating the rate of delayed polypectomy bleeding after colorectal endoscopic mucosal resection of lesions ≥ 20 mm (B).

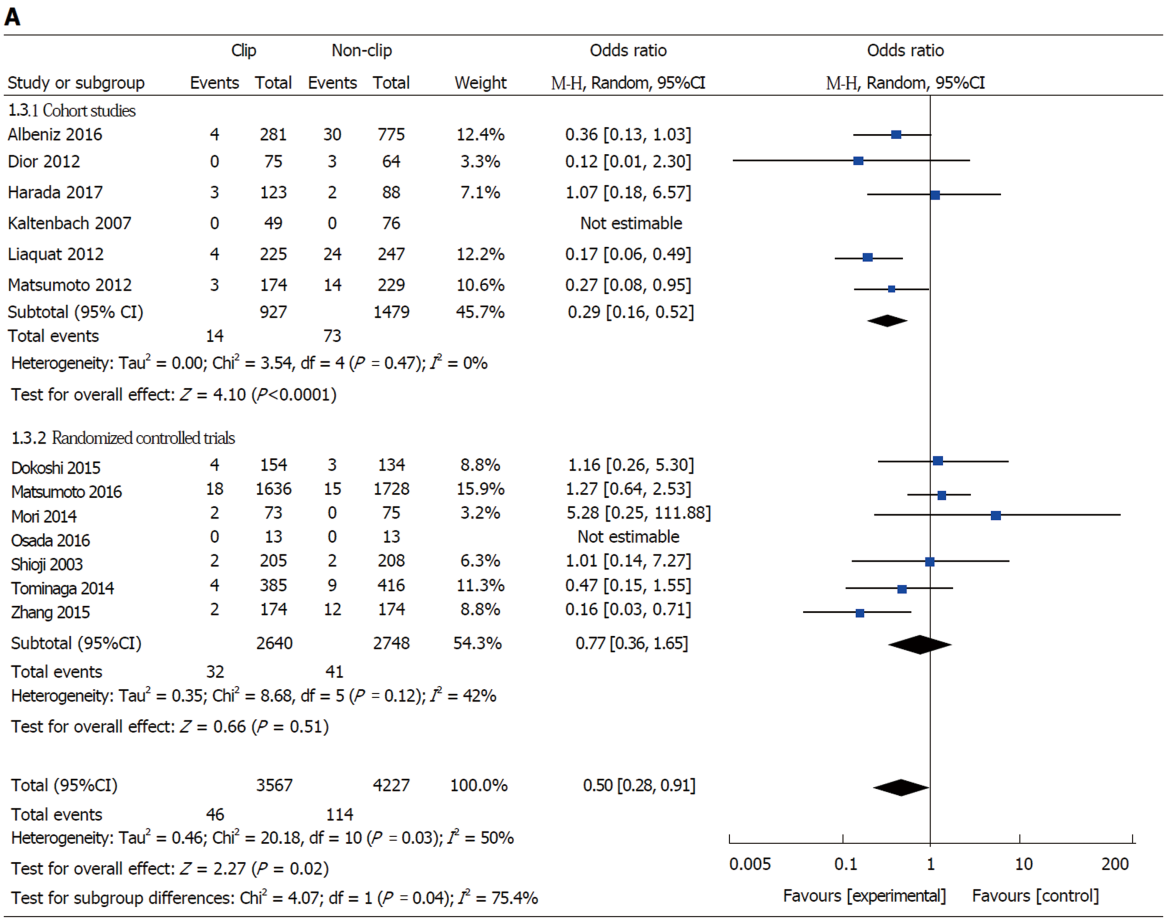

Figure 3 Forrest plots on the effect of prophylactic clipping on delayed polypectomy bleeding following colorectal endoscopic resection stratified by study type (A) and funnel plot of the included studies comparing the rate of delayed polypectomy bleeding between clipping *vs* no clipping (B).

