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EDITORIAL

# Fecal microbiota transplantation for irritable bowel syndrome: Current evidence and perspectives

Cong Dai, Yu-Hong Huang, Min Jiang

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## Abstract

In this editorial we comment on the article published in the recent issue of the *World journal of Gastroenterology*. We focus specifically on the mechanisms underlying the effects of fecal microbiota transplantation (FMT) for irritable bowel syndrome (IBS), the factors which affect the outcomes of FMT in IBS patients, and challenges. FMT has emerged as a efficacious intervention for clostridium difficile infection and holds promise as a therapeutic modality for IBS. The utilization of FMT in the treatment of IBS has undergone scrutiny in numerous randomized controlled trials, yielding divergent outcomes. The current frontier in this field seeks to elucidate these variations, underscore the existing knowledge gaps that necessitate exploration, and provide a guideline for successful FMT implementation in IBS patients. At the same time, the application of FMT as a treatment for IBS confronts several challenges.

**Key Words:** Fecal microbiota transplantation; Irritable bowel syndrome; Microbiota; Randomized controlled trial; Meta-analysis



**Core Tip:** Fecal microbiota transplantation (FMT) has emerged as a efficacious intervention for Clostridium difficile infection and holds promise as a therapeutic modality for irritable bowel syndrome (IBS). The utilization of FMT in the treatment of IBS has undergone scrutiny in numerous randomized controlled trials, yielding divergent outcomes. The current frontier in this field seeks to elucidate these variations, underscore the existing knowledge gaps that necessitate exploration, and provide a guideline for successful FMT implementation in IBS patients. At the same time, the application of FMT as a treatment for IBS confronts several challenges.

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## INTRODUCTION

The transplantation of the intestinal microbiome through feces obtained from a healthy individual with normal bowel function, commonly termed fecal microbiota transplantation (FMT), has historical roots dating back to the fourth century when the Chinese physician pioneered its application in addressing severe diarrhea and malaria[1]. In contemporary medical contexts, FMT has emerged as a efficacious intervention for Clostridium difficile infection (CDI) and holds promise as a therapeutic modality for various conditions, including irritable bowel syndrome (IBS)[2-4]. The utilization of FMT in the treatment of IBS has undergone scrutiny in numerous randomized controlled trials (RCTs), yielding divergent outcomes[5-10]. The current frontier in this field seeks to elucidate these variations, underscore the existing knowledge gaps that necessitate exploration, and provide a guideline for successful FMT implementation in IBS patients.

Halkjær *et al*[11] conducted a comprehensive meta-analysis to evaluate the efficacy and safety of FMT in the treatment of IBS. The study revealed that, compared to a placebo, FMT yielded no statistically significant benefits in alleviating IBS symptoms at three months post-treatment (RR 1.19, 95%CI: 0.68-2.10). Additionally, FMT exhibited no discernible enhancement in the quality of life for individuals with IBS (MD -6.30, 95%CI: -13.39-0.79). Notably, a subgroup analysis indicated a noteworthy amelioration of symptoms with endoscopic FMT delivery, whereas capsules did not elicit a comparable effect. Adverse events were documented in 97 participants within the FMT group, contrasting with 45 participants in the placebo group (RR 1.17, 95%CI: 0.63-2.15). The incongruent findings across the included studies can be attributed to variations in the selection of IBS patient subtypes, the frequency and route of transplant delivery, FMT-content, pre-treatment protocols, and the number of donors.

While the studies included in the analysis did not report major adverse effects linked directly to FMT, it is imperative to maintain a low threshold for toxicity evaluation, especially in non-life-threatening conditions. It becomes incongruent when proponents selectively reference murine transplant experiments highlighting the role of the microbiota in IBS pathogenesis while neglecting analogous studies suggesting the potential transferability of phenotypes. Consequently, the risks associated with FMT extend beyond infection or transmission of antibiotic-resistant organisms, encompassing the theoretical transfer of inflammatory, metabolic, and even behavioral phenotypes from donor to recipient. Although meticulous donor selection can mitigate these risks, certain microbiotas may harbor latent dangers associated with the future onset of colon cancer. Then I will provide a detailed introduction to the mechanisms, the influencing factors, and challenges of FMT for IBS.

### *Mechanisms of FMT for IBS*

Enteroendocrine cells, mast cells, and fecal Short Chain Fatty Acids (SCFAs) in individuals with IBS exhibit distinctions from those in healthy subjects, and these variances are thought to be pivotal in the pathophysiology of IBS[12-14]. Butyrate, an SCFA, serves as an energy source for colonic epithelial cells, modulates immune responses, mitigates oxidative stress, reduces intestinal motility, and decreases cell permeability. Notably, butyrate also regulates colonic hypersensitivity, and its intake has been associated with diminished abdominal pain in IBS patients. While it is premature to definitively delineate the mechanisms underpinning the effects of FMT, available data propose that the amelioration of IBS symptoms through FMT may be attributed to alterations in enteroendocrine cells, mast cells, and SCFAs including butyrate.

The majority of bodily serotonin is situated in the gut, with only 10% residing in the enteric nervous system (ENS), and the remaining portion contained in the enterochromaffin (EC) cells dispersed among the gastrointestinal epithelial cells. Serotonin plays a crucial role in gastric emptying and intestinal peristalsis[15]. Additionally, serotonin activates the sensory nerve endings of submucosal ENS, transmitting gut sensations to the central nervous system. Upon exerting its effects at serotonin receptors, serotonin is transported into intestinal epithelial cells by the serotonin-selective reuptake transporter (SERT), where it undergoes degradation. In individuals with IBS, reported lower densities of EC cells and diminished SERT immunointensity in the gut suggest impaired serotonin uptake and degradation. Specific bacteria, such as Corynebacterium, Streptococcus, and Enterococcus spp., alongside indigenous spore-forming bacteria, have been identified as serotonin producers. Furthermore, Clostridium ramosum regulates serotonin release from EC cells. The altered intestinal bacterial composition induced by FMT may influence the serotonin-regulating system.

## THE INFLUENCING FACTORS OF FMT FOR IBS

### *FMT donor selection*

Considerable variability in the outcomes of FMT for IBS has been noted among studies, a phenomenon largely ascribed to differences in donor selection. The designation "superdonor" is bestowed upon a donor eliciting a robust response to FMT [16]. Attempts to predict superdonors have involved pooling feces from multiple donors to augment the chances of patients receiving superdonor feces. However, this strategy proved unsuccessful, likely due to the dilution of superdonor

feces and subsequent inadequate dosing for recipients. The divergent outcomes in RCTs of FMT in IBS can be predominantly traced to variations in donor selection criteria. Notably, the RCT conducted by El-Salhy *et al*[17], which demonstrated positive FMT responses, established the strict selection of superdonor. But some RCTs omitted these criteria for donor selection. The temporal stability of the donor's intestinal bacterial composition is another pivotal consideration when selecting a superdonor.

Clinical criteria for superdonor selection have been grounded in factors known to impact the intestinal microbiota, including age (> 50 years), smoking habits, birth by cesarean section, formula feeding during infancy, antibiotic use, nonantibiotic drug consumption, and lifestyle factors such as regular exercise and diet. Genetic considerations also underscore the need for superdonors to be non-first-degree relatives of recipients. The superdonor identified in El-Salhy *et al*'s RCT adhered to a profile of a healthy young male with a normal body mass index, born vaginally, breastfed, nonsmoking, medication-free, with infrequent antibiotic use, regular exercise, and a healthy diet[17]. Fecal microbiota analysis of this donor revealed high microbial diversity, with deviations in bacterial composition from the norm of 165 healthy subjects, predominantly in the Firmicutes phylum. This included increased abundance of beneficial bacteria including Lactobacillus, Streptococcus, and Ruminococcaceae spp. Importantly, the composition of fecal microbiota from the superdonor can remain stable for over 18 months. Despite efforts to ensure superdonor presence by pooling feces from multiple donors, this strategy yielded no response or only transient improvement.

IBS patient inclusion.

Caution must be exercised in generalizing the outcomes of RCTs of FMT for IBS, as the patient cohorts included in these trials represent specific subsets of the IBS population. Notably, five RCTs exclusively enrolled patients with diarrhea-predominant IBS (IBS-D), mixed-diarrhea-and-constipation IBS (IBS-M), or IBS unclassified (IBS-U). In contrast, three other RCTs encompassed all IBS subtypes, encompassing IBS-D, constipation-predominant IBS (IBS-C), IBS-M, and IBS-U. Additionally, the RCT conducted by El-Salhy *et al*[17] found that the patients who live with IBS patients for two days may exhibit moderate to severe IBS symptoms, even if they completely follow the National Institute for Health and Care Excellenc dietary regimen. The RCT by Holster *et al*[8] specifically included patients characterized by low levels of fecal butyrate-producing bacteria. Furthermore, Holvoet *et al*[10] exclusively enrolled refractory IBS patients with severe bloating who are ineffective in conventional therapies for at least 3 d. These variations in patient selection criteria underscore the need for cautious interpretation and application of the outcomes of FMT trials to the broader spectrum of individuals with IBS.

### Route of administration and dose of FMT

FMT can be performed through upper gastrointestinal pathways including gastroscope and nasojejunal tube, as well as lower gastrointestinal pathways including colonoscope. Both routes of administration have demonstrated efficacy. But a noteworthy placebo effect was observed in 43%-44% and 23.6%-26% of patients who received FMT in the large and small intestine, respectively[8,10]. This placebo effect might be more pronounced in patients receiving FMT in the colon, potentially attributable to the favorable impact of bowel preparation for colonoscopy on IBS symptoms. In contrast to its success in treating CDI, the administration of FMT *via* capsule ingestion proved ineffective in IBS. Possible factors contributing to this ineffectiveness include donor selection, a low transplant dose, or the pooling of donors.

The dose of the fecal transplant appears to influence FMT outcomes, suggesting a dose-dependent response. Notably, 70% of patients unresponsive to a 30 g FMT dose exhibited a positive response to a 60 g FMT dose. The majority of included studies utilized a dose of at least 30 g[8,10]. Concurrently, further investigations are imperative to assess the comparative efficacy of single versus repeated FMT administrations.

### Challenges of FMT for IBS

Although FMT is a promising treatment for gastrointestinal and nongastrointestinal diseases, the application of FMT as a treatment for IBS confronts several challenges. Firstly, the absence of a definitive microbial signature and the diverse dysbiosis patterns in IBS. Pathobiont proliferation, commensal loss, and reduced microbial diversity contribute to the complexity of the condition in IBS. At the same time, some factors such as infection, inflammation, dietary influences, xenobiotics, genetics, and familial transmission exert dynamic effects on the composition of the intestinal microbial community.

Secondly, the lack of a universally defined normal microbiome limits the application of FMT. FMT has many complex mechanisms of action, including direct effects on the host, reinstatement of missing network interactions, and microbiome modulation. However, it is crucial to acknowledge that FMT is not a curative measure, as its benefits persist for a median duration of four months and gradually diminish over a year.

Thirdly, FMT responsiveness varies in IBS patients, with distinct and stable subsets of responders and non-responders. While initial responders often regain a positive response after re-FMT, those who failed to respond initially generally continue to exhibit disappointing outcomes with subsequent FMT attempts from different donors. This raises the prospect that disruptions in the microbiota may offer a pathway for stratifying IBS patients. While symptoms play a central role in IBS diagnosis, their questionable value in stratification is evident as patients categorized as IBS-C, IBS-D, or IBS-M may transition between these patterns over time. Additionally, microbiota studies do not support stratification based on constipation or diarrhea. Integrating microbiota analysis including microbiome composition and functional analysis before and after FMT into the design and monitoring of future FMT trials for IBS appears reasonable. This does not imply exclusive responsiveness in those with disturbed microbiota but holds the potential to predict varying degrees of responsiveness, offering a refined rationale for patient selection and valuable data for result interpretation.

Fourth, the risks of FMT should also be evaluated. Improving the screening of FMT donors including testing the donor stool for beta-lactase extended-spectrum beta-lactamase, *E. coli*, and severe acute respiratory syndrome coronavirus 2 would reduce the risks of infection by known agents. Moreover, restricting the selection of patients with IBS for FMT to

those without systemic disease, immune deficiency, treatment with immune-modulating medication, and severe illness would reduce the risks.

## CONCLUSION

FMT is a promising treatment for both gastrointestinal and nongastrointestinal diseases. There is currently a lot of evidence to prove that it can improves the IBS symptoms, fatigue, and quality of life. However, several questions remain to be answered, and further investigations are needed before FMT can be applied for IBS treatment in clinical practice. The criteria to apply when selecting an effective donor for FMT remain unclear, including the administration route, the optimal dose, and the frequency of treatment. Moreover, it is not clear whether FMT is effective for all IBS patients, or certain subsets of IBS patients. At the same time, there is some concern regarding the long-term side effects of FMT.

## FOOTNOTES

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EDITORIAL

# MicroRNAs in inflammatory bowel disease: What do we know and what can we expect?

Ellen Cristina Souza de Oliveira, Ana Elisa Valencise Quaglio, Thais Gagno Grillo, Luiz Claudio Di Stasi, Ligia Yukie Sassaki

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## Abstract

MicroRNAs (miRNAs), small non-coding RNAs composed of 18–24 nucleotides, are potent regulators of gene expression, contributing to the regulation of more than 30% of protein-coding genes. Considering that miRNAs are regulators of inflammatory pathways and the differentiation of intestinal epithelial cells, there is an interest in exploring their importance in inflammatory bowel disease (IBD). IBD is a chronic and multifactorial disease of the gastrointestinal tract; the main forms are Crohn's disease and ulcerative colitis. Several studies have investigated the dysregulated expression of miRNAs in IBD, demonstrating their important roles as regulators and potential biomarkers of this disease. This editorial presents what is known and what is expected regarding miRNAs in IBD. Although the important regulatory roles of miRNAs in IBD are clearly established, biomarkers for IBD that can be applied in clinical practice are lacking, emphasizing the importance of further studies. Discoveries regarding the influence of miRNAs on the inflammatory process and the exploration of their role in gene regulation are expected to provide a basis for the use of miRNAs not only as potent biomarkers in IBD but also as therapeutic targets for the control of inflammatory processes in personalized medicine.

**Key Words:** MicroRNAs; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Biomarker; Therapy

**Core Tip:** MicroRNAs (miRNAs) function in the regulation of inflammatory pathways and the differentiation of intestinal epithelial cells. There is substantial evidence for the important regulatory roles of miRNAs in inflammatory bowel disease (IBD), suggesting that they may serve as biomarkers. Therefore, this editorial aims to present what is already known and what the expectations are regarding the role of miRNAs in IBD.

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## INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs composed of 18 to 24 nucleotides that are recognized as potent downregulators of gene expression or messenger RNA translation[1-3]. They regulate more than 30% of protein-coding genes and play important roles in cell survival, differentiation, proliferation, apoptosis, cell cycle control, and homeostasis[1,2,4].

Because miRNAs regulate inflammatory cellular signaling pathways and the intestinal epithelial cell differentiation, with an important role in the homeostasis of the intestinal mucosa[1,5,6], we explored the importance of miRNAs in inflammatory bowel diseases (IBD). IBD is a chronic disease of the gastrointestinal tract with a multifactorial and imbricated etiology, involving genetic, immune, and environmental factors. The main representatives of IBD are Crohn's disease (CD) and ulcerative colitis (UC), which differ both clinically and pathophysiologically[7].

In the last decade, several clinical and experimental studies of IBD have improved our understanding of miRNAs and contributed to the search for new and more accurate diagnostic markers and targets for treatment. Based on this, this editorial aims to present what is already known and what the expectations are regarding the role of miRNAs in IBD.

## WHAT DO WE KNOW ABOUT MIRNA IN IBD?

miRNAs play an important role as cellular and homeostasis regulators and may interfere with important inflammatory signaling pathways, such as the nuclear transcription factor kappa B (NF-κB), interleukin 23 (IL23)/IL23R, and IL-6/STAT3 pathways[8-11]. Therefore, alterations in the expression of certain miRNAs may be related to various immune diseases, including IBD. To evaluate their expression profiles in diseases, miRNAs can be quantified using samples of body fluids (circulating miRNAs), such as blood and feces, as well as through homogenized tissue biopsies (tissue miRNAs) using microarray profiling, quantitative real-time PCR, and next-generation sequencing techniques[12-15].

One of the first studies focusing on miRNAs in IBD identified three under expressed miRNAs (miR-192, miR-375, and miR-422b) and eight overexpressed miRNAs (miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, and Let-7f) in tissues from patients with active UC compared to tissues from healthy individuals[5]. Another study conducted by the same research group evaluated colonic tissues from patients with CD and identified three upregulated miRNAs (miR-23b, miR-106a, and miR-191) and two downregulated miRNAs (miR-19b and miR-629) when compared with levels in colonic tissues from healthy individuals[6]. Neither upregulated nor downregulated miRNAs in CD patients with were altered in UC patients[6], indicating that the miRNA expression profile differs between CD and UC.

These studies prompted researchers to investigate the role of miRNA dysregulation in IBD, both as regulators of inflammatory processes and as IBD biomarkers. Several studies have focused on the detection of miRNA biomarkers for IBD, revealing miR-223, miR-155, and miRNA-320a as key candidates.

A study using serum samples from IBD patients suggested that miR-223 is a potential biomarker, as levels of this miRNA were higher in both CD and UC samples than in healthy individuals[16]. Additionally, miR-223 expression is associated with the active phase of the disease[16]. Another study using the same sample type corroborated the increase in miR-223 expression in patients when compared with that in healthy individuals[17]. However, when active patients were compared with those in remission, miR-223 expression showed no significant differences[17]. Similarly, miR-155 expression differed between patients and healthy individuals[17], and this was corroborated by another study that demonstrated higher miR-155 expression levels in the colon tissues of patients than in samples from healthy individuals [18], suggesting that miR-155 is a potential biomarker of IBD activity. Similarly, a recent study on IBD demonstrated that miR-320a expression is higher in blood samples of IBD patients than in samples from healthy individuals and is significantly higher in patients with active IBD than in patients in remission[19], highlighting that miR-320a is another promising biomarker of disease activity in IBD patients.

The search for miRNA biomarkers of IBD has also led to the identification of the roles of miRNAs in inflammatory signaling pathways modulated by various drugs, such as prednisone, Janus kinase (JAK) inhibitors, and monoclonal antibodies, including infliximab, ustekinumab, and vedolizumab (Figure 1). Furthermore, some miRNAs can be regulated

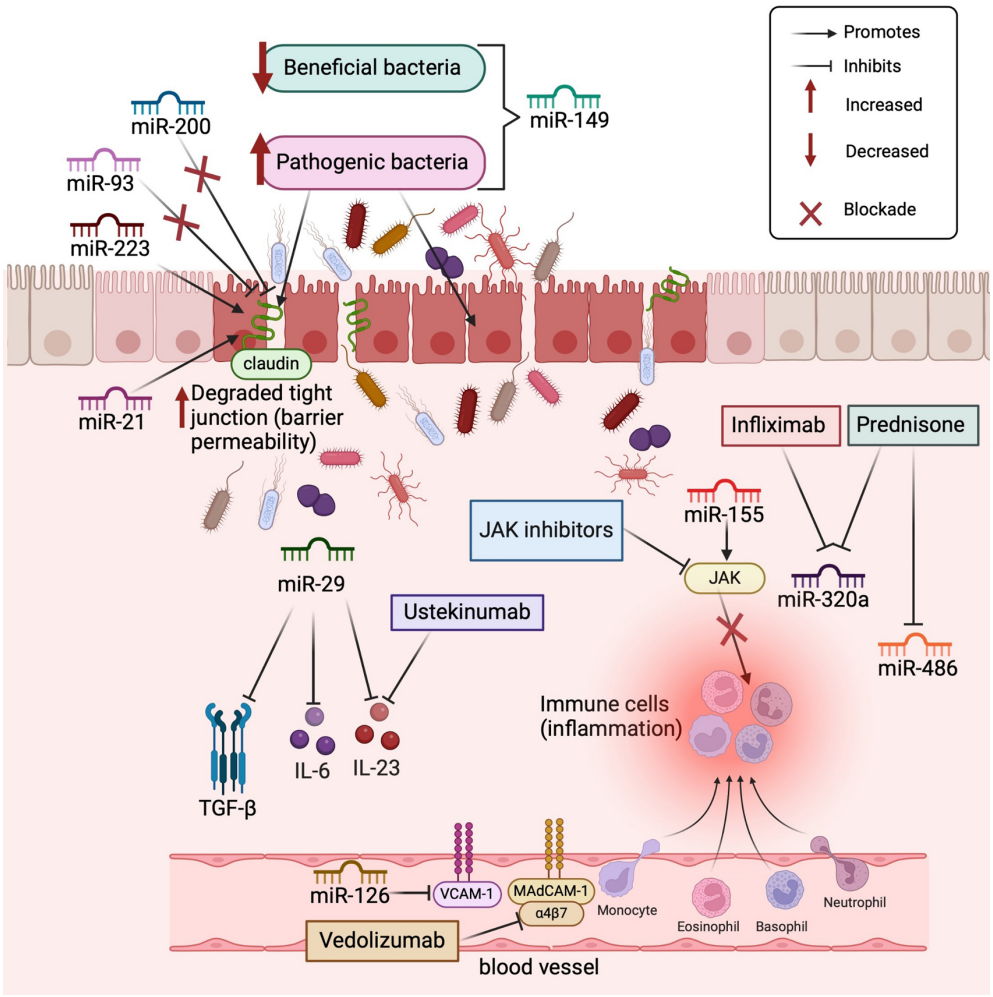


**Figure 1 Main microRNAs involved in intestinal inflammation and inflammatory pathways targeted by medications used to treat inflammatory bowel disease.** MicroRNA (miR)-21 and miR-223 inhibit tight junctions, whereas miR-200 and miR-93 maintain tight junctions. miR-149 promotes changes in the intestinal microbiota, favoring the growth of pathogenic bacteria. Prednisone reduces miR-486 expression, while miR320a expression is reduced by prednisone and infliximab. miR-29 reduces the expression of transforming growth factor β, interleukin (IL)-6, and IL-23, similar to ustekinumab, a biological inhibitor of IL12/23. miR-155 regulates the activity of Janus kinase (JAK), which activates immune cells, whereas JAK inhibitors exert the opposite effects. miR126 regulates vascular cell adhesion molecule-1 (VCAM-1), similar to vedolizumab, which regulates mucosal addressin cell adhesion molecule-1 (MAdCAM-1). α4β7: A4β7 integrin. Created with BioRender.com (Supplementary material).

when exposed to certain drugs, as demonstrated in the pediatric population, where it was observed that miR-146a, miR-146b, and miR-320a were reduced with the use of infliximab and prednisone, whereas miR-486 expression was reduced only with the use of prednisone[20].

In a study of dextran sodium sulfate (DSS)-induced intestinal inflammation in mice, tail vein injection of miR-29 linked to a nanoparticle significantly inhibited the intestinal anti-inflammatory process, which was related to reductions in transforming growth factor-β, IL-6, and IL-23 expression[21]. Notably, the cytokine IL-23 is the target of the monoclonal antibody ustekinumab, which is currently used to treat CD, indicating that miR-29 is a potential therapeutic target for IBD. In contrast, miR-155 targets regulatory proteins in the JAK signaling pathway, which controls immune cells and, consequently, inflammatory processes[22]. This mechanism is the same as that of JAK inhibitors, such as tofacitinib and upadacitinib, which are oral drugs used to treat UC.

*In vitro* and *in vivo* analyses have shown that miR-126 inhibits leukocyte adhesion to endothelial cells through the regulation of vascular cell adhesion molecule-1 (VCAM-1)[23]. Vedolizumab, indicated for the treatment of UC and CD, also inhibits the migration of leukocytes into inflamed intestinal tissue, blocking their interaction with mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) in the intestinal vasculature[24], a mechanism similar to that described for miR-126. The development of new drugs capable of modulating the expression of these miRNAs is a promising approach for controlling the inflammatory response.

Other studies have also reported an important relationship between miRNAs and intestinal permeability, considering that a loss of the intestinal barrier is one of the processes that triggers intestinal inflammation in IBD patients[25,26]. In this context, miR-21 plays an important role in regulating the intestinal epithelial barrier, as it blocks the production of the RhoB (Ras homolog gene family member B) protein[25]. A reduction in RhoB protein levels results in the loss of tight junctions, increasing intestinal permeability with subsequent increased exposure to antigens, which serves as a trigger for the intestinal inflammatory process[25].

Besides miR-21, other miRNAs destroy tight junctions and consequently weaken the intestinal epithelial barrier, including miR-191, miR-212, miR-675, miR-874, miR-122a, miR-34c, miR-150, and miR-01a[26]. In contrast, miR-200 and miR-93 assist in the protection of tight junctions, maintaining intestinal barrier function[26]. These recent findings indicate that several miRNAs are important targets for the development of treatments aimed at maintaining the integrity of the intestinal barrier, thereby preventing overexposure to antigens as a trigger in the inflammatory processes involved in IBD pathophysiology.

A recent review published by our research group[27] reported that some miRNAs modulate the intestinal microbiota and induce dysbiosis in IBD patients, whereas the intestinal microbiota can also regulate the expression of miRNAs, establishing a complex relationship between these taxa and their host[27]. For example, in a DSS-induced intestinal inflammation model, miR-149 deletion induces changes in the microbiota and promotes intestinal inflammatory processes [28]. Additionally, the use of probiotics in mice, in addition to improving dysbiosis, reduces the expression of miR-155, miR-223, miR-150, and miR-143, which act on both intestinal permeability and the pro-inflammatory response, improving intestinal inflammation[29].

## WHAT CAN WE EXPECT FROM MIRNAS IN IBD?

From the first studies on miRNAs to recent research, there has been a great evolution in our understanding of their functions in the immune system and inflammatory processes. Despite this, there is a lack of data to support the use of miRNAs as biomarkers for chronic diseases, such as IBD, limiting the application of scientific knowledge to clinical practice. To date, specific miRNAs have not been described as biomarkers for differentiating UC from CD, as markers of inflammatory activity, or as predictors of response to clinical treatment. However, research work has yielded new discoveries and insights into the roles of miRNAs in the pathogenesis and maintenance of the inflammatory process in IBD. In the near future, these findings are expected to lead to novel applications of these biomarkers in clinical practice.

Regarding IBD monitoring, few biomarkers for disease activity, such as fecal calprotectin, have been validated[30,31] and therefore colonoscopy is needed to visualize the intestinal mucosa. Considering that colonoscopy is invasive with inherent risks, new markers with high accuracy are required. miRNAs participate in various processes, including inflammation. Therefore, miRNAs can serve as appropriate biomarkers for the diagnosis and therapeutic monitoring of IBD patients. In addition to contributing to a more specific diagnosis and treatment, the identification of miRNAs as blood or fecal markers of IBD provides a less invasive[8], faster, and more accurate alternative to colonoscopy. The disadvantage of miRNA markers is the high cost of tests, which hinders their applicability in clinical practice.

Regarding the role of miRNAs in differentiating disease activity from remission, miR-223 expression is higher in the serum, tissue from the terminal ileum, and fecal samples of active CD compared to inactive CD[32]. Furthermore, miR-223 levels in serum, intestinal tissue, and fecal samples were correlated with Crohn´s Disease Activity Index, and fecal miR-223 was correlated with fecal calprotectin. These findings indicated that fecal miR-223 may be a novel, noninvasive biomarker for estimating disease activity in CD patients[32].

The Selecting Therapeutic Targets in IBD (Stride) II and IBD consensus established clinical response and remission as well as normalization of C-reactive protein as immediate and short-term targets, and endoscopic healing, restoration of quality of life, and absence of disability as long-term targets in IBD treatment, including mucosal healing as therapeutic goals[33,34]. The expectations include histological healing in UC and transmural healing in CD[33]. The identification of specific miRNAs correlated with mucosal healing, histological healing, or even transmural healing would be a revolutionary milestone in IBD, facilitating patient monitoring and the development of treat-to-target strategies using a simple blood marker.

Several miRNAs act on the same signaling pathways that are targets of drugs used to treat IBD[13,20-24], suggesting the potential use of miRNAs as IBD targets. The modulation of these miRNAs may positively interfere with patient responses to treatment, which is a promising strategy for drug development, as previously reported for miR-29[21], miR-155[22], and miR-126[23].

Regarding the modulation of miRNAs as a therapeutic strategy, a clinical trial is evaluating the efficacy of the small molecule drug candidate obefazimod for the treatment of moderate to severe active UC[35]. Obefazimod is the only known molecule that modulates miRNAs, as it enhances miR-124 expression, which is responsible for modulating inflammation and the innate immune response activated in IBD[35]. Results from this clinical trial are expected soon. Considering this mechanism of action, future studies should include other miRNAs as therapeutic targets; for example, the activation of miRNAs that help maintain the intestinal barrier, including miR-200 and miR-93, or inhibition of miRNAs that negatively regulate inflammatory processes, including miR-223 and miR-320a, should be evaluated.

In addition to their potential use as diagnostic markers and therapeutic targets, miRNAs have been studied as predictors of clinical and endoscopic responses in IBD patients. Reduced serum expression levels of let-7e at week 14 and miR-126 at week 54 were associated with clinical remission at weeks 14 and 54 and endoscopic remission at week 54 in 37 patients with CD treated with anti-tumor necrosis factor α therapy[36]. Another study found that increased let-7d and let-7e expression were associated with clinical remission at week 14 after infliximab induction therapy in CD, suggesting that these miRNAs are possible therapeutic biomarkers in CD patients treated with infliximab[37].

In severe acute colitis, a study evaluated tissue miRNAs associated with the response to intravenous (IV) steroids and in response to infliximab or cyclosporine in steroid-refractory patients[13]. Initially, 15 miRNAs associated with the response to IV steroids were identified (hp_hsa-mir-3934, hp_hsamir-3667, hp_hsa-mir-100, hsa-miR-603, hsa-miR-718, hsa-miR-4259, hp_hsa-mir-193b, hsa-miR-3150a-5p, hp_hsa-mir-1260b, hsa-miR-938, hsa-miR-3128, hsa-miR-4423-3p, hsa-miR-518b, hsa-miR-1468, and hsa-miR-3152-3p), in addition to six miRNAs associated with the response to infliximab

**Table 1 Roles of microRNAs in patients with inflammatory bowel disease**

| What is already known about miRNAs in IBD? | What can we expect from miRNAs in IBD? | Ref. |
|---|---|---|
| Regulate cellular processes and homeostasis | Discover new functions in the pathophysiology of IBD | [8-11] |
| Differentially expressed between patients with IBD and healthy controls | Can be used as diagnostic markers for IBD | [5,6,16-19] |
| Differentially expressed between UC and CD | Can be used to differentiate UC from CD | [6] |
| Differentially expressed with respect to disease activity | Can be used as biomarkers of inflammatory activity | [16,17,19,32] |
| Regulated in response to drug exposure | Can be used as markers of drug responses | [20] |
| Regulate the expression of inflammatory cytokines | Potential therapeutic targets in IBD | [21,22,23] |
| Act on the intestinal barrier | Potential therapeutic targets in IBD | [25,26] |
| Modulate the intestinal microbiota | Potential therapeutic targets in IBD | [27-29] |

CD: Crohn's disease; IBD: Inflammatory bowel disease; miRNA: microRNA; UC: Ulcerative colitis.

**Table 2 Barriers to the use of microRNAs in clinical practice**

| |
|---|
| High exam cost |
| Better definition of miRNAs as diagnostic markers in IBD (differentiation between UC and CD and diagnosis of disease activity) |
| Few validation studies of miRNAs as blood, fecal, and endoscopic biomarkers |
| Better definition of the differences between the expression of fecal, blood, and tissue miRNAs |
| Lack of evidence validating miRNAs as a tool for evaluating mucosal healing |
| Lack of evidence validating miRNAs as a tool for evaluating histological remission |
| Lack of evidence validating miRNAs predictors of clinical and endoscopic responses |
| Lack of evidence validating miRNAs in treatment monitoring |
| Lack of evidence validating miRNAs as predictors of severe disease |
| Insufficient data on the role of miRNAs in modulating the inflammatory response |
| Insufficient data on the effects of miRNAs on the intestinal barrier, intestinal microbiota, and the response to probiotics |

CD: Crohn's disease; IBD: Inflammatory bowel disease; miRNA: microRNA; UC: Ulcerative colitis.

(hsa-miR-4423-3p, hsa-miR-3128, hsa-miR-3152-3p, hp_hsa-miR-193b, hsa-mi-R938, and hp_hsa-miR-100) and four miRNA associated with the ciclosporin response (hsa-mi-R4423-3p, hsa-mi-R938, hsa-mi-R518b, and hp_hsa-miR-100). In a validation cohort study, among the miRNAs initially identified, only two were significantly differentially expressed between responders and non-responders: miR-3934 for IV steroids and miR-938 for second-line treatment (infliximab or cyclosporine)[13].

In the pediatric population, five serum miRNAs (miR-126, let-7c, miR-146a, miR-146b, and miR-320a) were associated with the clinical response; further studies are needed to validate these miRNAs as biomarkers of infliximab and glucocorticoid treatment response within this specific population[38].

In the future, it will be important to consider the modulation of miRNAs by drugs or probiotics and the use of miRNAs as treatment itself (Table 1). A recent review has highlighted promising results in preclinical cancer studies when using a single miRNA to target multiple genes[39]. Despite these advances, several limitations and challenges must be overcome to enable the use of miRNAs in IBD in clinical practice, as described in Table 2.

## CONCLUSION

Previous studies have demonstrated that miRNAs are important mediators of the inflammatory process in IBD patients and represent potential therapeutic targets for the development of new drugs. Experimental and clinical studies have focused on the modulation of miRNA expression, either by stimulating miRNA expression with anti-inflammatory functions or inhibiting miRNA expression with pro-inflammatory functions. In this editorial, we present the main findings and future perspectives regarding miRNAs and their roles in IBD patients. Currently, there is no specific miRNA biomarker for IBD, nor is there a specific marker for UC or CD. It is expected that in the future, miRNAs will be developed as sensitive and specific diagnostic, therapeutic, and prognostic biomarkers in IBD, providing a non-invasive

and accessible tool for effective monitoring.

## FOOTNOTES

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*EDITORIAL*

# Microplastics and microbiota: Unraveling the hidden environmental challenge

Jean Demarquoy

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## Abstract

This editorial explores the intricate relationship between microplastics (MPs) and gut microbiota, emphasizing the complexity and environmental health implications. The gut microbiota, a crucial component of gastrointestinal health, is examined in the context of potential microbial degradation of MPs. Furthermore, dysbiosis induced by MPs emerges as a consensus, disrupting the balance of gut microbiota and decreasing diversity. The mechanisms triggering dysbiosis, including physical interactions and chemical composition, are under investigation. Ongoing research addresses the consequences of MPs on immune function, nutrient metabolism, and overall host health. The bidirectional relationship between MPs and gut microbiota has significant implications for environmental and human health. Despite uncertainties, MPs negatively impact gut microbiota and health. Further research is essential to unravel the complex interactions and assess the long-term consequences of MPs on both environmental and human well-being.

**Key Words:** Microplastics; Microbiota; Gut; Dysbiosis



**Core Tip:** The intricate relationship between microplastics (MPs) and gut microbiota, as outlined in this article, emphasizes the growing concern for environmental health. Although the potential microbial degradation of certain MPs is recognized, the dysbiosis induced by these particles is widely acknowledged as a threat, impacting the balance and diversity of gut microbiota. Ongoing research aims to unravel these complex, bidirectional interactions, highlighting the need for a comprehensive understanding of their implications for both environmental ecosystems and human health.

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## INTRODUCTION

This editorial comments on an article published in a recent issue of *World Journal of Gastroenterology*, entitled "Impact of microplastics and nanoplastics on liver health: Current understanding and future research directions"[1]. We delve deeper into the connection between gut microbiota and plastic microparticles.

The interaction between microplastics (MPs) and microbiota is a subject of growing concern, especially in the context of environmental and human health. Overall, the interaction between MPs and microbiota is a complex issue that requires further research, particularly to understand the long-term health effects in both animals and humans. The growing body of evidence suggests that MPs could be a significant environmental health concern, impacting not just ecosystems but also the health of individual organisms by altering their gut microbiota.

## MPs

MPs can at least be categorized according to their size, origin, or chemical composition.

### *Classification by size*

MPs are tiny particles of plastic that measure less than 5 mm in length. Nanoplastics are particles smaller than 1 micrometer in size[2].

### *Two main categories based on their source*

MPs can be classified according to their source: Primary MPs and secondary MPs.

Primary MPs encompass various types of tiny plastic particles that directly contribute to environmental pollution. Microbeads, deliberately added to personal care items such as scrubs and toothpaste, have faced bans in several regions due to their detrimental impact. Nurdles, small plastic pellets used in plastic production, are released into the environment through accidental spills during transport. Microfibers from synthetic textiles during washing, become a notable source of microplastic pollution in water. Additionally, MPs are present in cosmetics and personal care products, including glitter and other small plastic particles used for aesthetic purposes, contributing to the broader issue of microplastic contamination[3].

Secondary MPs result from the breakdown of larger plastic items through environmental processes such as sunlight exposure, wind, and wave action. Over time, exposure to environmental factors such as sunlight, heat, and mechanical forces can lead to the breakdown of larger plastic items into smaller particles, eventually forming MPs. The deterioration of vehicle tires represents a noteworthy contributor, as minute particles released during this process contribute to the presence of MPs in both terrestrial and aquatic ecosystems[4]. Additionally, the degradation of paints, coatings, and finishes on various surfaces releases small plastic particles into the environment, further contributing to the issue of secondary MPs [5].

### *Chemical structure of MPs*

MPs consist of various synthetic polymers, each characterized by long chains of molecules formed from repeating subunits. The chemical composition of MPs varies according to the specific polymer used in their production. Noteworthy polymers found in MPs encompass Polyethylene (PE), prevalent in packaging materials, bottles, and various plastic products; Polypropylene, utilized in packaging, textiles, and plastic containers; PE terephthalate, commonly employed in beverage bottles, food containers, and synthetic fibers; Polyvinyl chloride, used in construction materials, pipes, and certain types of packaging; Polystyrene, commonly present in foam packaging, disposable utensils, and insulation materials; Polyurethane, employed in foams, coatings, adhesives, and flexible plastics; Nylon, found in textiles, fishing nets, and certain plastic components; and Acrylic, used in transparent plastics, lenses, and signage[6]. These polymers are not easily biodegradable, contributing to the persistence of MPs in the environment.

It's important to note that the chemical composition of MPs can also be influenced by additives and colorants used in the manufacturing process.

## THE GUT MICROBIOTA

The gut microbiota is a complex and dynamic ecosystem, primarily composed of bacteria, along with archaea, viruses, fungi, and protozoa, residing in the gastrointestinal tract. This diverse microbial community is predominantly composed of bacteria from the *Firmicutes* and *Bacteroidetes* phyla, with significant contributions from *Actinobacteria*, *Proteobacteria*,

and *Verrucomicrobia*. The specific composition varies widely among individuals due to factors such as diet, health status, age, and genetic background. Within these microbial groups, genera such as *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Clostridium*, and *Faecalibacterium* play crucial roles in maintaining gut health[7]. They contribute to nutrient absorption, synthesis of vitamins, protection against pathogens, and modulation of the immune system. The balance and diversity of the gut microbiota are essential for overall health, with imbalances linked to a range of diseases, including obesity, inflammatory bowel disease, diabetes, and allergies.

## INTERACTION MICROBIOTA/MPs

The relationship between microbiota and MPs can exhibit various forms of interaction. The microbiota, with its diverse ensemble of microorganisms, may possess the ability to degrade certain MPs, a mechanism beyond the capability of eukaryotic cells. Conversely, the gut microbiota, a complex and diverse community of microorganisms, holds the potential to contribute to the degradation of MPs; nonetheless, this aspect is not yet fully comprehended.

## DEGRADATION OF PLASTICS BY THE MICROBIOTA

Some microorganisms have been found to possess enzymes capable of breaking down certain types of plastics. These microorganisms, often bacteria or fungi, can metabolize or degrade plastic polymers to some extent under specific conditions.

Exploring the human digestion of MPs and their influence on colonic microbiota involves a range of methodologies, incorporating both in vitro and in vivo approaches. While investigations using animals and human trials are considered the standard due to their physiological relevance, they face limitations such as ethical concerns, high expenses, and the intricate nature of the multistage processes in human digestion. Consequently, there is a legitimate need for in vitro models that faithfully replicate the physiological conditions of human digestion.

Static models play a crucial role in identifying endpoints or kinetics of particular digestion phases, such as the biotransformation occurring in the stomach and small intestine. In contrast, dynamic models, despite their increased complexity, offer a more accurate representation of the physiological reality within the gastrointestinal tract[7]. Among these dynamic simulators, the simgi® system for instance, has been employed to investigate the effects of various foods, for example[8].

Numerous articles have discussed the existence of enzymes within the gut microbiota capable of breaking down MPs. However, these studies have not provided specific numbers regarding the percentage of MPs degraded by the microbiota, nor have they clarified whether the degradation process is complete. Additionally, there is a lack of information on the actual impact of the microbiota on eliminating MPs from the human environment. The diverse structure of MPs also suggests that the microbiota may not be totally capable of degrading all types of MPs. The work by Nugrahapraja *et al*[8] (2022) serves as an illustrative case of this issue. While the authors delineated enzymatic activities capable of degrading plastics within the human gut microbiota, their conclusion highlighted the challenge in quantifying the actual impact of the microbiota on the elimination of MPs.

The degradation of MPs by gut microbiota, if possible, would depend on several factors. These include the type of plastic, the size and shape of the MPs, the specific microbial species present, and the environmental conditions within the gut (such as pH, temperature, and oxygen levels)[9].

If gut microbiota can degrade MPs, it could have significant implications for reducing the environmental burden of plastic pollution and its impacts on health. However, the potential byproducts or consequences of such microbial degradation in the gut environment are not yet clear and would need to be thoroughly studied.

In summary, while there is potential for certain microorganisms to degrade plastics, the extent to which gut microbiota can break down MPs is still an open question in scientific research. More studies are needed to understand this interaction and its implications for environmental and human health. Regardless, it is evident that the microbiota within the human gut lacks the capability to break down all the MPs present in the food ingested by an individual.

## DYSBIOSIS INDUCED BY MPs

If there is a consensus regarding the interrelationships between MPs and the intestinal microbiota, it is indeed that of dysbiosis. The vast majority of publications related to the connections between MPs, and the microbiota conclude that MPs present in the intestine induce, in humans as well as in other species, particularly in fish[10], a modification of the microbiota composition, notably resulting in a decrease in its diversity. Numerous bibliographic references on this topic exist, and some reviews are available on this subject[11,12].

Dysbiosis induced by MPs represents a disruption in the delicate balance of the gut microbiota, a complex community of microorganisms residing in the gastrointestinal tract. MPs, being foreign entities, can interact with the gut environment in various ways, potentially triggering alterations in microbial composition, diversity, and function.

The precise mechanisms by which MPs trigger dysbiosis are still under investigation, with several factors currently under consideration. First, the physical presence of MPs may lead to direct interactions with gut microorganisms, influencing their growth, survival, and metabolic activities. Second, the chemical composition of MPs and any associated additives might have direct or indirect effects on the microbiota. Moreover, MPs may serve as carriers for other pollutants

or pathogens, further complicating their impact on gut microbial communities. The consequences of dysbiosis extend beyond the gut, potentially affecting immune function, nutrient metabolism, and overall host health. Long-term exposure to MPs and their influence on gut dysbiosis continue to be critical areas of research, with significant implications for both environmental and human health as contamination levels rise.

## CONCLUSION

Overall, MPs have no positive effect on gut microbiota and human health. The interactions between MPs and the gut microbiota are complex, given the diverse sources, sizes, shapes, and chemical structures of MPs. The relationships between plastics and the microbiota operate bidirectionally. While microbiota can, in certain conditions, be able to degrade and eliminate some MPs, simultaneously, MPs can alter the function of the microbiota, inducing dysbiosis, and subsequently may have health effects.

## FOOTNOTES

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*REVIEW*

# Mechanisms of tumor immunosuppressive microenvironment formation in esophageal cancer

Xiao-Jun Zhang, Yan Yu, He-Ping Zhao, Lei Guo, Kun Dai, Jing Lv

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## Abstract

As a highly invasive malignancy, esophageal cancer (EC) is a global health issue, and was the eighth most prevalent cancer and the sixth leading cause of cancer-related death worldwide in 2020. Due to its highly immunogenic nature, emerging immunotherapy approaches, such as immune checkpoint blockade, have demonstrated promising efficacy in treating EC; however, certain limitations and challenges still exist. In addition, tumors may exhibit primary or acquired resistance to immunotherapy in the tumor immune microenvironment (TIME); thus, understanding the TIME is urgent and crucial, especially given the importance of an immunosuppressive microenvironment in tumor progression. The aim of this review was to better elucidate the mechanisms of the suppressive TIME, including cell infiltration, immune cell subsets, cytokines and signaling pathways in the tumor microenvironment of EC patients, as well as the downregulated expression of major histocompatibility complex molecules in tumor cells, to obtain a better understanding of the differences in EC patient responses to immunotherapeutic strategies and accurately predict the efficacy of immunotherapies. Therefore, personalized treatments could be developed to maximize the advantages of immunotherapy.

**Key Words:** Esophageal cancer; Esophageal squamous cell carcinoma; Esophageal adenocarcinoma; Tumor immune microenvironment; Immunosuppression; Immunotherapy

**Core Tip:** Esophageal cancer (EC) is a significant global health issue, and immunotherapy holds promise for treating this disease. However, resistance to immunotherapy may occur, and is usually associated with the tumor immune microenvironment (TIME). Understanding the TIME, especially the suppressive TIME, is crucial. The aim of this review is to elucidate the underlying mechanisms of the suppressive TIME in EC, including cell infiltration, immune cell subsets, cytokines and signaling pathways, as well as the downregulated expression of major histocompatibility complex molecules in tumor cells. This summary may help predict EC patient responses to immunotherapies and facilitate personalized treatments to optimize immunotherapeutic outcomes.