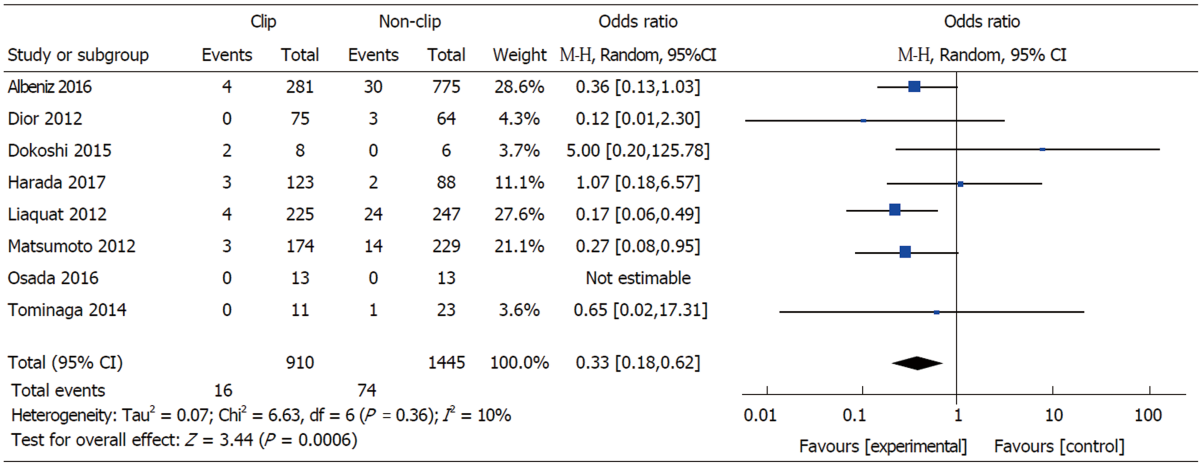

Figure 4 Forrest plot of the included studies evaluating the rate of delayed polypectomy bleeding for lesions ≥ 20 mm.

ARTICLE HIGHLIGHTS

Research background

The role of prophylactic clipping in the prevention of delayed polypectomy bleeding (DPB) is unclear.

Research motivation

Previous meta-analyses included a variety of polyp resection methods and all polyp sizes, our analysis used a more focused approach.

Research objectives

To assess the effect of prophylactic clip placement on DPB after endoscopic mucosal resection (EMR) of colorectal lesions 20mm or larger.

Research methods

We performed a systematic search of Medline through PubMed and the Cochrane Library database for studies investigating the effect of prophylactic clipping on DPB following EMR of colorectal lesions. We used the PRISMA protocol for our analysis and assessed the quality of included articles using the Newcastle-Ottawa scale. We used RevMan version 5 for the statistical analysis, using the random-effects model (DeSimonian-Laird method).

Research results

A total of 7794 polyps in 13 studies were analyzed, including 1701 cases of EMR of lesions ≥ 20 mm. We found that prophylactic clipping following EMR of lesions ≥ 20 mm was associated with a lower rate of DPB (1.4%) compared to no clipping (5.2%).

Research conclusions

Placement of clips prophylactically following EMR of colorectal lesions ≥ 20 mm may reduce rates of DPB and its associated morbidity and should be considered by practicing endoscopists in select patients.

Research perspectives

Future prospective studies on the effect of clipping for DPB after EMR should focus on lesions ≥ 20 mm since those represent the highest risk. Cost analyses must also be conducted to implement the most cost-effective strategies for DPB prevention.

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CASE REPORT

Recurrent renal cell carcinoma leading to a misdiagnosis of polycystic liver disease: A case report

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Abstract

BACKGROUND

Polycystic liver disease (PCLD) with a large cystic volume deteriorates the quality of life of patients through substantial effects on the adjacent organs, recurrent cyst infections, cyst rupture, and hemorrhage. Surgical or radiological intervention is usually needed to alleviate these symptoms. We report a rare case of the cystic metastasis of renal cell carcinoma (RCC), which was misdiagnosed as PCLD, as a result of the clinical and radiological similarity between these disorders.

CASE SUMMARY

A 74-year-old female who had undergone nephrectomy for papillary-type RCC (PRCC) was suffering from abdominal pain and the recurrent intracystic hemorrhage of multiple cysts in the liver. Imaging studies and aspiration cytology of the cysts showed no evidence of malignancy. With a diagnosis of autosomal dominant polycystic liver disease, the patient received hepatectomy for the purpose of mass reduction and infectious cyst removal. Surgery was performed without complications, and the patient was discharged on postoperative day 14. Postoperatively, the pathology revealed a diagnosis of recurrent PRCC with cystic formation.

CONCLUSION

This case demonstrates the importance of excluding the cystic metastasis of a cancer when liver cysts are observed.

Key words: Polycystic liver disease; Polycystic kidney disease; Cystic metastasis; Renal cell carcinoma; Case report

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Core tip: Polycystic liver disease (PCLD) usually exhibits typical presentations in imaging studies, but the diagnosis is sometimes challenging because of the late onset of this genetic disorder and the atypical presentations of other diseases. In a case of cystic metastasis of renal cell carcinoma, the disease could be misdiagnosed as PCLD due to the clinical and radiological similarity between these disorders. This case demonstrates that when multiple cystic lesions are observed in the liver, it is important to first exclude the cystic metastasis of a cancer. Additionally, some specific types of cancer can have different presentations at metastasis and recurrence.

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INTRODUCTION

Polycystic liver disease (PCLD) is defined as the presence of more than 20 cysts in the liver or the presence of more than 4 cysts in the liver with a family history of the disease. PCLD is a relatively rare disease, which is estimated to be present in 0.05%-0.53% of the total population[1]. PCLD manifests as clinical symptoms such as tiredness, fullness, shortness of breath, dissatisfaction with the abdomen size, limited mobility and early satiety, which significantly deteriorate the patient's quality of life[2]. PCLD is classified as one of two inherited disorders, *i.e.*, autosomal dominant polycystic kidney disease (ADPKD) and autosomal dominant polycystic liver disease (ADPLD). ADPLD is distinguished from ADPKD by the absence of polycystic kidneys. The mutations present in polycystic kidney disease (PKD1 and PKD2) are causative genes for ADPKD, while 20% of ADPLD is caused by mutations in the protein kinase C substrate 80K-H (PRKCSH) or SEC63, leaving the other 80% with unknown etiologies. PCLD, together with congenital hepatic fibrosis, is a type of disease that is characterized by the dysfunction of the primary cilium[3]. Current radiological and surgical interventions for "symptomatic" PCLD patients include aspiration-sclerotherapy, fenestration, hepatectomy and liver transplantation. Hepatectomy is usually indicated for Gigot type II PCLD, in which one liver segment is retained with unaffected liver parenchyma[4].

The diagnosis of PCLD is sometimes challenging. Typical differential diagnoses include ciliated hepatic foregut cysts, hepatobiliary cystadenomas, and parasitic cysts. However, in very rare cases, the cystic metastasis of a cancer becomes an important differential diagnosis that significantly changes the treatment strategy. The origins of cystic metastasis include colon, pancreas, ovary, kidney, neuroendocrine, and prostate cancer[1]. Here, we describe a rare case of the cystic metastasis of renal cell carcinoma (RCC), for which hepatectomy was performed due to the misdiagnosis of ADPLD.

CASE PRESENTATION

Chief complaints

A 74-year-old female complained of right upper quadrant abdominal pain when she presented at our hospital. Computed tomography (CT) with intravenous contrast demonstrated the local recurrence of RCC in the ipsilateral lymph nodes and multiple liver cysts.

History of present illness

The patient was diagnosed with left renal cancer and liver cysts in the bilateral lobes 4 years prior (Figure 1A). There was one large cyst and several small cysts, as demonstrated by a CT scan. The liver cysts appeared as well-demarcated and water-dense sacs without mural nodules. The patient had not received health screening for 20 years. Left nephrectomy with ipsilateral adrenalectomy was performed. Pathology revealed PRCC, G2, INF-α, pT2a, N0, M0, and v (-) (Figure 1B). No cysts were found

in the excision. Eight months prior to the surgery, the patient complained of right upper quadrant pain secondary to recurrent intracystic hemorrhage and received cyst aspiration and sclerotherapy. Aspiration cytology showed no evidence of malignancy, and the pain recurred soon after the treatment.

History of past illness

The patient had a medical history of hypertension and hyperlipemia.

Family history

The patient did not have a family history of PCLD.

Physical examination

The patient's temperature was 36.2 °C, heart rate was 82 bpm, respiratory rate was 14 breaths per minute, blood pressure was 140/99 mmHg and oxygen saturation in room air was 98%. A surgical scar was located in the left upper abdomen. In the abdominal examination, an abdominal mass was observed in the umbilical region, shifting dullness was present and liver cysts were palpable.