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## INTRODUCTION

According to the Global Cancer Statistics 2020 database (https://gco.iarc.fr/), approximately 20000000 people are diagnosed with cancer each year, and approximately 10000000 people die from cancer worldwide[1]. Esophageal cancer (EC) accounts for 3.1% of all new cancer cases and ranks eighth in incidence among all cancer types; however, EC accounts for 5.5% of all cancer-related deaths and ranks sixth in mortality[1]. There are two main histological types of EC: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC)[2]. Although nearly 90% of EC cases are ESCC, the incidence and mortality rates of EAC are gradually increasing and even surpass those of ESCC in some regions of North America and Europe[3,4]. The main risk factors for EAC include gastroesophageal reflux disease and obesity, and ESCC is associated with chemical carcinogen exposure, cigarette smoking and alcohol consumption, a diet with low amounts of fruits or vegetables, high consumption of pickled vegetables or processed meat, hot drinks, *etc* [3,5]. The five-year survival rate of patients with EC is usually between 20% and 30%, and mainly depends on the tumor stage at initial diagnosis and the therapeutic strategy, such as surgery combined with neoadjuvant therapy (radiotherapy and chemotherapy)[6-8]. Since conventional treatments have limited efficacy and potential adverse effects, more effective therapeutic strategies are urgently needed to improve the prognosis of patients with EC[9].

Cancer development is closely related to the accumulation of gene mutations, and researchers have focused on changes in cancer cells for quite a long time[10]. Recently, the tumor microenvironment (TME) has become a hot topic, and the regulation of immune cells in the TME has drawn much attention[11]. The immune system can recognize and eliminate tumor cells expressing specific antigens, a process known as cancer immunosurveillance[12]; while, cancer cells can escape or suppress attacks from the immune system by various mechanisms, including decreasing antigen presentation, upregulating the expression of apoptotic inhibitors, increasing the expression of inhibitory molecules on the cell surface, and enhancing the secretion of certain cytokines or recruitment of regulatory cells to create an immunosuppressive microenvironment[12]. As an important component of the TME, the tumor immune microenvironment (TIME) refers to the microenvironment involving interactions between host immune agents and tumor cells[13,14]; tumors may confront host immune systems by gradually forming immunosuppressive conditions, and the presence of protumor and antitumor factors in the TIME may determine cancer progression and response to treatments[14-16]. Therefore, a comprehensive understanding of the interactions between tumor cells and various immune cells or other immune components in the TIME is vital for further elucidating the mechanisms of EC immunotherapy[17-20].

In this review, we mainly summarize the mechanisms of immunosuppression in the TIME of EC, including immune cells, immune checkpoints, immunosuppressive cells and tumor cell-related immunosuppressive factors, to provide evidence for the maintenance of an immune-activated state in the TIME of EC, with the goal of improving immunotherapeutic efficacy.

## IMMUNOGENICITY OF ESOPHAGEAL CANCER

Esophageal epithelial tumor cells are the main constituents of EC and express tumor-associated antigens (TAAs)[19]. TAAs are a class of overexpressed molecules that are present mainly on the membrane of tumor cells, and are usually expressed at lower levels or undetected in normal cells[21]. T lymphocytes may recognize and bind the TAA peptides presented by major histocompatibility complex (MHC) molecules on tumor cells through the T-cell receptor, thereby initiating an immune response and triggering an attack on tumor cells[21]. In addition, natural killer (NK) lymphocytes and B lymphocytes play important roles in the regulation of immunoreactivity in EC[22,23]. For example, as a class of TAAs associated with 276 genes in more than 70 gene families, the antigen families formed by cancer-testis antigens (CTAs) are expressed mainly in ovarian granulosa cells and testicular germ cells, and are barely expressed in normal tissues[24-26]. Certain CTAs, such as New York ESCC 1 (NY-ESO-1) and melanoma-associated antigen-A (MAGE-A), have been reported to be highly expressed in EC, and specific immune responses targeting MAGE-A and NY-ESO-1 have been observed in EC patients[27-30]. MAGE-A3-specific $CD8^+$ T cells may kill HLA-$A2^+$/MAGE-$A3^+$ tumor cells in ESCC patients, and functional MAGE-C2-specific $CD8^+$ T cells may independently affect the prognosis of EC patients[27,31].

Since EC cells possess high immunogenicity, partially because of the presence of numerous antigens, these molecules could be potential targets for immunotherapy, and immunotherapy has been shown to be more effective in EC patients with an immuno-activated TME, leading to an improved prognosis[32]. However, current immunotherapeutic strategies have several limitations, *e.g.*, accompanying adverse effects and drug resistance cannot be avoided[33]. Therefore, a comprehensive understanding of the underlying mechanisms of the TIME in EC, especially the suppressive TIME, is pivotal and urgent for the management of EC patients.

## DYSFUNCTION OF IMMUNE CELLS

A suppressive TIME is usually accompanied by the reduced infiltration or exhaustion of immune cells, and is correlated mainly with the presence of immunosuppressive cells and coinhibitory signals[34]. Herein, we focused on the reduced infiltration and exhaustion of T cells and NK cells, which play important roles in the TIME. In addition, immunosuppressive cells, such as suppressive macrophages (M2 macrophages) and myeloid-derived suppressor cells (MDSCs), can inhibit the activities of immune cells through various mechanisms to participate in balancing immune reactions in the TIME[35], and their presence may influence immunotherapeutic efficacy in cancers. Thus, elucidating the underlying molecular mechanisms is highly important for improving the therapeutic efficacy of agents for cancer treatment.

### *T lymphocytes*

T cells are the major component of infiltrated immune cells in most solid tumors, and $CD8^+$ cytotoxic T cells (CTLs) and $CD4^+$ T helper cells (Ths) play crucial roles in eliminating tumor cells[36,37]. Specifically, activated CTLs may exert a cytotoxic effect on tumor cells by releasing cytotoxic substances, and Ths can promote or suppress host immune activities targeting tumor cells[36-38].

According to the single-cell sequencing results, the percentage of exhausted $CD8^+$ T cells positive for C-X-C motif chemokine ligand 13 (CXCL13) increased, as these cells are the main T-cell type in the TME of EAC patients[39]. In ESCC, the infiltration and proliferation of T-cell clones have also been observed, and an exhausted $CD8^+$ T-cell cluster (CD8-C7-TIGIT) and pre-exhausted CD8-C5-CCL5 and CD8-C6-STMN1 clusters accounted for high proportions of $CD8^+$ T-cell clusters[22]. The expression level of the E3 ubiquitin ligase MARCH7 in ESCC tissues has been shown to be significantly greater than that in nontumor tissues, and was negatively correlated with tumor-infiltrating immune cells, such as $CD8^+$ T cells[40]. Moreover, a subpopulation of $CD8^+$ T cells expressing SPRY1 has been found in ESCC tissues after neoadjuvant immune checkpoint blockade, and these cells may possess certain progenitor cell characteristics and exhibit an exhausted phenotype[41]. Additionally, fibroblast growth factor 2 derived from tumor fibroblasts can induce the expression of SPRY1 in infiltrating T cells and participate in T-cell exhaustion in EC[42].

**Immune checkpoints:** Activated T cells may express various inhibitory receptors, known as immune checkpoints, to prevent excessive immune responses, aiming to maintain an immunologic balance; however, tumor cells may exploit these checkpoints to induce coinhibitory signals in the TME and create an immunosuppressive TME, which plays a pivotal role in tumor immune escape[43,44]. Thus, medications such as immune checkpoint inhibitors have been investigated for their ability to block these checkpoints, subsequently enhancing the ability of the immune system to attack tumor cells[34,45].

Programmed cell death protein 1 (PD-1), which is expressed on the T-cell membrane, is a classic immune checkpoint that can transmit immune inhibitory signals when it interacts with its corresponding ligand programmed cell death ligand 1 (PD-L1), which is expressed on tumor cells[46,47]. These interactions can inhibit the cytotoxic activities of T cells and allow tumor cells to escape immune surveillance and attack, accounting for one of the mechanisms of tumor immune escape[46,47]. For instance, EC patients with high PD-L1/PD-L2 expression, particularly patients in advanced stages, may have a poor prognosis[20]. Therefore, inhibiting PD-1/PD-L1 by blocking their interaction may restore the vigor and cytotoxicity of T cells in the TIME[18]. In recent years, immunotherapy involving checkpoint blockade targeting PD-1/PD-L1 has developed rapidly, becoming a first-line treatment for many cancers[17,48], but the efficacy of PD-1/PD-L1 blockade largely depends on the expression levels of PD-1/PD-L1 in the TME[19,49,50].

The interaction between CD28 on T cells and B7-1 (CD80)/B7-2 (CD86) on antigen-presenting cells or target cells can provide costimulatory activating signals to T cells, and subsequently boost T-cell activation[51]. Cytolytic T lymphocyte-associated antigen-4 (CTLA-4), another important regulatory molecule primarily expressed on regulatory T cells (Tregs) and activated T cells, can competitively bind B7 and inhibit cellular signal transduction for T-cell activation, subsequently suppressing immune responses[52,53]. Therefore, CTLA-4 is also considered an immune checkpoint molecule, and CTLA-4 blockade could effectively enhance immune responses against tumor cells[52]. However, the efficacy and safety of CTLA-4 blockade in EC patients require further investigation due to the limited number of related clinical trials.

In addition to the coinhibitory molecules mentioned above[18,52,54,55], researchers have identified various other immune checkpoints, such as T-cell immunoglobulin (Ig) and mucin domain-containing protein-3 (TIM-3)[56,57], lymphocyte activation gene-3[57-59] and T-cell Ig and ITIM domain[60-63]; detailed information about the potential immune checkpoints involved in EC in Figure 1[63-66].

**Regulatory T lymphocytes:** Tregs are $CD4^+CD25^+Foxp3^+$ T cells that play an important role in suppressing the host immune response in the TME[67-70]. The infiltration of Tregs may be correlated with tumor invasion, progression, metastasis and poor survival after chemotherapy[68-71], and the infiltration of Tregs has also been shown to be negatively correlated with antitumor effector cells such as CTLs and NK cells in ESCC[72]. In addition, the hypomethylation-induced chemokine CCL20 in the TIME could affect the immune balance and promote the progression

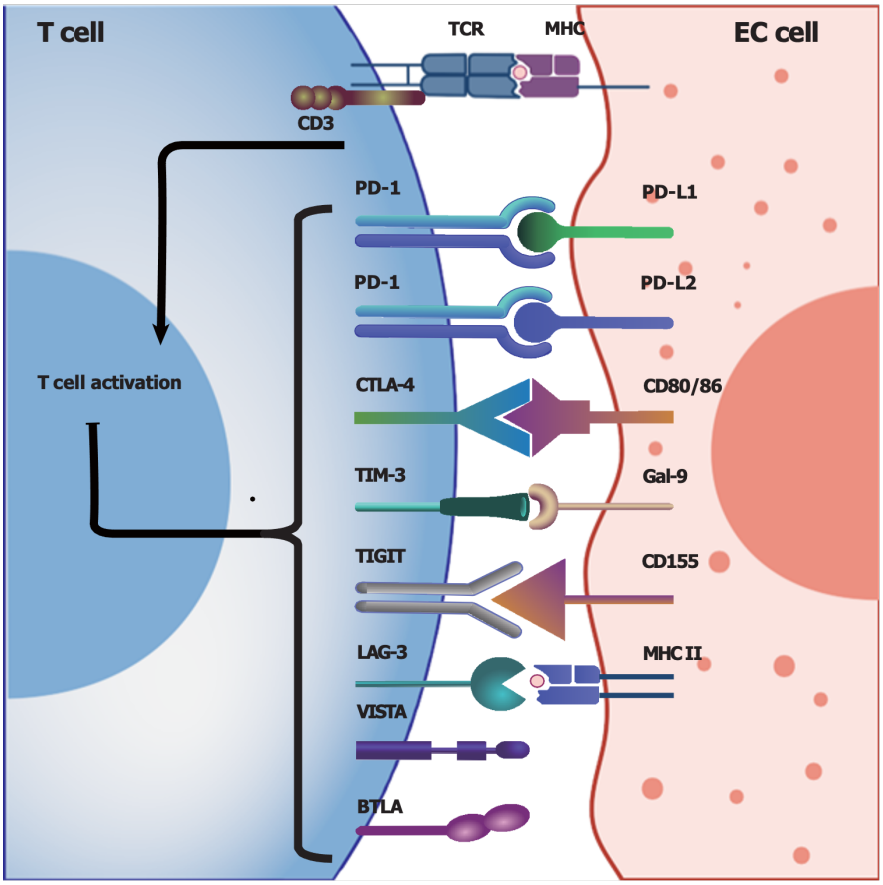


**Figure 1 Summary of potentially involved immune checkpoints in esophageal cancer.** T cells can be activated by interacting with major histocompatibility complexes expressed on esophageal cancer (EC) cells, and the presence and interaction of immune checkpoints with their ligands can suppress T-cell activation and function to achieve immunosuppression. Herein, we summarize the immune checkpoints and their ligands that are potentially involved in the tumor microenvironment of EC. Programmed cell death protein 1, cytolytic T lymphocyte-associated antigen-4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), lymphocyte activation gene 3 (LAG-3), V-domain Ig suppressor of T-cell activation and B- and T-lymphocyte attenuator are expressed on T cells, while TIM-3, TIGIT and LAG-3 are also expressed on natural killer cells. EC: Esophageal cancer; TCR: T cell receptor; MHC: Major histocompatibility complex; CD: Cluster of differentiation; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death ligand 1; PD-L2: Programmed cell death ligand 2; CTLA-4: Cytolytic T lymphocyte-associated antigen-4; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; Gal-9: Galectin-9; TIGIT: T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; LAG-3: Lymphocyte activation gene 3; VISTA: V-domain Ig suppressor of T-cell activation; BTLA: B- and T-lymphocyte attenuator.

of EC, possibly contributing to the infiltration of Tregs in ESCC[73]. Moreover, Han *et al*[74] showed that Tregs may have the highest interleukin (IL)-32 expression in the TME of ESCC patients, and this expression is positively correlated with that of Foxp3, potentially promoting tumor progression; in addition, IL-32 may also induce interferon (IFN)-γ secretion by $CD8^+$ T cells and facilitate antitumor immunity. Additionally, an imbalance in Th17/Treg cells has also been reported to occur during the development of Barrett's esophagus, the precursor of EAC, through the regulation of the release of certain inflammatory cytokines[75].

Generally, T cells may experience functional loss or exhaustion in the TIME through interactions with various coinhibitory factors, and Tregs could play a crucial role in immunosuppression in the TME. Therefore, elucidating the functions and interactions of T cells with other cells in the TIME and understanding the mechanism of Treg-mediated tumor immune escape could provide valuable insights into the mechanisms of tumor immune escape, and thus further provide important evidence for novel immunotherapeutic strategies aimed at overcoming tumor immune escape.

### *NK lymphocytes*

NK cells are another type of tumor cell-killing lymphocyte that has garnered significant attention in cancer immunotherapy[76]. Previous preclinical and clinical studies have shown promising results for NK cell-related immunotherapy, and provided a novel perspective on immunotherapeutic strategies for NK cell-related treatments[77]. However, NK cells often experience a reduction or exhaustion in the immunosuppressive TME similar to that of T cells, which may also limit their antitumor effects[76,78].

The number of NK cells has been shown to be significantly lower in ESCC tissues than in adjacent nontumor tissues; in addition, a specific subset of cells, NK-C3-KLRC1 has been shown to differentiate from NK-C1-NCR3, and the number of NK-C2-STMN1 cells was significantly increased in ESCC[22]. The NK-C1-NCR3 subset has been shown to express relatively high levels of NCR3, CD266, NKG7 and LAMP1, and the NK-C3-KLRC1 and NK-C2-STMN1 subsets have been

shown to express relatively high levels of KLRC1 and ITGA1[22]. As a cell surface receptor primarily expressed on NK cells and some types of T cells, NK group 2 member D (NKG2D) can interact with its ligands (NKG2DLs) to activate NK cells and T cells, and subsequently enhance immune surveillance and the clearance of tumor cells or infected cells[79,80]. Researchers have shown that the expression of NKG2DLs is significantly higher in ESCC tissues than in control tissues, and ESCC cells exhibit increased NKG2DL expression, thus providing a potential therapeutic target for ESCC *via* the use of NK cells[78]. Moreover, the inhibitory receptor NKG2A has been shown to be upregulated in NK cells in ESCC tissues compared to adjacent nontumor tissues[22], and a higher level of TIM-3 in tumor-infiltrating NK cells has been shown to be correlated with functional impairment and related to tumor invasion, lymph node metastasis and advanced stages in EC patients[56]. Notably, the expression of $CD16^{bright}CD56^{dim}$ may significantly decrease in NK cells in ESCC, leading to a weakened antibody-dependent cell-mediated cytotoxicity response mediated by cetuximab, which binds to the CD16 receptor on NK cells and targets the epidermal growth factor receptor (EGFR)[81,82].

Furthermore, numerous cytokines may also participate in regulating the immuno-activation of NK cells. For instance, transforming growth factor (TGF)-β partially contributes to the downregulation of CD16 expression on NK cells, resulting in impaired NK cell function[81]. A lack of IL-18 in ESCC tissues may induce the production of IFN-γ in NK cells and $CD8^+$ T cells, and potentially promote the clearance of tumor cells and improve the TME in patients with EC[83]. The expression level of IL-6, an important cytokine secreted by ESCC cells in the TME, has been shown to be higher in tumor tissues and blood circulation in ESCC patients, and may significantly upregulate the expression of CD39 on NK cells and impair the functions of NK cells, as well as be related to the poor prognosis of ESCC patients[84]. Another clinical study reported that IL-6 and IL-8 secreted by ESCC cells may downregulate the expression of certain activating receptors on NK cells and impair the function of NK cells by activating the signaling transducers and activators of transcription 3 (STAT3) signaling pathway[85]. Taken together, the above results demonstrate the decreased number and dysfunction of NK cells, effects that may disrupt immune surveillance in cancer patients, and pose a challenge for the investigation and clinical application of NK cell-related immunotherapy in ESCC patients.

### *Immune suppressive cells*

**Macrophages:** Macrophages are important components of the innate immune system, and play pivotal roles in recognizing and removing damaged cells, pathogens and other foreign matter, as well as regulating adaptive immune responses by secreting various cytokines and chemokines[86]. Based on their functions and phenotypes, tumor-associated macrophages (TAMs) can be classified into two types: M1 and M2 macrophages[86,87]. M1 macrophages have proinflammatory properties and primarily participate in clearing pathogens, whereas M2 macrophages promote cell proliferation and tissue repair[86,87]. M1 macrophages in the tumor stroma are involved mainly in inhibiting the migration and invasion of ESCC cells, and serve as good prognostic factors for ESCC patients[88,89].

As an element of immunosuppression, M2 macrophages enriched in the TME of ESCC may suppress cell-mediated immune responses, secrete immunosuppressive factors and promote tumor angiogenesis[22]. M2 polarization may increase the expression of PD-L2 in ESCC cells, and lead to tumor immune escape and progression *via* PD-1-related signaling pathways[89]. In addition, Lu *et al*[90] reported that the upregulation of S100A7, a member of the S100 superfamily, could promote macrophage infiltration and M2 polarization, facilitating tumor angiogenesis by enhancing the activation of the p-ErK and p-FAK signaling pathways in the TME of ESCC. IL-32, which is highly secreted by Tregs, may promote the formation of an immunosuppressive TME; in addition, researchers have shown that IL-32, which is secreted from ESCC cells *via* extracellular vesicles, may shuttle into macrophages to promote M2 polarization *via* the FAK-STAT3 signaling pathway, further contributing to ESCC metastasis[91]. Moreover, Wang *et al*[92] reported that ESCC $FOXO1^+$ cells may promote M2 polarization and recruitment to the TME in ESCC through the transcriptional regulation of CCL20 and CSF-1, and $FOXO1^+$ tumor-induced M2 macrophages could promote tumor proliferation through FAK-PI3K-AKT signaling, which could be blocked by the blockade of PI3K[92]. In a rodent ESCC model, researchers found that CCL18, a chemokine secreted by TAMs, may promote tumor cell proliferation through the Janus-activated kinase 2 (JAK2)/STAT3 signaling pathway, and higher CCL18 levels are correlated with poor prognosis in ESCC patients[93]. To investigate the potential therapeutic efficacy of CCL18, researchers synthesized a CCL18-blocking peptide (Pep3) and found that it could inhibit the proliferation of EC-109 cells, suggesting potential targets through which CCL18 represses the progression of ESCC[93]. CCL22, another chemokine produced by TAMs in ESCC, may activate the FAK/AKT pathway and facilitate the malignant progression of ESCC cells[94]. Moreover, M2 macrophages may transmit the long noncoding RNA (lncRNA) AFAP1-AS1 to ESCC cells *via* secreted exosomes, downregulating miR-26a expression and upregulating ATF2 expression, thereby promoting tumor cell invasion and metastasis in EC[95]. Furthermore, a recent study showed that exosomes secreted by M2 macrophages carrying LINC01592 could be transferred to EC cells, resulting in a decrease in MHC-I expression, thereby allowing tumor cells to escape from attacks by $CD8^+$ CTLs[96]. When the E2F6/NBR1/MHC-I signaling pathway was disrupted by small interfering RNAs or corresponding blocking antibodies, the tumor-promoting effects induced by LINC01592, as well as M2-driven tumor growth, were significantly inhibited[96]. In summary, M2 macrophages play an inhibitory role in the TIME of EC and can be recognized as key regulators of cancer occurrence, progression and metastasis. Therefore, targeting M2 macrophages and related signaling pathways may provide a promising perspective on therapeutic strategies for EC management.

**MDSCs**: MDSCs are widely accepted as a population of immature bone marrow cells, that can be classified into granulocyte-like MDSCs (G-MDSCs) and monocyte-like MDSCs (M-MDSCs)[97]. Both G-MDSCs and M-MDSCs play important roles in inhibiting immune cell activities in the TME, thus promoting tumor growth and metastasis[97]. It has been reported that the proportions of MDSCs and Tregs are significantly greater in EC patients than in controls, further suggesting an immunosuppressive role of MDSCs in EC[98]. Therefore, inhibiting the recruitment of MDSCs to the TME might be a promising approach for treating EC *via* immunotherapy. For example, TGF-β secreted by MDSCs in the TME

may induce the phosphorylation of Smad2/Smad3, and contribute to the increased expression of the cancer/testis-associated gene *Maelstrom* (*MAEL*) in EC cells[99]. MAEL may be correlated with increased IL-8 expression by regulating the Akt1/RelA signaling pathway, and IL-8, in turn, may guide the recruitment of MDSCs into the TME of ESSCs[99]. In addition, the expression of MAEL in ESCC cells has been shown to be associated with recurrence and poor prognosis[99]. Moreover, it has been shown that the gene developmentally downregulated 9, which is critical for maintaining the stemness phenotype of ESCC cells, can regulate the expression of *CXCL8* through the ERK signaling pathway, thereby contributing to the recruitment of MDSCs to the TME[100].

In addition to focusing on the recruitment of MDSCs, inhibiting MDSC function in the TME might be another important strategy. MDSCs with higher CD38 expression have been shown to be better able to inhibit activated T cells and promote tumor growth than MDSCs with lower CD38 expression[101]. This enhanced immunosuppressive capacity of $CD38^{high}$ MDSCs may be attributed to their increased production of inducible nitric oxide synthase (iNOS), since the upregulated iNOS may act as an immunosuppressive molecule to suppress the immune responses of T cells and contribute to tumor immune escape[101]. Moreover, EC patients exhibit increased numbers of MDSCs and Th17 cells in the peripheral circulation, as well as increased levels of plasma Arg1 and iNOS mRNA in peripheral blood mononuclear cells[102]. Additionally, the expression of myeloid cell markers in ESCC may be positively correlated with the increased expression of certain immune checkpoints, such as PD1, TIM3 and V-domain Ig suppressor of T-cell activation, as well as the development of ESCC[103]. However, the depletion of $Gr1^+$ MDSCs may reduce the number of MDSCs, decrease the expression levels of immune checkpoint molecules, and inhibit tumor growth, suggesting the potential roles of MDSCs in the immunosuppression and progression of ESCC[103]. Furthermore, another fundamental study reported higher levels of lnc-17Rik in MDSCs derived from the peripheral blood of EC patients, and indicated that lnc-17Rik may enhance tumor immunosuppression by increasing the expression and enhancing the activation of certain key genes involved in MDSC differentiation, such as arginase 1, cyclooxygenase 2, NOS2, and NADPH oxidase 2[104]. These findings highlight the significance of elucidating the functions of MDSCs in the TIME, and suggest potential targets for therapeutic interventions aimed at overcoming immunosuppression and improving therapeutic efficacy in patients with EC.

## TUMOR CELL-RELATED IMMUNOSUPPRESSIVE FACTORS

Although EC exhibits strong immune responsiveness, as previously mentioned, it may still achieve immune escape in the immunosuppressive TME through various mechanisms (Figure 2), including the downregulation of MHC expression, the secretion of immunosuppressive factors and alterations in tumor metabolism.

### *Downregulation of MHC expression*

Tumor immune escape is often accompanied by a decrease in or loss of MHC molecules, which play crucial roles in the recognition and killing of tumor cells by immune cells[105]. Notably, the expression of HLA-ABC molecules is usually decreased or even absent in ESSC tissues[106]. Specifically, a previous study reported that approximately 41% of EC patients had no HLA-ABC expression, more than half of the EC patients had weak expression, and only approximately 3% of the EC patients had strong HLA-ABC expression[106]. In addition, the reduced or absent expression of HLA-ABC in ESCC may be strongly correlated with the expression of certain molecules that participate in antigen processing, such as b2m, ATP binding cassette subfamily B member 1 (TAP1), TAP2, LMP2 and LMP7[107]. Moreover, allelic loss in the 6p21.3 region, observed in approximately 46.9% of ESCC patients in a Chinese study, has been shown to be associated with the downregulation of HLA class I antigens[108,109], and DNA hypermethylation may result in deficient expression of HLA class I genes in ESCC[110]. Numerous ncRNAs, such as miR-125a-5p and miR-148a-3p, may downregulate the expression of TAP2 and HLA-I to affect the antigen presentation process[111], and exosomal LINC01592 released from TAMs may also downregulate the expression of MHC-I in EC cells and promote malignant EC progression[96]. Downregulation of the expression of MHC molecules in the TME hampers antigen processing and presentation processes, thereby enabling tumor immune evasion in patients with EC. Investigating these underlying mechanisms is crucial for advancing innovative cancer immunotherapy focused on these molecules.

### *Secretion of immunosuppressive factors*

An immunosuppressive TME is partially generated by immunosuppressive factors secreted by tumor cells, immune cells and stromal cells[112], and these factors play crucial roles in tumor proliferation, angiogenesis and invasion, as well as in EC progression[113]. Some classic immunosuppressive cytokines, such as TGF-β and IFN-γ, may inhibit the functions of immune cells, thereby weakening the ability of the immune system to attack tumor cells[113,114].

The TGF-β signaling pathway could play a dual role in cancer development depending on the stage of disease[114]. Under pathological conditions, the overexpression of TGF-β may lead to epithelial mesenchymal transition, extracellular matrix deposition and the formation of cancer-associated fibroblasts, resulting in fibrotic diseases and cancers[115]. In addition, TGF-β can restrict the infiltration of T cells to the TME and decrease antitumor immunoactivity[116]. Moreover, TGF-β derived from MDSCs in the TME of ESCC may increase PD-1 expression in $CD8^+$ T cells, leading to resistance to immunotherapy *via* PD-1/PD-L1[27]. Furthermore, the combination of TGF-β and PD-L1 blockade has been shown to significantly increase the number of tumor-infiltrating T cells and reduce the tumor burden in EAC patients[116].

The IFN signaling pathway also plays a dual role in the TME. On the one hand, IFN-γ acts as a cytotoxic cytokine and induces tumor cell apoptosis, thus exerting antitumor effects[117]. On the other hand, IFN-γ may contribute to immunosuppression in the TIME by promoting the synthesis of immune checkpoint-related factors, such as PD-L1, thus

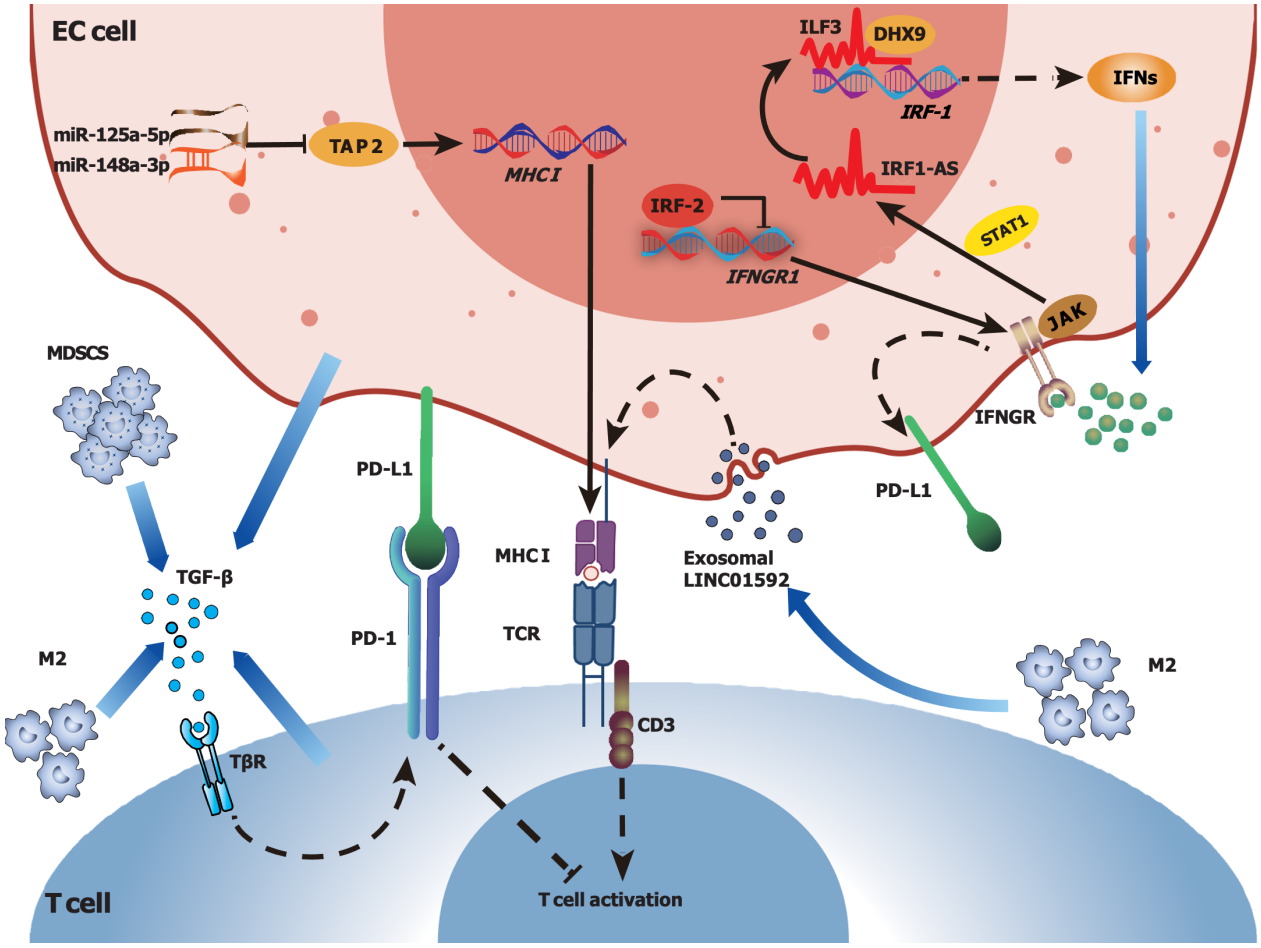


**Figure 2 Potential mechanisms of tumor immunosuppressive microenvironment formation in esophageal cancer: Major histocompatibility complex downregulation and immunosuppressive factors.** In esophageal cancer (EC) cells, miR-125a-5p and miR-148a-3p may downregulate ATP binding cassette subfamily B member 2 translation and major histocompatibility complex (MHC)-I expression, and exosomal LINC01592 released by M2 macrophages may also downregulate MHC-I expression. Transforming growth factor beta secreted by various cells can enhance programmed cell death protein 1 expression on T cells, and interferon (IFN)-γ can upregulate programmed cell death ligand 1 expression on EC cells, subsequently contributing to immunosuppression in the tumor microenvironment. Additionally, EC cells may acquire immune resistance by downregulating the expression of IFN-γ receptors and suppressing the activation of Janus-activated kinase signaling. EC: Esophageal cancer; TCR: T-cell receptor; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death ligand 1; MDSC: Myeloid-derived suppressor cell; M2: Type 2 macrophage/suppressive macrophage; TGF-β: Transforming growth factor beta; TβR: Transforming growth factor beta receptor; IFN: Interferon; IFNGR: Interferon gamma receptor; IRF-1: Interferon regulatory factor 1; IRF-2: Interferon regulatory factor 2; IRF1-AS: Interferon regulatory factor 1 antisense RNA; ILF3: Interleukin enhancer binding factor 3; DHX9: DExH-box helicase 9; STAT1: Signal transducer and activator of transcription 1; JAK: Janus-activated kinase; TAP2: ATP binding cassette subfamily B member 2.

allowing tumors to escape immune surveillance[117,118]. Notably, interferon regulatory factors (IRFs) play important roles in regulating the effects of IFN-γ: IRF-1 is generally considered a tumor suppressor, whereas IRF-2 is regarded as an oncogenic factor[119,120]. In addition, IRF-1 expression has been shown to be decreased, and IRF-2 expression has been shown to be increased in EC, contributing to the suppression of immune responses[121]. Most importantly, IFN-γ can interact with various factors. For instance, an IFN-induced lncRNA, IRF1-AS, has been shown to activate IRF-1 transcription by interacting with IL enhancer binding factor 3 and DExH-box helicase 9, thereby activating the IFN response[119]. However, IRF-2 may inhibit the transcription of IFN-γ receptor 1 (*IFNGR1*) by binding to specific motifs in the *IFNGR1* promoter, thereby reducing the sensitivity of EC cells to IFN-γ and enhancing the resistance of EC cells to IFN-γ[120]. IFNs can regulate the JAK-STAT signaling pathway, and the activation of STATs often facilitates tumor progression[122,123]. MAGE-C3 may enhance the interaction between IFNGR1 and STAT1 by binding to IFNGR1, which can activate IFN-γ signaling and upregulate PD-L1 expression, thus contributing to immunosuppression[118]. Moreover, the overexpression of MAGE-C3 may be associated with lymph node metastasis and poor survival in ESCC patients[118]. Therefore, various factors have been suggested to participate in the immunosuppression mediated by IFN-γ in EC, but the underlying mechanisms urgently need to be elucidated.

The interplay of cytokines and signaling pathways in the TIME of EC results in the construction of a complex network, and certain key cytokines, such as TGF-β and IFN-γ, play dual roles in tumor progression by promoting tumor growth and immune escape or exerting antitumor effects. Understanding the intricate interactions among these factors might provide insights into potential therapeutic targets for enhancing antitumor immunity in patients with EC. Further research is warranted to explore novel strategies for immune modulation and improving immunotherapeutic efficacy in

EC patients.

### *Tumor metabolism*

Tumor metabolism is usually characterized by high heterogeneity and constant remodeling due to the evolution of cancer cells, and metabolic reprogramming is a distinctive feature of malignant tumors[124]. The dynamic interactions among tumor cells and various immune cells could lead to metabolic competition within the tumor ecosystem, limiting the availability of nutrients for immune cells and resulting in acidification of the TME, thereby impairing the functions of immune cells[125]. In a previous study, ESCC patients were divided into high- and low-risk subtypes based on three genes associated with tumor metabolism, namely, *CD38*, *INPP5E* and *POLR3G*, and the high-risk subgroup exhibited decreased *CD38* and *POLR3G* expression and increased *INPP5E* expression[126]. Compared with patients in the low-risk subgroup, patients in the high-risk subgroup had increased Treg infiltration and decreased plasma cell infiltration in the TME, as well as significant metabolic differences in ESCC tissues[126]. Notably, ESCC was primarily associated with glycolysis, and EAC was strongly correlated with oxidative metabolism, glycolipid metabolism and the tricarboxylic acid cycle[127].

Under normoxic conditions, most tumors preferentially rely on glycolysis for energy, which is considered an advantage for survival and is known as the Warburg effect[128]. A recent study highlighted the inhibitory role of estrogen-related receptor gamma in the occurrence, proliferation and glycolytic activity of ESCC cells, and one of its specific agonists, DY131, could inhibit the proliferation and glycolytic activity of ESCC cells by modulating certain specific genes involved in the glycolytic pathway[128]. In addition, the combination of DY131 with PD-1 blockade may have a synergistic effect on the suppression of ESCC growth[128]. As a byproduct of glycolysis, lactate may play an important regulatory role in the development and progression of ESCC, and is closely correlated with immunosuppression in the TME[129]. Furthermore, intracellular hypoxia is also associated with the progression, treatment resistance and poor prognosis of various malignancies. Numerous genes associated with hypoxia, such as *PGK1*, *PGM1* and *SLC2A3*, have been shown to be correlated with poor prognosis in EAC patients; *EGFR* and *ATF3* may be correlated with poor prognosis in ESCC patients[130]. In addition, EAC patients with higher *PGK1* and *SLC2A3* expression and lower *PGM1* expression, and ESCC patients with higher *ATF3* expression and lower *EGFR* expression, may have increased infiltration of immunosuppression-associated cells, including memory-activated $CD4^+$ T cells, activated mast cells and M2 mac-rophages[130]. Furthermore, another clinical study focused on the co-expression of hypoxia-related genes and lncRNAs in digestive system pancancer, and identified 18 hypoxia-related lncRNAs (HRlncRNAs); patients with six of these identified lncRNAs (LUCAT1, MIR4435-2HG, LINC01711, AP000695.2, ADAMTS9-AS2, and AC087521.1) had increased infiltration of immune cells, such as B cells, cancer-associated fibroblasts, endothelial cells, monocytes, macrophages and bone marrow dendritic cells, in tumors, as well as a poor prognosis[131].

Certain metabolic pathways other than the glycolysis and hypoxia pathways are also involved in EC. Zhao *et al*[132] identified six genes associated with iron metabolism and iron death (*PRNP*, *SLC3A2*, *SLC39A8*, *SLC39A14*, *ATP6V0A1*, and *LCN2*) in ESCC, and these genes may be associated with the infiltration of immune cells, tumor mutational load and ESCC prognosis. In addition, lncRNAs such as LINC01068, TMEM92-AS1 and AC243967.2 have been reported to be correlated with iron metabolism and iron death, and be closely related to the infiltration of immune cells in ESCC[133]. Moreover, Zhang *et al*[134] reported that mitochondrial energy metabolism is associated with the TIME and poor prognosis in ESCC patients, and identified several fatty acid metabolism-related genes that are predictors of EC prognosis [135]. Additionally, tryptophan-derived metabolites have been shown to contribute to tumor immune escape, and been identified as biomarkers for EC metastasis and prognosis[136].

These insights emphasize the importance of metabolic alterations in the TME of patients with EC. Understanding the intricate metabolic interactions between tumor cells and immune cells could guide the development of targeted therapies for different subtypes of EC, and further research in these areas may open new avenues for the management of patients with EC.

## CONCLUSION

In this review, we mainly described the potential mechanisms of immunosuppression in the TME of patients with EC, which opens up an interesting and promising field of future immunotherapies. The presence of decreased immune cells and increased immunosuppressive cells, including exhausted $CD8^+$ T cells and NK cells, Tregs, M2 macrophages and MDSCs, in the TIME of EC is not rare, and these cells may contribute to tumor immune escape and tumor progression. Moreover, various other factors related to tumor cells also participate in the formation of an immunosuppressive microenvironment in EC, such as the downregulated expression of MHC molecules on tumor cells, the release of immunosuppressive cytokines by tumor cells and their surroundings, and altered tumor metabolism. With a deeper and more comprehensive understanding of the complexity and heterogeneity of the TME, such as tumor types, the distribution and function of infiltrating immune and nonimmune cell subsets, the expression of cytokines and the activation or inhibition of signaling pathways in the TME, we may better elucidate the mechanisms of the immunosuppressive microenvironment, better understand the differences in patient response to the same immunotherapeutic strategies, and accurately predict the efficacy of immunotherapeutic approaches; thus, personalized treatments can be developed to overcome the effects of immune suppressive factors, improve the efficacy of immunotherapy, and maximize the advantages of immunotherapy.

## FOOTNOTES

**Co-first authors:** Xiao-Jun Zhang and Yan Yu.

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*MINIREVIEWS*

# Laryngopharyngeal reflux disease: Updated examination of mechanisms, pathophysiology, treatment, and association with gastroesophageal reflux disease

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## Abstract

Laryngopharyngeal reflux disease (LPRD) is an inflammatory condition in the laryngopharynx and upper aerodigestive tract mucosa caused by reflux of stomach contents beyond the esophagus. LPRD commonly presents with symptoms such as hoarseness, cough, sore throat, a feeling of throat obstruction, excessive throat mucus. This complex condition is thought to involve both reflux and reflex mechanisms, but a clear understanding of its molecular mechanisms is still lacking. Currently, there is no standardized diagnosis or treatment protocol. Therapeutic strategies for LPRD mainly include lifestyle modifications, proton pump inhibitors and endoscopic surgery. This paper seeks to provide a comprehensive overview of the existing literature regarding the mechanisms, pathophysiology and treatment of LPRD. We also provide an in-depth exploration of the association between LPRD and gastroesophageal reflux disease.

**Key Words:** Laryngopharyngeal reflux; Gastroesophageal reflux; Head neck surgery; Laryngitis; Otolaryngology

**Core Tip:** The pathological mechanism underlying laryngopharyngeal reflux disease (LPRD) is still unclear. There is still a lack of unified standards for the diagnosis of LPRD. Comprehensive evaluation of multiple diagnostic methods is the most reasonable choice, and help clinician to systematically establish personalized treatment options, ranging from lifestyle/diet changes, to medication, and possibly even surgery. LPRD may be pathogenically associated with gastroesophageal reflux disease. As our ability to understand LPRD, and diagnose and classify patients on the basis of diagnostic tests improves, we hope to develop a more simplified approach to treat these patients.

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## INTRODUCTION

Laryngopharyngeal reflux disease (LPRD) is an inflammatory disease caused by gastroduodenal contents regurgitating into the pharynx, stimulating and damaging the pharyngeal mucosa, and remains one of the most complex and socially relevant problems in modern medicine[1]. Reflux of stomach contents into the upper aerodigestive tract causes many clinical symptoms, including hoarseness, cough, sore throat, a feeling of throat obstruction, and excessive throat mucus. The stomach contents usually include gastric acid, nonacid substances, bile and pepsin. Reflux of the upper respiratory tract mainly involves the pharynx, larynx and nasal cavity. The mucosal changes of the pharynx and larynx mainly include interarytenoid mucosal hyperemia and edema, posterior commissure hyperplasia and vocal cord edema. More serious conditions include ulcers, granulomas and laryngeal compartment disappearance, although these are rare. LPRD has become a societal burden in recent years[2]. It has been found that 15% of otorhinolaryngology outpatient patients have laryngeal reflux. In one study, 50% of patients with laryngeal and voice disorders were diagnosed with LPRD through 24-h dual-probe pH monitoring.

However, because of the variety of symptoms and signs, the current limited diagnostic methods and the lack sensitivity or specificity, LPRD is sometimes treated empirically and without a correct diagnosis. At the same time, its pathophysiological mechanism has not been clarified. In the reflux theory, factors that cause LPRD include inhaling pepsin, trace amounts of stomach and bile acids, which can damage the throat and cause inflammation. The resistance of the laryngeal forces to these substances is weak, so some patients may not respond well to proton pump inhibitors (PPIs) alone. In addition, in reflex theory, acidic stomach contents are postulated to stimulate the vagus nerve at the distal end of the esophagus to induce laryngeal. Therefore, different pathological mechanisms lead to differences in the clinical manifestations of LPRD, and there are also differences in treatment strategies. Recent studies have found that the disease is closely related to other common diseases, such as sinusitis, otitis media, asthma and laryngeal cancer[3]. Although LPRD has gradually developed in recent years, its prevalence has become more evident in this decade. Patients with LPRD who experience severe clinical manifestations can have increased physiological and psychological burden, and impaired quality of life and emotional state. LPRD is considered an extraesophageal manifestation of gastroesophageal reflux disease (GERD). The present article reviews the characteristics, pathophysiology, diagnostic work-up, and new therapeutic strategies for LPRD, and investigates the association between LPRD and GERD.

## PATHOPHYSIOLOGY OF LPRD

In 2002, the American Academy of Otolaryngology Head and Neck Surgery first proposed the concept of LPRD[4]. Currently, the definition of LPRD is still controversial and there is disagreement between specialists about its pathogenesis. Many researchers believe that LPRD is a form of extraesophageal symptoms of GERD, and its pathogenesis is roughly similar to GERD[5]. But, otolaryngologists consider LPRD to be an independent disorder. It is widely believed that the pathogenesis of LPRD includes reflux theory, reflex theory, behavioral changes and psychological factors.