Laboratory examinations

Blood analysis revealed anemia with a hemoglobin level of 7.8 g/dL, white blood cells at 2.3 × 10^9/L, with a normal hematocrit and platelet count. The prothrombin, partial thromboplastin times and d-dimers were normal. The serum albumin was low, at 3.2 mg/dL. In the blood biochemistry analysis, the lactate dehydrogenase was high, at 262 U/L, with a high alkaline phosphatase level, at 626 U/L, and a γ-glutamyl transpeptidase rate of 101 U/L. The alanine aminotransferase and aspartate aminotransferase were normal. The urine analysis was normal. The electrocardiogram, chest X-ray and arterial blood gas were also normal.

Imaging examinations

CT with intravenous contrast demonstrated the local recurrence of RCC in the ipsilateral lymph nodes and multiple liver cysts, which had increased in size and number (Figure 2A and B). The cysts were various in sizes, but the borders were clear, and there was no sign of enhancement in the cystic walls.

FINAL DIAGNOSIS

Considering the fact that multiple cysts existed, mainly in the right lobe, while normal liver areas were retained in the lateral section, the patient was classified as having Type II PCLD, based on Gigot's classification (Figure 2B and C).

TREATMENT

Tumor resection with lymphadenectomy was planned to ensure the complete resection of the locally recurrent RCC. Further, with a diagnosis of PCLD, right lobectomy combined with cyst fenestration was planned to provide an optimized method for alleviating the symptoms.

OUTCOME AND FOLLOW-UP

The surgery was completed without major intraoperative complications. In the pathological report of the liver specimens, cells located focally around the cysts formed tubule-papillary structures that were mostly lined with single-cuboidal or low-columnar epithelial cells with scant cytoplasm and uniform nuclei, which were morphologically similar to PRCC by hematoxylin-eosin staining (Figure 3A)[5]. Immunohistochemical staining showed the presence of $CD10^+$ cells in the edges of the cysts (Figure 3B), while $CK7^+$ and $CK19^+$ cells were absent (Figure 3B-D). Combined with the results of H&E staining, the diagnosis of PRCC liver metastasis was verified. The patient was discharged on postoperative day 14 and started sunitinib treatment 1 mo after the hepatectomy. It has been 2 years since the surgery was performed. The pain was relived after the surgery, and the patient is still alive. However, lymph node metastases and lung metastases appeared after a few months, and the cysts occupying the remnant liver are growing, causing recurrent abdominal pain. The patient is now managed with symptomatic treatment.

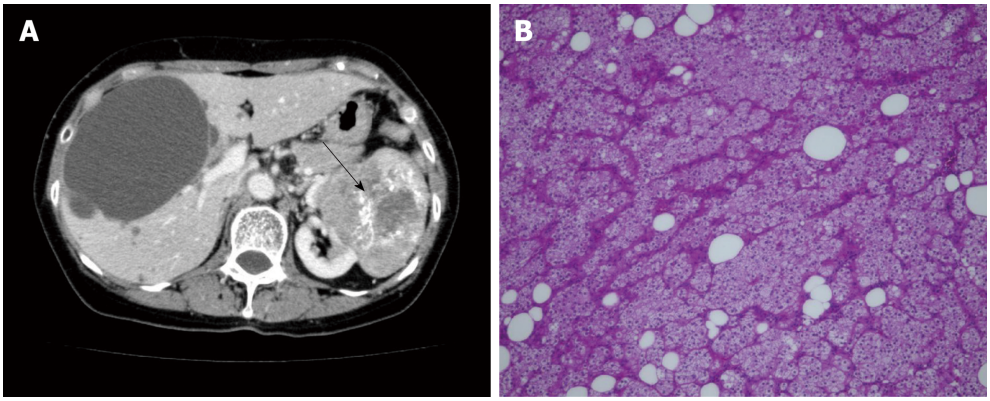

Figure 1 Abdominal computed tomography of the liver and pathologic findings from the primary tumor. Abdominal computed tomography demonstrated left renal cancer (arrow) and bilateral liver cysts. A: There was one large cyst and several small cysts. The borders of the liver cysts were clear, with no mural nodules. B: Pathology of the primary tumor revealed papillary renal cell carcinoma, G2, INF-α, T2a, N0, M0, v (-), × 20.