### *Reflux theory*

Reflux refers to the backflow of stomach contents to the pharynx and larynx, causing direct damage to mucosal tissue. Under physiological conditions, the body has an antireflux mechanism, including the upper esophageal sphincter (UES), lower esophageal sphincter, diaphragmic foot, esophageal peristalsis associated with swallowing, and acid resistance and clearance ability of the esophageal mucosa[6]. The antireflux mechanism prevents gastric contents from regurgitating into the pharynx and directly damaging the pharyngeal mucosal tissue. However, under pathogenic conditions, the antireflux mechanisms can be damaged individually or simultaneously, causing the gastric contents to regurgitate to the laryngopharyngeal mucosa. Studies have shown that the esophageal mucosa can resist 50 potential episodes of reflux per day without causing tissue damage[7]. However, the laryngopharyngeal mucosa can be damaged by four reflux episodes per day. This indicates that the laryngopharyngeal mucosa is more fragile and more sensitive to stimulation. At the same

time, the gastric contents of reflux are complex, including hydrochloric acid, pepsin, bile and trypsin. These different substances cause the symptoms and pathogenesis of laryngopharyngeal injury. This is why, although PPIs are the leading choice for drug treatment of LPRD, up to 40% of patients with LPRD do not obtain relief[8]. The effect of PPIs on non-acidic LPRD is not satisfactory. Therefore, the development of drugs targeting non-acidic LPRD is very urgent. A survey showed that the prevalence of non-acidic and mixed LPRD reached 25.4% and 35.5%, respectively[9]. Considering the complexity of LPRD, it is necessary to study the action mechanism of different reflux substances on LPRD. The main factors affecting the severity of LPRD include the composition, duration and frequency of regurgitation.

### Reflex theory

The pharynx is an essential organ in humans that connects the mouth with the respiratory and digestive systems. It is important for both breathing and swallowing. Because the esophageal and bronchial trees have the same embryonic origin, there is a common vagal reflex pathway between them. In the physiological state, if the nerve receptors in the pharynx are stimulated by acidic substances, it can cause bronchospasm, accumulation of sticky mucus, involuntary swallowing, glottal closure reflex and cough reflex, through nerve reflexes[10]. Once LPRD occurs, patients often cough and clear their throat in order to relieve throat discomfort. The above actions further aggravate throat mucosal edema and damage, resulting in throat sensory disorders, and then enter a vicious cycle of chronic persistent cough and throat clearing.

### Connection between LPRD and related respiratory diseases

LPRD is an inflammatory reaction that occurs in the mucosa of respiratory organs such as larynx and pharynx. Reflux of stomach contents outside the esophagus may cause diseases of the upper and lower airways. Current studies[11-13] have shown that LPRD is associated with a variety of upper and lower airway diseases, such as chronic pharyngitis, chronic laryngitis, laryngeal contact granuloma, paroxysmal laryngeal spasm, space edema, vocal cord leukoplasia, glottic laryngeal cancer, chronic cough, asthma, pediatric subglottic stenosis, secreted otitis media, sinusitis and sleep apnea hypopnea syndrome.

### LPRD and sinusitis

There is a relationship between LPRD and chronic recurrent sinusitis (CRS). Although CRS and LPRD have different courses, they can coexist in the same patient. In case-control studies, patients with LPRD were found to be at risk of developing sinusitis one year after diagnosis. DelGaudio e*t al*[14] found that patients with refractory CRS had a higher incidence of LPRD than control patients had. The pathogenesis of CRS is still unclear, but most scholars believe that it is related to mucosal injury caused by direct stimulation, pepsin action and autonomic hyper-responsiveness caused by reflux. The above factors all cause edema and retention of secretions in the mucosa of the nasal cavity and sinuses, and then secondary infection[15]. LPRD can cause sneezing, runny nose, nasal congestion and a series of similar allergic rhinitis symptoms of throat reflux rhinitis.

### LPRD and vocal fold polyps

Recent studies have shown that laryngeal reflux is associated with vocal cord polyps, but whether it is an independent risk factor is unclear. Kantas *et al*[16] found that antipharyngeal reflux played an important role in preventing the incidence of vocal cord polyps and reducing the recurrence rate of vocal cord polyps, and proposed that pharyngeal reflux was related to the damage of vocal cord regeneration. At present, research on laryngeal reflux and vocal cord polyps is limited, and the specific mechanism of laryngeal reflux causing vocal cord polyps is still unclear. The epithelium is the first barrier of the vocal cords for resistance to foreign irritants, and studies have shown that laryngeal epithelial injury caused by laryngeal reflux is the main pathogenesis[17]. First, gastric acid, pepsin, trypsin, bile salt and gastroduodenal protein directly stimulate the throat mucosa, but the larynx lacks the defense mechanism against gastric and duodenal reflux. Second, the reflux stimulates the distal mucosal chemoreceptors of the esophagus to cause vagal nerve reflex, which leads to indirect laryngeal injury such as coughing and throat clearing, but the mechanism is still controversial. Some studies have located pepsin in vocal cord polyps, and speculated that the mechanism of laryngeal reflux participating in the pathogenesis of vocal cord polyps may be that pepsin promotes the aggregation of immune cells and leads to the increase of local cytokines. Bile acids disrupt the epithelial barrier function of the esophagus by regulating the expression of claudin (CLDN)1 and CLDN4 and increasing the gap between epithelial cells[18]. However, the expression of CLDN1 and CLDN4 in vocal cord polyp tissue is increased, and the reflux bile acids may destroy the epithelial barrier of the vocal cords and induce the occurrence of vocal cord polyps.

### LPRD and asthma

In 40%-80% of patients with asthma, laryngoscopy also found LPRD[19]. There is a growing belief that sinusitis and asthma can co-occur, but often asthma symptoms caused by LPRD are overlooked. Therefore, with the exception of asthma and/or allergic rhinitis, pH probe detection is recommended for the diagnosis of LPRD patients. In addition, the use of beta agonists has become possible. Laryngeal reflux may be induced by reducing the contractile force of the lower esophageal sphincter (LES)[20]. Studies on this have been inconsistent. Some studies suggest that severe asthma may aggravate LPRD, but the incidence of LPRD is similar in children with asthma. Based on the current level of research, the feasibility of simultaneous treatment needs to be considered in the future for the reality that LPRD and asthma often occur together.

### *Relationship between LPRD and stomach contents*

Different substances of reflux have different pathological mechanisms. Compared nonacidic and mixed regurgitation of LPRD, the patients with mixed regurgitation of LPRD were more severe cough then nonacidic regurgitation[21].

### *Hydrochloric acid*

Hydrochloric acid is the main determinant of reflux symptoms. Acid in gastric juices regurgitate and contact the laryngeal tissues, causing damage and inflammation to the epithelium of the laryngeal mucosa. In general, the throat mucosa is more sensitive to acid stimulation than the esophagus. Small amounts of acid may also cause damage to the throat mucosa. Carbononic anhydrase III (CA III) secretes bicarbonate, regulates pH, and neutralizes stomach acid. In one study, it was found that some LPRD patients lacked CA III in the throat tissue, stomach acid could not be neutralized, and pH value was unbalanced[22]. E-cadherin is a transmembrane glycoprotein, which affects the intercellular adhesion of epithelial tissue and forms an anti-permeability barrier to prevent the penetration of solutes[23]. Hydrochloric acid can down-regulate the expression of E-cadherin, improve the intercellular permeability and weaken the barrier function of throat mucosa.

### *Pepsin*

The abnormal secretion and activation of pepsin may play an important role in the pathogenesis of LPRD. Pepsin, which is obtained by the conversion of pepsinogen, is the main factor that causes cell damage and protein hydrolysis. Normally, pepsin is not detected in the mucosa of the larynx in normal people. When gastric contents regurgitate into the throat, pepsin enters the throat, and at different pH environments, the signaling pathways that disrupt the integrity of the epithelial barrier include E-cadherin, CA III, nuclear factor (NF)-κB and interleukin (IL)-8[24]. It causes mucosal damage in the throat and induces inflammation. Roh *et al*[25] found that under acidic conditions (pH 1-2), pepsin and bile acids had more serious damage to subglottic tissue. In addition to damaging the laryngeal mucosa, pepsin may also cause chronic inflammation of surrounding tissues such as vocal cord polyps, tonsil hypertrophy, otitis media, and laryngeal tumors.

### *Bile acid*

Bile acid reflux is the main cause of laryngeal injury. Bile acids are normally secreted by the liver to maintain fat digestion and absorption, regulate inflammation, and affect intestinal flora. The bile acid will be protonated under the action of hydrochloric acid, enhancing their cytotoxic effects and can penetrate and dissolve cell membranes. The main mechanism of bile acid induced throat mucosal injury is as follows[26]. Bile acid induces cell epithelial–mesenchymal transformation (EMT) and induces transforming growth factor-β1 (TGF-β1), matric metalloproteinase (MMP)-9 and fibronectin to increase. It reduces expression of E-cadherin, leading to laryngotracheal scar formation and tracheal stenosis. Bile acids can cause NF-κB activation, DNA/RNA damage, and induce abnormal expression of tumor factors[27].

### *Trypsin*

The laryngeal mucosa is damaged by trypsin reflux[28]. Trypsin is secreted by pancreatic cells mainly in the form of proenzyme. And zymogen acts as the main activator of protease activating receptor-2 (PAR-2). PAR-2 affects the functionality of LES. LES dysfunction is considered to be the main factor inducing LPRD. Trypsin activates PAR-2, induces IL-8 and transient receptor potential vanilloid (TRPV) secretion, and causes epithelial barrier dysfunction in the throat[29].

Taken together, the pathogenesis of LPRD is caused by a variety of factors, including bile acids, pepsin, acids and trypsin, as shown in Figure 1. At present, the mechanism of the interaction of various reflux substances is not clear.

## CLINICAL FEATURES AND DIAGNOSIS OF LPRD

### *Clinical features of LPRD*

LPR lacks specific symptoms and signs, which can be manifested as sore throat globus pharyngeus, chronic throat clearing, and dysphonia[30]. The initial stages of LPR are characterized by hoarseness, globus pharyngeus, excessive mucus in the throat, chronic cough and persistent throat clearing. If LPRD is not treated in time, it will be complicated with laryngeal granuloma and vocal cord polyps. It can develop from GERD, along with the typical symptoms of GERD. GER may also be absent and present only with throat discomfort. Different from the characteristics of patients with esophageal reflux, LPRD mostly occurs when patients remain upright or during the day, while esophageal peristalsis and gastric acid clearance was within the normal range. However, regurgitation in GERD patients is usually in the supine position. At night, it is often accompanied by esophageal peristalsis disorder and prolonged exposure to gastric acid. The two have different clinical characteristics.

### *Diagnosis of LPRD*

LPRD is a common disease with a vast number of clinical symptoms that are sometimes treated empirically and without a correct diagnosis. There is disagreement in the diagnosis of LPRD between specialists in different area about its definition. The specificity of the diagnostic methods reported in the literature is not ideal, which faces great challenges [31]. Currently, diagnostic methods commonly used include medical history, physical examination, fibrolaryngoscopy, 24-h pH monitoring, 24-h multichannel intraluminal impedance (MII), esophageal manometry, biomolecular marker

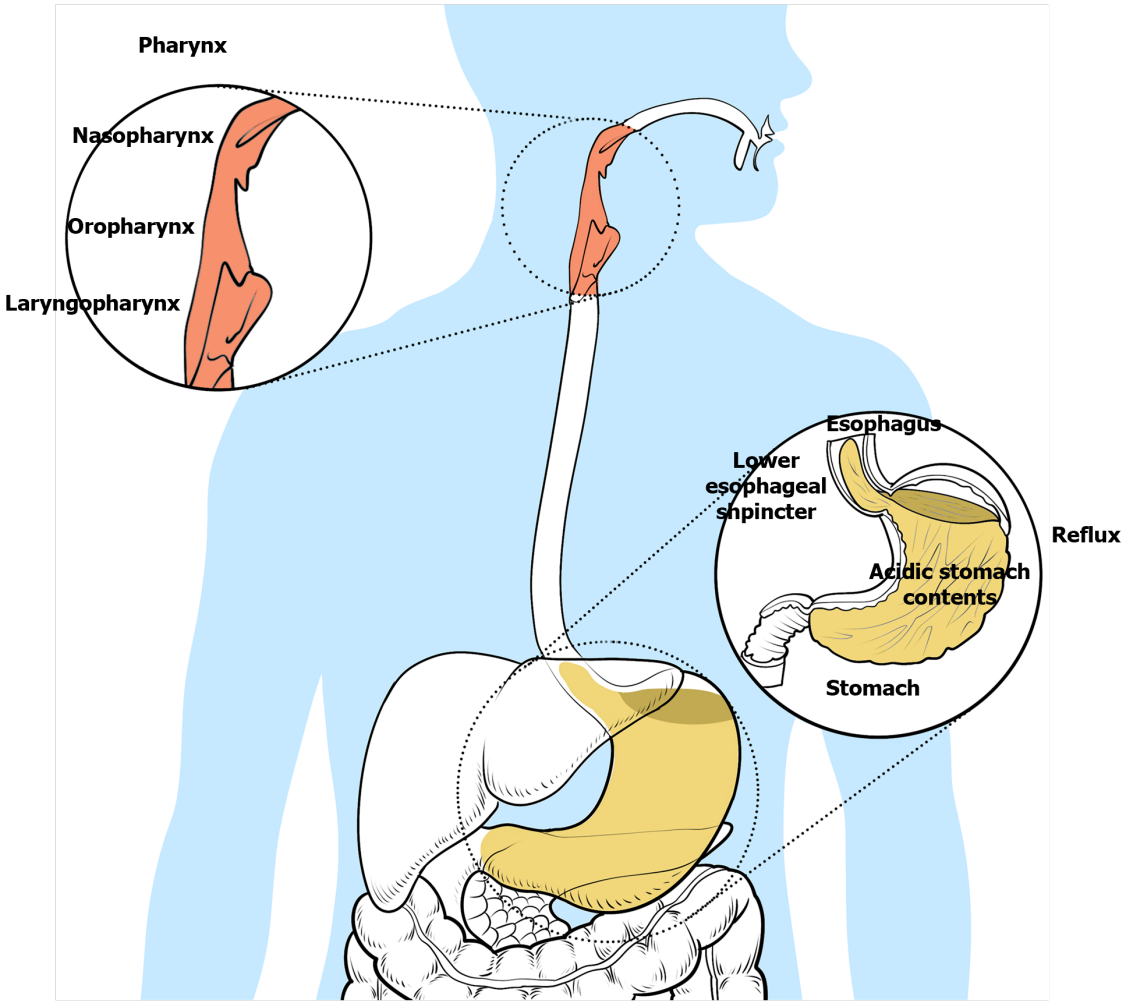


**Figure 1 Pathogenesis of laryngopharyngeal reflux.** Laryngopharyngeal reflux disease is an inflammatory disease of the upper aerodigestive tract caused by reflux of gastroduodenal content. The stomach contents usually include gastric acid, nonacid substances, bile and pepsin. Reflux of the upper respiratory tract mainly involves the pharynx, larynx and nasal cavity.

detection, pepsin detection nuclide scanning, esophagography, reflux scale scoring and experimental treatment. The 24-h MII can identify gas, liquid, or a mixture of both, and can detect acid reflux and non-acid reflux, so it is recommended as the preferred method for the diagnosis of LPRD. However, 24-h MII is expensive, limiting its popularity in the clinic. Pepsin, bile acid, and MMP are easy to detect. However in different research centers, their detection standards, sensitivity and specificity are different. Therefore, they are still not suitable for clinical diagnosis of LPRD. The pepsin-positive threshold and the timing of saliva specimen collection are inconclusive. Na *et al*[32] suggested that the best time to collect saliva is when LPRD patients have just woken. De Corso *et al*[33] believed that bile acid was the most suitable for the diagnosis of LPRD, and its sensitivity and positive predictive value both exceeded 80%. Salivary bile acid is one of the indexes to evaluate LPRD grade[34]. Hoppo *et al*[35] believed that Sep70 was a predictive indicator, and the absence of Sep70 meant that hypopharyngeal cells were damaged. In addition, the Sep70/pepsin ratio can be used to predict LPRD damage with a sensitivity of over 90%. The disadvantage is that the specificity is low, and further clinical research is needed. Moreover, the sensitivity and specificity of MMP-7 as a marker were found to be 71.43% and 79.75%[36], respectively. When MMP-7 is combined with pepsin, its sensitivity and specificity exceed 80%.

### *24-h pH monitoring*

Twenty-four-hour pH monitoring of the hypopharynx and esophagus is the gold standard for the diagnosis of LPRD[37]. A catheter with a pH monitoring probe is placed at the lower end of the esophagus through the patient's nose and oropharynx, and the catheter is fixed to monitor reflux and removed on the next day. It is a currently accepted form of diagnosis and has become an acceptable method for most researchers to significantly improve patient compliance. The greatest advantage of this method is that intermittent reflux can be recorded by daily measurement of patients, and the pH change in the esophagus can be objectively recorded in the physiological state, so as to determine whether there is reflux, and to distinguish physiological and pathological reflux. The hypopharyngeal–esophageal multichannel intraluminal impedance catheter with dual pH (HEMII-pH) has been used to monitor hypopharyngeal reflux in patients with LPR. HEMII-pH monitoring can differentiate LPR and GERD. There is no universal definition of pH for pharyngeal acid reflux. Some analysts believe that pH 5 can be judged as the defining diagnostic value. pH 5 indicates damaged laryngeal epithelium, and pH < 4 damaged cells in the esophageal epithelium. pH < 4 is considered to be highly sensitive

and specific.

### *Fibrolaryngoscopy*

Currently, fibrolaryngoscopy is the most commonly used method of examination (Figure 2). Fibrolaryngoscopy can also rule out other nasal and throat lesions such as laryngeal cancer, allergic rhinitis or sinusitis. Fibrolaryngoscopy has diagnostic value for LPRD. Endoscopic findings include subglottic edema, diffuse laryngeal edema, laryngeal ventricle disappearance, granuloma, contact ulcer and vocal cord lesion. Belafsky *et al*[38] proposed reflux discovery score (RFS) and reflux Symptom Index (RSI) for screening LPRD. The RSI scale includes nine symptoms. It is rated on a scale of 0–5 depending on the severity. The RFS scale is a score given by clinicians according to the characteristics of electronic laryngoscopy images. In LPRD patients, due to long-term repeated stimulation of reflux, the throat mucosal tissue is damaged, and various characteristics such as mucosal erythema, edema and posterior connective hyperplasia are shown by laryngoscopy. It is proved that RSI/RFS scale has high reliability and good clinical practical value. LPRD can be diagnosed by RSI > 13 and/or RFS > 7 points[39].

## TREATMENT OF LPR

### *Routine intervention – behavioral adjustment of life and diet*

Lifestyle behavior and dietary habits of patients can be adjusted to maximize improvement of LPR symptoms[40]. Patients who followed diet and lifestyle recommendations had significantly greater improvements in RSI compared with patients who did not. In a retrospective study, researchers found that patients with LPRD who took anti-reflux drugs and took behavioral change measures improved their RSI scores significantly (mean 32 d of first follow-up), while the RSI scores of patients in the control group taking only antireflux drugs (mean 62 d of first follow-up) did not improve significantly. Recommended lifestyle habits include: wearing loose clothing on a daily basis; standing as upright as possible for 30 min after eating; not eating or drinking 2–3 h before bedtime; chewing gum to increase saliva secretion; controlling blood pressure; quitting smoking; eating slowly; avoiding talking while eating; avoiding drugs such as aspirin, progesterone, corticosteroids, and nonsteroidal anti-inflammatory drugs and fried foods and fatty animal products such as chicken/fish and meat.

### *Drug treatment*

There is no reliable treatment for LPRD because its pathogenesis is not clear. To study the etiology of LPRD, it is necessary to consider the influence of self and environmental factors[3]. The acid-suppressing PPIs are currently the main drug used for the treatment of LPRD and are suitable for LPRD patients with GERD symptoms. PPIs combined with gastroenterokinetic drugs is the most common clinical treatment for LPRD, and they are recommended for at least 8 wk.

PPIs can inhibit the secretion of gastric acid, down-regulate the activity of pepsin, damage the throat mucosa, and finally achieve the treatment of lesions and alleviate symptoms. Some analysts believe that PPIs twice daily are the best choice to treat this disease[41]. Therefore, the study of pathogenesis of LPRD has become the research focus at home and abroad.

A recent multicenter study found that patients with different phenotypes, such as no LPRD or GERD, LPRD/GERD with hiatic hernia, reflex cough, LPRD with mild GERD, *etc*., were most responsive to PPIs[42]. This was followed by LPRD and LPRD with mild reflux and reflux cough. Therefore, it is speculated that classification based on phenotype may be more conducive to matching patients and corresponding treatment methods. Esophagopharyngeal reflux may not be entirely acidic. Previous research has suggested that acid reducers may not be effective for patients with nonacid reflux events. A mixed response to PPIs depends on their underlying complicating disease pathology. Despite the failure of empiric PPI treatment, 24-h MI-pH testing is still considered necessary. If significant acid reflux occurs, the PPI regimen needs to be optimized, for example, by increasing the dose, extending the duration of treatment and adjusting the time of administration. Patients with acid reflux who do not respond to PPIs may try switching to potassium-competitive acid blockers[43].

H2 receptor antagonists are commonly used in the treatment of LPRD. Due to the short duration of action of the drug, the acid inhibition efficacy is lower, and its status is slightly lower than that of PPIs[44]. The study found that 51 percent and 54 percent of anti-reflux prod patients who took 20 mg of omeprazole and 20mg of famotidine at night experienced relief from symptoms, respectively. Eighty-three percent of patients improved after taking 20mg of omeprazole at night.

Alginate is an oral drug. It prevents reflux of gastric acid or non-acid substances by forming a viscous mechanical barrier on the surface of the stomach contents, ultimately reducing the contact of the stomach contents with the esophagus or pharynx[45]. It works regardless of whether the reflux is acidic or nonacidic. In addition, alginate inhibits pepsin and bile salts. Alginate also improves symptoms in patients with LPRD. In a randomized controlled trial, patients treated with alginate showed significant improvement in symptoms at 2, 4 and 6 months after treatment.

Baclofen can inhibit LES relaxation and prevent acidic and non-acidic reflux[46]. In one study, Baclofen was found to significantly reduce the duration of reflux, the incidence of GER, and the incidence of LES relaxation compared to placebo.

Considering the different mechanisms of action of reflux substances in LPRD, a variety of receptor antagonists and enzyme inhibitors have emerged as new inhibitors, such as trypsin inhibitors, protease activating receptor 2 (PAR-2) antagonists, pepsin inhibitors or receptor antagonists, NF-κB antagonists, TRPV1 antagonists and MMP inhibitors. Hossain thinks PAR-2 may play an important role in unresolved heartburn symptoms after PPI treatment. TRPV1 and PAR-2 antagonists have the potential to be targeted agents for ameliorating LPRD-induced heartburn and pain[47].

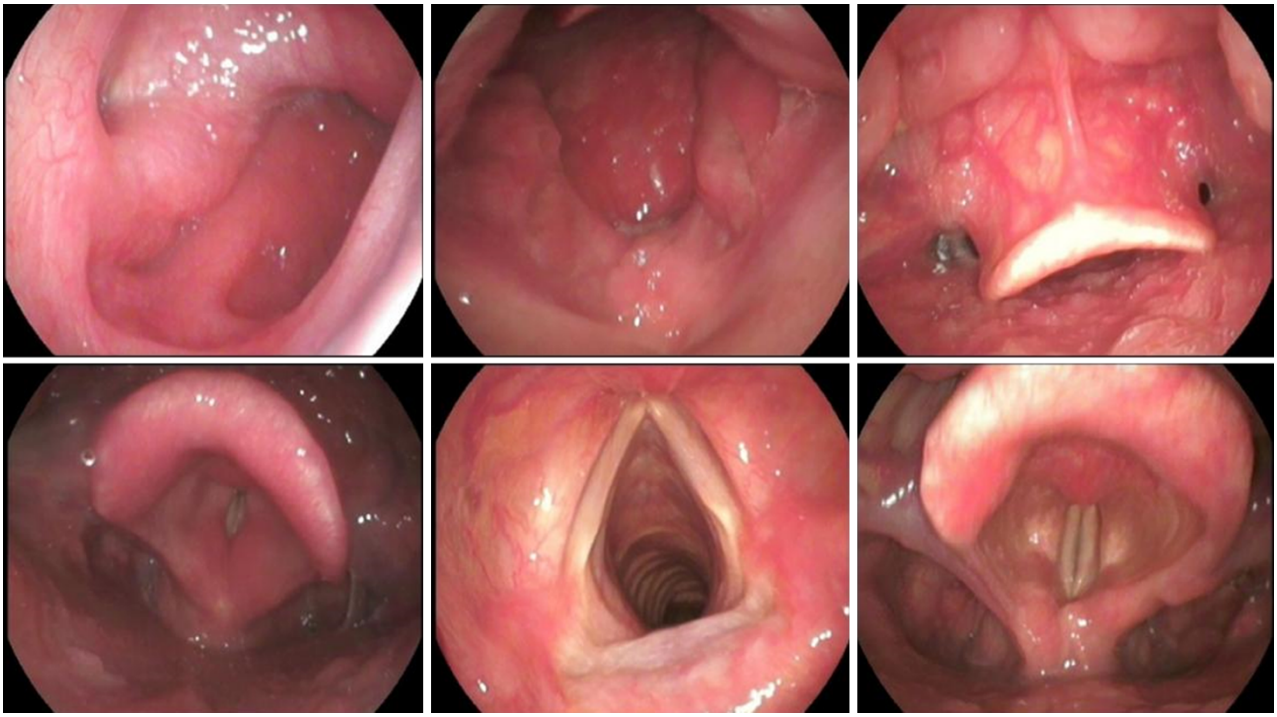

**Figure 2 Fibrolaryngoscopy diagnostic value for laryngopharyngeal reflux disease.** Fibrolaryngoscopy showed granulations of the posterior wall of the nasopharynx and edema of the retrocricoid region, epiglottis erythema, and posterior commissure hypertrophy, and laryngeal erythema.

Yoshida *et al*[48] found that MEK inhibitors and p38 inhibitors reduce IL-6 or IL-8 secretion through MAPK signaling pathway, thereby reducing esophageal inflammation and achieving the treatment of LPRD.

### *Surgical intervention*

Experts suggest that surgery may be considered for patients with refractory extraesophageal symptoms treated with medication in the latest American Gastroenterological Association guidelines[49].

In a retrospective controlled trial, Swoger *et al*[50] evaluated the difference between surgical treatment and PPI. They found significant differences in fundus dilation. Fundus dilation is associated with improved RSI scores and is expected to be an effective treatment for patients with LPR. However, how to judge the effect of surgery and accurately select the right patients for surgical treatment is a difficult problem, worthy of further study. It would be irresponsible to recommend that every LPR patient undergo major abdominal surgery. Previous studies often assessed the effect of treatment according to the subjective judgment of patients, which has great subjectivity and individual differences. Therefore, when selecting and evaluating the effect of treatment, patient-reported results must be given priority in a patient-centered approach, and reflux and symptoms must be evaluated in detail in combination with pH monitoring and RSI score. Although fundoplication is more effective than PPI, it is more risky. The most common complaint is dysphagia. In one study[51], all patients experienced dysphagia after surgery. These patients may have dysphagia in the initial postoperative period, but it resolves spontaneously after 2 wk, and 13 patients (4.53%) reported prolonged dysphagia after surgery. And the second most common complaint was postoperative gas/abdominal distension. One study[52] reported that abdominal distension occurred in all 12 patients during the first 2 wk. Sahin *et al*[53] found that postoperative complications mainly included emphysema (10.8%), intraoperative hemorrhage (4.4%), pleural displacement (2.9%), *etc*. 2.4% patients needed a second operation due to postoperative complications, and 0.4% patients needed a second operation due to surgical failure.

For granulomas in the laryngeal cavity that are large and may affect normal breathing, surgical treatment is required and antacid therapy can be performed after surgery. A number of recent data have confirmed that esophageal diseases such as hiatal hernia can lead to an increase in the incidence of LRPD and refractory extraesophageal symptoms. The main symptoms of primary esophageal diseases are significantly improved after receiving standard laparoscopic surgery. The fundoplication is achieved by reconstructing the gastroesophageal junction area and re-establishing the barrier function[54]. Currently, fundus folding has relatively obvious value. Overall, endoscopic and surgical interventions are considered as the last line of care for patients with LPRD, and only some patients should be considered for surgical or endoscopic interventions.

### *Nonsurgical treatment*

The UES external pressure device is a new treatment for LPRD[55]. Reflux Band (Somna Therapeutics, Germantown, WI, United States) has received United States Food and Drug Administration approval. It has been reported that patients with typical reflux symptoms and supresophageal symptoms have impaired esophageal and UES responses that mimic reflux and are therefore at greater risk for esophagopharyngeal reflux. After wearing an external UES compression device at

night for 2–4 wk, RSI scores improved significantly.

### *Speech therapy*

In addition to medication, speech therapy is proven to be effective and is recommended by CHEST guidelines[56]. The treatment is achieved through speech and breathing training. In one study[57], it was found that compared with a control group, patients who received 6 months of PPI therapy and 3-5 sessions of breathing therapy and after treatment with speech therapy and guidance on a healthy lifestyle such as relaxation, exercise, diet, and stress management, patients showed significant improvement in upper respiratory tract breathing and cough. The results suggest that speech therapy may potentially improve laryngeal allergy symptoms in patients with chronic cough. It was found that 100% of patients experienced an improvement in their cough symptoms. Further research in this field is still needed in the future.

### *Behavioral therapy*

In cognitive behavioral therapy[58], psychiatrists use a range of behavioral therapies, including stress management, cognitive reconstruction, coping strategies, problem solving, and anxiety management, to improve patients' throat symptoms, which have been shown to be a safe and effective treatment option. It was reported that hypnotherapy was used to treat patients with allergic laryngeal symptoms and foreign body sensation in the pharynx[59,60]. The patients experienced a significant reduction in the severity and symptoms of throat discomfort after relaxation breathing therapy, which involves adjusting breathing and relaxing muscles, and esophageal-oriented hypnotic-assisted relaxation therapy. In another study, nine patients with functional heartburn who received esophageal directed hypnotherapy seven times a week experienced significant improvements in heartburn symptoms, visceral anxiety, and quality of life. In a recent study, it was found that, based on the available evidence, hypnotherapy for patients presenting with dysphagia, foreign body sensation, indigestion, and functional heartburn has been comprehensively studied in patients with bowel disease as a form of cognitive behavioral therapy (CBT). Future research is needed in behavioral therapy for laryngeal hypersensitivity and laryngeal dysfunction.

## ASSOCIATION BETWEEN LPRD AND GERD

LPRD is considered to be a substantially part of the extraesophageal manifestations of GERD. Koufman[61] first reported an important epidemiological study on LPRD in 1991. In that study, LPR was initially differentiated from GERD based on dual-probe pH monitoring in the esophagus and pharynx. Many existing studies have shown that the prevalence of LPRD is generally higher in GERD patients[62]. Clinical symptoms of GERD include regurgitation and heartburn, resulting from esophageal mucosal disruption caused by gastric content reflux. However, some patients with GERD have no symptoms of LPRD. According to the results of pH monitoring, the incidence of GERD in LPRD was 52.7%, while the incidence of LPRD in GERD was 46.3%[42]. Otolaryngologists believe that LPRD is an independent, but common in their practice, upper airway disorder. GER and LPR have similar pathological mechanisms, but their clinical manifestations and 24-h double pH-probe monitoring are different. Patients with GERD have a lower proportion of nonacid and mixed LPRD compared with LPRD/GERD patients. The recent use of HEMII-pH monitoring gave evidence that physiology may differ between LPRD and GERD. LPRD is induced by daytime and upright gaseous weakly or nonacid hypopharyngeal reflux. A series of symptoms beyond the esophagus may be caused by nonacid content such as bile, pepsin and trypsin l[6]. However, the presence of GERD is strongly associated with acid reflux in the esophagus. However, due to the reflux of gastric contents, the treatment plan with acid suppression with PPIs is similar. *Helicobacter pylori* (*H. pylori*) is found in many sites, including laryngeal mucosa and interarytenoid region[63]. For cases complicated with *H. pylori* infection, this should be considered as a cause of LPRD.

## PROSPECT

For LPRD caused by different regurgitation substances, 24-h MII-pH test, salivary pepsin and bile acid test are usually performed clinically. On the one hand, it can determine the cause of the disease, on the other hand, it can determine the cause of the disease, record the therapeutic effect of the drug, and then provide the best treatment plan for the patient. For patients with refractory LPRD, multidisciplinary evaluation is required in conjunction with otolaryngologists and gastroenterologists. The efficacy of PPIs in some patients is not satisfactory, indicating that the pathogenesis of non-acidic components in throat mucosal injury needs further study. Furthermore, the reliability of biomarkers such as pepsin and bile acids for the diagnosis and prognosis of LPRD needs to be further evaluated. At the same time, more clinical prospective studies are needed to evaluate the selection of laparoscopic surgical treatment methods and indications in order to provide more effective treatment strategies for the patients.

## CONCLUSION

The pathological mechanism leading to LPRD is still unclear. There is still a lack of unified standards for the diagnosis of LPRD. Comprehensive evaluation of multiple diagnostic methods is still the most reasonable choice. The treatment of LPRD also presents new challenges for clinicians. Although empirical PPI trials are the first-line treatment, nearly half of

patients do not respond well. Therefore, it will help clinicians to systematically understand LPRD and develop rational, personalized treatment options to help patients, ranging from lifestyle/diet changes, to medication, and possibly even surgery. LPRD may be pathogenically associated with GERD. Future clinical and experimental studies are still needed to investigate the association between LPRD and GERD in LPRD patients with or without GERD, through extensive gastric content analysis and impedance-pH monitoring. Eventually, as our ability to understand LPRD, and diagnose and classify patients on the basis of diagnostic tests improves, we hope to develop a more simplified approach to help these complex patients.

## FOOTNOTES

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*MINIREVIEWS*

# Drug-induced mucosal alterations observed during esophagogastroduodenoscopy

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## Abstract

Several features of drug-induced mucosal alterations have been observed in the upper gastrointestinal tract, *i.e.*, the esophagus, stomach, and duodenum. These include pill-induced esophagitis, desquamative esophagitis, worsening of gastroesophageal reflux, chemotherapy-induced esophagitis, proton pump inhibitor-induced gastric mucosal changes, medication-induced gastric erosions and ulcers, pseudomelanosis of the stomach, olmesartan-related gastric mucosal inflammation, lanthanum deposition in the stomach, zinc acetate hydrate tablet-induced gastric ulcer, immune-related adverse event gastritis, olmesartan-associated sprue-like enteropathy, pseudomelanosis of the duodenum, and lanthanum deposition in the duodenum. For endoscopists, acquiring accurate knowledge regarding these diverse drug-induced mucosal alterations is crucial not only for the correct diagnosis of these lesions but also for differential diag-nosis of other conditions. This minireview aims to provide essential information on drug-induced mucosal alterations observed on esophagogastroduodenoscopy, along with representative endoscopic images.

**Key Words:** Diagnosis; Esophagogastroduodenoscopy; Non-neoplastic lesions; Esophageal lesions; Gastric lesions; Duodenal lesions

**Core Tip:** Various lesions associated with medication use are detected during esophagogastroduodenoscopy, including pill-induced esophagitis, desquamative esophagitis, deteriorating gastroesophageal reflux, chemotherapy-induced esophagitis, proton pump inhibitor-induced gastric mucosal changes, medication-induced gastric erosions and ulcers, pseudomelanosis of the stomach, olmesartan-related gastric mucosal inflammation, lanthanum deposition in the stomach, zinc acetate hydrate tablet-induced gastric lesions, immune-related adverse event gastritis, olmesartan-associated sprue-like enteropathy, duodenal pseudomelanosis, and lanthanum deposition. Endoscopists must diagnose these mucosal alterations by acquiring pertinent knowledge regarding medication-induced lesions, concomitant with inquiries concerning patient medication history.

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## INTRODUCTION

The primary purpose of screening esophagogastroduodenoscopy (EGD) is to comprehensively examine the esophagus, stomach, and duodenum to detect neoplasms. Furthermore, EGD provides invaluable information for disease diagnosis, assessment of disease state, and treatment planning in symptomatic patients. This procedure can reveal a spectrum of conditions, including cancer, and can also enable the identification of mucosal changes attributed to medications taken by the patient[1,2]. Although gastric and duodenal ulcers caused by nonsteroidal anti-inflammatory drugs (NSAIDs) have long been known as drug-induced upper gastrointestinal lesions[3,4], the advent of various medications on the market has led to the emergence of new types of mucosal injuries and alterations. Despite the inclusion of information on some drug-induced upper gastrointestinal mucosal lesions in the package inserts of medications, not all prescribing physicians are acquainted with these conditions due to their infrequency. Therefore, endoscopists should acquire accurate knowledge regarding diverse drug-induced mucosal alterations for appropriate diagnosis. This knowledge is also crucial for the differential diagnosis of other conditions, including neoplastic lesions. Herein, we review articles associated with drug-induced mucosal alterations in the esophagus, stomach, and duodenum, and present endoscopic images of representative lesions detected on EGD.

## LITERATURE REVIEW

### *Search strategy*

We conducted a systematic search of the PubMed database to retrieve all peer-reviewed articles published between January 1, 2013, and August 3, 2023, without imposing any study design filters. To augment our search results, we manually screened additional relevant articles using a reference list of selected publications that met our eligibility criteria. Our search used the keywords "drug-induced" and "esophagus", "stomach", or "duodenum", and was performed by the principal investigator Iwamuro M. The inclusion criteria were as follows: (1) Peer-reviewed articles describing cases of drug-induced upper gastrointestinal tract lesion; and (2) Review articles, original articles, case series, and case reports. Articles were excluded if they: (1) Did not focus primarily on drug-induced upper gastrointestinal tract lesion; (2) Were animal or cell studies; (3) Were letters, editorials, or correction notices; or (4) Were written in languages other than English. All the eligible articles were evaluated.

### *Search results*

Figure 1 presents a flow diagram summarizing the identification, screening, eligibility, and exclusion processes of the literature search. The keywords "drug-induced" and "esophagus" yielded 45 papers of which 19 were excluded for the following reasons: Not primarily focused on drug-induced upper gastrointestinal tract lesion ($n$ = 14); animal or cell studies ($n$ = 2); and written in languages other than English ($n$ = 3). The keywords "drug-induced" and "stomach" yielded 173 papers, of which 149 papers were excluded for the following reasons: Not primarily focused on drug-induced upper gastrointestinal tract lesion ($n$ = 65); animal or cell studies ($n$ = 72); and studies written in languages other than English ($n$ = 12). The keywords "drug-induced" and "duodenum" yielded 30 articles, of which 19 were excluded due to the following reasons: Not primarily focused on drug-induced upper gastrointestinal tract lesion ($n$ = 8); animal or cell studies ($n$ = 8); and studies written in languages other than English ($n$ = 3). Finally, 61 articles were retrieved from the initial PubMed search after applying the exclusion criteria. After a manual screening, 33 additional articles were deemed relevant and included. A total of 94 articles were reviewed in detail.

Virtually all drugs may cause adverse events, including those involving the digestive tract, and various changes in the gastrointestinal mucosa due to different drugs have been reported. Drugs reported in two or more papers are presented in Table 1. In the subsequent sections, we elucidate the discernible categories of drug-induced mucosal alterations accompanied by illustrative EGD images.

**Table 1 Drugs described in two or more papers**

| Esophagus | | Stomach | Duodenum |
|---|---|---|---|
| NSAIDs | Warfarin | PPIs | Olmesartan |
| Bisphosphonates | DOACs | NSAIDs | Iron tablets |
| Iron tablets | SSRIs | Steroids | Diuretics |
| Doxycycline | Benzodiazepine | Bisphosphonates | Lanthanum carbonate |
| Tetracycline | Phenytoin | Iron tablets | |
| Ciprofloxacin | Pinaverium | Doxycycline | |
| Clindamycin | Ascorbic acid | Diuretics | |
| Amoxicillin | L-arginine | Olmesartan | |
| Metronidazole | Opiates | Lanthanum carbonate | |
| Rifaximin | 5-fluorouracil | Zinc acetate | |
| Potassium chloride | Bleomycin | Immune checkpoint inhibitors | |
| Antihypertensives | Dactinomycin | | |
| Nitrates | Methotrexate | | |
| Quinidine | Cytarabine | | |
| Acetaminophen | Vincristine | | |
| Colchicine | | | |

NSAIDs: Nonsteroidal anti-inflammatory drugs; DOACs: Direct oral anticoagulants; SSRIs: Selective serotonin reuptake inhibitors; PPIs: Proton pump inhibitors.

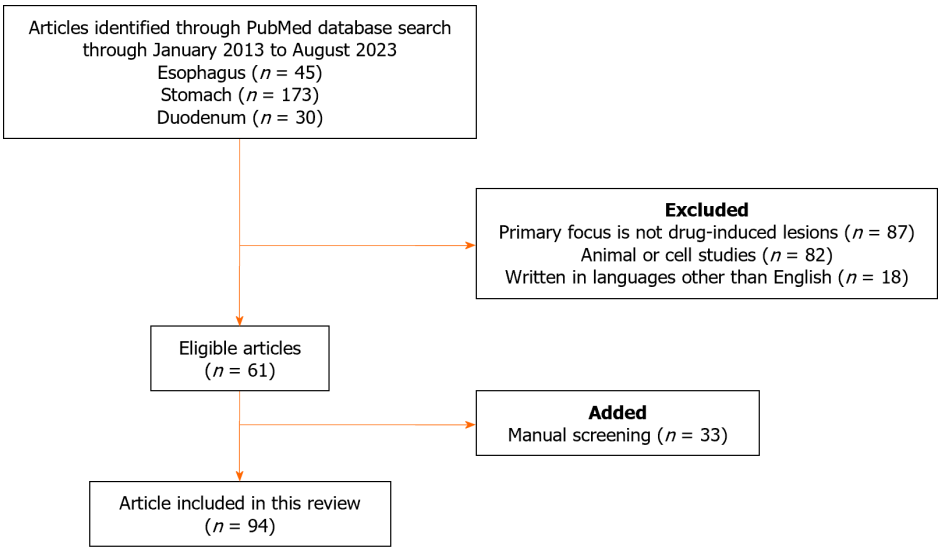


**Figure 1 Flow diagram summarizing the identification, screening, eligibility, and exclusion processes of the literature search.**

## PILL-INDUCED ESOPHAGITIS

Given that tablets are ingested in a supine posture or preceding sleep, accompanied by inadequate water intake, the entrapment of medication within the esophagus may result in the release of deleterious agents, imparting noxious constituents capable of inflicting damage to the esophageal wall. The mucosal injury to the esophagus due to the retention of such medications is also referred to as pill-induced esophagitis[5-19]. Esophageal injury can be caused by over hundred distinct substances consumed in the form of oral pharmaceuticals. Principal contributors include antibiotics, notably tetracycline and doxycycline, along with other agents such as bisphosphonates[20], NSAIDs, potassium chloride[21], and iron pills. Acetaminophen, warfarin, colchicine, ascorbic acid, L-arginine, pinaverium, antihypertensives, and antiarrhythmic agents may also induce esophagitis. These pharmaceutical agents are believed to

exert a corrosive effect on the esophageal mucosa, thereby instigating processes that lead to inflammation, irritation, erosion, and ulceration within the esophagus. Pill-induced esophagitis manifests as dysphagia, pain during swallowing, thoracic discomfort, heartburn, and general esophageal irritation. To attenuate the risk of esophageal injury, it is imperative for patients to ingest medications with a copious volume of plain water and concurrently adopt an upright posture (either sitting or standing) for a minimum of 30 min following the intake of the medication.

## DESQUAMATIVE ESOPHAGITIS

Desquamative esophagitis, also known as esophagitis dissecans superficialis, or sloughing esophagitis, is an infrequent, unique endoscopic finding characterized by mucosal sloughing into the esophageal lumen. Desquamative esophagitis occurs in patients taking direct oral anticoagulants, which are commonly prescribed for the prevention and treatment of blood clots. While dabigatran is frequently implicated[22-26], rivaroxaban, apixaban, and edoxaban can also induce this condition. A typical appearance is depicted in Figure 2, illustrating the presence of diffuse white membranous deposits in the mid to distal esophagus. Endoscopic biopsy of the white membranous deposits reveals a degenerated squamous epithelium accompanied by inflammatory cell infiltration[26]. A previous study found that the use of psychoactive agents, particularly selective serotonin reuptake inhibitors or serotonin-norepinephrine reuptake inhibitors, was prevalent in patients with desquamative esophagitis[27]. Other medications, such as benzodiazepines, opioids, and antiepileptic agents, similarly contribute to the occurrence of desquamative esophagitis[8,28]. Such esophageal mucosal injuries are believed to occur through a mechanism similar to that of pill-induced esophagitis, in which damage arises from the retention of medication in the esophagus. Therefore, for prevention, it is crucial to take medication with a full glass of water while in an upright position to ensure smooth passage into the stomach.

## WORSENING OF GASTROESOPHAGEAL REFLUX

In gastroesophageal reflux, the primary precipitant of mucosal injury is the refluxed gastric acid. However, various medications may exacerbate or trigger the onset of gastroesophageal reflux[13,14]. Nitrates such as nitroglycerin are commonly used to treat conditions such as angina by relaxing and dilating blood vessels. This relaxation effect is not specific to the blood vessels in the heart, but also extends to other smooth muscles, including the lower esophageal sphincter (LES), allowing stomach acid to flow back into the esophagus. Calcium channel blockers, anticholinergic medications, sedatives, tranquilizers, and theophylline may also relax the LES and contribute to acid reflux. In symptomatic individuals, it is imperative not only to administer proton pump inhibitors (PPIs), but also to evaluate the potential exacerbating effects of pharmacological agents on gastroesophageal reflux. Therefore, it is important to promptly discontinue or modify medication accordingly.

## CHEMOTHERAPY-INDUCED ESOPHAGITIS

Chemotherapy-induced esophagitis refers to inflammation and irritation of the esophagus, which occurs as a side effect of chemotherapy drugs. These potent medications used to treat cancer can inadvertently damage the esophageal lining, leading to a range of symptoms and complications[8,11,18]. Drugs such as 5-fluorouracil, bleomycin, dactinomycin, methotrexate, cytarabine, and vincristine have been identified as causative agents.

## PPI-INDUCED GASTRIC MUCOSAL CHANGE

PPIs, a class of medications that reduce stomach acid production, are commonly prescribed to treat conditions such as gastroesophageal reflux disease and peptic ulcers. Although PPIs are generally regarded as safe with a low incidence of adverse effects, emerging evidence suggests that their long-term use can elicit diverse endoscopic and histopathological alterations in the gastric mucosa[29,30]. These include multiple white and flat elevated lesions, fundic gland polyps, hyperplastic polyps, cobblestone-like mucosa, black spots, and a white globe appearance.

The term "multiple white and flat elevated lesions" was proposed in 2011 to describe a new type of polyp associated with PPI use that was observed in the gastric cardia, fornix, or corpus[31-34] (Figure 3A-C). These lesions manifest as circumscribed and sharply demarcated areas characterized by a whitish appearance, exhibiting a round morphology and slight elevation of the mucosa with a smooth surface. Multiple white and flat elevated lesions were more easily identified on narrow band imaging than on normal white-light observation. Pathologically, a straight, enlarged, and hyperplastic foveolar epithelium was observed, which is a typical feature of this lesion.

Fundic gland polyps are one of the most prevalent types of gastric polyps, with an estimated incidence ranging from approximately 2% to 11%, albeit subject to variation among diverse populations (Figure 3D and E)[35]. Notably, their occurrence tends to diminish in patients with *Helicobacter pylori* infection, but conversely increases in individuals undergoing PPI therapy. Fundic gland polyps reportedly regress after cessation of PPIs in some patients[36-40].

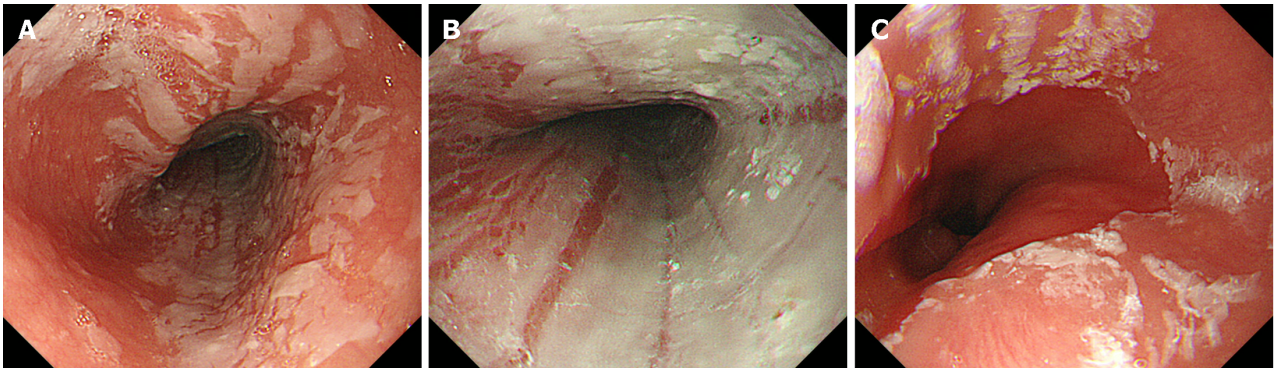


**Figure 2 Dabigatran-induced desquamative esophagitis.** A-C: White membranous material is observed in the middle to lower esophagus of a 73-year-old woman taking dabigatran.

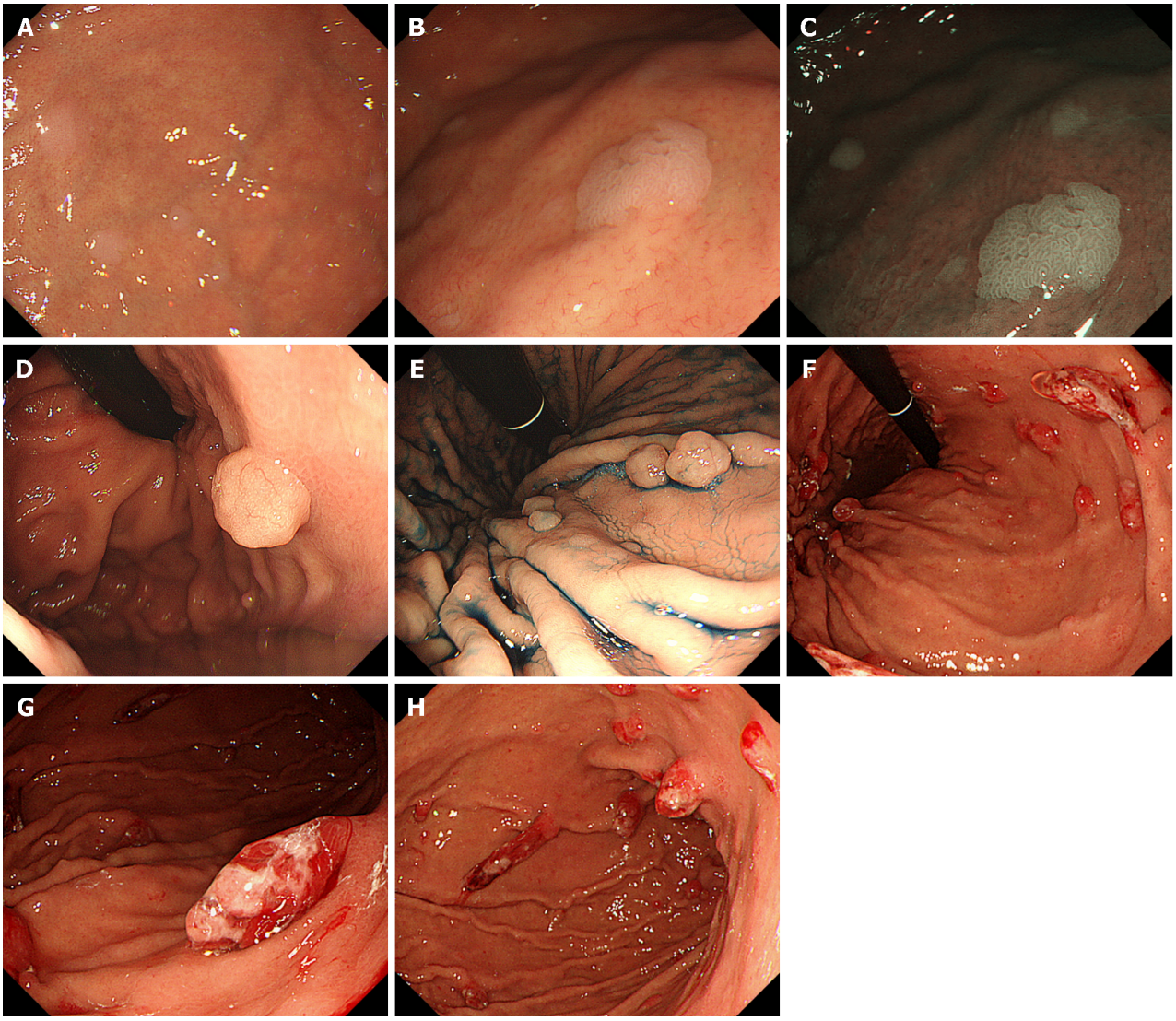


**Figure 3 Proton pump inhibitor-induced gastric mucosal changes.** A-C: Multiple white and flat elevated lesions. Whitish slight elevations are observed in the gastric fornix of a patient taking proton pump inhibitor (PPI). Lesions are easily identified on narrow-band imaging observation (C); D and E: Fundic gland polyps in a PPI user. After indigo carmine dye spraying (E); F-H: Hyperplastic polyps in the stomach. Multiple reddish, friable, long polyps are seen.

Several studies have explored the potential association between PPI use and the development of hyperplastic polyps in the stomach (Figure 3F-H). Some studies suggest that the long-term use of PPIs may be associated with an increased risk of gastric polyps[41]. The frequency of hyperplastic polyps exhibited a propensity for elevation among individuals testing positive for *Helicobacter pylori*. Similar to fundic gland polyps, hyperplastic polyps reportedly regress in some patients following the discontinuation of PPIs[36,42].

Cobblestone-like mucosa refers to the manifestation of numerous, approximately 3-5 mm-sized, irregular, elevated mucosal lesions in the gastric body[34,43,44] (Figure 4A-C). This distinctive mucosal pattern has a similar coloration as that of contiguous mucosa and is typically discerned as interspersed among the gastric folds. This represents a histopathological alteration attributable to prolonged PPI use. The histopathological characterization of the cobblestone-like mucosa involves the presence of parietal cell protrusions and cystic dilatation of the fundic glands, with these changes particularly accentuated in non-atrophic gastric regions.

Prolonged usage of PPIs induces the formation of black spots in the gastric mucosa, which are discerned as diminutive, dark, dot-like lesions on EGD (Figure 4D and E)[45]. Histopathologically, these spots are characterized by the entrapment of brownish substances within the dilated lumina of the expanded fundic gland cysts. The cystic dilatation of fundic gland cysts induced by the use of PPIs is strongly posited as a key etiological factor contributing to the development of black spots.

The term "white globe appearance" is defined as a small (≤ 1 mm) white globe-shaped feature located beneath the gastric epithelium, observed during magnifying endoscopic observation with narrow band imaging[46]. This feature is associated with early gastric cancers and is often detected near the demarcation line. It indicates cancers with a differentiated component. Conversely, a white globe appearance has also been noted in the gastric mucosa of non-cancer patients with autoimmune gastritis or during PPI use[47,48] (Figure 4F and G).

## MEDICATION-INDUCED GASTRIC EROSIONS AND ULCERS

Gastric mucosal damage caused by NSAIDs has long been recognized. The mechanism involves several complex interactions[1,3,4,49]. NSAIDs inhibit cyclooxygenase and subsequently reduce the synthesis of prostaglandins, which play a protective role in maintaining the integrity of the gastric mucosa. NSAIDs cause vasoconstriction and thereby reduce the blood flow, which compromises the delivery of oxygen and nutrients to the gastric mucosa, resulting in mucosal damage. Some NSAIDs have direct toxic effects on the gastric mucosa. Epidemiological investigations have shown that the relative risk for the development of gastrointestinal complications escalates in patients concomitantly administered with corticosteroids and NSAIDs[50,51]. Similar to pill-induced esophagitis, bisphosphonates, iron tablets, and doxycycline directly irritate the gastric mucosa due to the chemical properties of the drug and its direct contact with the lining of the stomach.

## PSEUDOMELANOSIS OF THE STOMACH

Pseudomelanosis is an infrequent and benign pathological condition in which a dark pigment accumulates within macrophages located in the lamina propria. Unlike melanosis coli, the onset of gastric pseudomelanosis is unrelated to laxative use, but is thought to be associated with diuretics, beta-blockers, and iron supplementation. While gastric pseudomelanosis induces alterations in mucosal coloration, patients are devoid of accompanying clinical symptoms and do not manifest mucosal damage such as erosions or ulcers[52-55]. Deemed a benign condition, a diagnosis of gastric pseudomelanosis does not necessarily mandate any modification in the prescribed medication.

## OLMESARTAN-RELATED GASTRIC MUCOSAL INFLAMMATION

Olmesartan, an angiotensin II receptor antagonist commonly used to treat hypertension, induces enteropathy with sprue-like symptoms. Although infrequent, olmesartan has been reported to induce lymphocytic, collagenous, or chronic gastritis[18,56-59].

## LANTHANUM DEPOSITION IN THE STOMACH

Lanthanum carbonate is used for the therapeutic management of hyperphosphatemia, primarily in patients with chronic renal insufficiency. White lesions are characteristic endoscopic features indicative of gastric lanthanum deposition[60-67]. These whitish deposits are easily discernible through narrow band or blue laser imaging. We have elucidated that the endoscopic manifestations of gastric lanthanum deposition vary between mucosa with and without atrophy. In non-atrophic mucosa, lanthanum was initially deposited on the posterior wall to the greater curvature of the gastric body, presenting as diffuse white lesions, the extent of which increased over time (Figure 5A and B). The susceptibility of the posterior wall to the greater curvature of the gastric body suggests that the active ingredient of the orally ingested lanthanum remains in prolonged contact with this region. Conversely, in atrophic mucosa, particularly with intestinal

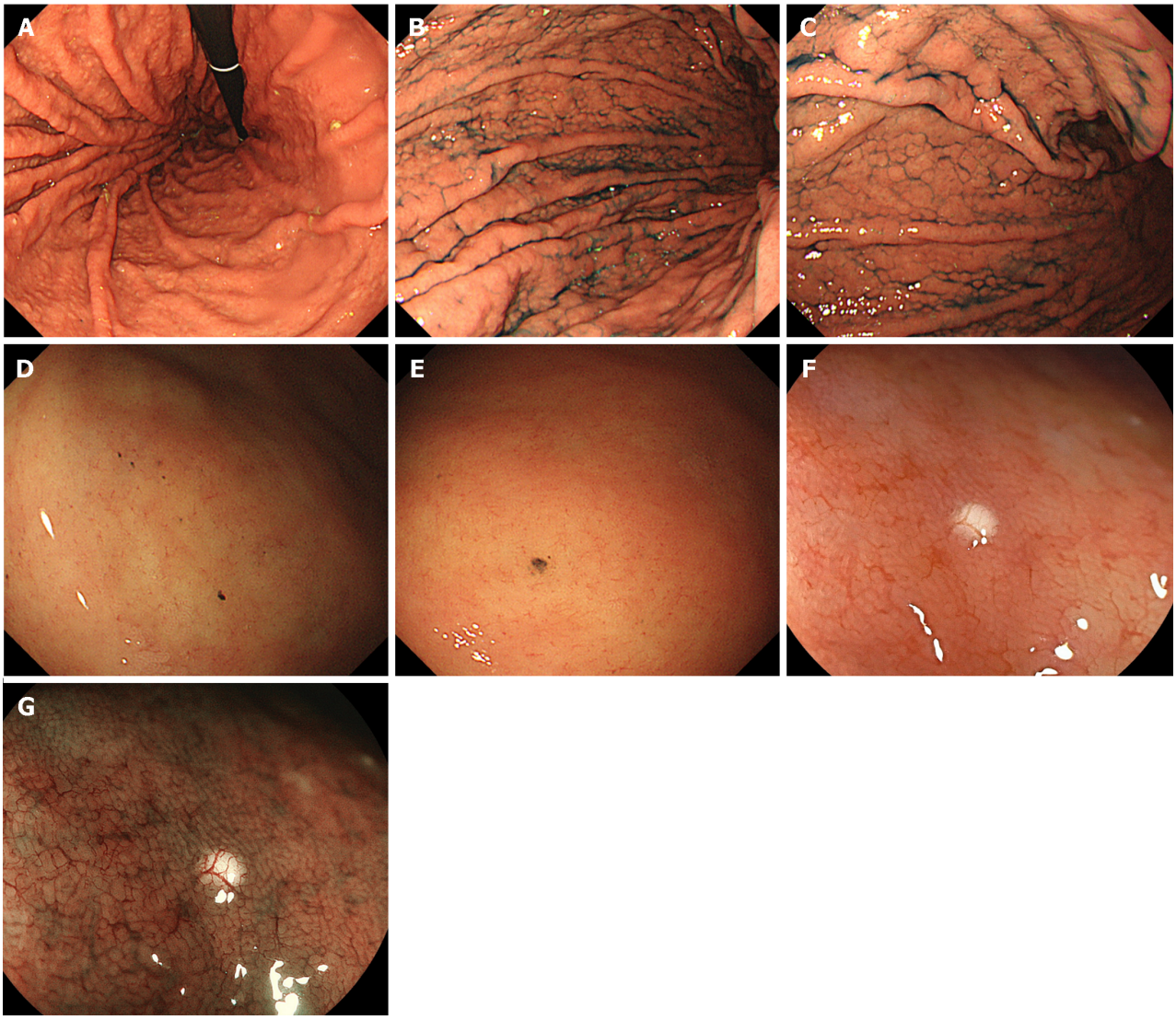


**Figure 4 Proton pump inhibitor-induced gastric mucosal changes.** A-C: Cobblestone-like mucosa. Numerous, approximately 3-5 mm-sized, elevated mucosal lesions are seen in the gastric body of a proton pump inhibitor user. After indigo carmine dye spraying (B and C); D and E: Black spots. Dark, dot-like spots are observed in the gastric body; F and G: White globe appearance. A small, round, white deposit observed during esophagogastroduodenoscopy. Magnifying endoscopic observation with blue laser imaging emphasized the lesion (G).

metaplasia, lanthanum deposition manifested as circular or granular white lesions (Figure 5C and D), and the extent of lanthanum deposition increased concurrently with the expansion of the intestinal metaplasia. The increased permeability of lanthanum in areas with intestinal metaplasia compared to that in normal mucosa may facilitate its deposition on the gastric mucosa. We speculate that the multifocal occurrence and mosaic-like distribution of intestinal metaplasia result in the circular or granular appearance of lanthanum deposition. Confirming a history of ingestion of lanthanum carbonate is essential for diagnosing this condition.

The pathological significance of lanthanum deposition in the human gastric mucosa remains unclear. To date, there have been no reports of health impairments associated with gastric lanthanum deposition, suggesting that the diagnosis of gastric lanthanum deposition does not necessarily mandate the discontinuation of lanthanum carbonate intake. However, the long-term prognosis of this condition is currently unknown, and ongoing follow-up of individual cases is desirable.

## ZINC ACETATE HYDRATE TABLET-INDUCED GASTRIC LESIONS

Zinc acetate tablets are used to treat zinc deficiency and Wilson's disease. We found that approximately two-thirds of the patients subjected to oral administration of zinc acetate tablets manifested gastric mucosal injuries characterized by mucosal erythema, erosions, white patches, and ulcers[68] (Figure 6). Localization occurred predominantly in the middle third region, followed by the upper third region. Owing to the potential occurrence of hemorrhagic gastric ulcers, patients undergoing oral administration of zinc acetate hydrate should be monitored for gastric mucosal damage.

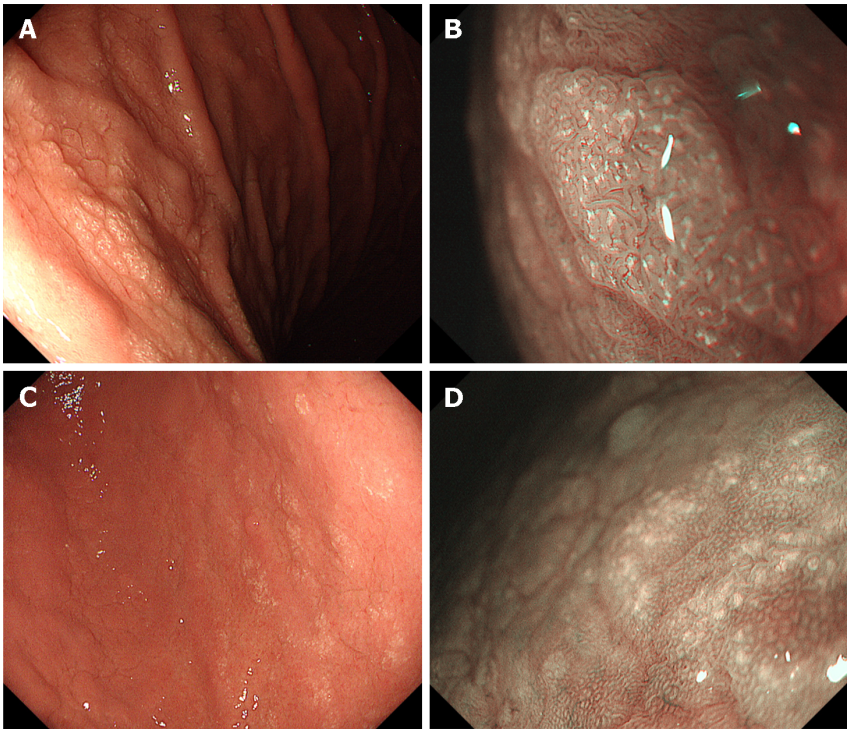


**Figure 5 Lanthanum deposition in the stomach.** A and B: Lanthanum deposition shows diffuse white lesions in non-atrophic mucosa. Magnifying observation with narrow-band imaging reveals tiny whitish depositions within the gastric mucosa (B); C and D: Multiple circular white lesions are seen in the gastric antrum with atrophic change. Magnifying observation with narrow-band imaging of the circular white lesions (D).

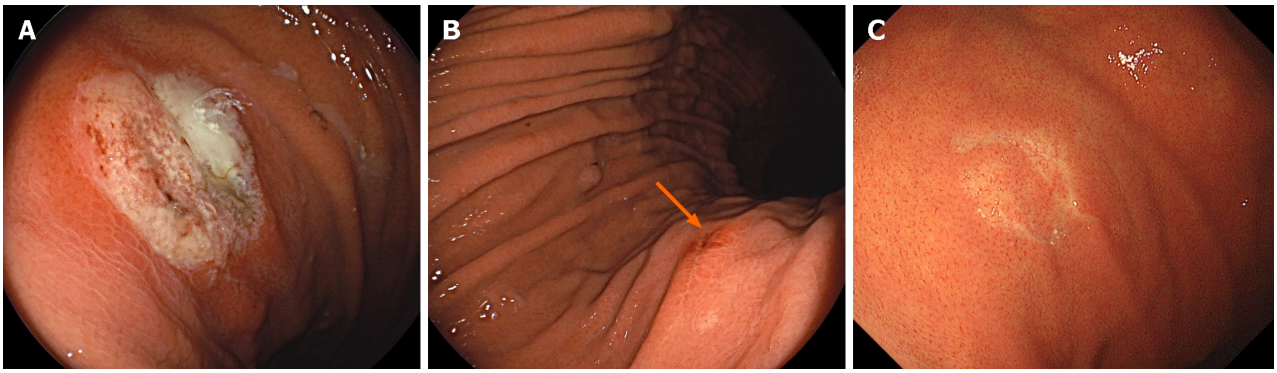


**Figure 6 Zinc acetate hydrate tablet-induced gastric lesions.** A 73-year-old Japanese woman had been taking zinc acetate dihydrate tablets for eight months to treat dysgeusia and hypozincemia. A: A round erosion with adhesion of the white coat is observed; B: Linear erosions are also seen in the gastric body (arrow); C: Esophagogastroduodenoscopy performed two months after cessation of zinc acetate hydrate tablet shows a resolution of erosions.

## IRAE GASTRITIS

Immune checkpoint inhibitors can alleviate T-cell deactivation by reinstating the immune response against tumor cells. However, systemic activation of immune cells simultaneously induces self-reactive T cells in organs other than the tumor, potentially leading to the onset of immune-related adverse event (irAE) in various organs. Among the immune checkpoint inhibitor-induced gastrointestinal injuries, irAE colitis is well recognized[69]. Although the incidence of irAE gastritis is presumed to be lower than that of irAE colitis, endoscopic features of erythema, white exudates, and friable mucosa have been documented (Figure 7)[70-77]. The destruction of the glandular structure is visible upon magnifying observation with narrow band imaging[78]. If such lesions are observed after the administration of immune checkpoint inhibitors, the possibility of irAE gastritis should be considered. In irAEs, the prompt cessation of the causative agent does not consistently lead to rapid symptom amelioration and often necessitates the administration of steroids.

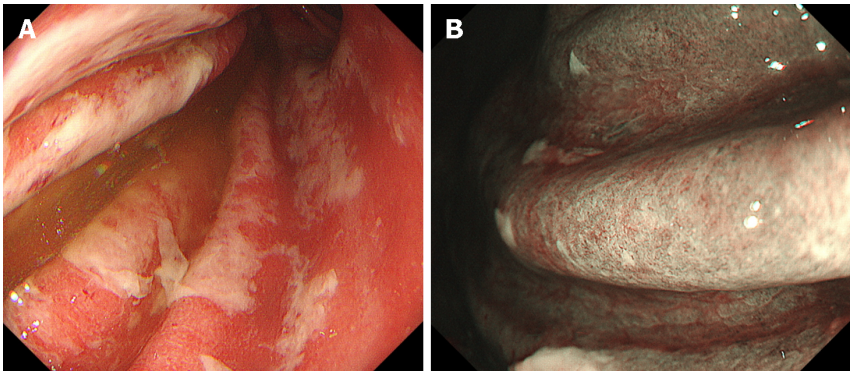


**Figure 7 Immune-related adverse events gastritis.** Esophagogastroduodenoscopy images after 16 wk of pembrolizumab administration in a 57-year-old female. A: White exudate and coarse mucosa are observed in the gastric body; B: Magnifying observation with narrow-band imaging shows that the glandular structures are absent.

## OLMESARTAN-ASSOCIATED SPRUE-LIKE ENTEROPATHY

Olmesartan-associated sprue-like enteropathy denotes a condition associated with the usage of olmesartan, an angiotensin II receptor blocker. Sprue-like enteropathy is characterized by symptoms resembling those of celiac disease, such as chronic diarrhea, weight loss, and malabsorption of nutrients. Unlike in celiac disease, these symptoms persist even with a gluten-free diet. Villous atrophy, crypt hyperplasia, and inflammation are discernible in biopsied specimens [79-81]. Consequently, if a patient presents with persistent diarrhea, weight loss, and malabsorption, particularly when using olmesartan, duodenal biopsy, along with EGD, is essential for the evaluation of these characteristic pathological features and for diagnosis. Discontinuation of olmesartan typically resolves the symptoms and mucosal changes.

## PSEUDOMELANOSIS OF THE DUODENUM

A black to dark brown pigmentation can be observed in the duodenum, termed duodenal pseudomelanosis (Figure 8)[54, 82-85]. This condition is often observed in patients with chronic diseases such as hypertension, chronic kidney failure, and diabetes. A history of oral iron supplementation, antihypertensive agents (hydralazine), diuretics (thiazides, furosemide), and beta-blockers was noted in most cases. As mentioned in the section on gastric pseudomelanosis, this condition is considered benign and a change in oral medications is not necessarily required.

## LANTHANUM DEPOSITION IN THE DUODENUM

Lanthanum deposition in the duodenum refers to the accumulation of lanthanum in patients with chronic kidney disease taking lanthanum carbonate as a phosphate binder to treat elevated phosphate levels. The representative endoscopic feature is the presence of whitish discoloration of the villi, displaying numerous pinpoint or dot-like white deposits (Figure 9)[86-88]. Although this discoloration is a notable finding, the clinical significance of lanthanum deposition in the duodenum is still not fully understood, and its presence does not necessarily indicate pathology or adverse effects.

## CONCLUSION

Diagnosing drug-induced mucosal alterations in the upper gastrointestinal tract is important for several reasons. First, in cases where a specific drug is identified as causing alterations in the esophageal, gastric, and duodenal mucosa, reassessment of treatment strategies is imperative. Discontinuation of the causative medication is generally recommended for patients presenting with symptoms or displaying evident mucosal damage, such as ulcers. If discontinuation of the causative agent proves challenging, dose reduction or transitioning to a medication with similar effects should be considered. Additionally, in the presence of lesions such as ulcers or erosions, acid-suppressing agents and mucosal protective agents may be administered. Second, establishing a diagnosis enables discerning whether gastrointestinal symptoms are attributable to a particular drug, prevents unnecessary examinations aimed at excluding other diseases, and facilitates the identification of appropriate interventions. In conclusion, the diagnosis of drug-induced upper gastrointestinal tract lesion is crucial for ensuring patient safety and facilitating appropriate medical management. Understanding the characteristic endoscopic images presented in this paper and conducting a thorough diagnosis will enable the implementation of suitable treatments and preventive measures.

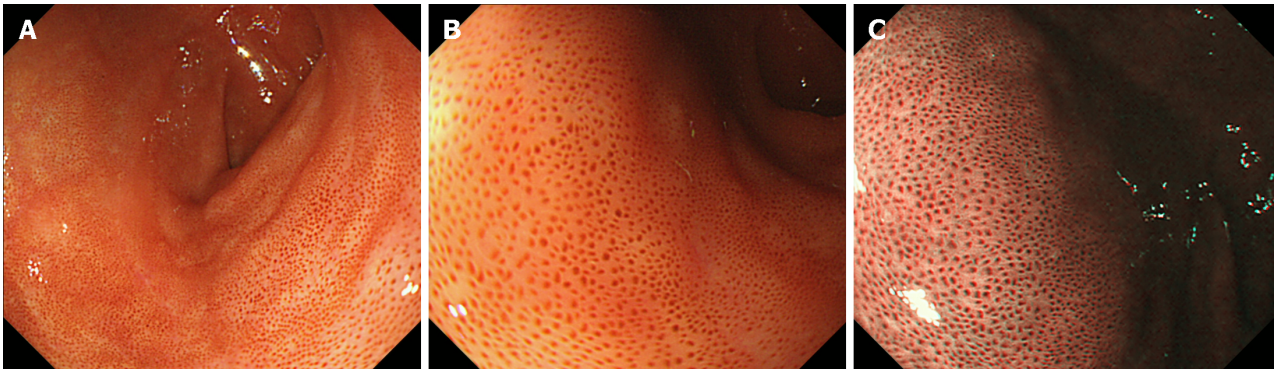


**Figure 8 Pseudomelanosis of the duodenum.** A-C: A dark brown pigmentation is observed in the duodenal bulb. Narrow-band imaging (C).

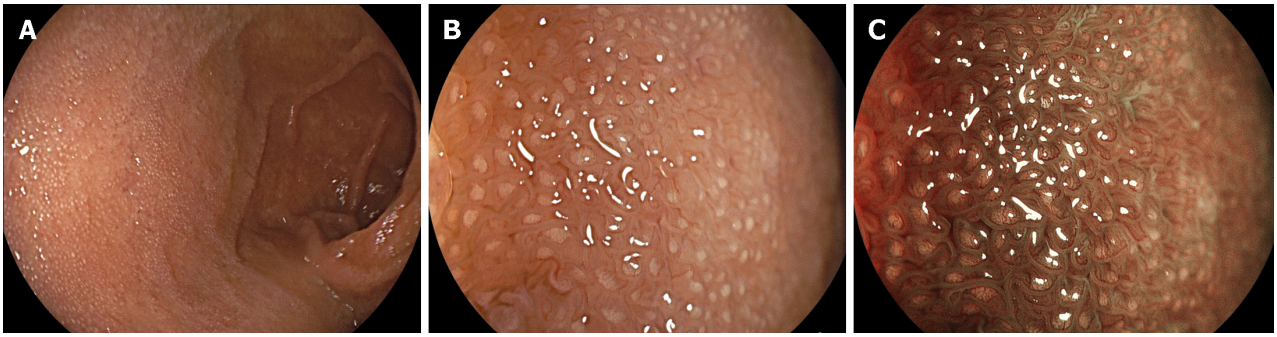


**Figure 9 Lanthanum deposition in the duodenum.** A: The duodenal mucosa is whitish; B: Magnifying observation reveals numerous dot-like white deposits in the duodenal villi; C: Magnifying observation with blue laser imaging emphasized the white deposits.

## FOOTNOTES

**Author contributions:** Iwamuro M designed the study and wrote the paper; Iwamuro M and Kawano S collected the data; Kawano S and Otsuka M critically reviewed the manuscript for important intellectual content; and Otsuka M approved the manuscript.

**Conflict-of-interest statement:** Iwamuro M has received lecture fees from Otsuka Pharmaceutical Co., Ltd. and Ono Pharmaceutical Co., Ltd., as well as grants from Taisho Pharmaceutical Co., Ltd., Zeria Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., and Abbott Japan Co., Ltd. Kawano S has received lecture fees from Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Company Limited, Astellas Pharma Inc., AstraZeneca PLC, Viatris Inc., Zeria Pharmaceutical Co., Ltd., and EA Pharma Co., Ltd., along with grants from Taisho Pharmaceutical Co., Ltd., Zeria Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., and Abbott Japan Co., Ltd. Otsuka M has received lecture fees from Chugai Pharmaceutical Co., Ltd., Gilead Sciences, Inc., Merck & Co., Inc., Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Company Limited, AbbVie GK, EA Pharma Co., Ltd., and Kowa Company, Ltd., along with grants from Taisho Pharmaceutical Co., Ltd., Zeria Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., and Abbott Japan Co., Ltd.



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ORIGINAL ARTICLE

Retrospective Study

# Preoperative prediction of perineural invasion of rectal cancer based on a magnetic resonance imaging radiomics model: A dual-center study

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## Abstract

### *BACKGROUND*

Perineural invasion (PNI) has been used as an important pathological indicator and independent prognostic factor for patients with rectal cancer (RC). Preoperative prediction of PNI status is helpful for individualized treatment of RC. Recently, several radiomics studies have been used to predict the PNI status in RC, demonstrating a good predictive effect, but the results lacked generalizability. The preoperative prediction of PNI status is still challenging and needs further study.

### *AIM*

To establish and validate an optimal radiomics model for predicting PNI status preoperatively in RC patients.

### *METHODS*

This retrospective study enrolled 244 postoperative patients with pathologically confirmed RC from two independent centers. The patients underwent preoperative high-resolution magnetic resonance imaging (MRI) between May 2019 and August 2022. Quantitative radiomics features were extracted and selected from oblique axial T2-weighted imaging (T2WI) and contrast-enhanced T1WI (T1CE) sequences. The radiomics signatures were constructed using logistic regression analysis and the predictive potential of various sequences was compared (T2WI, T1CE and T2WI + T1CE fusion sequences). A clinical-radiomics (CR) model was established by combining the radiomics features and clinical risk factors. The internal and external validation groups were used to validate the

proposed models. The area under the receiver operating characteristic curve (AUC), DeLong test, net reclassification improvement (NRI), integrated discrimination improvement (IDI), calibration curve, and decision curve analysis (DCA) were used to evaluate the model performance.

***RESULTS***

Among the radiomics models, the T2WI + T1CE fusion sequences model showed the best predictive performance, in the training and internal validation groups, the AUCs of the fusion sequence model were 0.839 [95% confidence interval (CI): 0.757-0.921] and 0.787 (95%CI: 0.650-0.923), which were higher than those of the T2WI and T1CE sequence models. The CR model constructed by combining clinical risk factors had the best predictive performance. In the training and internal and external validation groups, the AUCs of the CR model were 0.889 (95%CI: 0.824-0.954), 0.889 (95%CI: 0.803-0.976) and 0.894 (95%CI: 0.814-0.974). Delong test, NRI, and IDI showed that the CR model had significant differences from other models ($P < 0.05$). Calibration curves demonstrated good agreement, and DCA revealed significant benefits of the CR model.

***CONCLUSION***

The CR model based on preoperative MRI radiomics features and clinical risk factors can preoperatively predict the PNI status of RC noninvasively, which facilitates individualized treatment of RC patients.

**Key Words:** Rectal cancer; Perineural invasion; Magnetic resonance imaging; Radiomics; Nomogram



**Core Tip:** We constructed radiomics predictive models, clinical predictive model and clinical-radiomics (CR) model based on preoperative magnetic resonance imaging images of rectal cancer (RC), and independent clinical risk factors, to predict the preoperative perineural invasion (PNI) status of RC patients. The reliability and repeatability of the established predictive models were analyzed using internal and external validation groups. The CR model had the best stable neutral performance in both the internal and external validation groups. Therefore, the CR model was able to predict the PNI status of RC noninvasively before surgery, thereby providing support for the individualized treatment of RC patients.

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## INTRODUCTION

Rectal cancer (RC) is one of the most common gastrointestinal malignancies worldwide, and its incidence is increasing annually. Most patients with RC are diagnosed in the middle and late stages[1]. Perineural invasion (PNI), a potential pathway for the metastatic spread of RC, has been used as an important pathological indicator and independent prognostic factor for patients with RC since the 7th edition of the cancer staging system developed by the American Joint Commission on Cancer (AJCC)[2].

PNI refers to the invasion of nerves by tumor cells, followed by the spread of tumor cells along the nerve sheath. It is categorized as tumor cell invasion of endoneural, neuronal sheath, and epineural membrane layers, or tumor cells surrounding the nerve and wrapping around > 33% of the epineural membrane[3]. Multiple studies have shown that the PNI status is correlated with postoperative recurrence rates and poor prognosis of RC[4-8]. Preoperative stratification of RC patients according to the PNI status facilitates individualized treatment and improves the prognosis of RC patients. Studies have shown that neoadjuvant chemoradiotherapy (nCRT) can significantly reduce the incidence of PNI[9-12]. Preoperative assessment of PNI is helpful in decision-making regarding nCRT, especially in patients with stage II RC. Currently, the use of nCRT for stage II patients is controversial. Stage II RC is a heterogeneous disease; therefore, distinct clinicopathological features may lead to different clinical outcomes and should be treated differently[13]. The latest European Society of Medical Oncology RC Guidelines indicate that PNI is a key factor in determining whether stage II patients will benefit from nCRT and postoperative adjuvant chemotherapy[14,15]. In addition, nCRT combined with total mesorectum excision is regarded as the standard treatment for locally advanced RC. Although the local recurrence rate is reduced to < 10%, the distant metastasis rate is still more than 20%-30%. Postoperative adjuvant chemotherapy is used to prevent distant metastasis, and the PNI status is an indicator of postoperative adjuvant chemotherapy[14-16]. However, nCRT can significantly reduce the incidence of PNI in patients with RC, promoting the downward phase of the tumor. Therefore, the postoperative pathological conditions do not fully reflect the patient's status[17]. The preoperative evaluation of PNI is helpful for guiding the use of postoperative adjuvant chemotherapy for patients with locally advanced RC.

Currently, PNI status cannot be assessed based on conventional preoperative biopsy or magnetic resonance imaging (MRI), but can only be determined through postoperative pathological examination. Conventional preoperative biopsy only obtains the mucosal and submucosal layers, whereas peripheral nerves mostly exist outside the mucosal muscle layer or even outside the intestinal wall. Additionally, MRI cannot display small nerves. The efficiency and timeliness of postoperative pathological testing limit the use of PNI[13].

Radiomics can extract quantitative features that reflect tumor heterogeneity, allowing the extraction of deep mining data from images and analyzing noninvasive clinical predictors to provide detailed information for personalized treatment and patient management[18]. This technique is widely used for auxiliary diagnosis, pathological staging, prediction of treatment outcomes, and prognostication. Several radiomics studies have been used to predict PNI status in RC, demonstrating a good predictive effect. However, most studies were based on computed tomography or single sequences of MRI and were conducted at single centers without external validation. Therefore, the results lacked generalizability[13,19-23].

This two-center study aimed to construct a radiomics prediction model for the PNI status of RC based on T2-weighted imaging (T2WI) and contrast-enhanced T1WI (T1CE) sequences of high-resolution MRI and to systematically compare the performance of different radiomics predictive models. The optimal radiomics predictive model combined with PNI-related clinical features was selected to construct the final clinical-radiomics (CR) model, which provided the basis for individualized management strategies for RC patients.

## MATERIALS AND METHODS

### Patients

This two-center retrospective study was approved by the Ethical Review Committee of the Affiliated Hospital of North Sichuan Medical College (AHNSMC, file number: 2022ER431-1), which waived the need for informed consent from participants.

The study inclusion criteria were: (1) Postoperative pathologically confirmed RC; (2) high-resolution contrast-enhanced MRI of rectum performed using 1.5 T MRI within 1 wk before the operation, and a complete TNM staging report was obtained; and (3) preoperative peripheral blood carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels were detected within 1 wk before the operation.

The exclusion criteria included: (1) nCRT and other antitumor treatments were administered preoperatively; (2) postoperative pathological report did not record the PNI status; and (3) poor image quality, which made it difficult to segment the image of the lesion.

A total of 613 patients who underwent surgical resection of rectal RC were recruited between May 2019 and August 2022. Finally, 244 patients from two independent centers were enrolled. Among them, 170 consecutive patients from Center 1 (Affiliated Hospital of North Sichuan Medical College) were randomly divided into a training group ($n$ = 118) and an internal validation group ($n$ = 52) at a ratio of 7:3, whereas 74 consecutive patients from Center 2 (Second Clinical School of North Sichuan Medical College) served as an external validation group. The detailed workflow of the study is presented in Figure 1.

Preoperative information on clinical and radiological variables was collected retrospectively from our medical records and Picture Archiving and Communication System (PACS). The clinical variables included age, gender, CEA level (< 5 or ≥ 5 ng/mL), CA19-9 level (< 30 or ≥ 30 U/mL), histopathological grade, and clinical M stage. The radiological variables included the distance between the tumor and anal margin, MRI T stage, MRI N (mN) stage, clinical TNM (cTNM) stage, MRI-based circumferential resection margin (mCRM), and MRI-based extramural vascular invasion (mEMVI). PNI status data were derived from postoperative pathological analysis of hematoxylin and eosin (HE)-stained sections and immunohistochemical analysis of the resected specimens.

### MRI

All patients in Center 1 were scanned using Siemens Aera 1.5 T MRI, and all patients in Center 2 were scanned using GE Sigma HDxt 1.5 T MRI. The sequences included high-resolution oblique axial T2WI and axial T1CE. T1CE sequences were obtained after intravenous administration of gadolinium contrast agent using an MRI-compatible power injector at a rate of 2 mL/s and a dose of 0.2 mL/kg, followed by flushing 20 mL of saline using a high-pressure syringe. The detailed sequence parameters are presented in Table 1.

### Imaging analysis and segmentation

MR images were derived from PACS in the Digital Imaging and Communications in Medicine format. Two experienced radiologists, with 8 and 10 years of work experience, independently evaluated the MR images while being blinded to the postoperative pathological findings. Discrepancies among the readers were resolved through discussion. The diagnostic methods were based on the 8th AJCC staging system[24].

In cases with unknown pathological findings, Reader 1 used the 3D slicer software (version 4.11.2; https://www.slicer.org) to manually delineate the lesion layer-by-layer on oblique axial T2WI and axial T1CE sequences, thereby obtaining the volume of interest (VOI) of the tumor. Subsequently, the 3D mask of the tumor was exported. One month after completing the image segmentation of all patients by Reader 1, 30 patients were randomly selected, for whom Readers 1 and 2 independently performed repeated segmentation of the lesion and output masks. Repeatability analysis was conducted within and between observers to evaluate the stability of subsequent radiomics features.

**Table 1 Magnetic resonance imaging parameters of each sequence**

| Scanner | Sequence | Orientation | TR (ms) | TE (ms) | FOV (mm²) | Matrix | Thickness (mm) |
|---|---|---|---|---|---|---|---|
| Siemens Aera 1.5T | T2WI | Oblique axial | 4480 | 87 | 190 × 190 | 320 × 320 | 3 |
| | T1CE | Oblique axial | 7 | 3 | 210 × 210 | 256 × 256 | 3 |
| GE Signa HDxt 1.5T | T2WI | Oblique axial | 4120 | 70 | 180 × 180 | 256 × 192 | 3 |
| | T1CE | Oblique axial | 6 | 3 | 200 × 200 | 288 × 160 | 3 |

TR: Repetition time; TE: Echo time; FOV: Field of view; T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI.

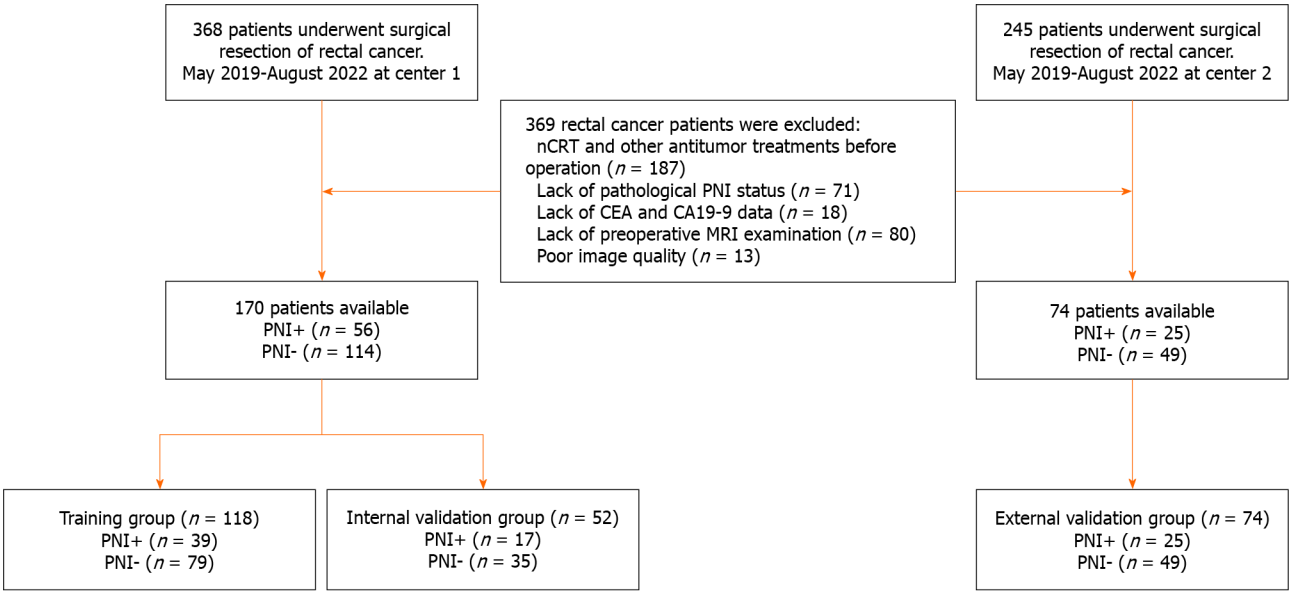


**Figure 1 Flowchart of participant selection.** nCRT: Neoadjuvant chemoradiotherapy; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; MRI: Magnetic resonance imaging; PNI: Perineural invasion.

### *Radiomics feature extraction and selection*

The Radiomics package of the 3D-Slicer software (version 4.11.2; https://www.slicer.org) was used for original image preprocessing and radiomics feature extraction. To minimize the image extraction of radiomics features using different MRI models, the preprocessing of the original images included the following steps: (1) Voxel spacing was standardized, and all images were resampled to a volume of 1 mm × 1 mm × 1 mm; and (2) the image noise and normalized voxel intensity were controlled, and the voxel intensity values were discretized using a fixed bin width (25 HU)[25].