DISCUSSION

PRCC is the second most frequent RCC subtype, followed by clear cell-type RCC (CRCC). PRCC is known to metastasize less frequently than CRCC, with a reported incidence of 5.7%-11%[6]. The lung and bone are common sites of metastases, whereas the metastasis of the liver is less frequent (16.1%)[6,7]. PRCC can be classified as having solid or cystic masses. Cystic-type PRCC may be a result of its inherent architecture or of cystic degeneration[6]. Cystic metastasis of RCC is very rare[7]. Some cases with cystic lymph node metastases or dissemination have been previously reported in the literature[8-11].

Liver cysts can be classified as developmental, neoplastic, inflammatory, or miscellaneous[12]. The differential diagnosis of liver cysts is sometimes challenging, and excluding metastasis is one of the highest priorities. In typical cases of cystic metastatic cancers, the borders of the cystic lesions are typically heterogeneous and ill-defined[13]. The cystic walls are irregular, and the vessels are amputated; however, these characteristics are not observed in cases of PCLD. Cystic metastases usually have a peripheral enhancing rim on the arterial phase of a CT scan and magnetic resonance imaging, while PCLD usually does not have this feature[14]. Peribiliary cysts, which are also seen with high incidence, are usually located at the hilum and adjacent to the hepatic ducts, and the cyst sizes are smaller than 10 mm[13,14]. The misdiagnosis of cystic liver disease can be critical since the treatment strategy is completely different.

We described a rare case of the cystic metastasis of PRCC, which was misdiagnosed as PCLD. This case is important in that it demonstrates that PRCC can manifest with different presentations at metastasis and recurrence, i.e., multiple cyst formation, imitating PCLD[7,13]. In our case, the preoperative images of the liver cysts indicated that there were clear borders with no signs of enhancement in the cystic walls, which were characteristically similar to the cysts of PCLD, and the aspiration cytology was negative for cancer cells. Pathologically, the cystic walls were irregular, and the cells around the cysts were morphologically compatible with RCC. The diagnosis of RCC was confirmed by the presence of $CD10^+$ cells around the cysts, which is a characteristic surface marker of RCC. It was quite unlikely that cystic liver metastasis occurred to a liver affected by PCLD, since all the cysts that were pathologically investigated showed the malignant characteristics described above. Since CA 50 was reported as a potential tumor marker for cystic RCC[15], we could have tested the CA 50 in the blood and the cystic fluid from repeated fine-needle aspiration. Further, ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography/CT (PET/CT) is highly sensitive and accurate, with a sensitivity of 97% and a specificity of 75% for hepatic metastasis[10]. It has been reported to show intense uptake in cystic liver metastasis compared with that in benign cysts[16]. We could have checked PET/CT scan to confirm the malignancy of the cysts if we had taken metastasis into consideration.

The treatment for metastatic renal cancer is debated, and the therapeutic options are limited. The National Health Service of England Guidelines stated that sunitinib and pazopanib are first-line systemic therapies for metastatic RCC[17]. Complete resection is the only choice for patients with metastatic RCC to have a satisfactory prognosis, with 5-year overall survival rates of 15 to 60%[18]. However, patients with bilateral multiple metastatic RCC, such as our patient, are not candidates for hepatectomy because radical resection cannot be performed, and the surgery will not