All VOIs were imported into the 3D-Slicer software for feature extraction. In total, 944 radiomics features were extracted for each VOI. The image types of the extracted radiomics features included the following: (1) A total of 107 radiomics features extracted from the original image after image preprocessing [including shape features, first-order statistical features, gray-level co-occurrence matrix (GLCM) features, gray-level dependence matrix features, gray-level run length matrix (GLRLM) features, gray-level size zone matrix (GLSZM) features, and neighboring gray tone difference matrix features]; (2) a total of 93 radiomics features of images extracted (including first-order statistical, GLCM, gray-level dependence matrix, GLRLM, GLSZM, and neighboring gray tone difference matrix) using the Laplace operator of the Gaussian filter and the image derived from the transformation of 1σ (0.5); and (3) a total of 744 radiomics features extracted from eight image types derived from wavelet transformation (including first-order statistical features, GLCM features, gray-level dependence matrix features, GLRLM features, GLSZM features, and neighboring gray tone difference matrix features). The specific radiomics feature types extracted based on the abovementioned images are shown in Supplementary Table 1. For further details, please refer to http://pyradiomics.readthedocs.io/en/Latest/features.html.

Based on the training group data, the radiomics features were reduced and screened. First, dimensionality reduction, Z-score standardization, and maximum-minimum normalization of radiomics eigenvalues were used to remove irrelevant and redundant features. Then, radiomics features were screened using the following steps: (1) Radiomics features with inter- and intraclass correlation coefficients > 0.75 were screened to ensure their stability; (2) radiomics features with significant differences ($P < 0.05$) were screened using univariate logistic regression analysis; and (3) the least absolute shrinkage and selection operator regression algorithm was used to adjust the penalty parameters using 10-fold crossvalidation, and the regression coefficients of each radiomics feature were compressed, according to the principle of the simplest model. The radiomics features with non-zero coefficients were screened to establish the Rad-score.

### Model construction and evaluation

Based on the training group data, univariate logistic regression analysis was carried out for all clinical and MRI factors. According to our results and clinical practice, relevant factors were selected for multifactorial logistic regression analysis. We constructed a clinical predictive model and three radiomics predictive models, namely T2WI, T1CE and T2WI + T1CE fusion sequence. Finally, the best radiomics predictive model was selected and combined with selected clinical and MRI risk factors to construct a combined CR predictive model.

The above predictive models were tested in the internal and external validation groups to determine their differentiation, calibration and clinical effectiveness. The area under the receiver operating characteristic (ROC) curve (AUC), Delong test, net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were used to compare the differentiation of each predictive model. Calibration curves were used to evaluate the calibration degree of each predictive model; that is, the consistency between the predicted and actual results. Decision curve analysis (DCA) was used to evaluate the efficacy of each predictive model. Finally, the optimal model was selected to construct a nomogram and an online interactive nomogram. The detailed research process is presented in Figure 2.

### Statistical analysis

The clinical and MRI factors were analyzed using SPSS software version 26.0. Categorical variables are expressed as percentages and analyzed using $\chi^2$ or Fisher's exact tests. Continuous variables with normal data distribution were expressed as mean ± SD, whereas those with non-normal distribution were expressed as median (interquartile range). $P < 0.05$ (two-tailed) was considered statistically significant. R version 4.2.1 was used for feature screening, construction and evaluation of the predictive models. Several R packages were used for data analysis, including caret, irr, car, MASS, glmnet, pROC, nricens, rms, DynNom, rsconnect, rmda, plot, and ggplot2.

## RESULTS

### Patient characteristics

In this study, two hundred and forty-four patients were enrolled and categorized into the training ($n = 118$), internal validation ($n = 52$) and external validation ($n = 74$) groups. Table 2 summarizes the detailed clinical and radiological characteristics of the patients. There were no significant differences in the proportion of PNI-positive patients between the training group and the two validation groups (32.1, 32.7 and 33.8%, respectively; $P > 0.05$). There were no significant differences between the training, internal validation and external validation datasets ($P > 0.05$). In contrast, the training group dataset demonstrated significant differences between PNI-positive and PNI-negative patients in terms of CA19-9 level, mN, mCRM, mEMVI and histological grade ($P < 0.05$). In the internal validation group, there were significant differences in the CEA and CA19-9 levels between the PNI-positive and PNI-negative patients ($P < 0.05$). In the external validation group, there were significant differences in CEA, mN and mEMVI between the PNI-positive and PNI-negative patients ($P < 0.05$).

### Model construction and evaluation

In this study, twelve features were retained from the three sequences: T2WI ($n = 5$), T1CE ($n = 2$) and T2WI + T1CE fusion sequence ($n = 5$). The detailed information regarding these features is presented in Figure 3 and Supplementary Table 2. The radiomics scores were calculated by multiplying the selected features with the corresponding coefficients for each modality as follows: T2WI_Rad-score = 14.86872 + (0.03702 × original_glcm_Correlation) + (0.10289 × original_glcm_MCC) + (8.2082 × original_glszm_ZoneEntropy) + (9.15542 × wavelet-LLL_glcm_MCC) + (-3.61471 × wavelet-LLL_glrlm_GrayLevelNonUniformityNormalized). T1CE_Rad-score = -26.133 + (2.919 × original_shape_Maximum²DDiameterSlice) + (22.566 × original_gldm_DependenceEntropy). (T2WI + T1CE)_Rad-score = -7.0772 + (-0.1798 × T2WI_original_glcm_MCC) + (0.7251 × T2WI_original_glszm_ZoneEntropy) + (7.5941 × T2WI_wavelet-LLL_glcm_MCC) + (-4.442 × T2WI_wavelet-LLL_glrlm_GrayLevelNonUniformityNormalized) + (2.3265 × T1CE_original_shape_ Maximum²DDiameterSlice).

Three radiomics predictive models were constructed based on the Rad-score using logistic regression. In the training group, the AUCs of the T2WI sequence radiomics predictive model, T1CE sequence radiomics predictive model, and T2WI + T1CE fusion sequence radiomics predictive model were 0.817, 0.798 and 0.839, respectively, and these values were 0.763, 0.689 and 0.787 in the internal validation group, respectively, and 0.759, 0.841, and 0.836 in the external validation group, respectively. Detailed information regarding the predictive models is presented in Table 3 and Figure 4.

Univariate and multivariate logistic regression analyses of training group data demonstrated that cTNM [odds ratio (OR): 42.002; 95% confidence interval (CI): 2.913-605.511] ($P = 0.006$) and histological grade (OR: 0.113; 95%CI: 0.020-0.658) ($P = 0.015$) were independent risk factors for PNI in RC. Further details are presented in Table 4. The AUCs of the clinical predictive models for the training, internal validation and external validation groups were 0.804, 0.828 and 0.813, respectively. Further details are presented in Table 3.

The Delong test for the training group data showed no significant differences among the three radiomics predictive models ($P = 0.476$). However, the NRI and IDI indices demonstrated that the T2WI + T1CE fusion sequence radiomics predictive model had significantly higher predictive ability compared to the T2WI and T1CE sequence radiomics predictive models (NRI index > 0, IDI index > 0, $P < 0.05$). The T2WI + T1CE fusion sequence radiomics predictive model was superior to the T2WI and T1CE sequence radiomics predictive models. Further details are presented in Table 5. The T2WI + T1CE_Rad-score and independent clinical risk factors (cTNM and histological grade) were selected to construct a

**Table 2 Characteristics of patients in the training, internal validation and external validation groups, *n* (%)**

| Characteristics | Training group (*n* = 118) | | *P* value | Internal validation group (*n* = 52) | | *P* value | External validation group (*n* = 74) | | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| | PNI+ (*n* = 39) | PNI- (*n* = 79) | | PNI+ (*n* = 17) | PNI- (*n* = 35) | | PNI+ (*n* = 25) | PNI- (*n* = 49) | |
| Sex | | | 0.854 | | | 0.882 | | | 0.640 |
| Male | 25 (64.1) | 52 (65.8) | | 12 (70.6) | 24 (68.6) | | 16 (64.0) | 34 (69.4) | |
| Female | 14 (35.9) | 27 (34.2) | | 5 (29.4) | 11 (31.4) | | 9 (36.0) | 15 (30.6) | |
| Age (yr) | 68 (56.5-73) | 68 (58.5-75) | 0.479 | 66.59 ± 12.57 | 64.17 ± 10.32 | 0.497 | 65.24 ± 11.70 | 66.84 ± 9.85 | 0.562 |
| CEA | | | 0.150 | | | 0.027 | | | 0.005 |
| Negative (< 5 ng/mL) | 23 (59.0) | 57 (72.2) | | 9 (52.9) | 30 (85.7) | | 14 (56.0) | 42 (85.7) | |
| Positive (≥ 5 ng/mL) | 16 (41.0) | 22 (27.8) | | 8 (47.1) | 5 (14.3) | | 11 (44.0) | 7 (14.3) | |
| CA19-9 | | | 0.034 | | | 0.019 | | | 1.000 |
| Negative (< 30 U/mL) | 30 (76.9) | 72 (91.1) | | 12 (70.6) | 34 (97.1) | | 23 (92.0) | 46 (93.9) | |
| Positive (≥ 30 U/mL) | 9 (23.1) | 7 (8.9) | | 5 (29.4) | 1 (2.9) | | 2 (8.0) | 3 (6.1) | |
| DIS | | | 0.050 | | | 0.444 | | | 0.823 |
| High | 24 (61.5) | 31 (39.2) | | 9 (52.9) | 15 (42.9) | | 12 (48.0) | 22 (44.9) | |
| Mid | 3 (7.7) | 16 (20.3) | | 5 (29.4) | 9 (25.7) | | 5 (20.0) | 13 (26.5) | |
| Low | 12 (30.8) | 32 (40.5) | | 3 (17.6) | 11 (31.4) | | 8 (32.0) | 14 (28.6) | |
| mT | | | 0.211 | | | 0.885 | | | 0.314 |
| mT1-2 | 3 (7.7) | 15 (19.0) | | 6 (35.3) | 10 (28.6) | | 5 (20.0) | 9 (18.4) | |
| mT3 | 24 (61.5) | 47 (59.5) | | 8 (47.1) | 18 (51.4) | | 14 (56.0) | 32 (65.3) | |
| mT4 | 12 (30.8) | 17 (21.5) | | 3 (17.6) | 7 (20.0) | | 6 (24.0) | 8 (16.3) | |
| mN | | | 0.003 | | | 0.063 | | | 0.028 |
| mN0 | 5 (12.8) | 27 (34.2) | | 3 (17.6) | 18 (51.4) | | 6 (24.0) | 19 (38.8) | |
| mN1 | 13 (33.3) | 33 (41.8) | | 5 (29.4) | 7 (20.0) | | 7 (28.0) | 21 (42.9) | |
| mN2 | 21 (53.8) | 19 (24.1) | | 9 (52.9) | 10 (28.6) | | 12 (48.0) | 9 (18.4) | |
| cM | | | 0.070 | | | 1.000 | | | 0.064 |
| cM0 | 34 (87.2) | 77 (97.5) | | 17 (100.0) | 34 (97.1) | | 22 (88.0) | 49 (100.0) | |
| cM1 | 5 (12.8) | 2 (2.5) | | 0 (0.0) | 1 (2.9) | | 3 (12.0) | 0 (0.0) | |
| cTNM | | | 1.000 | | | 1.000 | | | 1.000 |
| I | 1 (2.6) | 29 (36.7) | | 0 (0.0) | 16 (45.7) | | 0 (0.0) | 18 (36.7) | |
| II | 9 (23.1) | 25 (31.6) | | 5 (29.4) | 13 (37.1) | | 3 (12.0) | 12 (24.5) | |
| III | 25 (64.1) | 23 (29.1) | | 11 (64.7) | 5 (14.3) | | 19 (76.0) | 19 (38.8) | |
| IV | 4 (10.3) | 2 (2.5) | | 1 (5.9) | 1 (2.9) | | 3 (12.0) | 0 (0.0) | |
| mCRM | | | 0.002 | | | 0.374 | | | 0.236 |
| Negative | 14 (35.9) | 52 (65.8) | | 9 (52.9) | 23 (65.7) | | 15 (60.0) | 36 (73.5) | |
| Positive | 25 (64.1) | 27 (34.2) | | 8 (47.1) | 12 (34.3) | | 10 (40.0) | 13 (26.5) | |
| mEMVI | | | 0.016 | | | 0.935 | | | 0.009 |
| Negative | 15 (38.5) | 49 (62.0) | | 10 (58.8) | 21 (60.0) | | 10 (40.0) | 35 (71.4) | |
| Positive | 24 (61.5) | 30 (38.0) | | 7 (41.2) | 14 (40.0) | | 15 (60.0) | 14 (28.6) | |
| Histological grade | | | 0.000 | | | 0.920 | | | 0.894 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Well differentiated | 4 (10.3) | 27 (34.2) | 2 (11.8) | 11 (31.4) | 4 (16.0) | 13 (26.5) |
| Moderately differentiated | 26 (66.7) | 49 (62.0) | 12 (70.6) | 23 (65.7) | 18 (72.0) | 34 (69.4) |
| Poorly differentiated | 9 (23.1) | 3 (3.8) | 3 (17.6) | 1 (2.9) | 3 (12.0) | 2 (4.1) |

*P* value represents analysis of PNI-positive and PNI-negative datasets between each group. PNI: Perineural infiltration; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; mCRM: MRI-based circumferential resection margin; mEMVI: MRI-based extramural vascular invasion; mT: MRI T stage; mN: MRI N stage; cM: Clinical M stage; cTNM: Clinical TNM stage; DIS: The distance between tumor and anal margin, Low (0-5 cm from the anal verge), middle (5.1-10 cm from the anal verge), and high (10.1-15 cm from the anal verge).

**Table 3 Performance of various predictive models in the training, internal validation and external validation groups**

| Models | Training group | Internal validation group | External validation group |
|---|---|---|---|
| T2WI | | | |
| AUC (95%CI) | 0.817 (0.733-0.901) | 0.763 (0.626-0.900) | 0.759 (0.644-0.875) |
| Sensitivity | 0.564 | 0.294 | 0.480 |
| Specificity | 0.899 | 0.886 | 0.857 |
| Positive predictive value | 0.733 | 0.556 | 0.632 |
| Negative predictive value | 0.807 | 0.721 | 0.764 |
| T1CE | | | |
| AUC (95%CI) | 0.798 (0.707-0.890) | 0.689 (0.521-0.857) | 0.841 (0.752-0.930) |
| Sensitivity | 0.487 | 0.471 | 0.480 |
| Specificity | 0.937 | 0.857 | 0.878 |
| Positive predictive value | 0.792 | 0.615 | 0.667 |
| Negative predictive value | 0.787 | 0.769 | 0.768 |
| T2WI + T1CE | | | |
| AUC (95%CI) | 0.839 (0.757-0.921) | 0.787 (0.650-0.923) | 0.836 (0.735-0.937) |
| Sensitivity | 0.641 | 0.529 | 0.560 |
| Specificity | 0.899 | 0.914 | 0.939 |
| Positive predictive value | 0.758 | 0.750 | 0.824 |
| Negative predictive value | 0.835 | 0.800 | 0.807 |
| Clinical model | | | |
| AUC (95%CI) | 0.804 (0.727-0.881) | 0.828 (0.719-0.937) | 0.813 (0.724-0.903) |
| Sensitivity | 0.718 | 0.706 | 0.800 |
| Specificity | 0.747 | 0.829 | 0.694 |
| Positive predictive value | 0.583 | 0.667 | 0.571 |
| Negative predictive value | 0.843 | 0.829 | 0.872 |
| CR model | | | |
| AUC (95%CI) | 0.889 (0.824-0.954) | 0.889 (0.803-0.976) | 0.894 (0.814-0.974) |
| Sensitivity | 0.692 | 0.647 | 0.760 |
| Specificity | 0.924 | 0.886 | 0.899 |
| Positive predictive value | 0.818 | 0.733 | 0.792 |
| Negative predictive value | 0.859 | 0.838 | 0.880 |

T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI; CR: Clinical-radiomics prediction model; AUC: Area under receiver operating characteristic curve.

**Table 4 Results of univariate and multivariate logistic regression analyses**

| Variables | Univariate logistic regression | | Multivariate logistic regression | |
|---|---|---|---|---|
| | OR (95%CI) | *P* value | OR (95%CI) | *P* value |
| Gender | 0.927 (0.418-2.098) | 0.854 | NA | NA |
| Age | 0.976 (0.944-1.008) | 0.138 | NA | NA |
| CEA | 1.802 (0.801-4.045) | 0.152 | NA | NA |
| CA19-9 | 3.086 (1.056-9.375) | 0.040[a] | NA | NA |
| DIS | 1.487 (0.968-2.329) | 0.075 | NA | NA |
| mT | 1.717 (0.919-3.312) | 0.096 | NA | NA |
| mN | 2.507 (1.467-4.495) | 0.001[a] | NA | NA |
| cM | 5.662 (1.158-40.921) | 0.044[a] | NA | NA |
| cTNM | 3.705 (2.139-7.056) | 0.000[a] | 42.002 (2.913-605.511) | 0.006[a] |
| mCRM | 3.439 (1.562-7.838) | 0.003[a] | NA | NA |
| mEMVI | 2.613 (1.199-5.852) | 0.017[a] | NA | NA |
| Histological grade | 0.229 (0.092-0.496) | 0.001[a] | 0.113 (0.020-0.658) | 0.015[a] |

[a]$P < 0.05$.

CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; DIS: The distance between tumor and anal margin; mT: MRI T stage; mN: MRI N stage; cM: Clinical M stage; cTNM: Clinical TNM stage; mCRM: MRI-based circumferential resection margin; mEMVI: MRI-based extramural vascular invasion; OR: Odds ratio; CI: Confidence interval; NA: Not available.

**Table 5 Performance of various radiomics predictive models in the training group as evaluated using the Delong test, integrated discrimination improvement index and net reclassification improvement index**

| Radiomics prediction models | AUC (95%CI) | Delong test *P* value | IDI (95%CI) | IDI index *P* value | NRI (95%CI) | NRI index *P* value |
|---|---|---|---|---|---|---|
| T2WI + T1CE | 0.839 (0.757-0.921) | | | | | |
| T2WI | 0.817 (0.733-0.901) | 0.252 | 0.081 (0.031-0.131) | 0.001 | 0.510 (0.104-0.865) | 0.008 |
| T1CE | 0.798 (0.707-0.890) | 0.196 | 0.127 (0.064-0.190) | 0.000 | 0.536 (0.255-1.018) | 0.005 |

AUC: Area under receiver operating characteristic curve; IDI: Integrated discrimination improvement; NRI: Net reclassification improvement; T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI.

combined CR predictive model through logistic regression. The combined CR model demonstrated AUCs of 0.889, 0.889 and 0.894 in the training, internal validation and external validation groups, respectively. Further details are presented in Table 3.

### *Construction and validation of models*

Figure 4 shows the ROC curves of each model in the training, internal validation and external validation groups. The CR model demonstrated the best discrimination ability in each group (Table 3).

Further comparisons were made between the CR, clinical predictive and T2WI + T1CE fusion sequence radiomics predictive models. Based on the training group data, the Delong test showed no significant differences ($P > 0.05$) between the clinical predictive model and T2WI + T1CE fusion sequence radiomics predictive model. The CR model demonstrated a significant difference ($P < 0.05$) compared to the clinical predictive model and T2WI + T1CE fusion sequence radiomics predictive model. NRI and IDI indices further revealed that the CR model had significantly higher predictive ability compared to the clinical predictive model and T2WI + T1CE fusion sequence radiomics predictive model (NRI index > 0, IDI index > 0, $P < 0.05$) (Table 6).

The calibration curves of the three models are shown in Figure 5. The CR model had the best calibration ability in the training, internal validation and external validation groups (i.e., the calibration curve was closest to the reference line). The DCA curve is shown in Figure 6. The CR model achieved the highest clinical net benefit compared to the clinical predictive model and T2WI + T1CE fusion sequence radiomics predictive model.

Based on the final results of model construction and validation, the CR model with the best model performance was selected to construct a concise nomogram (Figure 7) and an online interactive dynamic web page nomogram (Figure 8)

**Table 6 Performance of the clinical model, T2-weighted imaging + contrast-enhanced T1WI fusion sequence radiomics model, and clinical-radiomics model in the training group, as evaluated using the Delong test, integrated discrimination improvement index, and Net reclassification improvement index**

| Models | AUC (95%CI) | Delong test *P* value | IDI (95%CI) | IDI index *P* value | NRI (95%CI) | NRI index *P* value |
|---|---|---|---|---|---|---|
| CR model | 0.889 (0.824-0.954) | | | | | |
| Clinical | 0.804 (0.727-0.881) | 0.009 | 0.210 (0.130-0.290) | 0.000 | 0.588 (0.271-0.904) | 0.000 |
| T2WI + T1CE | 0.839 (0.757-0.921) | 0.019 | 0.075 (0.026-0.124) | 0.002 | 0.447 (0.164-0.731) | 0.002 |

AUC: Area under receiver operating characteristic curve; IDI: Integrated discrimination improvement; NRI: Net reclassification improvement; CR: Clinical-radiomics; T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI.

for visualization.

## DISCUSSION

Noninvasive assessment of the prognosis of RC is challenging and has always been a research hotspot[26,27]. Research has shown that PNI is not only characterized by tumor cell infiltration and growth along the nerves but also involves an interaction between various neurotrophic and chemotactic factors released from tumor cells and the surrounding microenvironment. This process induces tumor invasion, local recurrence, and metastasis, leading to poor prognosis[28-30]. Preoperative prediction of PNI status is helpful for individualized treatment of RC. For example, PNI-positive RC patients should receive more aggressive treatment, such as nCRT[9-12].

The present study constructed radiomics predictive models, clinical predictive models and CR models based on preoperative high-resolution MRI of RC, as well as independent clinical risk factors (cTNM and histological grade), to predict the preoperative PNI status of RC patients. The reliability and repeatability of the established predictive models were analyzed using internal and external validation groups. Compared to the radiomics and clinical predictive models, the CR model had the best discrimination, calibration and clinical net benefit, with stable neutral performance in both the internal and external validation groups.

We constructed three radiomics predictive models based on T2WI and T1CE sequences: T2WI, T1CE and T2WI + T1CE fusion sequence. In the training and internal validation groups, the AUCs of the fusion sequence model were 0.839 (95%CI: 0.757-0.921) and 0.787 (95%CI: 0.650-0.923), which were higher than those of the T2WI and T1CE sequence models. In the external validation group, the AUC of the fusion sequence model was 0.836 (95%CI: 0.735-0.937), which was lower than that of the T1CE sequence model (0.841, 95%CI: 0.752-0.930). Although the AUCs of the fusion sequence models in the training group were higher than those of the T2WI and T1CE sequences, there were no significant differences between the models in the Delong test ($P > 0.05$). The contradictory results may have been due to two reasons: first, the study sample size was not sufficiently large. Further research with larger sample sizes is needed. Second, there are inherent limitations of the Delong test. Although it is widely used to compare models, it may not be sufficiently sensitive to the incremental changes in the predictive ability of the model. Therefore, we also used the NRI and IDI indexes for evaluation[31]. The final results showed that the fusion sequence model had a significantly higher predictive ability compared to the T2WI and T1CE sequence models (NRI index > 0, IDI index > 0, $P < 0.05$). Therefore, a CR model was constructed using Rad-score fusion sequences and independent clinical risk factors.

The Rad-score value of the T2WI + T1CE fusion sequence radiomics predictive model consisted of five radiomics features, including one shape feature, two GLCM features, one GLRLM feature and one GLSZM feature. The shape feature is the maximum-2D-diameter-slice based on the original image of the T1CE sequence, which reflects the tumor size. This may be because larger tumors have a higher probability of contact with nerves, leading to a higher probability of PNI[32,33]. The GLCM features include Matthews' correlation coefficient (MCC) based on original T2WI and wavelet transformation of T2WI sequence wavelet-LLL images. The GLCM features mainly reflect the probability of voxel values appearing at a given direction and distance in the spatial arrangement relationship between voxel gray levels of an image. MCC is primarily used to determine the binary classification, and it comprehensively considers true-positive, true-negative, true-positive, true-negative, false-positive and false negative cases, making it a balanced indicator. The GLRLM feature is the gray-level nonuniformity normalized (GLNN) based on the T2WI sequence wavelet-LLL image. This feature reflects the spatial arrangement of voxels in the image, and the voxel length with the same gray level continuously appearing in the specified direction. GLNN is primarily used to determine the similarity of gray-level intensity values in the image. The GLSZM feature is the zone entropy (ZE) based on the original T2WI, which reflects the area of continuous voxel values quantified in the spatial arrangement of voxels and gray scales in the image. ZE represents the uncertainty or randomness of the measurement area and grayscale distribution. The higher the value, the greater the nonuniformity of the texture pattern.

Among the five radiomics features, two are wavelet-transformed features, which decompose the original image into different frequency domains and then extract features from each wavelet image individually. Therefore, multi-frequency domain and multi-scale image information can be obtained, allowing the obtained features to reflect the spatial hetero-

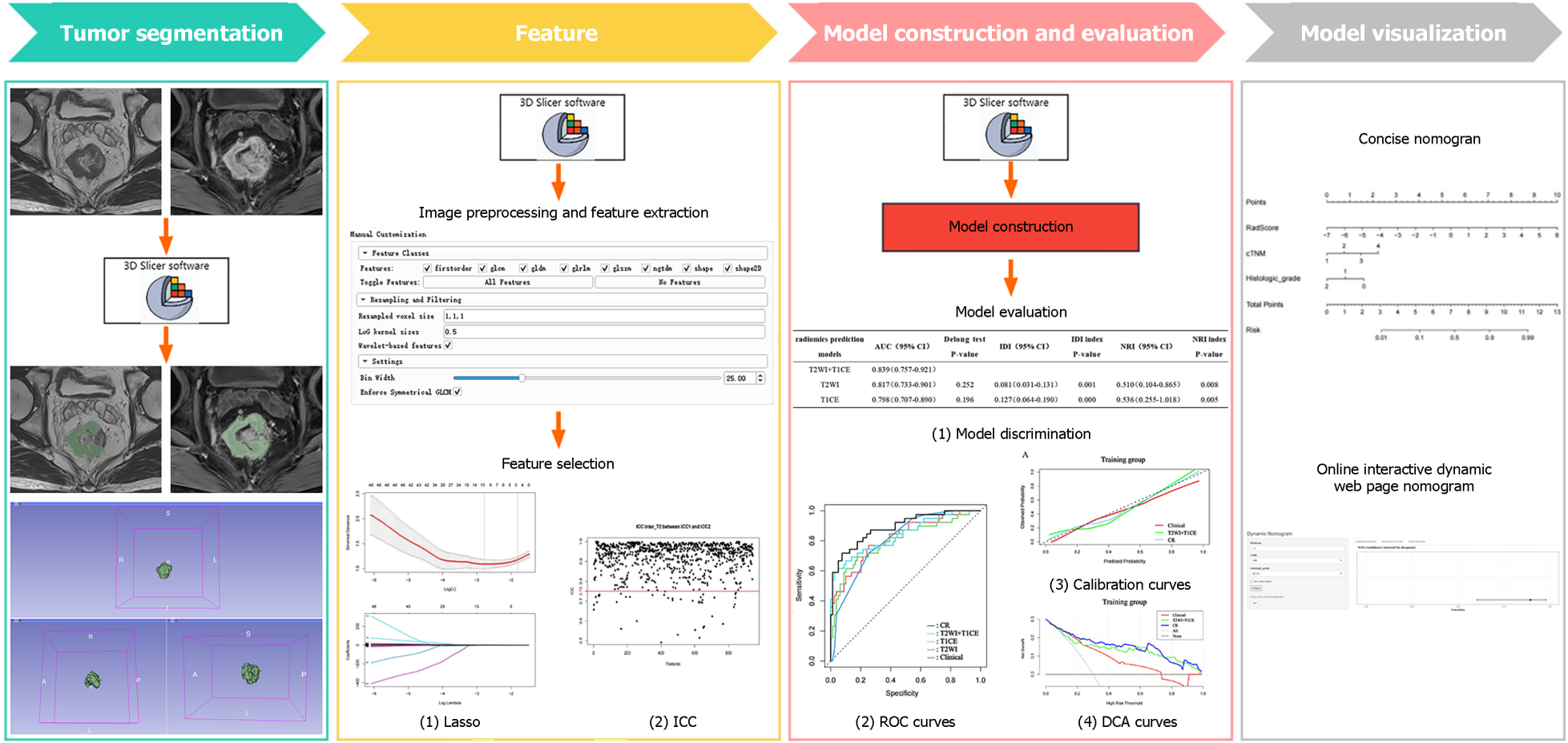


**Figure 2 Radiomics workflow.**

geneity of tumors more effectively. The selected GLCM and GLSZM features of MCC and ZE mainly reflect the heterogeneity of the extracted VOI, which is inconsistent with the specific features extracted by Guo *et al*[23] and Huang *et al*[20]. These findings may be explained by the differences in scanning and reconstruction parameters, as well as inconsistencies between the methods of model construction. Nevertheless, our results were consistent for some of the extracted radiomics features, reflecting tumor heterogeneity. This also confirms the results of previous studies, which found that the higher the tumor heterogeneity, the more aggressive the tumor is likely to be[34]. In addition, the GLRLM feature of GLNN mainly reflects the tumor homogeneity, which is negatively correlated with the Rad-score, confirming our conclusion.

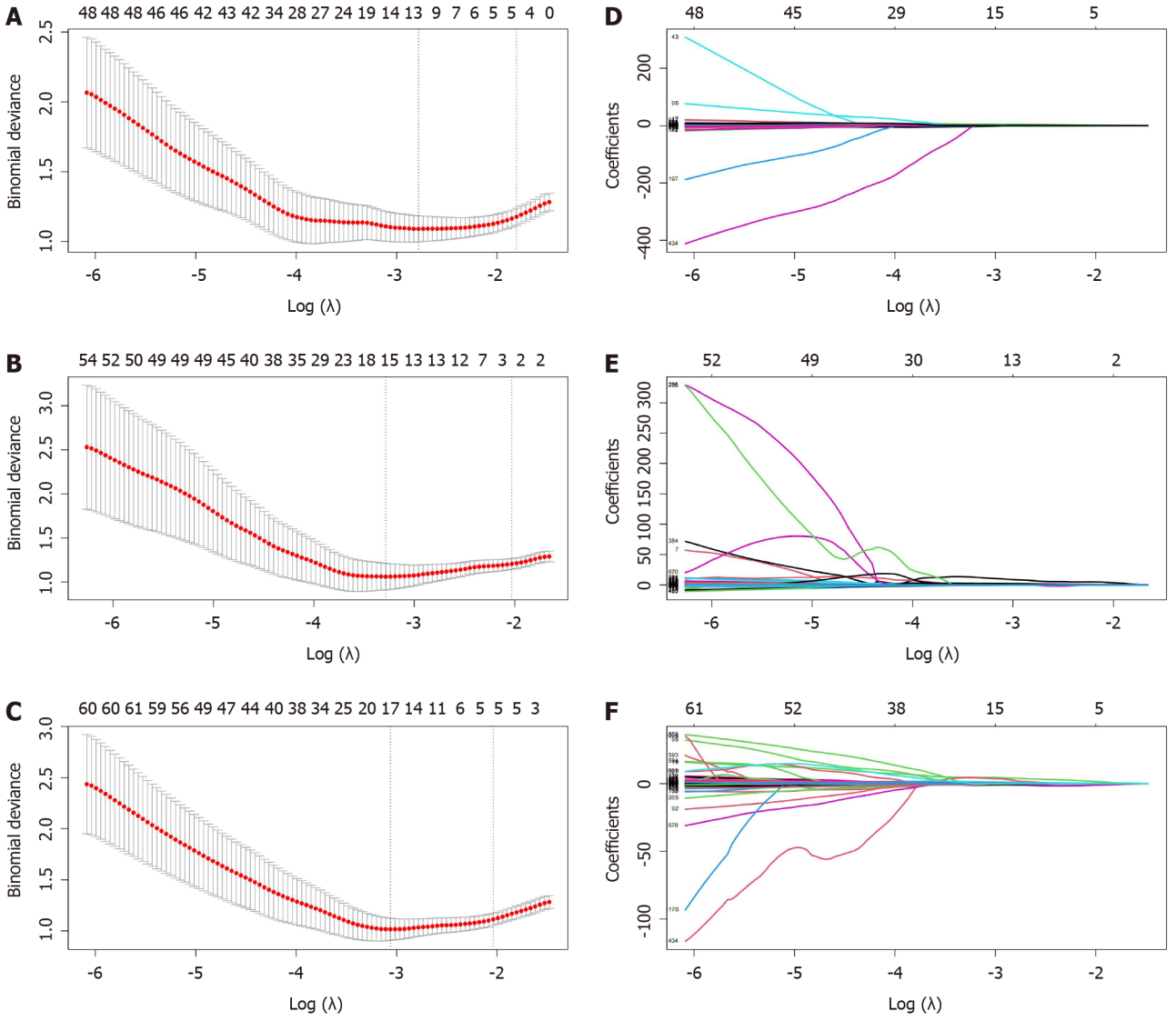


**Figure 3 The process of feature selection and dimensionality reduction through least absolute shrinkage and selection operator.** A-C: The process of 10-fold crossvalidation penalty parameter λ selection, according to the rule of the simplest model. The abscissa corresponding to the lowest point of model deviation is the optimal λ; D-F: Each colored line in the variation curve of the characteristic coefficient with value of λ, which was used to determine the non-zero coefficient characteristic parameters based on λ obtained in the upper panel of the figure. T2WI: T2-weighted imaging; T1CE: contrast-enhanced T1WI.

Univariate and multivariate logistic regression analyses of the clinical predictive model showed that cTNM and histological grade were independent risk factors for the PNI status of RC, which is in line with the results of Alotaibi *et al* [32] and Poeschl *et al*[33]. The explanation for these findings may be that a higher TNM stage and lower histological grade correlate with a more malignant and aggressive tumor that is more likely to invade the nerves around the tumors. Previous studies have also shown that a high T stage, high N stage, distant metastasis, positive circumferential resection margin status, positive extramural vascular invasion status, high CEA level (≥ 5 ng/mL), and tumor budding are also independent risk factors for PNI. However, in the present study, the selection of patients and variables was biased due to the study design. Multivariate logistic regression analysis did not reveal significant results. Finally, the clinical predictive model based on cTNM and histological grade was selected, which demonstrated AUCs of 0.804 (95%CI: 0.727-0.881), 0.828 (95%CI: 0.719-0.937) and 0.813 (95%CI: 0.724-0.903) in the training, internal validation and external validation groups, respectively, as well as satisfactory model stability. Compared with the T2WI + T1CE fusion sequence radiomics predictive model, the Delong test showed that there were no significant differences in the differentiation ability among the models. Therefore, the combined CR model was constructed, and showed the best performance. The AUCs of the CR model in the training and internal and external validation groups were 0.889 (95%CI: 0.824-0.954), 0.889 (95% CI: 0.803-0.976) and 0.894 (95%CI: 0.814-0.974), respectively.

Compared with previous similar studies, the present study had certain strengths. First, this study extracted 944 radiomics features from each sequence of the following images: Raw images, Gaussian transformed images, and wavelet transformed images. The number of features was significantly higher than in previous studies, which may reflect the spatial heterogeneity of tumors more effectively. Second, this study used internal and external validation groups to evaluate the models, whereas previous studies were based on a single-center internal validation group, lacking independent external validation. Third, the CR model demonstrated good stability in both the internal and external

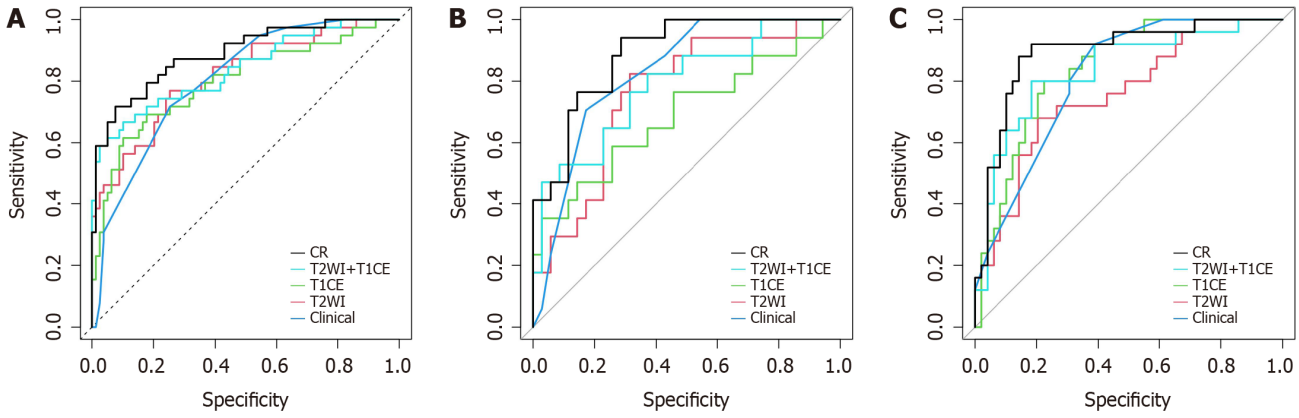


**Figure 4 Receiver operating characteristic curves for perineural invasion prediction of different models.** A: Receiver operating characteristic (ROC) curves in the training group; B: ROC curves in the internal validation group; C: ROC curves in the external validation group. CR: Clinical-radiomics predictive model; T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI.

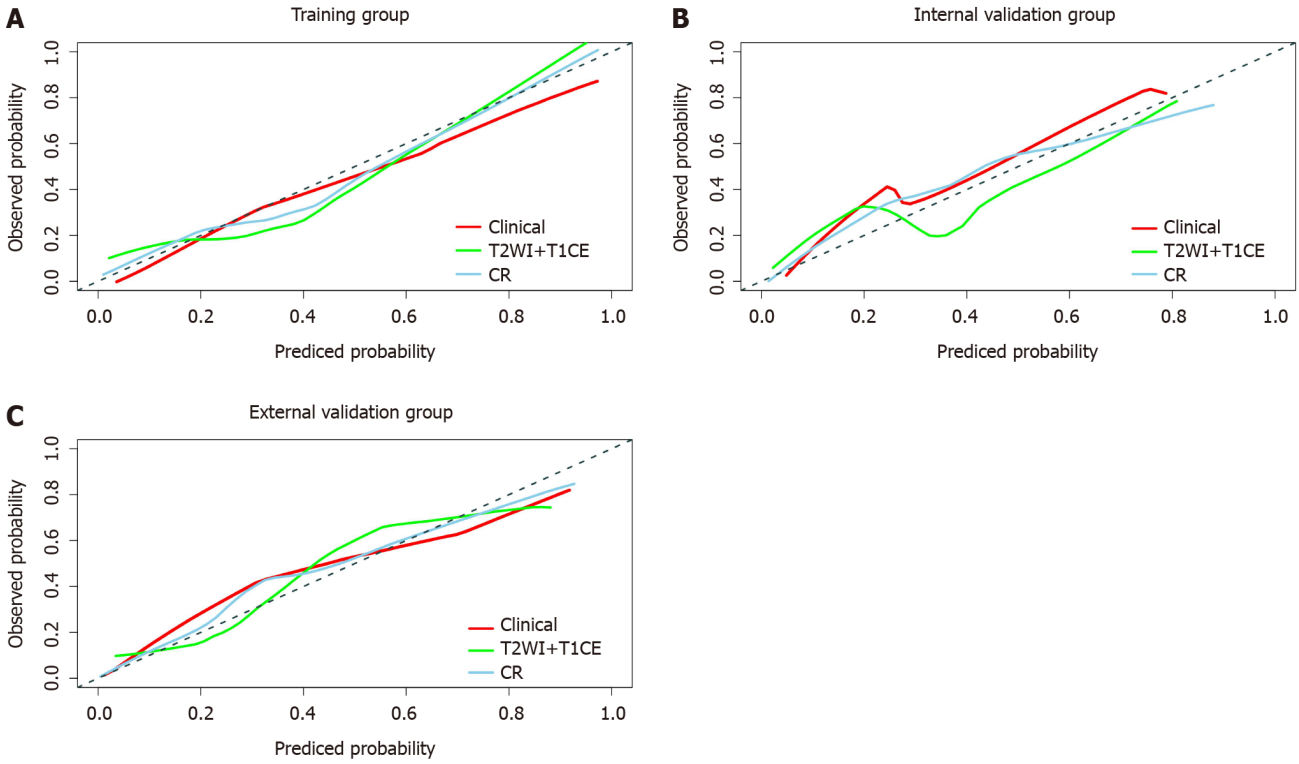


**Figure 5 Calibration curves of the clinical model, T2-weighted imaging + Contrast-enhanced T1WI fusion sequence radiomics model and clinical-radiomics prediction model.** Diagonal lines serve as reference lines, representing the most suitable model. The clinical-radiomics prediction model shows optimal calibration. A: Calibration curves in the training group; B: Calibration curves in the internal validation group; C: Calibration curves in the external validation group. CR: Clinical-radiomics prediction model; T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI.

validation groups, with greater repeatability than models proposed in previous studies. Fourth, the PNI status of 244 RC patients was evaluated based on HE staining and immunohistochemical analysis of postoperative resection specimens. A previous study demonstrated that immunohistochemical analysis can significantly improve the PNI detection rate, which is 2-3 times higher than the detection rate using HE staining alone[5]. However, some previous studies did not provide a detailed explanation of their specimen analysis methods[13,19,21,22], whereas others evaluated the PNI status based on HE staining only of postoperative specimens[20,23].

There were some limitations to this study. First, this was a retrospective study with possible selection bias. Second, although the external validation group included patients from an independent center based on strict eligibility criteria, further validation studies are needed to reduce the impact of data bias. Third, the VOI was manually delineated layer by layer, which takes a long time in clinical settings and may lead to interobserver variability. Therefore, further studies are needed to determine the feasibility of applying deep learning to automatically delineate the VOI[35,36].

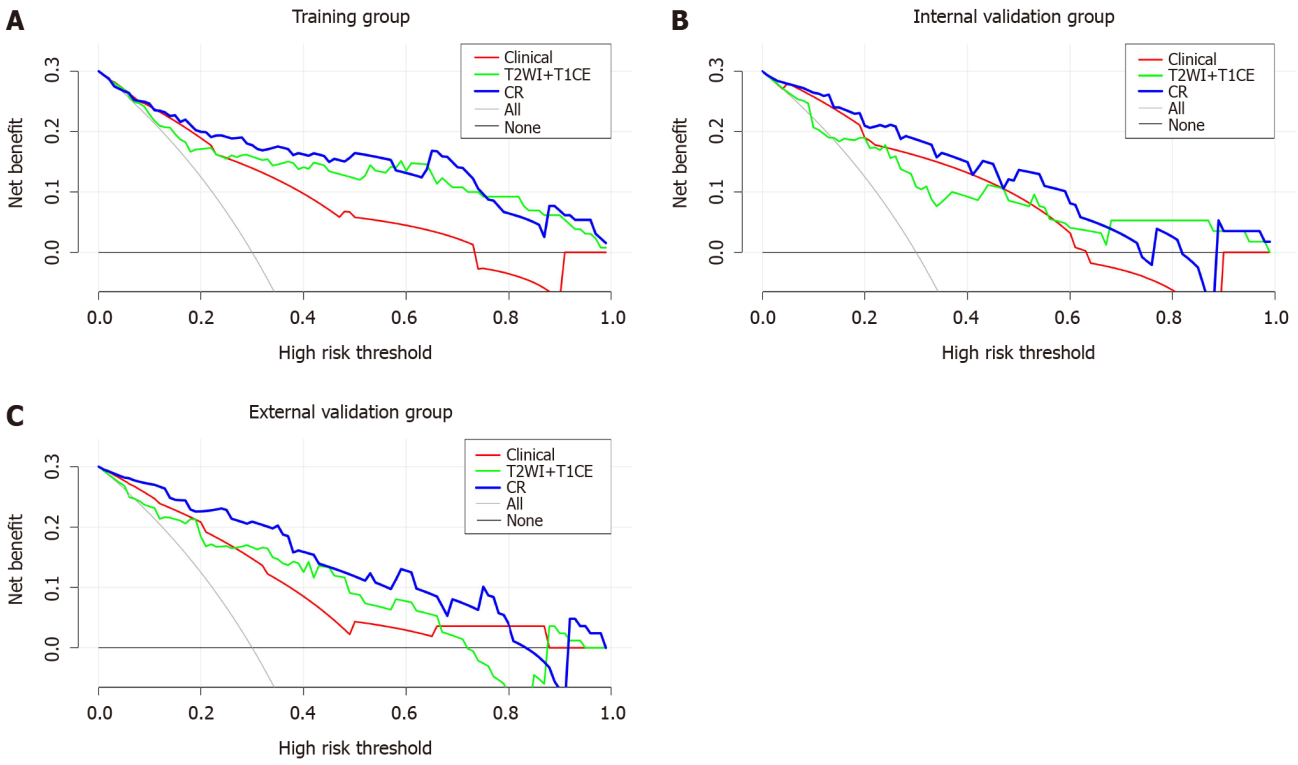


**Figure 6 Decision curve analysis of the clinical model, T2-weighted imaging + contrast-enhanced T1WI fusion sequence radiomics model and clinical-radiomics prediction model.** The clinical-radiomics prediction model exhibits the greatest net clinical benefit. A: Decision curve analysis (DCA) in the training group; B: DCA in the internal validation group; C: DCA in the external validation group. CR: Clinical-radiomics prediction model; T2WI: T2-weighted imaging; T1CE: Contrast-enhanced T1WI.

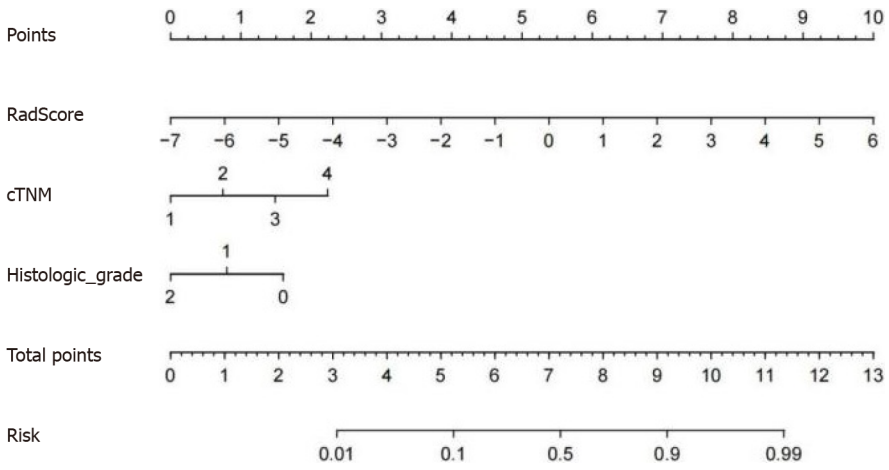


**Figure 7 Concise nomogram was developed in the training group.** cTNM: clinical TNM.

## CONCLUSION

In conclusion, the radiomics model based on preoperative MRI was found to be useful for predicting the PNI status in RC. The CR model combined with the clinical risk factors of PNI was able to predict the PNI status of RC noninvasively before surgery, thereby providing support for the individualized treatment of RC patients.

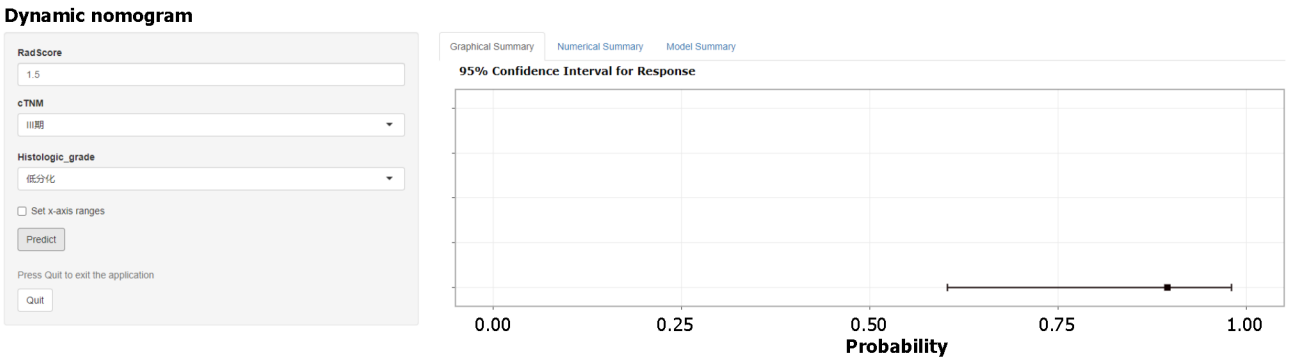


**Figure 8 Online interactive dynamic web page nomogram based on the clinical-radiomics model was constructed to predict the perineural invasion status of patients.** The Online tool is available at https://ly1070007554.shinyapps.io/dynnomapp/.

## ARTICLE HIGHLIGHTS

### *Research background*

Perineural invasion (PNI), is a potential pathway for the metastatic spread of rectal cancer (RC), and has been used as an important pathological indicator and independent prognostic factor. Preoperative stratification of RC patients according to PNI status facilitates individualized treatment and improves the prognosis of RC patients.

### *Research motivation*

Nowadays, the preoperative predicton of PNI status is still challenging and needs further study.

### *Research objectives*

To evaluate the usefulness of a model based on preoperative magnetic resonance imaging (MRI) radiomics for predicting PNI status in patients with RC and establishing and validating an optimal nomogram model for predicting PNI status preoperatively in RC patients.

### *Research methods*

We enrolled 244 RC patients from two independent centers from May 2019 to August 2022. The patients from Center 1 were randomly divided into a training group ($n$ = 118) and an internal validation group ($n$ = 52), whereas 74 patients from Center 2 served as an external validation group. Extracted and selected quantitative radiomics features and clinical risk factors to establish and validate the radiomics predictive model and clinical-radiomics (CR) model.

### *Research results*

We extracted 944 radiomics features from T2-weighted imaging and contrast-enhanced T1-weighted imaging sequences, combined with PNI-related clinical features (clinical TNM and histological grade) to construct the final CR model, and used internal and external validation groups to evaluate the models. The final CR model showed good performance to predict PNI status, the area under the curve of the CR model in the training and internal and external validation groups were 0.889, 0.889 and 0.894, respectively.

### *Research conclusions*

The CR model based on MRI radiomics features and clinical risk factors was able to predict the PNI status of RC noninvasively, showed stable performance, which can provide support for individualized treatment of RC patients.

### *Research perspectives*

Further external verification is needed to optimize the model, and explore the feasibility of applying deep learning to automatically describe volume of interest, reduce the difference between observers, and improve the applicability of the model.

## FOOTNOTES

**Author contributions:** Liu Y data acquisition and analysis, drafting and writing of the manuscript; Sun BJT data collection and data analysis; Zhang C, Li B and Yu XX language editing and revisions to the manuscript; Du Y work concept or design and important revisions to the manuscript; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Observational Study

# Association between childhood obesity and gut microbiota: 16S rRNA gene sequencing-based cohort study

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## Abstract

***BACKGROUND***

This study aimed to identify characteristic gut genera in obese and normal-weight children (8-12 years old) using 16S rDNA sequencing. The research aimed to provide insights for mechanistic studies and prevention strategies for childhood obesity. Thirty normal-weight and thirty age- and sex-matched obese children were included. Questionnaires and body measurements were collected, and fecal samples underwent 16S rDNA sequencing. Significant differences in body mass index (BMI) and body-fat percentage were observed between the groups. Analysis of gut microbiota diversity revealed lower α-diversity in obese children. Differences in gut microbiota composition were found between the two groups. *Prevotella* and *Firmicutes* were more abundant in the obese group, while *Bacteroides* and *Sanguibacteroides* were more prevalent in the control group.

***AIM***

To identify the characteristic gut genera in obese and normal-weight children (8-12-year-old) using 16S rDNA sequencing, and provide a basis for subsequent

mechanistic studies and prevention strategies for childhood obesity.

***METHODS***
Thirty each normal-weight, 1:1 matched for age and sex, and obese children, with an obese status from 2020 to 2022, were included in the control and obese groups, respectively. Basic information was collected through questionnaires and body measurements were obtained from both obese and normal-weight children. Fecal samples were collected from both groups and subjected to 16S rDNA sequencing using an Illumina MiSeq sequencing platform for gut microbiota diversity analysis.

***RESULTS***
Significant differences in BMI and body-fat percentage were observed between the two groups. The Ace and Chao1 indices were significantly lower in the obese group than those in the control group, whereas differences were not significant in the Shannon and Simpson indices. Kruskal-Wallis tests indicated significant differences in unweighted and weighted UniFrac distances between the gut microbiota of normal-weight and obese children ($P <$ 0.01), suggesting substantial disparities in both the species and quantity of gut microbiota between the two groups. *Prevotella*, *Firmicutes*, *Bacteroides*, and *Sanguibacteroides* were more abundant in the obese and control groups, respectively. Heatmap results demonstrated significant differences in the gut microbiota composition between obese and normal-weight children.

***CONCLUSION***
Obese children exhibited lower α-diversity in their gut microbiota than did the normal-weight children. Significant differences were observed in the composition of gut microbiota between obese and normal-weight children.

**Key Words:** Childhood obesity; Gut microbiota; 16S rDNA sequencing; Diversity analysis; Genus identification; Body mass index



**Core Tip:** This study used 16S rDNA sequencing to identify characteristic gut genera in obese and normal-weight children. The findings revealed lower α-diversity in the gut microbiota of obese children compared to normal-weight children. Significant differences were observed in the composition of gut microbiota between the two groups, with *Prevotella* and *Firmicutes* being more abundant in the obese group, and *Bacteroides* and *Sanguibacteroides* more prevalent in the control group. These results provide insights into the potential role of gut microbiota in childhood obesity and may contribute to future mechanistic studies and prevention strategies.

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## INTRODUCTION

Childhood obesity is a growing global health concern with an increasing prevalence worldwide. The World Health Organization defines childhood obesity as "a condition in which excess body fat negatively affects children's health and well-being". It is associated with various adverse health outcomes, including type 2 diabetes, cardiovascular diseases, and psychological disorders. The complex etiology of childhood obesity involves genetic, environmental, and behavioral factors. However, emerging evidence suggests that the gut microbiota, a community of microorganisms residing in the gastrointestinal tract, plays a significant role in the development of obesity. The human gut microbiota consists of trillions of microorganisms, including bacteria, viruses, fungi, and archaea, which interact with the host physiology, metabolism, and immune system. Recent advances in sequencing technologies, particularly 16S rRNA gene sequencing, have enabled researchers to comprehensively explore the composition and function of gut microbiota. Studies in adults have highlighted the association between alterations in the gut microbiota and obesity; however, research in pediatric populations is essential to understand the early-life factors contributing to obesity.

Approximately $3.9 \times 10^{13}$ bacteria in the human body, with the majority residing in the intestines, where approximately $10^{11}$ bacteria are found per gram of wet feces[1]. Studies have identified nearly 10 million non-redundant genes in the human intestinal tract, which is 150 times the size of the human genome, leading to the use of the term "human second genome" to describe the human intestinal microbiota. Therefore, human intestinal microbiota constitutes a unique ecological system with distinct microenvironments. The metabolic capacity of the gut microbiota far exceeds that of human cells and plays crucial roles in human health, including digestion, nutrition, metabolism, and immunity. Imbalances in the intestinal microbiota are associated with various diseases, including obesity and underweight status. A

causal relationship exists between disruptions in the gut microbiota and obesity or underweight conditions. Multiple animal experiments have demonstrated the role of the host gut microbiota in energy acquisition and storage, potentially leading to obesity or underweight conditions. For instance, germ-free mice fed a high-fat diet exhibited lower weight gain than conventionally fed mice fed the same diet[2]. Germ-free mice transplanted with fecal microbiota from obese individuals exhibit obesity symptoms[3], whereas mice colonized with microbiota from malnourished children show symptoms of poor development and are underweight[4]. Numerous studies have indicated alterations in the composition and diversity of the gut microbiota in obese adults compared to those with normal weight[5,6]. However, inconsistencies exist regarding changes in the *Firmicutes*/*Bacteroidetes* ratio in the gut microbiota of obese individuals compared to those with normal weight[7].

Multiple studies have found that the composition, quantity, and proportion of gut microbiota in obese children undergo changes. Abdallah *et al*[8] reported that when comparing the gut microbiota of obese children to that of a control group, there were more bacteria from the phylum *Firmicutes* and fewer from the phylum *Bacteroidetes*. However, after controlling for dietary factors, when body weight decreased, the abundance of these two bacterial phyla was reversed. This suggests that the changes in the ratio of *Bacteroidetes* to *Firmicutes* in the gut are associated with childhood obesity. Kalliomäki *et al*[9] conducted a prospective study of 7-year-old children with an increased body mass index (BMI) and found that, compared to age-matched children with a normal BMI, children with a higher BMI had a reduced abundance of *Bifidobacteria* and an increased abundance of *Enterobacteriaceae* in their gut microbiota. Gao *et al*[10] discovered that, compared to school-aged children with normal weight, school-aged obese children had a decreased abundance of *Bifidobacteria* and an increased abundance of *Escherichia coli* in their feces. This resulted in a lower ratio between the two bacterial groups. These findings suggest a correlation between childhood obesity and an imbalance in the gut microbiota. *Bifidobacteria* are typical representatives of beneficial probiotics in the gut, whereas *Escherichia coli* can serve as a representative of pathogenic bacteria. An increased abundance of *Escherichia coli* is considered an important warning sign of the gut microbiota structure shifting towards a less favorable state for overall health. Both *Bifidobacteria* and *Escherichia coli* are commonly found in childhood gut microbiota, and their ratio can be used to assess the condition of the gut microbiota structure.

In obesity, there is an increase in taxa within the *Bacteroidales* order, such as *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., and *Enterococcus* spp., as well as an elevated ratio of *Firmicutes* to *Bacteroidetes* and *Enterobacteriaceae*, while taxa within the *Clostridia* class, including *Clostridium leptum* and *Enterobacter* spp., are decreased[11-13]. Numerous studies suggest that an increased *Firmicutes* to *Bacteroidetes* ratio at the phylum level is a notable feature of the gut microbiota in individuals with obesity. Families such as *Christensenellaceae* and orders like *Methanobacteriales*, as well as genera including *Lactobacillus*, *Bifidobacteria*, and *Akkermansia*, are commonly regarded as probiotics, and their relative abundance typically correlates negatively with obesity. The gut microbiota regulates obesity by modulating energy absorption, central appetite, fat storage, chronic inflammation, and circadian rhythms[14]. The composition of the gut microbiota profoundly influences nutrient acquisition and energy regulation in the body, thus playing a pivotal role in the onset and progression of obesity and associated conditions[15,16]. Notably, the microbiota composition varies between infants and adults, as well as between obese and lean individuals. For instance, calorie-restricted diets can reduce the *Firmicutes* to *Bacteroidetes* ratio in the gut, while vegetarian diets have been found to increase *Bacteroidetes* and decrease *Firmicutes*, *Bifidobacterium* spp., *Escherichia coli*, *Enterobacteriaceae*, and *Clostridia*[17]. Consequently, targeting the gut microbiota presents a promising therapeutic avenue for addressing obesity[18].

This study aimed to investigate changes in gut microbiota composition in children with obesity using 16S rRNA gene sequencing technology. Understanding the relationship between childhood obesity and alterations in gut microbiota can shed light on potential therapeutic interventions and preventive strategies. Moreover, it may offer insights into the role of gut microbiota in childhood obesity-related metabolic disturbances.

## MATERIALS AND METHODS

### Study design

This study employed a prospective cohort design to investigate the relationship between childhood obesity and the composition of the gut microbiota. Data collection will span a two-year period, allowing for the observation of long-term microbiota dynamics. A total of 60 children, aged 8 to 12 years, were recruited, and stratified into two age- and sex-matched groups: The obese group (defined as having a BMI percentile greater than or equal to the 95$^{th}$ percentile) and the normal-weight group.

### Subject recruitment and data collection

The inclusion criteria for the obese group were based on BMI percentile, while the normal-weight group was selected to match for age and sex. Comprehensive baseline data, including age, sex, and lifestyle information, were collected from all participants. Clinical parameters, such as height, weight, waist circumference, and other relevant measurements were recorded. Stool samples will be collected using standardized procedures and immediately stored at -80 °C to preserve microbial DNA integrity.

### Sample processing and DNA extraction

Total microbial DNA was extracted from the fecal samples using a high-efficiency soil DNA extraction kit. The extraction procedure strictly followed the instructions provided in the manual. The extracted DNA was stored at -80 °C for future use.

**Table 1 Basic indicators of obese and normal-weight children**

| Indicator | Control (*n* = 30) | Obese group (*n* = 30) | *P* value |
|---|---|---|---|
| Age (yr) | 10.67 ± 1.36 | 10.29 ± 1.84 | > 0.05 |
| Height (cm) | 116.82 ± 1.97 | 118.27 ± 1.25 | > 0.05 |
| Weight (kg) | 23.90 ± 0.82 | 33.89 ± 0.17 | < 0.05 |
| Body mass index | 18.96 ± 0.19 | 24.35 ± 0.79 | < 0.05 |
| Percentage of body fat (%) | 16.49 ± 0.81 | 23.74 ± 0.63 | < 0.05 |

### *Application of 16S rRNA gene sequencing technology*

Forty microliters of DNA from each sample were used for high-throughput sequencing on an Illumina MiSeq platform. Paired-end sequencing of the V3-V4 region of bacterial 16S rRNA was performed. The concentration and purity of DNA samples were evaluated using a UV spectrophotometry and agarose gel electrophoresis, respectively. Samples with DNA quantities greater than 500 ng were considered as qualified. The qualified samples were subjected to PCR amplification of the 16S rRNA gene V4 region using forward primer 347F (5′-CCT ACG GRR BGC ASC AGK VRV GAA T-3′) and reverse primer 806R (5′-GGA CTA CNV GGG TWT CTA ATC C-3′). PCR products were verified for specificity through agarose gel electrophoresis, and the purified PCR products were sequenced using an Illumina MiSeq M300 sequencer (Illumina Inc., United States) for paired-end 250 bp sequencing.

### *Bioinformatics analysis*

Raw sequencing data were filtered using VSEARCH software to remove low-quality fragments. The PCR products were assembled, and duplicate, tag, and primer sequences were removed to obtain the optimized sequences. Operational taxonomic units (OTUs) were clustered with 97% sequence similarity. Representative sequences and corresponding taxonomic information for each OTU were extracted using the QIIME software. Alpha diversity indices, namely Ace, Chao1, Shannon, and Simpson indices, were calculated using R software to assess the diversity and richness of the gut microbiota within each sample. Beta diversity, which describes the diversity between samples, was analyzed using various tools, including R, QIIME, and Mothur software, considering OTU abundance, bacterial alpha diversity, beta diversity, and taxonomic composition at different taxonomic levels.

### *Data analysis methods*

Diversity analyses, including alpha diversity (*e.g.*, Shannon diversity index) and beta diversity (*e.g.*, Bray-Curtis distance matrix), were performed using QIIME 2 and R packages. The resulting data were used for species annotation and classification to generate the OTU or ASV tables. Differential abundance analysis was conducted using statistical tools such as DESeq2 or LEfSe to identify significant differences in microbial communities between the obese and normal-weight groups. Functional prediction of the gut microbiota can be achieved using PICRUSt or other relevant tools. Correlation analysis was performed using Pearson or Spearman correlation methods to explore the associations between microbial composition and clinical parameters.

## RESULTS

### *Basic information*

To investigate the differences in gastrointestinal microbiota between obese and normal-weight children, 30 samples each from healthy children and obese children were collected from the hospital. BMI, which is widely used to measure obesity, continues to be a practical tool for large-scale population studies and clinical screening. Specific information regarding the children is presented in Table 1, which shows the differences in BMI values and body fat content between the two groups of children.

### *Intestinal microbiota analysis*

In the control group of healthy children, there were 1791 OTUs, with 61 unique OTUs. In contrast, the obese group contained 1759 OTUs, with 29 unique OTUs (Figure 1A). Comparatively, the richness of OTUs in the obese children group decreased at various taxonomic levels compared to that in the control group. Alpha diversity analysis of the gut microbiota in the study subjects showed that, in comparison to the control group, obese children had lower Ace and Chao1 indices, while the differences in the Shannon and Simpson indices were not statistically significant (Figure 1B).

The relative abundances of the top five genera in the gut microbiota of the control group (normal-weight children) and obese group were analyzed. These data highlight the significant differences in the gut microbiota composition between the control and obese groups. In the control group, *Bacteroides* and *Faecalibacterium* had higher relative abundances, whereas *Prevotella* and *Firmicutes* were more abundant in the obese group (Table 2). These differences indicate distinct compositional variances in the gut microbiota of obese children compared with normal-weight children, possibly related to the development of obesity and its associated metabolic disorders.

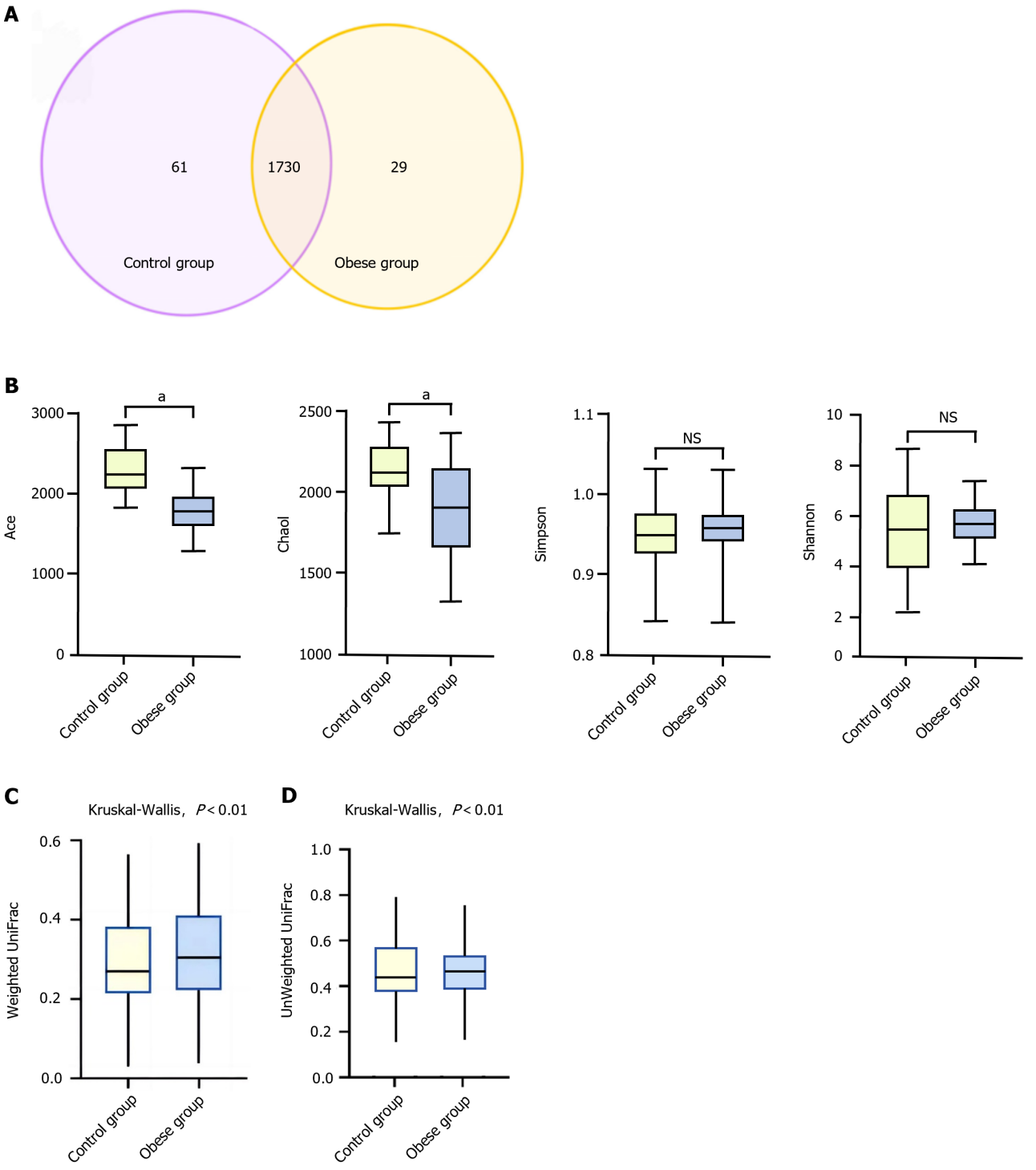


**Figure 1 Basic analysis of gut microbiota.** A: Venn diagram illustrating the richness of operational taxonomic units in children; B: Alpha diversity analysis of gut microbiota in the control group and obese group children; C and D: Beta diversity distance comparison. $^{a}P < 0.001$.

The overall structure of the gut microbiota in normal-weight and obese children was analyzed based on two different beta diversity metrics: Unweighted UniFrac and Weighted UniFrac (Figure 1C and D). UniFrac distances were calculated based on the evolutionary tree of each OTU, reflecting differences in the gut microbiota between samples based on their evolutionary relationships. Unweighted UniFrac distances consider only the presence or absence of OTUs in the microbial community, without considering their abundance. In contrast, weighted UniFrac distances incorporate OTU abundance into the calculations. Kruskal-Wallis tests revealed significant differences ($P < 0.01$) in Unweighted UniFrac and Weighted UniFrac distances between the gut microbiota of normal-weight and obese children, indicating significant variations in both the species and quantity of gut microbiota between these two groups.

### *Trends in gut microbiota changes in obese and normal-weight children*

There were significant differences in the gut microbiota between the obese and normal-weight children. The results indicate that in obese children, the relative abundance of *Firmicutes* bacteria increases, while *Bacteroidetes* bacteria

**Table 2 Top 5 genera in the gut microbiota of control and obese group children**

| Control group | Obese group |
|---|---|
| *Bacteroides* | *Prevotella* |
| *Sanguibacteroides* | *Firmicutes* |
| *Faecalibacterium* | *Bacteroides* |
| *Pseudoramibacter* | *Peptoclostridium* |
| *Plesiomonas* | *Faecalibacterium* |

decrease, leading to an elevated *Firmicutes*/*Bacteroidetes* ratio. Additionally, there was an increase in *Clostridia* (involved in cellulose breakdown), a decrease in beneficial probiotics such as *Bifidobacteria* and *Lactobacilli*, and a reduction in butyrate-producing bacteria, which are responsible for producing beneficial short-chain fatty acids (SCFAs), in obese children. In cases of metabolic syndrome, there is an increase in the *Enterobacteriaceae* family. Conversely, *Akkermansia muciniphila*, a bacterium usually beneficial for gut health, decreased in obese children (Figure 2). These observations highlight the potential role of the gut microbiota in the development of obesity. However, further research is required to explore the specific impact of these changes on health.

Apart from these trends, some gut microbiota showed no significant differences between the obese and normal-weight children. These relatively stable microbiota included *Ruminococcus*, *Faecalibacterium*, *Blautia*, *Dorea*, *Collinsella*, *Fusobacterium*, *Parabacteroides*, *Veillonella*, *Haemophilus*, *Oscillospira*, *Enterococcus*, and *Alistipes*. Although some of these may exhibit minor changes in different studies, the magnitude of these changes is typically small, making them difficult to confirm. This stability emphasizes the complexity of gut microbiota, with different bacterial groups showing individual variations that are likely influenced by factors such as lifestyle and dietary habits. Therefore, when studying the microbiota of obese children, it is crucial to comprehensively consider this diversity to gain a more holistic understanding of the relationship between the gut microbiota and obesity development.

## DISCUSSION

The results of this study revealed significant differences in the gut microbiota of obese and normal-weight children. The gut microbiota of obese children exhibits multifaceted changes that may play a crucial role in the development of obesity and related metabolic disorders. First, we observed an increase in the relative abundance of *Firmicutes* and a decrease in *Bacteroidetes* in obese children, leading to an elevated *Firmicutes*/*Bacteroidetes* ratio. This alteration is commonly associated with obesity, suggesting that energy metabolism in obese children may be influenced by gut microbiota[19]. Increased abundance of *Firmicutes* is typically linked to more efficient energy absorption from food, potentially contributing to energy intake and storage in obese children[20]. Second, the proliferation of *Clostridia* bacteria may accelerate cellulose breakdown, further enhancing energy absorption in obese children. Conversely, the reduction in beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli*, as well as a decrease in butyrate-producing bacteria, could weaken the intestinal barrier function, disrupt the immune system, and increase chronic inflammation. These factors may create favorable conditions for the development of obesity and its related metabolic disorders[21-23]. Additionally, with the progression of metabolic syndrome, there was a significant increase in *Enterobacteriaceae* family, further elevating the risk of chronic inflammation [24-26]. Simultaneously, *Akkermansia muciniphila*, a bacterium that is usually beneficial for intestinal health, decreases in obese children. This reduction may lead to mucosal layer damage and exacerbation of intestinal inflammation. However, it is worth noting that some gut microbiota showed no significant differences between the obese and normal-weight children. This stability emphasizes the complexity of gut microbiota, suggesting that individuals may react differently to obesity. Such variations may be influenced by various factors, including individual lifestyle and dietary habits.

The prevalence of overweightness and obesity in children and adolescents is becoming an increasingly serious public health concern. Childhood overweight and obesity are caused by multiple factors including genetic background, diet, and lifestyle. Furthermore, the gut microbiota and its metabolites play crucial roles in the progression of childhood overweight and obesity. Alpha diversity plays a significant role in the study of gut microbiota in obese children. Alpha diversity primarily reflects the abundance and diversity of the microbial species within an individual. According to studies conducted both domestically and abroad, the alpha diversity of the gut microbiota in obese adults is typically low, which has been widely confirmed[27]. Similarly, in the present study, we observed that the ACE and Chao1 indices (used to estimate the total number of species in a community) were significantly lower in obese children than those in normal-weight children, particularly in obese boys, where the Chao1 index exhibited a more significant decrease. However, consistent with some research findings[28], differences in the Shannon and Simpson indices between the obese and control groups were not significant.

Alterations in the gut microbiota reveal an imbalance in the ecosystem in obese children. Our results demonstrated significant differences in the gut microbiota composition between obese and normal-weight children. In obese children, there was an increase in the relative abundances of *Firmicutes* and *Clostridia*, whereas those of *Bacteroidetes*, *Bifidobacteria*, and *Lactobacilli* decreased. These changes could stem from various factors; for instance, obese children often undergo more antibiotic treatments in early life, which may have a lasting impact on the composition of their gut microbiota[29].

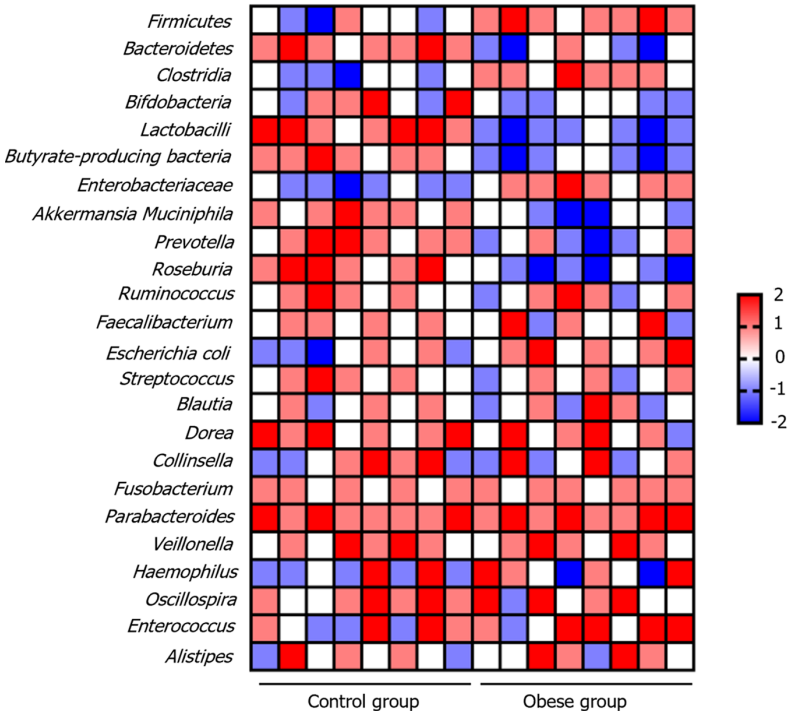


**Figure 2 Comparative heat map of gut microbial communities in obese and normal-weight children.**

These alterations may lead to mucosal barrier dysfunction in the intestines of obese children, including the S100 calcium-binding proteins S100A8 and S100A9[30]. Simultaneously, the abnormal production and absorption of specific metabolites such as SCFAs and bile acids can affect energy metabolism and weight control[19]. The gut microbiota and its metabolites differ significantly between obese and normal-weight individuals. The reduced abundance of various *Akkermansia* species that metabolize glutamate is associated with a higher risk of obesity. Additionally, the gut microbiota of obese adolescents exhibits enhanced carbohydrate oxidation capabilities[31]. Changes in the gut microbiota have been linked to childhood obesity and non-alcoholic fatty liver disease. The biosynthesis of SCFAs, amino acids, and lipopolysaccharides is negatively correlated with insulin resistance (IR), whereas peptidoglycan biosynthesis pathways are positively correlated with IR[32]. Therefore, studying alterations in the gut microbiota of obese children is of great significance.

The study revealed significant differences between obese and normal-weight children, including higher BMI and body-fat percentage in obese children. While the Ace and Chao1 indices indicated lower species richness in the obese group, the Shannon and Simpson indices showed no significant diversity differences. Moreover, Kruskal-Wallis tests highlighted significant dissimilarities in both unweighted and weighted UniFrac distances between the gut microbiota of normal-weight and obese children ($P < 0.01$). *Prevotella* and *Firmicutes* were more abundant in obese children, while *Bacteroides* and *Sanguibacteroides* were prevalent in normal-weight children, as evidenced by heatmap results. These findings suggest distinct microbial profiles associated with obesity in children, implicating the potential for targeted interventions to modulate gut microbiota composition and inform individualized treatment strategies for childhood obesity. Longitudinal monitoring of gut microbiota alongside BMI changes may offer insights into intervention effectiveness and guide adjustments to treatment plans over time. Although the study made some important findings in comparing the gut microbiota of obese and normal-weight children, there are several limitations. Firstly, the sample size of the study was relatively small, including only 30 obese children and 30 normal-weight children, which may limit the generalizability and statistical significance of the results. Secondly, the study only utilized 16S rDNA sequencing technology for microbial composition analysis, which may restrict the understanding of microbial functions and metabolic activities. Additionally, the study did not comprehensively control for children's dietary habits, lifestyles, and environmental factors, which could influence the composition and abundance of the gut microbiota. Furthermore, the study did not explore the causal relationship between gut microbiota and childhood obesity, making it unclear whether changes in gut microbiota are the cause or the result of obesity. Therefore, future research needs larger sample sizes, more in-depth methods, and comprehensive controls to validate and expand these findings, thus enhancing our understanding of the relationship between childhood obesity and gut microbiota.

## CONCLUSION

In summary, our study revealed the diversity and complexity of the gut microbiota in obese children. These microbial changes may affect energy metabolism, the immune system, and intestinal barrier function in obese children, providing new insights into the development of obesity and related metabolic diseases. However, further research is needed to

elucidate the specific relationship between these changes and the pathological processes related to obesity, and whether they can serve as targets for intervention strategies.

## FOOTNOTES

**Co-first authors:** Xu-Ming Li and Qing Lv.

**Author contributions:** Li XM, Lv Q, and Xiong X proposed the concept of this study; Chen YJ validated this study; Li XM and Lv Q jointly wrote the initial draft; Yan LB has made contributions to data collection; Xiong X has made contributions to formal analysis; Liu XM, Lv Q, and Xiong X participated in the survey; Chen YJ and Li XM have contributed to these methods; Lv Q contributed to the visualization of this study; and all authors jointly guide the research, review, and edit the manuscript. Liu XM and Lv Q, as the first authors, made equal contributions to this work. After discussion among all authors, it has been decided to designate Li XM and Lv Q as the first authors for three main reasons. Firstly, this study was conducted as a collaborative effort, and it is reasonable to designate a joint first author. The author accurately reflects the distribution of responsibilities and burdens related to the time and effort required to complete the research and final manuscript. Designating two co first authors will ensure effective communication and management of post submission matters, thereby improving the quality and reliability of the paper. Secondly, the co-first authors of the research team possess diverse professional knowledge and skills from different fields, and their appointments best reflect this diversity. It also promotes the most comprehensive and in-depth exploration of research topics, ultimately enriching readers' understanding by providing various expert perspectives. Thirdly, Li XM and Lv Q made substantial and equal contributions throughout the entire research process. Choosing these researchers as co-first authors, acknowledging, and respecting their equal contributions, demonstrates the spirit of collaboration and teamwork in this study. We believe that designating Li XM and Lv Q as co-first authors are suitable for our manuscript, as it accurately reflects the collaborative spirit, equal contribution, and diversity of our team.

**Institutional review board statement:** This study has been approved and reviewed by the Ethics Committee of the Obstetrics and Gynecology Hospital affiliated with Nanjing Medical University.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** We declare that there is no conflict-of-interest disclosure relationship.

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

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



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ORIGINAL ARTICLE

Basic Study

# Chitin-glucan improves important pathophysiological features of irritable bowel syndrome

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## Abstract

### *BACKGROUND*

Irritable bowel syndrome (IBS) is one of the most frequent and debilitating conditions leading to gastroenterological referrals. However, recommended treatments remain limited, yielding only limited therapeutic gains. Chitin-glucan (CG) is a novel dietary prebiotic classically used in humans at a dosage of 1.5-3.0 g/d and is considered a safe food ingredient by the European Food Safety Authority. To provide an alternative approach to managing patients with IBS, we performed preclinical molecular, cellular, and animal studies to evaluate the role of chitin-glucan in the main pathophysiological mechanisms involved in IBS.

### *AIM*

To evaluate the roles of CG in visceral analgesia, intestinal inflammation, barrier function, and to develop computational molecular models.

***METHODS***

Visceral pain was recorded through colorectal distension (CRD) in a model of long-lasting colon hypersensitivity induced by an intra-rectal administration of TNBS [15 milligrams (mg)/kilogram (kg)] in 33 Sprague-Dawley rats. Intracolonic pressure was regularly assessed during the 9 wk-experiment (weeks 0, 3, 5, and 7) in animals receiving CG ($n$ = 14) at a human equivalent dose (HED) of 1.5 g/d or 3.0 g/d and compared to negative control (tap water, $n$ = 11) and positive control (phloroglucinol at 1.5 g/d HED, $n$ = 8) groups. The anti-inflammatory effect of CG was evaluated using clinical and histological scores in 30 C57bl6 male mice with colitis induced by dextran sodium sulfate (DSS) administered in their drinking water during 14 d. HT-29 cells under basal conditions and after stimulation with lipopolysaccharide (LPS) were treated with CG to evaluate changes in pathways related to analgesia (μ-opioid receptor (MOR), cannabinoid receptor 2 (CB2), peroxisome proliferator-activated receptor alpha, inflammation [interleukin (IL)-10, IL-1b, and IL-8] and barrier function [mucin 2-5AC, claudin-2, zonula occludens (ZO)-1, ZO-2] using the real-time PCR method. Molecular modelling of CG, LPS, lipoteichoic acid (LTA), and phospholipomannan (PLM) was developed, and the ability of CG to chelate microbial pathogenic lipids was evaluated by docking and molecular dynamics simulations. Data were expressed as the mean ± SEM.

***RESULTS***

Daily CG orally-administered to rats or mice was well tolerated without including diarrhea, visceral hypersensitivity, or inflammation, as evaluated at histological and molecular levels. In a model of CRD, CG at a dosage of 3 g/d HED significantly decreased visceral pain perception by 14% after 2 wk of administration ($P$ < 0.01) and reduced inflammation intensity by 50%, resulting in complete regeneration of the colonic mucosa in mice with DSS-induced colitis. To better reproduce the characteristics of visceral pain in patients with IBS, we then measured the therapeutic impact of CG in rats with TNBS-induced inflammation to long-lasting visceral hypersensitivity. CG at a dosage of 1.5 g/d HED decreased visceral pain perception by 20% five weeks after colitis induction ($P$ < 0.01). When the CG dosage was increased to 3.0 g/d HED, this analgesic effect surpassed that of the spasmolytic agent phloroglucinol, manifesting more rapidly within 3 wk and leading to a 50% inhibition of pain perception ($P$ < 0.0001). The underlying molecular mechanisms contributing to these analgesic and anti-inflammatory effects of CG involved, at least in part, a significant induction of MOR, CB2 receptor, and IL-10, as well as a significant decrease in pro-inflammatory cytokines IL-1b and IL-8. CG also significantly upregulated barrier-related genes including muc5AC, claudin-2, and ZO-2. Molecular modelling of CG revealed a new property of the molecule as a chelator of microbial pathogenic lipids, sequestering gram-negative LPS and gram-positive LTA bacterial toxins, as well as PLM in fungi at the lowesr energy conformations.

***CONCLUSION***

CG decreased visceral perception and intestinal inflammation through master gene regulation and direct binding of microbial products, suggesting that CG may constitute a new therapeutic strategy for patients with IBS or IBS-like symptoms.

**Key Words:** Chitin-glucan; Irritable bowel syndrome; Abdominal pain; Inflammation; Intestinal barrier; Molecular modelling; Microbial cell walls chelation



**Core Tip:** Currently available irritable bowel syndrome (IBS) treatments are often inadequate. Chitin-glucan is a novel, well-tolerated, non-digestible prebiotic considered a safe food ingredient by the European Food Safety Authority. This study suggests new capacities of chitin-glucan to target most pathophysiological mechanisms of IBS and its therapeutic potential as a promising new generation of prebiotics for patients with IBS or IBS-like symptoms.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder with a worldwide prevalence of 5%-10%. It is associated with annual direct and indirect costs of more than 20 billion USD/year in the United States and is one of the leading causes of work absenteeism[1-3]. Although IBS represents a major burden for patients, the therapeutic strategies recommended by several gastroenterology societies (European, American, Canadian, Japanese, and British societies)[4-10] are often inadequate, leading to dissatisfaction among several patients with standard medical care[11,12].

Chitin-glucan is a novel, non-digestible dietary compound considered a safe food ingredient by the European Food Safety Authority (EFSA)[13]. It is the major component of the cell walls of the mycelium of *Aspergillus niger* (*A. niger*) fungi and is mainly composed of a branched β-1,3/1,6 glucan that is bound to chitin *via* a β-1, 4 linkage. Previous preclinical studies in rodent models[14,15], functional *in vitro* evaluation using the Simulator of the Human Intestinal Microbial Ecosystem model[16], and clinical exploration in healthy volunteers[17] have highlighted that oral administration of chitin-glucan at the EFSA-recommended dosage[13] induces a microbial signature of a prebiotic. Briefly, chitin-glucan is slowly fermented in all colon segments, leading to significant changes in gut microbiota composition, with a particular increase in the butyrate-producing genus *Roseburia spp.* and the *Faecalibacterium* genus, known as a fiber fermenter with strong intestinal anti-inflammatory properties[18].

To propose chitin-glucan treatment as an alternative approach for managing patients with IBS, we performed preclinical molecular, cellular, and animal studies to evaluate the role of chitin-glucan in the main physiopathological mechanisms responsible for IBS symptoms, including visceral analgesia, intestinal inflammation, and barrier function, together with the development of a computational molecular model.

## MATERIALS AND METHODS

### *Chitin-glucan*

CG from the cell wall of *A. niger* was obtained from Kitozyme SA (Herstal, Belgium). Rodents received a dose of 25 mg/kg body weight (BW)/d or 50 mg/kg BW/d of chitin-glucan [corresponding to a human equivalent dose (HED) of respectively 1.5 g/d and 3.0 g/d for a 70 kg man] by oral gavage once per day[14]. For *in vitro* studies, HT-29 cells were incubated with chitin-glucan at 500 micro (μ) g/mL or 1000 μg/mL corresponding to estimated luminal ileal concentrations of chitin-glucan calculated in healthy volunteers receiving the compound respectively at 1.5 g/d or 3.0 g/d.

### *Model of TNBS-induced long-lasting visceral hypersensitivity in rats*

TNBS-induced long-lasting visceral hypersensitivity is a reference model for screening novel treatments for visceral pain originating in the gastrointestinal tract[19].

**Rats:** Animal experiments were performed in accredited facilities at the Institut Pasteur, Lille, according to governmental guidelines. All studies were approved by the local investigational Ethics Review Board (Nord-Pas-de-Calais CEEA N°75, Lille, France; protocol reference numbers 352012 and 19-2009R), and the French government agreement *n*° APAFIS#16100-2018070309443695 v4 (colorectal distension, CRD) and APAFIS#9148-201901101416384 v1 (colitis). Three animals were housed per cage and had free access to standard rodent chow (Safe A04 P2,5) and tap water.

Male Sprague-Dawley rats, aged 5 wk and weighing 175 g to 200 g, were obtained from Janvier labs (France). Rats were randomized into different groups using a manual procedure and acclimated to the study conditions for at least 7 d before the beginning of the pre-treatment period. Upon completion of treatment, the animals were euthanized by cervical dislocation after gaseous anesthesia (isoflurane).

**TNBS-induced visceral hypersensitivity:** Rats were anesthetized for 2 h using a subcutaneous injection of xylazine at 12.5 mg/kg (Bayer, Rompun 2%) and ketamin at 25.0 mg/kg, (Virbac, Ketamin 1000; 100 mg/mL). Colitis was induced by intrarectal injection of TNBS (15 mg/kg) 8 cm from the anus. Using this dose of TNBS, rats develop transitory colitis followed by a wound healing period of 4-8 wk, during which macroscopic and histological inflammation disappears but hypersensitivity to CRD persists[20].

**Evaluation of visceral pain by CRD:** Nociception in the animals was assessed by measuring the intracolonic pressure required to induce a behavioral response during CRD caused by the inflation of the balloon introduced in the colon. This response is characterized by an elevation of the hind part of the animal's body and clearly visible abdominal contractions corresponding to severe contractions[21-23]. Briefly, rats were anesthetized with volatile anesthesia (2% isoflurane), a balloon[21-23] was inserted intrarectally in a minimally invasive manner 7 cm from the anus, and the catheter was taped to the base of the tail. After 5 min, rats were placed in the middle of a 40 cm × 40 cm Plexiglas box, and the catheter was connected to an electronic barostat apparatus (Distender Series IIR™, G&J Electronics). Increasing pressure was continuously applied until pain behavior was displayed or a cut-off pressure of 80 mmHg was reached.

**Experimental design:** Animals were weighed and randomly distributed into five groups: One control group ($n$ = 3); four groups with colitis, including a negative control group receiving tap water ($n$ = 8); two groups of rats treated daily by oral gavage of chitin-glucan at 1.5 g/d ($n$ = 5) or 3.0 g/d ($n$ = 9); and a positive control group consisting of eight rats treated under the same conditions with phloroglucinol at 1.5 g/d (Figure 1). Visceral sensitivity was assessed at regular intervals throughout the 9-week experiment, with the first evaluation at week-2 corresponding to the basal condition, week 0 just before colitis induction, and weeks 3, 5, and 7 after colitis induction (Figure 1).