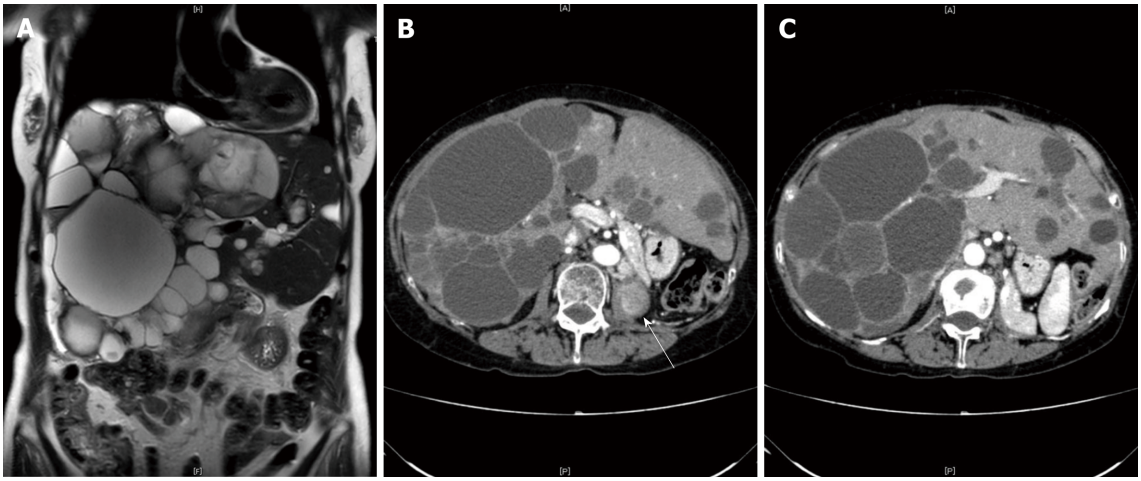

Figure 2 Images from abdominal computed tomography. Local recurrence of renal cell carcinoma was detected in the ipsilateral lymph nodes and in the left lumbar vertebra (B, white arrow). There was a diffuse involvement of the liver parenchyma, with multiple cysts and large areas of noncystic liver parenchyma remaining (C); A: coronal view; B and C: transverse view.

prolong the overall survival time of the patients[19,20]. In our case, since the patient suffered from acute abdominal pain and recurrent intracystic hemorrhage, we performed hepatectomy following the diagnosis of PCLD. However, as the cysts were located diffusely in the bilateral lobe, the patient would have had no indication for hepatectomy if she had been correctly diagnosed with PRCC preoperatively. We should have started with chemotherapy and palliative care, using opioids and additional adjuvants to alleviate the abdominal symptoms[21]. This case reminds us of the following: first, when multiple cystic lesions are observed throughout the liver parenchyma in patients with cancer, it is important to first exclude metastasis; and second, some specific types of cancer can show different presentations at metastasis and recurrence, which could cause preoperative misdiagnosis.

CONCLUSION

In a patient with cystic neoplasms of the liver, the diagnosis remains challenging in everyday practice. Cystic lesions may present as solitary or multiple cysts and may range from benign to malignant. To the best of our knowledge, this is the first report of cystic liver metastasis from PRCC. The most important implication of this case is that PCLD must be distinguished from the cystic metastasis of a cancer, which can contribute to a profoundly better prognosis as a result of the use of an optimal treatment approach.

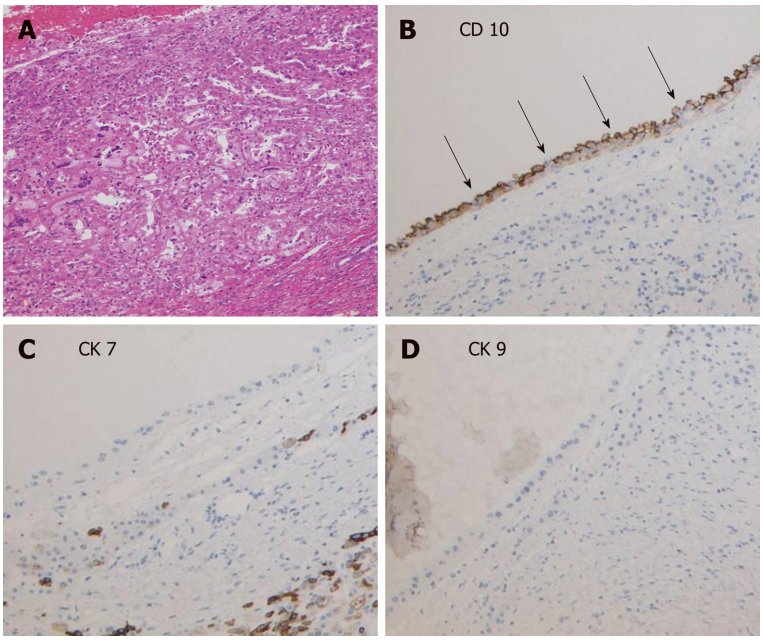

Figure 3 Pathological findings from the metastatic liver cysts. A: Cells around the cysts showed a basophilic morphologic appearance with low-grade nuclear features that were morphologically consistent with papillary-type renal cell carcinoma cells, × 20. B-D: Immunohistochemical staining showed the occasional presence of $CD10^+$ cells in the edges of the cysts, while $CK7^+$ and $CK19^+$ cells were not observed, × 20.

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