### *Model of dextran sodium sulfate-induced colitis in mice*

**Mice:** Nine-week-old C57BL/6 mice were obtained from Janvier labs (France). Animal experiments were conducted in accredited facilities at the Institut Pasteur, Lille, following governmental guidelines. All studies were approved by the local investigational Ethics Review Board (Nord-Pas-de-Calais CEEA N°75, Lille, France; protocol reference numbers 352012 and 19-2009R) and received French government agreement n° APAFIS#7542-20 17030609233680). Five animals were housed per cage with free access to standard rodent chow and tap water.

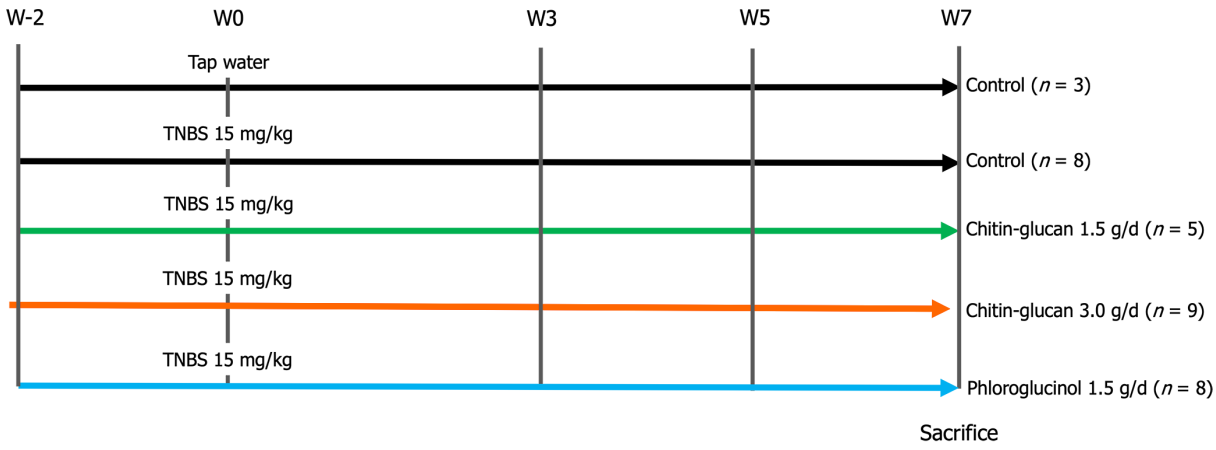


**Figure 1 Long-lasting visceral hypersensitivity in rats.** Chitin-glucan analgesic effect evaluated by pain thresholds at week (W)-2-0-3-5-7.

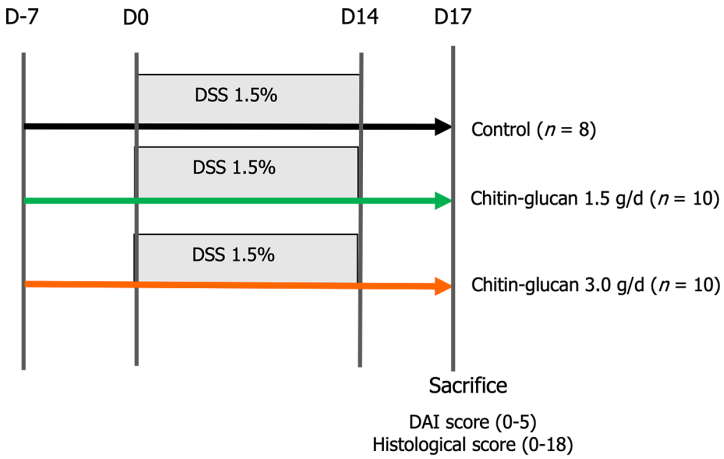


**Figure 2 Dextran sodium sulfate-induced colitis in C57BL/6 mice.** DSS: Dextran sodium sulfate; DAI: Disease activity index.

**Induction of colitis by dextran sodium sulfate:** C57BL/6 mice received 1.5% dextran sodium sulfate (DSS, 45kD; TDB Consultancy AB, Uppsala, Sweden, Ref DB001-42) in their drinking water for 14 d (from day 0 to day 14), followed by 3 d of regular water after the last administration of DSS.

**Experimental design:** Mice were weighed and randomly distributed into 3 groups with colitis including a negative control receiving tap water ($n$ = 8), and 2 groups of rats treated by daily oral gavage of chitin-glucan at 1.5 g/d ($n$ = 10) or 3.0 g/d ($n$ = 10). Preventive treatment with chitin-glucan was started 7 d before colitis induction on day 0 and continued until sacrifice at day 17[24] (Figure 2).

**Clinical evaluation:** The Disease Activity Index (DAI, 0-5) is a simple scoring system ranging from 0 to 5 used to determine the severity of colitis in mice[24]. It is calculated based on the evaluation of body weight loss compared to baseline at day 0 (0: No weight loss; 1: ≤ 10%; 2: > 10%), stool consistency (0: Well-formed pellets; 1: Soft pellets; 3: Liquid stools), and the presence of blood or occult blood in the feces (0-1) (Table 1). DAI was assessed by an investigator blinded to the protocol on day -7, day 0, and day 14. The presence of occult blood was recorded using the hemoccult method.

**Histologic score (0-18):** Transparietal colon samples were embedded in paraffin and stained with May-Grunwald-Giemsa stain. Multiparametric histologic scoring (0-10), as described by Dieleman *et al*[25], was performed blindly by two investigators. This score grades the severity and extent of inflammation, the intensity of cellular infiltrate in the mucosa, its extension in sub-mucosa layers, and the presence of epithelial lesions (Table 2).

### *Cells*

HT-29 cells (ATCC® HTB-38™, Molsheim, France), a human colon carcinoma-derived epithelial cell line, were cultured separately in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L) containing 10% fetal calf serum (DMEM 10% FCS, PAA Laboratories, Les Mureaux, France), and antibiotics (streptomycin 100 μg/mL and penicillin 100 units/mL, Sigma Aldrich, St. Quentin Fallavier, France).

**Table 1 Disease activity index (0-5)**

| Parameters | Scores |
|---|---|
| Weight loss | 0: No; 1: < 10%; 2: ≥ 10% |
| Stool consistency | 0: Regular; 1: Soft; 2: Diarrhea |
| Blood occurrence | 0: Absence; 1: Presence |

**Table 2 Histological score (0-18)**

| Parameters | Score | Description |
|---|---|---|
| Severity of inflammation | 0 | None |
| | 1 | Slight |
| | 2 | Moderate |
| | 3 | Severe |
| Extent | 0 | None |
| | 1 | Mucosa |
| | 2 | Mucosa and submucosa |
| | 3 | Transmural |
| Regeneration | 4 | No tissue repair |
| | 3 | Surface epithelium not intact |
| | 2 | Regeneration with crypt depletion |
| | 1 | Almost complete regeneration |
| | 0 | Complete regeneration or normal tissue |
| Crypt damage | 0 | None |
| | 1 | Basal 1/3 damaged |
| | 2 | Basal 2/3 damaged |
| | 3 | Only surface epithelium intact |
| | 4 | Entire crypt and epithelium lost |
| Involvement | 1 | 1%-25% |
| | 2 | 26%-50% |
| | 3 | 51%-75% |
| | 4 | 76%-100% |

**Experimental design:** The biological effects of chitin-glucan were evaluated under basal conditions after incubating the cells with chitin-glucan for 3 h at 500 μg/mL or 1000 μg/mL. To mimic inflammatory conditions, HT-29 cells were incubated with lipopolysaccharide (LPS) at 10 ng/mL for 24 h, as previously described[26]. After washing, cells were incubated with culture medium containing chitin-glucan (500 μg/mL or 1000 μg/mL) and (LPS 10 nanog/mL) for an additional 3 h. All experiments were performed in triplicate and repeated thrice for reproducibility. Cell viability was determined using the trypan blue exclusion assay.

**mRNA quantification in cells:** Cells were homogenized, and the mRNA expression of the main analgesic-related genes [μ-opioid receptor (MOR), cannabinoid receptor 2 (CB2), peroxisome proliferator-activated receptor alpha (PPARa), inflammation-related genes interleukin (IL)-1b, IL-8, and IL-10], and intestinal barrier-related genes [MUC2, MUC5AC, zonula occludens (ZO)-1, ZO-2, and claudin-2] were assessed by quantitative RT-PCR. Briefly, total RNA was extracted using the Nucleospin RNA Kit (Macherey-Nagel, Hoerdt, France). After RNAse inactivation, total RNA was cleaned of traces of genomic DNA *via* DNase treatment and eluted in RNAse- and DEPC-free water. The RNA purity was evaluated by UV spectroscopy using a Nanodrop system at wavelengths ranging from 220 nm to 350 nm. One microgram of total RNA was used to perform quantitative RT-PCR using LightCycler FastStart DNA Master SYBR Green I (Roche diagnostics, Indianapolis, IN, United States), according to the manufacturer's protocol. The sequences and relative NCBI reference sequences of the primer sets are listed in Table 3. For each reaction, a critical threshold cycle (Ct) value

**Table 3 Sequences of the primers**

| Human genes | Primer sequences (5'-3') |
|---|---|
| *GAPDH* | F: 5′-GAC ACC CAC TCC TCC ACC TTT-3′ |
| | R: 5′-TTG CTG TAG CCA AAT TCG TTG T- 3 |
| *IL-1b* | F: 5′-GAT GCA CCT GTA CGA TCA CT-3′ |
| | R: 5′-GAT GCA CCT GTA CGA TCA CT-3′ |
| *IL-8* | F: 5′-AAA TCA GGA AGG CTG CCA AGA-3′ |
| | R: 5′-AAG GAA CCA TCT CAC TGT GTG TAA AC-3′ |
| *IL-10* | F: 5′-ACTTTAAGGGTTACCTGGGTTGC-3′ |
| | R: 5′-TCACATGCGCCTTGATGTCTG-3′ |
| *PPARa* | F: 5′-CCA GTA TTT AGG AAG CTG TCC-3′ |
| | R: 5′-AAG TTC TTC AAG TAG CCC TCG-3′ |
| *MOR* | F: 5′-GAT CAT GGC CCT CTA CTC CA-3′ |
| | R: 5′-TGG TGG CAG TCT TCA TCT TG-3′ |
| *CB2* | F: 5′-GCT AAG TGC CCT GGA GAA CGT-3′ |
| | R: 5′- TCA GCC CCA GCC AAG CT-3′ |
| *ZO-1* | F: 5′-AGA GCA ATG GAG GAA ACA GC-3′ |
| | R: 5′-CCC CAC TCT GAA AAT GAG GA-3′ |
| *ZO-2* | F: 5′-GGA GCT GTC AGG TTG GCT C-3′ |
| | R: 5′-GTC TCT GCC TCC GGA CAC T-3′ |
| *Claudin2* | F: 5′-AAG GCT CTG CAA AGA ACT GC-3′ |
| | R: 5′-CTG CCA GGC TGA CTT CTC TC-3′ |
| *MUC2* | F: 5′-CTT CGA CGG ACT CTA CTA CAG C-3′ |
| | R: 5′-CTT TGG TGT TGT TGC CAA AC-3′ |
| *MUC5AC* | F: 5′-AGAGTGGGAGCTGGGAGAGAG-3′ |
| | R: 5′-AGCTCA-GAGGACATATGGGAGGT-3′ |

indicating the cycle number at which DNA amplification was determined. The relative gene expression value was calculated as $E = 2^{-\Delta Ct}$, where ΔCt is the difference in crossing points between GAPDH and each gene.

**Molecular modelling of chitin-glucan:** Molecular modelling studies were performed using SYBYL software version 7.2 (SYBYL; 7.2 ed.; Tripos Associates Inc., 1699 South Hanley Road, St. Louis, MO 63144, United States). A three-dimensional model of the compounds was built from a standard fragment library, and their geometries were subsequently optimized using the Tripos force field[27], including the electrostatic term calculated from the Gasteiger and Hückel atomic charges. The Powell method available in the Maximin 2 procedure was used for energy minimization until the gradient value was < 0.001 kcalorie/mol.Å.

A chitin-glucan model composed of a chitin moiety containing six units of N-acetyl-D-glucosamine (33%) covalently linked to a ß-D-glucan moiety containing 14 units of D-glucose (67%) was built. Its ability to chelate LPS, lipoteichoic acid (LTA), and phospholipomannan (PLM) was evaluated using classical molecular dynamics (MD) simulations performed at a constant temperature (300 K) in vacuo for a total period of 2100 ps. The target temperature was achieved by slowly heating the system to 50 K for 4 ps. The atomic velocities were initialized to a Maxwell distribution consistent with the selected interval temperatures. The Verlet algorithm was used with time steps of 0.001 ps, and bond lengths were constrained to their equilibrium values using the SHAKE algorithm. The results (coordinates, energies, and velocities) were collected every 0.025 ps during the simulations. The last 2000 ps (corresponding to 80000 conformers) were analyzed. The most stable complexes were energy-minimized. Molecular surfaces were calculated using the MOLCAD module implemented in the SYBYL software.

### *Statistical analysis*

All comparisons were performed using a Permutation Test for two independent samples. Statistical analyses were carried out using StatXact software (Cytel Inc., Cambridge, MA, United States). Differences were considered statistically significant if the *P* value was < 0.05.

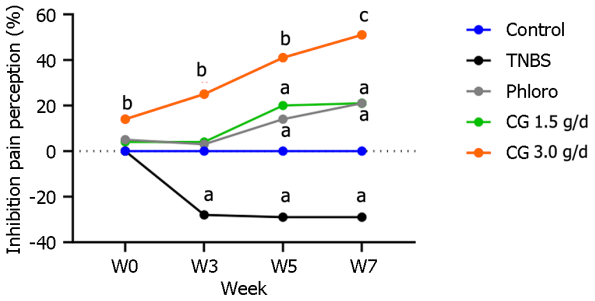


**Figure 3 Time- and dose-related analgesic effects of chitin-glucan in rats with long-lasting TNBS-induced hypersensitivity.** Inhibition of pain perception in % at week (W) 0-3-5-7 compared to W-2 in untreated animals receiving tap water (Control in blue), untreated animals sensitized by TNBS (TNBS in black), TNBS sensitized rats treated with phloroglucinol (Phloro in grey), TNBS sensitized rats treated with chitin-glucan at 1.5 g/d [chitin glucan (CG) 1.5 g/d in green], TNBS sensitized rats treated with chitin-glucan at 3.0 g/d (CG 3.0 g/d in orange). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. CG: Chitin glucan.

## RESULTS

### *Antinociceptive effects of chitin-glucan*

In control rats receiving tap water, a mean pressure of 46.0 mmHg ± 0.9 mmHg was required to induce pain. The two-week treatment with chitin-glucan at 3 g/d decreased normal visceral perception with a significant 14% increase in the pain threshold compared to untreated rats (52.5 mmHg ± 1.1 mmHg *vs* 46.0 mmHg ± 0.9 mmHg, $P < 0.01$). No significant modification of pain thresholds was observed in animals treated under the same conditions with chitin-glucan at 1.5 g/d (47.5 mmHg ± 0.8 mmHg *vs* 46.0 mmHg ± 0.9 mmHg, NS) or phloroglucinol (49.4 mmHg ± 1.2 mmHg *vs* 46.0 mmHg ± 0.9 mmHg, NS) compared to controls rats (Figure 3).

Compared to control rats, animals exposed to TNBS developed a significant and long-lasting hypersensitivity observed 3 wk after TNBS administration (36.2 mmHg ± 1.1 mmHg *vs* 50.0 mmHg ± 0.0 mmHg, -28%) ($P < 0.01$) and maintained at week 5 (34.4 mmHg ± 1.1 mmHg *vs* 48.3 mmHg ± 1.7 mmHg, -29%) ($P < 0.01$) and week 7 (33.1 mmHg ± 0.9 mmHg *vs* 46.7 mmHg ± 1.7 mmHg, -29%) ($P < 0.01$). A dose- and time-dependent analgesic effect of chitin-glucan was observed compared to control animals beginning from week 5 at 1.5 g/d (41.2 mmHg ± 1.2 mmHg *vs* 34.4 mmHg ± 1.1 mmHg, +20%) ($P < 0.01$) and from week 3 at 3.0 g/d (45.5 mmHg ± 1.4 mmHg *vs* 36.20 mmHg ± 1.12 mmHg, +25%) ($P < 0.001$). At the end of the experiment, the decrease in pain perception reached 51% in rats receiving chitin-glucan at 3.0 g/d (50.0 mmHg ± 1.6 mmHg *vs* 33.1 mmHg ± 0.9 mmHg, $P < 0.0001$). The kinetics and amplitude of the analgesic effects observed in rats receiving phloroglucinol were similar to those observed in animals treated with chitin-glucan at 1.5 g/d (Figure 3).

### *Anti-inflammatory effects of chitin-glucan in DSS-induced colitis mice*

**Disease activity index:** Untreated mice with colitis had a DAI score of 2.0 ± 0.3, characterized by soft stools and episodic blood or occult blood in their stools. No significant weight loss was observed. Compared to untreated animals, chitin-glucan at 1.5 g/d or 3.0 g/d significantly improved the DAI score, leading to 50% decrease, with a restoration of stool consistency in most animals (Figures 4A).

**Histological scores:** Histologic lesions (5.7 ± 1.7) in untreated animals receiving DSS were characterized by slight to moderate inflammation, mainly located in the mucosa and submucosa, with damage to basal crypts and only partial tissue repair with crypt deletion. A dose-dependent decrease in histologic scores was observed in animals treated with chitin-glucan at 1.5 g/d (4.5 ± 1.0, NS) and 3.0 g/d (2.3 ± 0.4, $P = 0.04$) leading to a 50% decrease in inflammation severity scores (0.6 ± 0.1 *vs* 1.2 ± 0.4; $P = 0.10$), and limited inflammation to the epithelium (0.7 ± 0.2 *vs* 1.2 ± 0.3; $P = 0.10$) with complete regeneration of the mucosa without crypt damage (0.0 ± 0.0 *vs* 1.3 ± 0.5; $P = 0.02$) (Figure 4B).

### *Molecular mechanisms contributing to chitin-glucan effects*

**Modulation of analgesic-related receptors (MOR, CB2, and PPARa):** Compared to untreated cells, a significant and similar increase of MOR mRNA levels was observed in HT-29 cells incubated with chitin-glucan at 500 µg/mL ($5.39 \times 10^4 \pm 1.99 \times 10^4$ *vs* 1.00 ± 0.16, $P = 0.017$) or 1000 µg/mL ($6.07 \times 10^4 \pm 2.43 \times 10^4$ *vs* 1.00 ± 0.16, $P = 0.002$) (Figure 5A). This increased expression of MOR mRNA was also observed at a similar level when LPS-stimulated HT-29 cells were incubated with chitin-glucan at 500 µg/mL ($3.35 \times 10^4 \pm 8.95 \times 10^3$ *vs* 1.00 ± 0.27, $P = 0.017$) or 1000 µg/mL ($4.50 \times 10^4 \pm 1.13 \times 10^4$ *vs* 1.00 ± 0.27, $P = 0.004$) (Figure 5B). Irrespective of the inflammatory status of HT-29 cells, a similar induction of CB2 mRNA expression was found in HT-29 cells after incubation with chitin-glucan at 500 µg/mL ($2.51 \times 10^4 \pm 9.54 \times 10^3$ *vs* 1.00 ± 0.12, $P = 0.002$) or 1000 µg/mL ($2.52 \times 10^4 \pm 8.43 \times 10^3$ *vs* 1.00 ± 0.12, $P = 0.002$) (Figures 5A and B). No relevant modification of PPARa mRNA levels was observed in quiescent or LPS-stimulated HT-29 cells after incubation with chitin-glucan, irrespective of the concentration used (500 µg/mL or 1000 µg/mL).

**Modulation of inflammatory-related cytokines (IL-1b, IL-8, and IL-10):** Treatment of HT-29 cells with CG at 500 µg/mL or 1000 µg/mL significantly and similarly decreased the basal production of inflammatory cytokines IL-1b (95% and 92%, respectively) and IL-8 mRNA (97% for both concentrations), along with a 4-log increase in the expression of IL-10 mRNA ($3.4 \times 10^4 \pm 1.1 \times 10^4$; $P = 0.002$ and $4.1 \times 10^4 \pm 1.6 \times 10^4$; $P = 0.002$, respectively) (Figure 6A). Moreover, incubation of LPS-

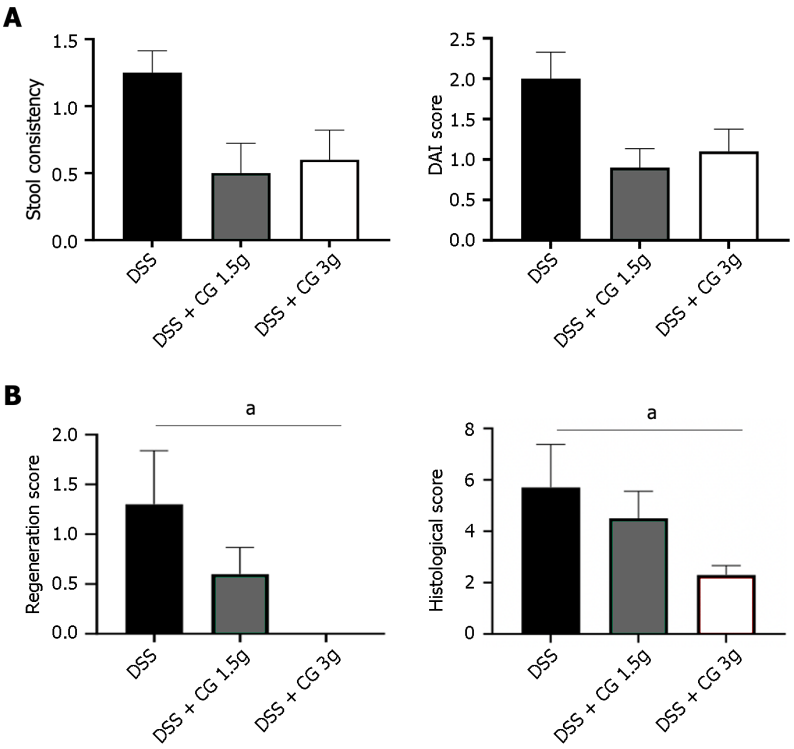


**Figure 4 Improvement of clinical and histological scores in animals with colitis receiving chitin-glucan treatment.** A: Stool consistency in mice with colitis [Dextran sodium sulfate (DSS), black] was improved by chitin glucan (CG) treatment at a concentration of 1.5 g/d (DSS + CG: 1.5 g, grey), or 3 g/d (DSS + CG: 3.0 g, white); B: Disease activity index (DAI) in mice with colitis (DSS, black) was improved by CG treatment at 1.5 g/d (DSS + CG: 1.5 g, grey), or 3.0 g/d (DSS + CG: 3.0 g, white); C: Regeneration score in mice with colitis (DSS-induced, black) was improved by CG treatment at 1.5 g/d (DSS + CG: 1.5 g, grey), or 3.0 g/d (DSS + CG: 3.0 g, white); D: Histological score in mice with colitis (DSS, black) was improved by CG treatment at 1.5 g/d (DSS + CG: 1.5 g, grey), or 3.0 g/d (DSS + CG 3.0 g, white). $^{a}P < 0.05$. DSS: Dextran sodium sulfate; CG: Chitin glucan; DAI: Disease activity index.

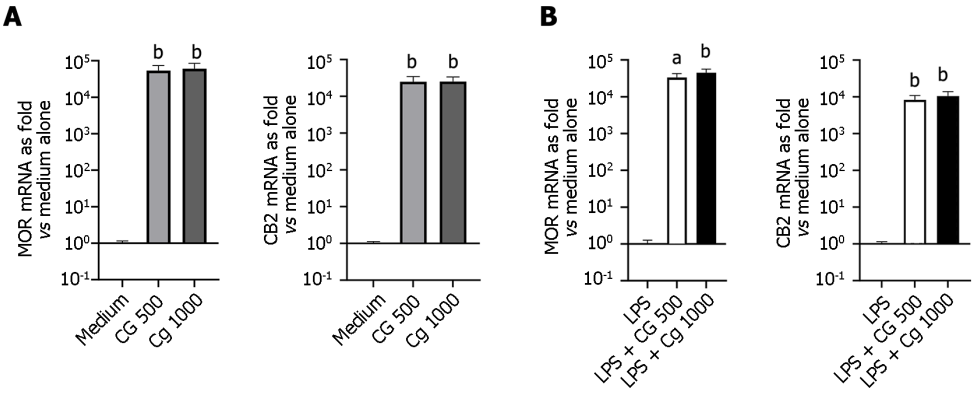


**Figure 5 Modulation of analgesic-related receptors in HT-29 cells incubated with chitin-glucan at different concentrations, with or without lipopolysaccharide stimulation.** A: Modulation of analgesic-related receptors in non-stimulated HT-29 cells incubated for 3 h in medium alone (medium), chitin-glucan at 500 microgram per milliliter [chitin glucan (CG) 500, grey], or chitin-glucan at 1000 microgram per milliliter (CG 1000, dark grey); B: Modulation of analgesic-related receptors in HT-29 cells stimulated during 24 h with lipopolysaccharide (LPS) and incubated 3 additional hours in medium alone (LPS), chitin-glucan at 500 microgram per milliliter (LPS + CG: 500, white), or chitin-glucan at 1000 microgram per milliliter (LPS + CG: 1000, black). $^{a}P < 0.05$, $^{b}P < 0.01$. MOR: Mu-opioid receptor; CB2: Cannabinoid receptor; CG: Chitin glucan; LPS: Lipopolysaccharide.

stimulated HT-29 cells with chitin-glucan at 500 μg/mL or 1000 μg/mL prevented the increased production of IL-1b and IL-8 mRNA while maintaining a strong induction of IL-10 mRNA expression (Figure 6B).

**Modulation of intestinal barrier-related molecules (MUC2/5AC, ZO-1/2, and claudin-2):** Compared to untreated HT-29 cells, those treated with chitin-glucan used at 500 or 1000 μg/mL induced the expression of MUC5AC (86.50 ± 22.80 *vs* 1.00 ± 0.10, $P = 0.002$ and 98.90 ± 10.70 *vs* 1.00 ± 0.05, $P = 0.002$, respectively), ZO-2 (56.60 ± 5.90 *vs* 1.00 ± 0.10, $P = 0.002$ and 150.60 ± 29.30 *vs* 1.00 ± 0.10, $P = 0.005$, respectively) and claudin-2 mRNA (18.00 ± 4.50 *vs* 1.00 ± 0.01, $P = 0.008$ and 22.50 ± 3.60 *vs* 1.00 ± 0.01, $P = 0.008$, respectively) (Figure 7A). Similar induction was observed with chitin-glucan in LPS-stimulated HT-29 cells (Figure 7B). No significant modification of MUC2 and ZO-1 mRNA levels was observed after

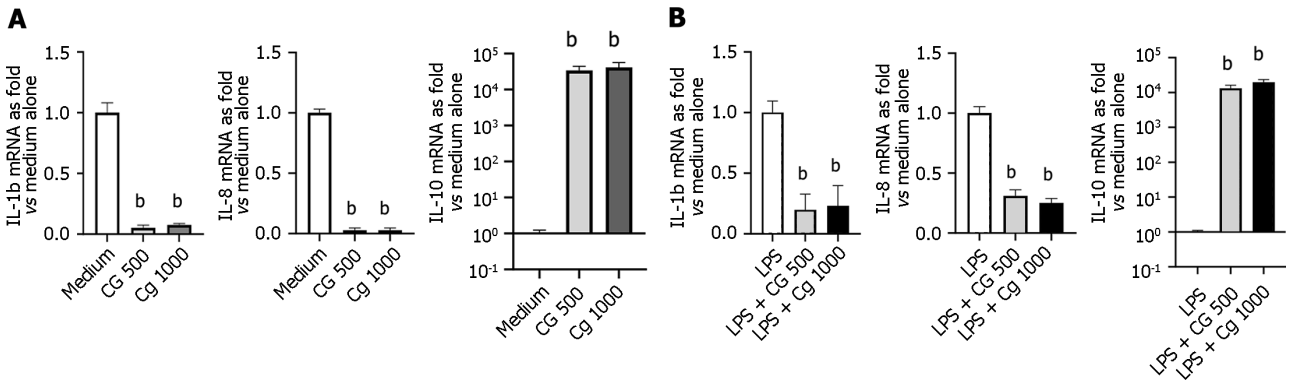


**Figure 6 Modulation of inflammatory-related cytokines in HT-29 cells incubated with chitin-glucan at different concentrations, with or without lipopolysaccharide stimulation.** A: Modulation of inflammatory-related cytokines in non-stimulated HT-29 cells incubated for 3 h in medium alone (medium, white), chitin-glucan at 500 microgram per milliliter [chitin glucan (CG): 500, grey)], or chitin-glucan at 1000 microgram per milliliter (CG 1000, dark grey); B: Modulation of inflammatory-related cytokines in HT-29 cells stimulated during 24 h by lipopolysaccharide (LPS) and incubated 3 additional hours in medium alone (LPS, white), chitin-glucan at 500 microgram per milliliter (LPS + CG 500, grey), or chitin-glucan at 1000 microgram per milliliter (LPS + CG 1000, black). $^{b}P < 0.01$. IL-1b: Interleukin-1 beta; IL-8: Interleukin-8; IL-10: Interleukin-10; CG: Chitin glucan; LPS: Lipopolysaccharide.

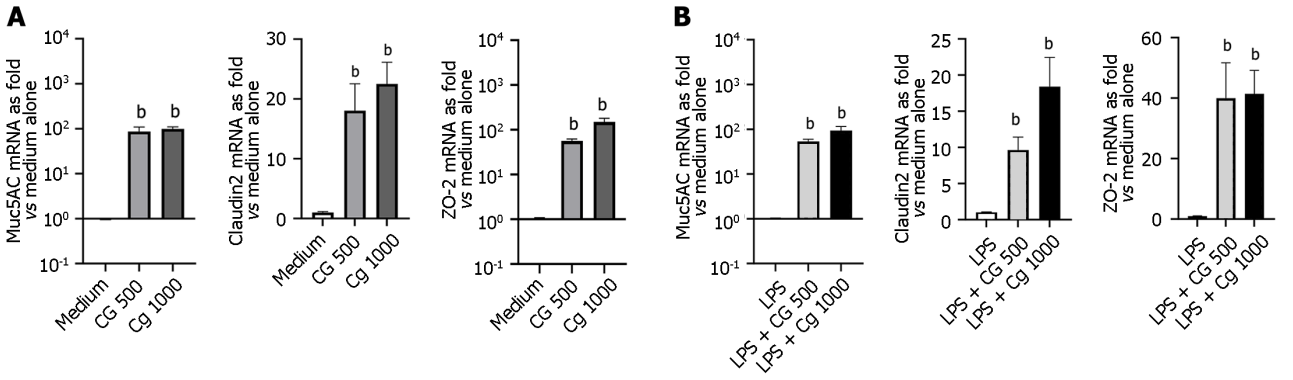


**Figure 7 Modulation of intestinal barrier-related molecules in HT-29 cells incubated with chitin-glucan at different concentrations, with or without lipopolysaccharide stimulation.** A: Modulation of intestinal barrier-related molecules in non-stimulated HT-29 cells incubated for 3 h in medium alone (medium, white), chitin-glucan at 500 microgram per milliliter [chitin glucan (CG): 500, grey)], or chitin-glucan at 1000 microgram per milliliter (CG 1000, dark grey); B: Modulation of intestinal barrier-related molecules in HT-29 cells stimulated during 24 h by lipopolysaccharide (LPS) and incubated 3 additional hours in medium alone (LPS, white), chitin-glucan at 500 microgram per milliliter (LPS + CG: 500, grey), or chitin-glucan at 1000 microgram per milliliter (LPS + CG: 1000, black). $^{b}P < 0.01$. MUC5AC: Mucin-5AC; ZO-2: Zonula occludens-2; CG: Chitin glucan; LPS: Lipopolysaccharide.

treatment with CG (500 μg/mL or 1000 μg/mL), independently of the inflammatory status of HT-29 cells (Figure 7).

### *Chelation of LPS, LTA, and PLM by chitin-glucan complexes*

The lowest energy conformations from the MD trajectories showed the ability of chitin-glucan to chelate/complex both bacterial toxins (LPS and LTA) and fungal toxin (PLM). The high flexibility of the polysaccharide chain allowed it to wrap around LPS (Figure 8), LTA (Figure 9), and PLM (Figure 10). For LPS, the chitin-glucan folded into a hairpin to form a cavity hosting LPS in the middle (Figure 8B). LPS interacts with chitin-glucan *via* a network of 18 hydrogen bonds, mainly between the hydrophilic glucan moiety of chitin-glucan and the hydrophilic antigen O moiety of LPS (Figure 8C). The terminal part of the chitin-glucan moiety was wrapped around the lipophilic lipid A moiety of LPS (Figure 8C). Concerning LTA, chitin-glucan folded to form a small hole between the chitin and glucan moieties, occupied by the shortest lipid side chain of LTA (Figure 7B), and interacted with chitin-glucan *via* four hydrogen bonds (Figure 9C). For PLM, chitin-glucan formed a cavity hosting the PLM in its middle (Figure 10B). PLM was distributed on both sides of the cavity according to the degree of lipophilicity of its moieties (Figure 10C) and interacted with chitin-glucan *via* a network of 13 hydrogen bonds distributed around the cavity (Figure 10D).

## DISCUSSION

IBS is a chronic functional gastrointestinal disorder diagnosed according to symptom-based criteria defined by the ROME IV classification[28]. IBS is a heterogeneous disorder with multiple physiopathological mechanisms[29]. Exposure to

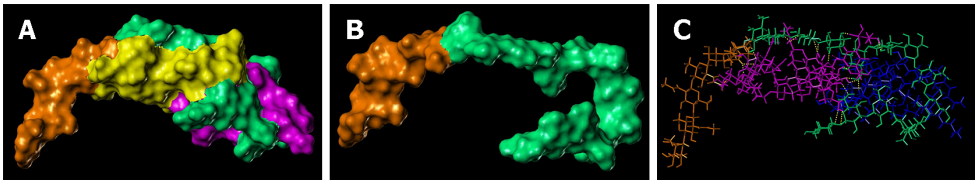


**Figure 8 Molecular modelling of interaction between lipopolysaccharide and chitin-glucan.** A: Trapping of lipopolysaccharide (LPS) (O antigen moiety colored in yellow and lipid A moiety colored in magenta) by chitin-glucan (CG) (chitin moiety colored in orange and glucan moiety colored in green); B: Folding of CG into a hairpin showing the cavity that hosts LPS in its middle (chitin moiety colored in orange and glucan moiety colored in green); C: Interactions of LPS (O antigen moiety colored in magenta and lipid A moiety colored in blue) with CG (chitin moiety colored in orange and glucan moiety colored in green) *via* a network of 18 hydrogen bonds (yellow dashed line).

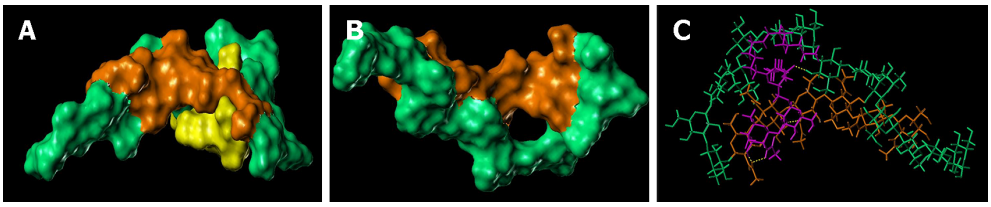


**Figure 9 Molecular modelling of interaction between lipoteichoic acid and chitin-glucan.** A: Trapping of lipoteichoic acid (LTA) (colored in yellow) by chitin-glucan (CG) (chitin moiety colored in orange and glucan moiety colored in green); B: Folding of CG showing a small hole occupied by the shortest lipidic side chain of LTA (chitin moiety colored in orange and glucan moiety colored in green); C: Interactions of LTA (colored in magenta) with CG (chitin moiety colored in orange and glucan moiety colored in green) *via* four hydrogen bonds (yellow dashed line).

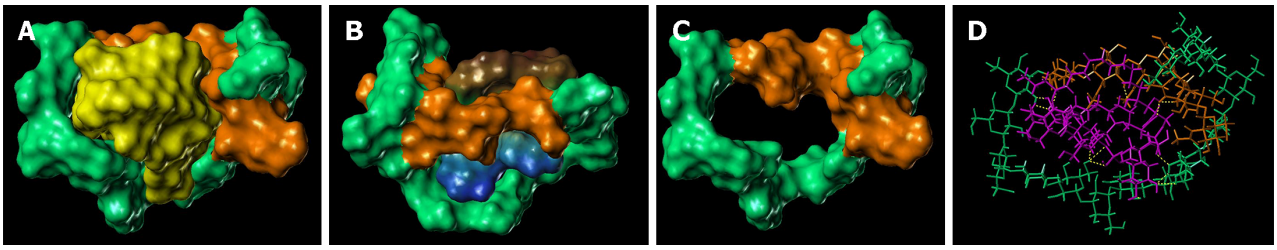


**Figure 10 Molecular modelling of interaction between phospholipomannan and chitin-glucan.** A: Trapping of phospholipomannan (PLM) (colored in yellow) by chitin-glucan (CG) (chitin moiety colored in orange and glucan moiety colored in green); B: Folding of CG showing the large cavity that hosts PLM in its middle (chitin moiety colored in orange and glucan moiety colored in green); C: Lipophilic potential surfaces (according to the lipophilicity scale from blue for the minimum until brown for the maximum) of PLM trapped by CG (chitin moiety colored in orange and glucan moiety colored in green); D: Interactions of PLM (colored in magenta) with CG (chitin moiety colored in orange and glucan moiety colored in green) *via* a network of 13 hydrogen bonds (yellow dashed line).

pathogenic organisms, changes in host-microbiota interactions, and disruption of the intestinal barrier can affect the gut-brain axis, triggering locally persistent low inflammation and altering visceral sensitivity[29]. Studies on the basic molecular mechanisms that enhance IBS management and facilitate the development of new, specific targeted treatments are important. In the present study, we investigated the functional and molecular gastrointestinal responses to chitin-glucan administration, particularly in relation to the main pathophysiological mechanisms of IBS. We showed that chitin-glucan decreased visceral pain perception and intestinal inflammation rapidly and significantly through master gene regulation in pain, inflammation, and intestinal barrier function, and may neutralize harmful substances in the intestinal lumen, such as microbial cell walls.

Visceral hypersensitivity is a key symptom of IBS. CRD in rats is the most widely used method for assessing visceral pain due to its ease of use and robust reproducibility[23,30]. In this model, we evaluated the functional characteristics of chitin-glucan, a novel dietary prebiotic used in humans at recommended dosages of 1.5 g/d and 3.0 g/d[13]. In untreated rats, a mean CRD of 46.0 mmHg ± 0.9 mmHg was required to induce pain, characterized by clearly visible abdominal contractions and elevation of the hind part of the animal's body[21-23]. Oral administration of chitin-glucan at a HED of 3 g/d for 15 d decreased normal visceral perception, allowing for a significant 14% increase in the pain threshold under normal conditions. To more accurately evaluate the functional characteristics of chitin-glucan in the modulation of visceral pain, we induced intestinal hypersensitivity by local administration of TNBS. Compared to rats under normal conditions, intra-rectal administration of a low concentration of TNBS induces higher and long-lasting visceral hypersensitivity, making this model the reference for screening novel treatments for visceral pain originating within the gastrointestinal tract[19]. In rats with visceral hypersensitivity, oral administration of chitin-glucan (1.5 g/d decreased visceral perception by 20% five weeks after TNBS administration with a dose- and time-related analgesic effect leading, at 3.0 g/d, to a rapid 25% inhibition of pain perception three weeks after colon sensitization, reaching up to 50% at seven

weeks. In the present study, we used phloroglucinol as a positive control under the same conditions as chitin-glucan. Phloroglucinol is a well-tolerated phenol derivative with antispasmodic properties that has long been used in clinical practice for painful gastrointestinal conditions, particularly in patients with IBS[31-33]. Using the most relevant, robust, and reliable animal model of chronic visceral pain to screen promising therapies to alleviate visceral pain[19], chitin-glucan at 3.0 g/d showed more rapid and two-fold superior analgesic effects compared to the positive control treatment phloroglucinol used at conventional dosages[31].

There are few effective analgesic options to manage chronic visceral pain, except for opioids, which require parenteral administration and have numerous adverse side effects. Compared to a previous study[23], the administration of chitin-glucan at 3.0 g/d resulted in an antinociceptive effect of the same magnitude as that induced by the subcutaneous administration of 3 mg morphine per kg body weight. Therefore, we hypothesized that Chitin-glucan induces the expression of receptors on epithelial cells, which locally control the transmission of nociceptive information to the intestinal nervous system. Opioid[34,35] and cannabinoid[36] systems are important pathways involved in visceral sensory signaling and intestinal motility. Most ligands activating the MOR and CB2R signal also target several other pathways involving PPAR-a to mediate synergistic antinociceptive activities[37,38]. Since these receptors are highly expressed in epithelial cells[23,39], we conducted a series of *in vitro* experiments using human intestinal HT-29 epithelial cells showing increased expression of MOR and CB2R mRNA, starting after cell incubation with different concentrations of chitin-glucan, without significant modification of PPARa mRNA levels.

Gut inflammation is a major risk factor for developing long-lasting visceral hypersensitivity[40]. Within the gastrointestinal tract, low-grade mucosal inflammation with variable-intensity immune activation is present in patients with IBS[41], particularly in those with post-infection IBS or IBS with diarrhea[5]. In rats with long-lasting visceral hypersensitivity induced by intra-rectal administration of TNBS, active inflammation of the colon is present a few days after TNBS exposure, followed by a recovery period of 4-8 wk, where overt signs of inflammation disappear, but hypersensitivity to CRD persists[19]. To gain insight into the mechanisms underlying the improvement of TNBS-induced visceral hypersensitivity in rats by chitin-glucan, we analyzed the effects of chitin-glucan specifically on colitis in one of the most commonly used models of intestinal inflammation induced by the oral administration of DSS[24,25]. We showed that oral administration of chitin-glucan provides a therapeutic benefit in established DSS-induced inflammatory lesions of the colon, decreasing the intensity of inflammation by 50%, leading to complete regeneration of the colonic mucosa without crypt damage, resolution of clinical manifestations, and restoration of stool consistency in most animals. *In vitro*, using human intestinal HT-29 epithelial cells, chitin-glucan used at clinically and biologically relevant concentrations was able to increase the expression of IL-10 mRNA, which is an important player in the regulation of intestinal inflammation [42]. Furthermore, the anti-inflammatory activity of chitin-glucan was investigated in LPS-stimulated HT-29 epithelial cells, showing potent inhibition of major inflammatory cytokine genes, such as IL-1b and IL-8. Regarding the importance of developing new treatments for IBS guided by physiopathology, the anti-inflammatory properties of chitin-glucan are potentially important, as they target a relevant physiopathological mechanism associated with IBS[29].

Increased intestinal permeability is a pathophysiological observation in IBS, observed mostly in the diarrhea-predominant patient subgroup[43,44]. The intestinal epithelium is the main protective barrier used to regulate the contact between luminal antigens, including microbe-derived molecules, and immune cells located below the lamina propria and submucosa. This epithelial barrier comprises occlusive intracellular tight junctions consisting of transmembrane proteins, such as claudins and zonula occludens molecules, as well as a dense film of mucus containing glycosylated glycoproteins called mucins, where MUC2, MUC3, MUC4, and MUC5AC are among the most expressed in the human colon or small bowel[45]. We previously demonstrated the protective effect of chitin-glucan fermentation products on inflammation-induced epithelial barrier disruption and the production of inflammatory cytokines[16]. In the present study, the incubation of HT-29 epithelial cells with chitin-glucan resulted in increased levels of MUC5AC, claudin-2, and ZO-2 mRNA. HT-29 cells share similarities with enterocytes of the human small intestine, and this cell line is a valuable *in vitro* model for studying molecules constituting tight junctions, mucin expression, and the intestinal epithelial response to bacterial infection[46]. Taken together, these findings suggest that in addition to chitin-glucan fermentation products, chitin-glucans may also have a direct effect on epithelial cells to preserve barrier integrity.

Microbial cell walls contain pathogenic lipids, including LPS and LTA, in gram-negative and gram-positive bacteria, and PLM in fungi[47]. These compounds are present in large quantities in the intestinal lumen and act as major ligands for Toll-like receptors (TLR-2-4-6) and other innate immune receptors that trigger inflammatory responses[48]. Therapeutic strategies involving intestinal sequestration and clearance of these pathogenic lipids have been successfully evaluated in patients with IBS using the mineral clay diosmectite[49]. Other pathogenic lipid-sequestering molecules have already been identified in marine chelicerates and crustaceans[50-53]. Chitin is found in the exoskeletons of marine invertebrates, insects, arachnids, and the cell walls of various fungi and algae[52]. Although chitin-glucan has antimicrobial activity against a large number of microorganisms[53], the mechanisms underlying these antibacterial and antifungal activities remain unknown. Therefore, we developed a molecular model of chitin-glucan and performed molecular docking and molecular dynamic simulations to evaluate the ability of chitin-glucan to chelate LPS, LTA, and PLM. *In silico* findings revealed that the high flexibility of chitin-glucan formed a cavity around LPS through a network of 18 hydrogen bonds interacting with the lipid A structure of LPS, which is the active and most conserved component of LPS, acting as a pathogen-associated molecular pattern[54]. Similar interactions were observed between chitin-glucan complexes and LTA or PLM. Collectively, these results suggest that the antimicrobial activity of chitin-glucan may be involved, at least in part, in a new property of the complex as a chelator of pathogenic lipids.

## CONCLUSION

Current IBS treatments are often inadequate, and only a small percentage of patients are on prescription therapies, underscoring the potential market and need for additional therapeutic options. The multifactorial pathogenesis of IBS has led to the development of diverse treatment strategies. In the present study, the prebiotic chitin-glucan produced potent and non-tolerance-forming anti-nociception, together with the modulation of intestinal inflammation through intestinal intestinal master gene regulation and chelation of harmful microbial products in the gastrointestinal tract. These results advance our understanding of the mechanisms of action of chitin-glucans in the gut and augment the implementation of evidence-based chitin-glucan treatments in patients with IBS or IBS-like symptoms.

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## FOOTNOTES

**Author contributions:** Valibouze C, Dubuquoy C, Rousseaux C, and Chavatte P acquired the data; Genin M supervised the statistical analyses; Valibouze C, Desreumaux P, and Rousseaux C drafted the manuscript; and all the authors interpreted the data and critically reviewed the manuscript; Intestinal Biotech Development supervised study coordination, data collection, and analysis; Chitin glucan was obtained from Kitozyme (SA).

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ORIGINAL ARTICLE

Basic Study

# Optimization of tracheoesophageal fistula model established with T-shaped magnet system based on magnetic compression technique

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## Abstract

***BACKGROUND***

The magnetic compression technique has been used to establish an animal model of tracheoesophageal fistula (TEF), but the commonly shaped magnets present limitations of poor homogeneity of TEF and poor model control. We designed a T-shaped magnet system to overcome these problems and verified its effectiveness *via* animal experiments.

***AIM***

To investigate the effectiveness of a T-shaped magnet system for establishing a TEF model in beagle dogs.

***METHODS***

Twelve beagles were randomly assigned to groups in which magnets of the T-shaped scheme (study group, $n$ = 6) or normal magnets (control group, $n$ = 6)

were implanted into the trachea and esophagus separately under gastroscopy. Operation time, operation success rate, and accidental injury were recorded. After operation, the presence and timing of cough and the time of magnet shedding were observed. Dogs in the control group were euthanized after X-ray and gastroscopy to confirm establishment of TEFs after coughing, and gross specimens of TEFs were obtained. Dogs in the study group were euthanized after X-ray and gastroscopy 2 wk after surgery, and gross specimens were obtained. Fistula size was measured in all animals, and then harvested fistula specimens were examined by hematoxylin and eosin (HE) and Masson trichrome staining.

***RESULTS***

The operation success rate was 100% for both groups. Operation time did not differ between the study group (5.25 min ± 1.29 min) and the control group (4.75 min ± 1.70 min; $P = 0.331$). No bleeding, perforation, or unplanned magnet attraction occurred in any animal during the operation. In the early postoperative period, all dogs ate freely and were generally in good condition. Dogs in the control group had severe cough after drinking water at 6-9 d after surgery. X-ray indicated that the magnets had entered the stomach, and gastroscopy showed TEF formation. Gross specimens of TEFs from the control group showed the formation of fistulas with a diameter of 4.94 mm ± 1.29 mm (range, 3.52-6.56 mm). HE and Masson trichrome staining showed scar tissue formation and hierarchical structural disorder at the fistulas. Dogs in the study group did not exhibit obvious coughing after surgery. X-ray examination 2 wk after surgery indicated fixed magnet positioning, and gastroscopy showed no change in magnet positioning. The magnets were removed using a snare under endoscopy, and TEF was observed. Gross specimens showed well-formed fistulas with a diameter of 6.11 mm ± 0.16 mm (range, 5.92-6.36 mm), which exceeded that in the control group ($P < 0.001$). Scar formation was observed on the internal surface of fistulas by HE and Masson trichrome staining, and the structure was more regular than that in the control group.

***CONCLUSION***

Use of the modified T-shaped magnet scheme is safe and feasible for establishing TEF and can achieve a more stable and uniform fistula size compared with ordinary magnets. Most importantly, this model offers better controllability, which improves the flexibility of follow-up studies.

**Key Words:** Magnetic surgery; Magnetic compression technique; Tracheoesophageal fistula; Magnet; Animal model; Beagles



**Core Tip:** The magnetic compression technique has been successfully used to establish animal models of tracheoesophageal fistula (TEF) in beagle dogs. However, for TEF, use of the common circular magnet shape is associated with poor homogeneity and poor controllability of model. In this study, we used a modified T-shaped magnet system to establish a TEF, and the results showed this approach could obtain a more stable and uniform fistula size compared with standard magnets. Most importantly, the proposed model offers better controllability, which improves the flexibility of subsequent studies.

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## INTRODUCTION

Tracheoesophageal fistula (TEF) is a rare condition in clinic that is difficult to treat. Methods for treatment of TEF include endoscopic treatment and surgical treatment[1-4], and no uniform and standardized surgical method has been developed. Good animal models hold great significance for studying the occurrence and development of human diseases, pathophysiological conditions, and prognosis after treatment. In small animals, TEF can generally be established by puncture injury[5], while larger animal models of TEF, such as those in pigs and dogs, are generally established by surgical operation[6,7]. The operation for establishing TEF animal models *via* the surgical method is complicated and time-consuming.

With the magnetic compression technique, upon placement of magnets in the empty organs where anastomosis is to occur, the magnets are attracted together, and the compressed tissue undergoes pathological changes of ischemia, necrosis, and shedding[8], resulting in the establishment of a new channel. The magnetic compression technique can be used for vascular anastomosis[9-11], digestive anastomosis[12-15] and ureteral anastomosis[16,17]. Five years ago, we first proposed application of the magnetic compression technique for establishing animal models of TEF in beagle dogs. Subsequent animal experiments fully verified the feasibility of the method and its advantages of a simple operation, little

trauma, and high success rate[18]. However, in continued research, we found that this method is limited by poor controllability of model establishment time and time-limited requirements for subsequent therapeutic intervention.

To overcome the above shortcomings, we developed an improved T-shaped magnet design scheme on the basis of our previous research, and in the present study, we investigated the feasibility of this scheme for establishing a TEF model compared with the use of ordinary shaped magnets in beagle dogs.

## MATERIALS AND METHODS

### Ethical statement

The experimental protocol was approved by the Committee for Ethics of Animal Experiments of Xi'an Jiaotong University (license No. XJTUAE2023-2207). Twelve beagles (6 males and 6 females), > 1 year old and weighing 10-15 kg, were obtained from the Laboratory Animal Center of the Xi'an Jiaotong University (Xi'an, China). The research protocol and all experimental procedures were strictly in accordance with the Guidelines for the Care and Use of Experimental Animals, issued by the Xi'an Jiaotong University Medical Center. We fully safeguarded animal welfare and minimized animal suffering in this research.

### Magnet devices

The control group used cylindrical magnets. The magnet system of the control group consisted of two parts: The daughter magnet and the parent magnet. The daughter magnet was a cylindrical magnet with a diameter of 6 mm, a height of 6 mm, and a central hole with a diameter of 2 mm. The parent magnet also was a cylinder with a diameter of 9 mm, a height of 6 mm, and a central hole with a diameter of 3 mm. Both the daughter magnet and the parent magnet were fabricated from N50 sintered NdFeb, with saturation magnetization in the height direction and nitriding coating on the surface (Figure 1A-C).

A composite magnet design scheme was used in the study group. The T-shaped magnet comprised a magnet part and a base part. The magnet part was a cylindrical magnet with a diameter of 6 mm, a height of 6 mm, and a central hole with a diameter of 2 mm. The magnet was fabricated from N50 sintered NdFeb material with saturation magnetization in the height direction and titanium nitride coating on the surface. The base was a 3D-printed plastic ring with an outer diameter of 10.0 mm, an inner diameter of 6.5 mm, and a height of 2.0 mm. One end of the magnet was inserted into the central hole of the plastic ring and fixed firmly with a binder (Figure 1D-F). The daughter magnet in the study group was identical in size and structure to the parent magnet. The maximum magnetic force between the daughter and parent magnets was 11 N in the study group and 10 N in the control group.

### Study design

The 12 beagles were randomly divided into two groups: the study group (3 males and 3 females) and the control group (3 males and 3 females). The modified T-shaped magnet scheme was applied in the study group, and normal cylindrical magnets were used in the control group.

### Surgical procedures

The beagles were fasted for 12 h before surgery. For the operation, they were anesthetized by intravenous injection of 3% pentobarbital sodium (1 mL/kg) and fixed in the supine position. Once loss of the paw withdrawal reflex was confirmed, the dogs were placed on an operating table in the supine position. The procedure and method for magnet placement were the same as those described in our previous article[18]. Briefly, the glottis was sufficiently exposed, and the daughter magnet was inserted into the trachea (Figure 2A). Then the parent magnet was inserted into the cervical esophagus, and the parent magnet and daughter magnet automatically attracted together (Figure 2B). Neck X-ray and gastroscopy were used to confirm the target location and accurate coupling of the parent and daughter magnets. After a period of time, the parent magnet and daughter magnet are expected to fall off into the distal esophagus, and TEF can be established (Figure 2C). The T-shaped magnet scheme components were inserted into the trachea and esophagus in the study group using the same method as the control group (Figure 2D and E). TEF formation was observed after magnet removal under endoscopy (Figure 2F).

The operation time, operation success rate, and occurrence of accidental injury were recorded. Dogs in the control group were euthanized after X-ray and gastroscopy to confirm the establishment of TEF after coughing, and gross specimens of TEF were obtained. Dogs in the study group were euthanized after X-ray and gastroscopy at 2 wk after surgery, and gross specimens of TEF were obtained.

### Postoperative care

All dogs were managed in a single cage after emergence from anesthesia. Pethidine hydrochloride (1 mg/kg) was injected intramuscularly every 12 h for 3 d after operation for analgesia. After the operation, the general conditions of the experimental dogs, including the presence and timing of cough and the time at which the magnets left the neck were observed. X-ray examinations are usually performed every other day beginning on the fourth day after surgery to determine whether the magnet has departed from its initial position. Of course, if the dog has a cough during the observation period, X-ray examination is performed immediately to determine whether the magnet has moved from its initial position.

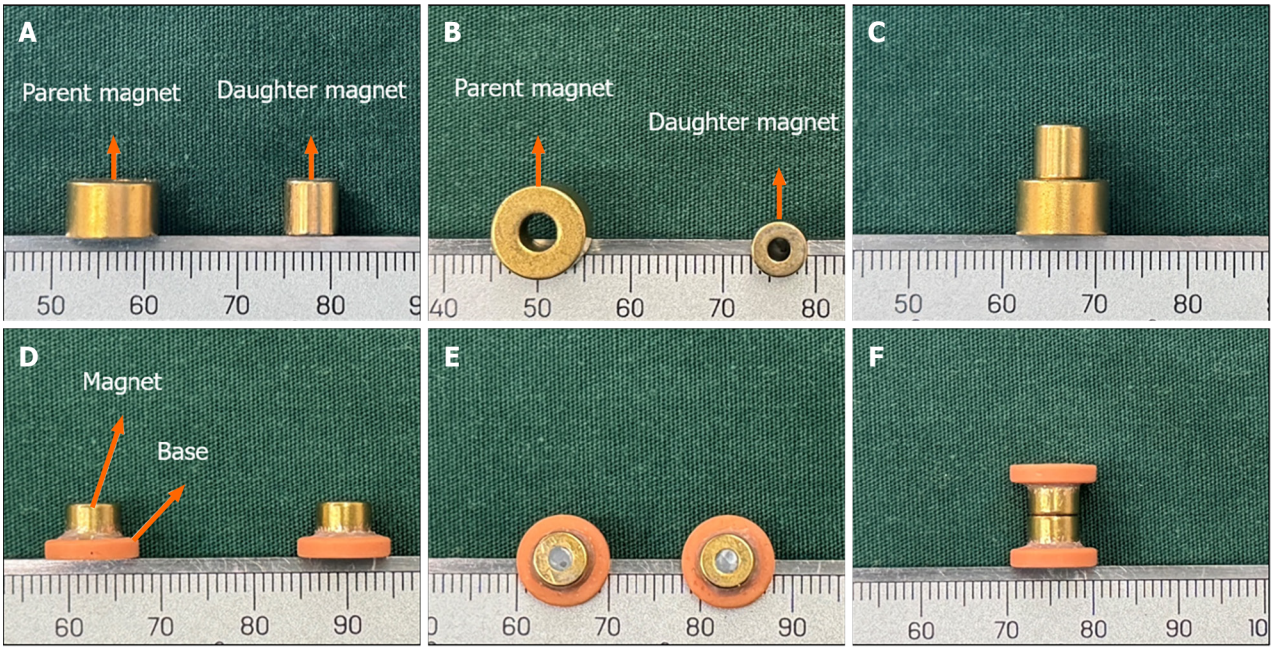


**Figure 1 Conventional cylindrical magnets and T-shaped magnet scheme used for the magnetic compression technique.** A-C: The daughter magnet and parent magnet used in the control group; D-E: The T-shaped magnets used in the study group.

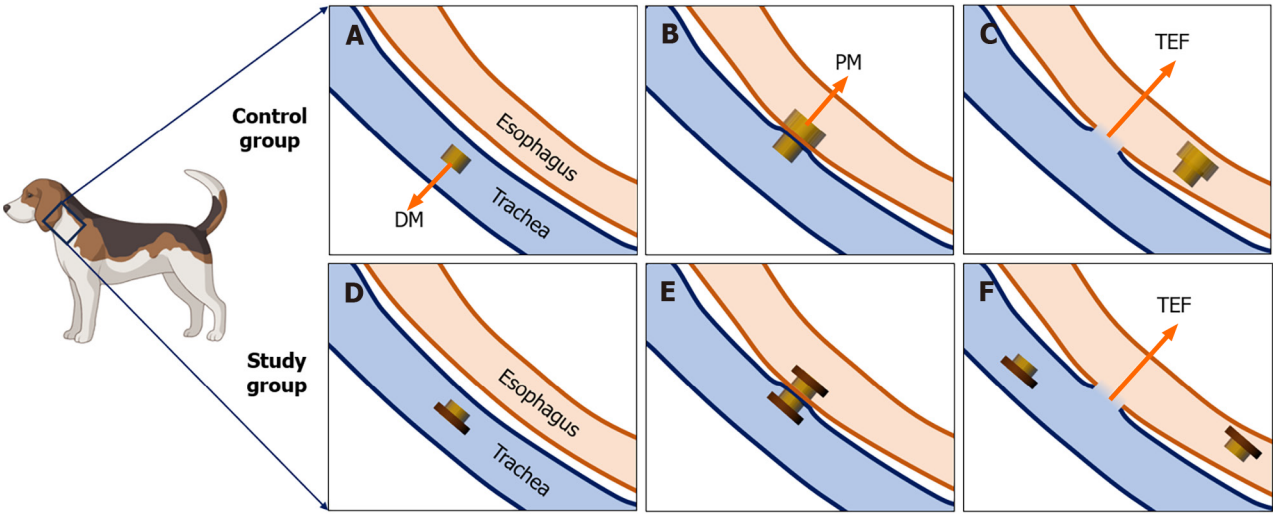


**Figure 2 Schematic diagram of the magnetic compression process.** A: The daughter magnet was inserted into the trachea of control dogs; B: The parent magnet was inserted into the esophagus of the control dog where it was then attracted with the daughter magnet; C: The parent magnet and daughter magnet fall off into the distal end of the esophagus, and the tracheoesophageal fistula (TEF) is established in the control group; D: The T-shaped magnet was inserted into the trachea of study dogs; E: Another T-shaped magnet was inserted into the esophagus of the study dog where it attracted with the magnet in the trachea; F: The endotracheal magnet was removed under endoscopy; the endoesophageal magnet entered the distal end of the esophagus; and the TEF was established in the study group. DM: Daughter magnet; PM: Parent magnet; TEF: Tracheoesophageal fistula.

### *Tissue analysis*

All harvested TEFs were visually inspected and measured before processing for pathological examination by hematoxylin and eosin (HE) and Masson trichrome staining.

For fixation, the TEF specimens were soaked overnight in 10% formalin. After fixation, each specimen was embedded in paraffin, and 4-μm-thick sections were prepared from the anastomosis. The sections were stained with HE and Masson trichrome stains and examined under a bright-field microscope.

### *Statistical analysis*

All data were analyzed using SPSS statistical 20.0 software. The normality of the study variables was assessed by Shapiro-Wilk test. Normally distributed quantitative data are presented as mean ± SD values, while data that did not conform to a normal distribution are described as median values. Differences between the study and control groups were determined

using an independent sample *t*-test or a nonparametric test, with $P < 0.05$ indicating a significant difference.

## RESULTS

### Procedural parameters

The magnet placement operations were successful in all 12 dogs (Figure 3A, B, E, and F), and no bleeding, asphyxia, or injury of the trachea and esophagus occurred during the operation. The operation time did not differ significantly between the study group (5.25 min ± 1.29 min; range, 4.00-7.50 min) and the control group (4.75 min ± 1.70 min; range, 3.50-8.00 min; $P = 0.331$). Cervical X-ray showed that the magnets attracted well (Figure 3C, D, G, and H). The operation success rate was 100% in both groups.

### Survival rate and postoperative observation

None of the animals exhibited cough after recovery from anesthesia, and all dogs ate generally well after surgery. The dogs in the control group developed coughs 6-9 d after surgery. X-ray examination indicated that the magnets had left the esophagus and entered the stomach (Figure 4A and B). Further gastroscopy showed the TEF formation (Figure 4C and D), and thus, the animals were euthanized for collection of gross specimens of TEF (Figure 4E-G). None of the dogs in the study group had obvious coughs after operation. X-ray examination of the neck indicated that the magnets remained in good position (Figure 5A and B). The magnets were removed under gastroscopy 2 wk after operation, and the TEF was well formed (Figure 5C and D). The dogs in the study group were euthanized, and the gross specimens of TEF were obtained (Figure 5E-G).

### Gross and histological appearance of anastomosis

In the gross specimens, the esophagus and trachea showed normal morphology in both the study group and the control group. Except for the fistula between the trachea and esophagus, no obvious tissue adhesion was observed between the trachea and esophagus, and no obvious thickening of the mucosa of the trachea was observed. The shape and patency of fistulas in the study group were better than those in the control group. The diameter of fistulas in the study group was 6.11 mm ± 0.16 mm (range: 5.92-6.36 mm), which was greater and showed less variability in shape than that in the control group of 4.94 mm ± 1.29 mm (range, 3.52-6.56 mm; $P < 0.001$). Histological observation showed that the trachea and esophageal mucosa converged along the medial surface of the fistula (Figure 6).

## DISCUSSION

In addition to inducing digestive tract anastomosis, the magnetic compression technique can also be used to create "therapeutic" fistulas. Uygun *et al*[19-21] reported experimental studies on the use of the magnetic compression technique in gastrostomy[19], colostomy[20], and cystostomy[21] in rats. The essence of endoscopic gastrointestinal bypass anastomosis is also enterostomy[22-26]. Although animal models generated using the magnetic compression technique represent pathological ostomy, the histopathological changes are the same.

The results of the present study indicate that the magnetic compression technique can be applied to establish a canine TEF model in a minimally invasive manner, and that the T-shaped magnet scheme offers an improved system compared with traditional round magnets. Compared with the control procedure, the newly developed procedure using the T-shaped magnet scheme showed distinct advantages, such as after the establishment of the TEF, the magnets remained at the fistula, where they would not cause serious complications, such as coughing and lung infection in the short term. At the time of further interventional treatment, the magnets can be removed from the fistula. In comparison, a disadvantage of the control procedure is that after the formation of TEF, an interventional operation must be performed as soon as possible to prevent serious lung infection caused by aspiration, which can lead to death of the animal. In general, the newly developed approach provides researchers with more flexible experimental arrangements than the control procedure. The magnet used in this study has a unit price of $10, which is a low-cost experimental consumable.

In this study, with the use of the T-shaped magnet scheme for esophageal and tracheal compression, the size of the fistula was equal to the outer diameter of the magnet, and the base of the magnet was larger than the diameter of the fistula. Therefore, after the formation of the fistula, the magnets would not fall off by themselves as in the control group, but rather would stay in the TEF. Such a design has two effects: First, it avoids aspiration by the experimental dogs, which would accelerate lung infection and death; and secondly, retention of the magnets in the fistula for a period of time is beneficial to the size of the fistula and the stability of the internal surface tissue of the fistula. In the preparation of an animal model of rectovaginal fistula, previous studies found that the fistula has a tendency to self-heal in the animal model, and thus, it is necessary to insert foreign bodies in the fistula mouth to promote the stability of the fistula[27,28]. The T-shaped magnet scheme introduced in this study can play exactly such a role.

In the present study, the time at which gross specimens of TEF were obtained was inconsistent between the study group and the control group. The dogs in the control group were euthanized and killed immediately after the establishment of TEF, because in the early period the TEF was established, the dogs would cough violently after eating and drinking, which was painful to the experimental animals. Therefore, to reduce the suffering of animals during the experiment and follow the ethical norms for animal experiments, we euthanized these dogs immediately after confirming TEF establishment. In the study group, the specially designed T-shaped magnet scheme avoided the onset of such

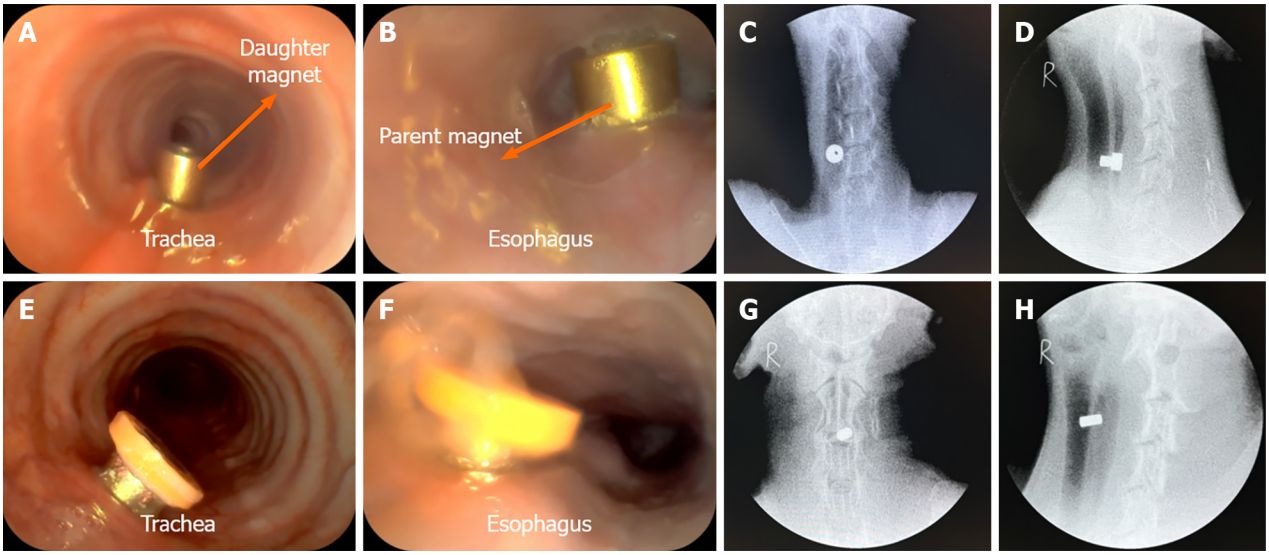


**Figure 3 Surgical procedure.** A: Endotracheal daughter magnet seen under endoscopy in the control group; B: Endoscopic view of the esophageal parent magnet in the control group; C and D: Fluoroscopy showing that the parent and daughter magnets were coupled and retained in the target location in the control group; E: Endotracheal magnet seen under endoscopy in the study group; F: Endoscopic view of the esophageal magnet in the study group; G and H: Fluoroscopy showing that the magnets were coupled and retained in the target location in the study group.

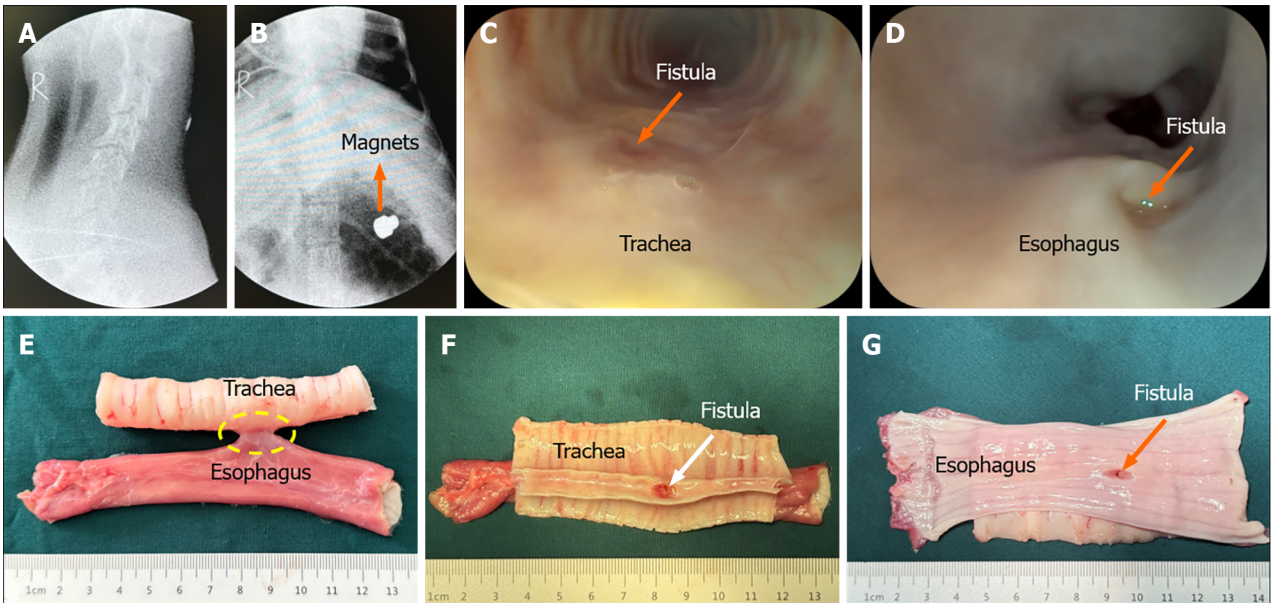


**Figure 4 Gross tracheoesophageal fistula specimens from the control group.** A and B: At 6-9 d after surgery, the magnets left the neck and entered the stomach; C: Bronchoscopy showing a fistula located in the posterior wall of the trachea; D: Gastroscopy showing a fistula located in the anterior wall of the esophagus; E: Gross specimen of tracheoesophageal fistula; F: Gross specimen showing the fistula in the trachea; G: Gross specimen showing the fistula in the esophagus.

coughing, allowing us to extend the time to obtain specimens. According to our research results from 5 years ago, the time for the magnetic compression technique to establish TEF is 4-6 d[18]. The control group in this study showed that TEF was established 6-9 d after surgery. Therefore, the TEF should be established within about 10 d in the study group. Therefore, we assumed that 2 wk is enough time for TEF establishment in the study group. Accordingly, we chose to euthanize the dogs in the study group at 2 wk to obtain TEF specimens. Of course, the purpose of our design of T-shaped magnets was to provide researchers with more flexible options for further treatment of TEF. Thus, anything longer than 10 d is appropriate.

One limitation of the present study is the small number of experimental animals. Additionally, the observation time after TEF formation was shorter for the control group than the study group, and the observation time could be appropriately extended if effective methods could be applied to reduce the choking and pain of the control animals. The anatomical characteristics of the trachea and esophagus in pigs are more similar to those in humans, and thus, an animal model of TEF that is more similar to human TEF may be obtained if experimental pigs are adopted.

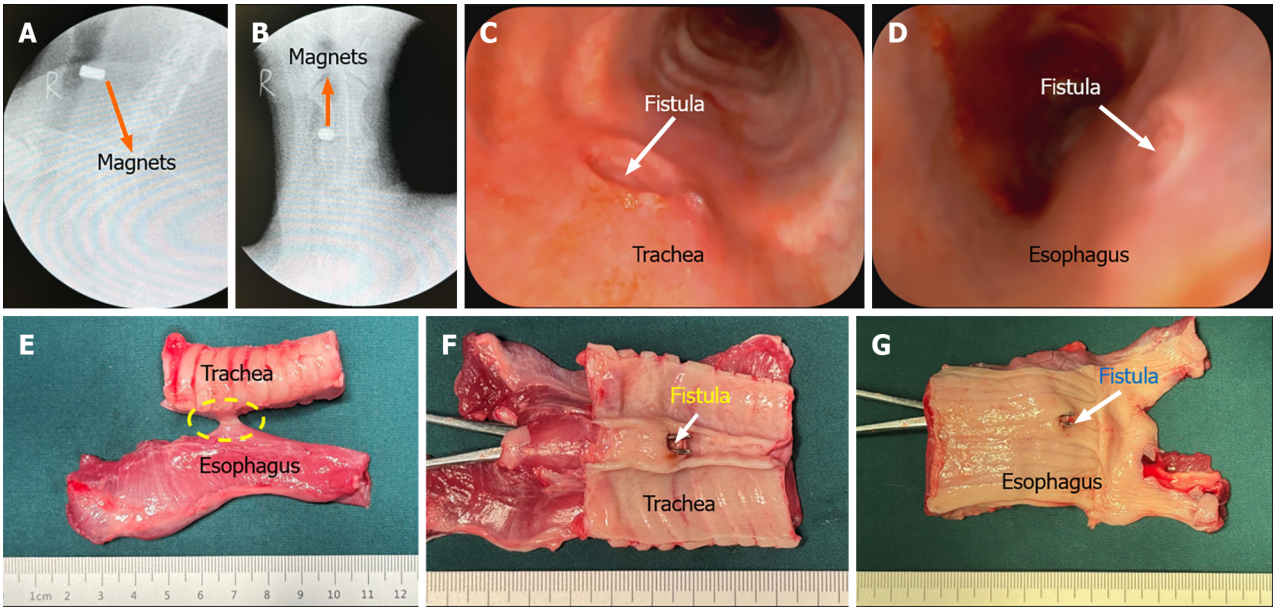


**Figure 5 Gross tracheoesophageal fistula specimens from the study group.** A and B: At 2 wk after surgery, the magnet positions had not changed; C: Bronchoscopy showing a fistula located in the posterior wall of the trachea; D: Gastroscopy showing a fistula located in the anterior wall of the esophagus; E: Gross specimen of tracheoesophageal fistula; F: Gross specimen showing the fistula in the trachea; and G: Gross specimen showing the fistula in the esophagus.

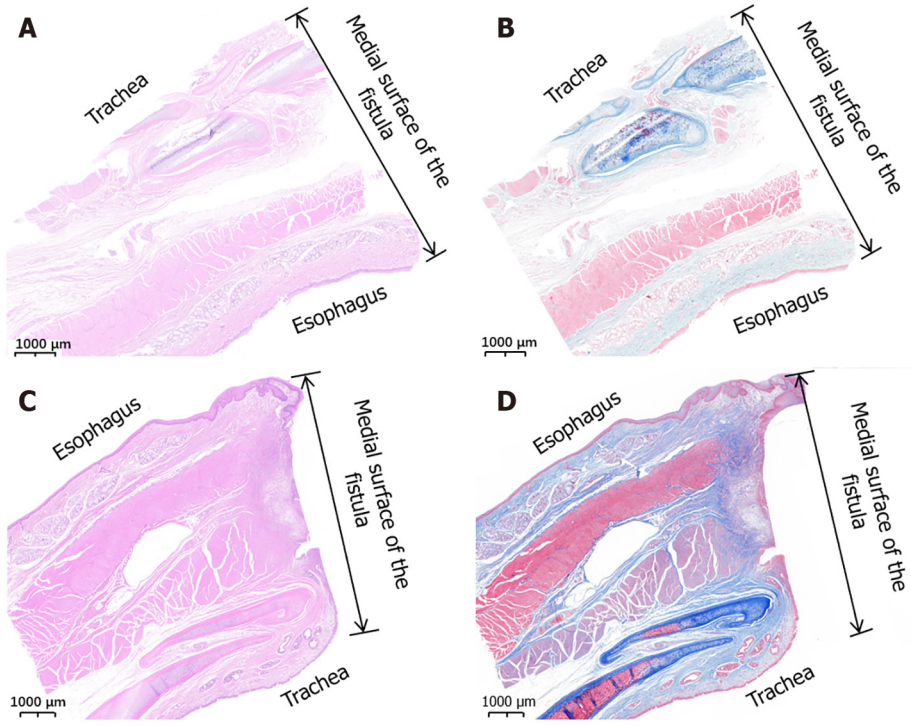


**Figure 6 Histological analysis of tracheoesophageal fistula.** A and B: Hematoxylin and eosin (HE) and Masson trichrome staining of tracheoesophageal fistula from the control group (1.5 ×); C and D: HE and Masson trichrome staining of tracheoesophageal fistula from the study group (1.3 ×).

To date, few reports have described the establishment of animal models of TEF. In one example, a rat model of TEF was prepared by injecting mitomycin into pregnant rats[29]. For larger animals, surgical methods have been required, and a pig TEF model was established by surgical methods[6]. A disadvantage of this model is that the operation is complicated and traumatic, which is not conducive to the next stage of intervention surgery. The successful establishment of a TEF model in a larger animal by applying the magnetic compression technique is beneficial to the development of intervention and treatment measures.

## CONCLUSION

The present study demonstrated the feasibility of establishing TEF using the magnetic compression technique and revealed the advantages of using a T-shaped magnet scheme rather than traditional cylindrical magnets.

## FOOTNOTES

**Co-first authors:** Miao-Miao Zhang and Jian-Qi Mao.

**Co-corresponding authors:** Xiao-Peng Yan and Yi Lyu.

**Author contributions:** Lyu Y and Yan XP designed and coordinated the study; Zhang MM, Mao JQ, Shen LX, Lyu X, Ma J, and Yan XP performed the research and acquired the data; Zhang MM, Mao JQ, and Shen LX analyzed the data; Zhang MM and Shi AH tested and analyzed the magnetic force; Zhang MM and Lyu X wrote the manuscript; Yan XP and Lyu Y conceived of the study and contributed to the study design, the interpretation of the results, and the critical revision of the manuscript; and all authors read and approved the final manuscript.

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LETTER TO THE EDITOR

# Ability of *Helicobacter pylori* to internalize into *Candida*

Zheng-Hong Chen, Jian-Chao Sun, Ting-Xiu Yang, Gu-Zhen Cui

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## Abstract

The following are our views regarding the "letter to the editor" (*Helicobacter* is preserved in yeast vacuoles! Does Koch's postulates confirm it?) by Alipour and Gaeini, and the response "letter to the editor" (*Candida* accommodates non-culturable *Helicobacter pylori* in its vacuole-Koch's postulates aren't applicable) by Siavoshi and Saniee. Alipour and Gaeini rejected the methods, results, discussion, and conclusions summarized in a review article by Siavoshi and Saniee. The present article reviews and discusses evidence on the evolutionary adaptation of *Helicobacter pylori* (*H. pylori*) to thrive in *Candida* cell vacuoles and concludes that *Candida* could act as a Trojan horse, transporting potentially infectious *H. pylori* into the stomach of humans.

**Key Words:** *Helicobacter pylori*; *Candida* yeast; Intracellular presence; *Helicobacter pylori*-specific gene; *Helicobacter pylori* transmission

**Core Tip:** The authors of the "letter to the editor" (*Helicobacter* is preserved in yeast vacuoles! Does Koch's postulates confirm it?) described "shortcomings" of the review article "Vacuoles of *Candida* yeast behave as a specialized niche for *Helicobacter pylori*" published in the *World Journal of Gastroenterology*. Here, we present our view that *Candida* spp. can indeed serve as reservoirs for *Helicobacter pylori*.

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## TO THE EDITOR

We read with interest the December 2017 letter to the editor "*Helicobacter* is preserved in yeast vacuoles! Does Koch's postulates confirm it?" by Alipour and Gaeini[1], and the February 2018 response letter "*Candida* accommodates non-culturable *Helicobacter pylori* in its vacuole-Koch's postulates aren't applicable" by Siavoshi and Saniee[2]. The argument originated from the review article "Vacuoles of *Candida* yeast behave as a specialized niche for *Helicobacter pylori*" by Siavoshi and Saniee[3]. To ensure clarity and comprehension, we have designated the authors of this review[3] and the response letter[2] as proponents, while referring to the authors of the December 2017 letter[1], who hold a contrasting viewpoint, as opponents.

The review article[3] presented the research findings on *Helicobacter pylori* (*H. pylori*) infection in *Candida* cells. Moving bacterium-like bodies inside the vacuoles of *Candida* spp. isolated from feces[4], vaginal discharge, and oral samples[5] were observed by light microscopy. Because bacterial cells can't be cultured from disrupted *Candida* cells, *H. pylori*-specific *16S rRNA*, *ureAB*, *vacA s1*, and *ahpC* genes from the whole DNA of *Candida* isolates similar in size to those of the control *H. pylori* were amplified[5,6] by PCR to reveal their bacterial nature. *H. pylori*-specific proteins in the protein pool of Candida cells were assessed by Western blot analysis using IgY-*H. pylori* raised in hens and IgG1-*H. pylori* infections in mice[7]. Antigen of *H. pylori* within the vacuoles of *Candida* was detected using direct immunofluorescence[8]. To avoid bacterial contamination, *H. pylori*-specific gene- or protein-positive *Candida* isolates were passaged several times on yeast extract-glucose agar with chloramphenicol. The presence of *H. pylori* genes and proteins persisted in the subcultures of *Candida* isolates, indicating that the transmission of the bacterium is integral to the transfer of *Candida* vacuolar content. The proponents concluded that *Candida* yeast serves as a host that protects *H. pylori* against stress outside the stomach, provides nutrients for the survival of *H. pylori*, and mediates its transmission[3].

The opponents[1], however, have argued that the review article[3] was not prepared in a scientific manner, and the methodology used was inadequate; therefore, they felt that the conclusion reached was incorrect. Subsequently, 11 months later, the proponents published a "letter to the editor" in response to all comments by the opponents[2].

The internalization of *H. pylori* into *Candida* may be crucial for *H. pylori* transmission and potentially change our understanding of the transmission route of this bacterium; therefore, most of our experiments focused on the internalization of *H. pylori* into *Candida* reported in the review[3]. Our results are consistent with those reported in the articles[4-8] analyzed by the proponents[3]. In the present study, *Candida*, including gastric, intestinal, and vaginal isolates from patients diagnosed with *H. pylori* infection were subcultured for more than 10 generations. *H. pylori*-specific 16S rDNA, *cagA* gene fragments, and *H. pylori*-specific antigens were still detectable, and some *H. pylori* 16S rDNA-positive *Candida* strains exhibited urease activity. We previously published a paper titled "Intracellular presence and genetic relationship of *Helicobacter pylori* within neonates' fecal yeasts and their mothers' vaginal yeasts"[9].

Hence, we agree with Farideh Siavoshi's (proponent) opinion that the establishment of *H. pylori* inside the ubiquitous yeast might explain why such fastidious bacteria can survive outside the stomach and remain highly prevalent in certain human populations, with yeast acting as a Trojan horse, carrying the potentially infectious *H. pylori* into the stomach[2,3]. Moreover, based on our experimental results, we present our opinion on the "shortcomings" of the article written by the opponents[1].

The opponents stated that the criteria established by Robert Koch for identifying a specific microorganism or pathogen were not adopted in the methodology used in the proponents' study[1]. However, the proponents[3] have mentioned that their study aimed to show that yeast cells can serve as a specialized niche and environmental reservoir for *H. pylori*. Because *H. pylori* in *Candida* cells is not culturable, Koch's postulates were not applicable. Moreover, we took into account the perspective highlighted by Fredricks and Relman[10]: "The power of Koch's postulates comes not from their rigid application, but from the spirit of scientific rigor that they foster. Proof of disease causation rests on the concordance of scientific evidence, and Koch's postulates serve as guidelines for collecting this evidence". Evans[11], who interpreted Koch's postulates with a modification describing the use of immunologic evidence for proof of disease causation, noted that "failure to fulfill the Henle-Koch postulates does not eliminate a putative microorganism from playing a causative role in a disease. Postulates of causation must change with the technology available to prove them and with our knowledge of the disease". These postulates have been invoked for sequence-based identification of bacterial pathogens, for resolving outbreaks of infectious diseases, and for defining the causation of certain noninfectious diseases[12]. Although no live *H. pylori* have been cultured from *Candida* cells positive for *H. pylori*-specific genes, released *H. pylori* from *Candida* cells can be grasped using magnetic beads coated with anti-*H. pylori* antibody[13]. Moreover, in our study,

**Table 1 Divergent perspectives about the intracellular occurrence of *Helicobacter pylori* in *Candida***

| Experimental facts and conclusions from Siavoshi and Saniee (proponents)[2-8] | The opposing view from Alipour and Gaeini (opponents)[1] | Experimental facts and conclusions from our lab[9] |
|---|---|---|
| The yeast cell can serve as a specialized niche and environmental reservoir for *H. pylori*. Koch's postulates are not applicable | Since Koch's postulates were not practiced in the study of *H. pylori* internalizing *Candida*, the hypothesis that the yeast can act as a vehicle to transfer *H. pylori* into humans is incorrect | Although no live *H. pylori* have been cultured from *Candida* cells, *H. pylori*-specific genes, antigens, and urease activity are positive in these *Candida* strains. The potency of *H. pylori* -internalized *Candida* in disease transmission and pathogenicity can be determined by molecular Koch's postulates |
| The IgY-*H. pylori* antibody has been used as a marker for localizing *H. pylori* inside yeast vacuoles | The presence of *H. pylori* in yeast cells demonstrated by IgY-*H. pylori* is inaccurate | The presence of *H. pylori* in vaginal and fecal *Candida* has been determined through immunofluorescence microscopy with IgG-*H. pylori* |
| The intracellular occurrence of *H. pylori* in the vaginal yeast of pregnant mothers provides potency for the transmission of *H. pylori* to newborns through vaginal yeast | The intracellular occurrence of *H. pylori* inside yeast is not reliable. If yeast can host *H. pylori*, the prevalence of *H. pylori* infection should be higher in females than in males owing to the higher yeast infection rate in the female population. However, the situation is the other way around | The intracellular occurrence of *H. pylori* in vaginal *Candida* of mothers and fecal *Candida* of newborns has been determined and suggests the transmission of *H. pylori* to newborns through vaginal yeast |

Our experimental results and perspectives are consistent with Siavoshi and Saniee (proponents). *H. pylori*: *Helicobacter pylori*.

*H. pylori-16S rDNA*- and *ureA*-positive *Candida* strains isolated from vaginal or fecal samples expressed urease activity, whereas *H. pylori*-negative *Candida* strains were urease-negative. Therefore, the significance of *H. pylori*-internalized *Candida* in disease transmission, latency, and pathogenicity cannot be excluded.

The opponents stated that IgY is not accurate enough for such an experiment. To our knowledge, we agree with the proponents that IgY-*H. pylori* demonstrated precise and specific interactions with *H. pylori* antigens. Our current study utilized IgG-*H. pylori* to detect *H. pylori* antigens in vaginal *Candida* and fecal *Candida* isolated from mothers and their newborns, respectively[9], as well as in gastric *Candida* strains isolated from patients. Our results showed that *H. pylori* antigen in *Candida* subcultures could be detected using immunofluorescence microscopy.

The opponents believe that the prevalence of *H. pylori* infection should be higher in women than in men because of the higher yeast infection rate in the female population. However, actual scenarios contradict this expectation[1]. As refuted by the proponents[2], the relationship between yeast-positive individuals and frequency of *H. pylori* infection has not been discussed in their articles. Therefore, we agree with their hypothesis. In our experiment, *Candida* was isolated not only from vaginal discharge but also from feces, and no difference in the positivity rate was noted for *H. pylori*-specific genes or antigens in gastrointestinal *Candida* isolated from males or females infected with *H. pylori*.

Table 1 presents the divergent perspectives of the proponents and opponents, along with the viewpoints derived from the outcomes of our experiments. In conclusion, based on our experimental results, we agree with the proponents Siavoshi *et al*[6] that *Candida* could be a reservoir for *H. pylori*. Nevertheless, more in-depth studies are needed to elucidate the internalization process of *H. pylori* in *Candida* cells, its significance in the spread of *H. pylori* among humans, and long-term colonization of *H. pylori* in the gastric epithelium.

## FOOTNOTES

**Author contributions:** Chen ZH analyzed the literature and wrote the letter; Sun JC and Yang TX performed the research mentioned in the letter; Cui GZ proposed the idea and revised the letter; and all authors have read and approved the final manuscript.

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LETTER TO THE EDITOR

# Transjugular intrahepatic portosystemic shunt: A promising therapy for recompensation in cirrhotic patients

Ya-Ni Jin, Wei Zhang

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## Abstract

This is a retrospective study focused on recompensation after transjugular intrahepatic portosystemic shunt (TIPS) procedure. The authors confirmed TIPS could be a treatment for recompensation of patients with cirrhosis according to Baveno VII. The paper identified age and post-TIPS portal pressure gradient as independent predictors of recompensation in patients with decompensated cirrhosis after TIPS. These results need to be validated in a larger prospective cohort.

**Key Words:** Cirrhosis recompensation; Transjugular intrahepatic portosystemic shunt; Portal pressure gradient; Predictor factor; Baveno VII



**Core Tip:** Approximately one-third of the patients experienced cirrhosis recompensation following transjugular intrahepatic portosystemic shunt (TIPS) and post-TIPS portal pressure gradient reduction below 12 mmHg contributes to the occurrence of recompensation. The selection of a smaller diameter (6 mm) stent may be an effective measure to reduce portal vein pressure while decreasing the incidence of postoperative hepatic encephalopathy.

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## TO THE EDITOR

Cirrhosis recompensation, as defined by the Baveno VII consensus, refers to a condition in which patients with decompensated cirrhosis exhibit stable improvement in liver function without any episodes of decompensation over an extended period, following effective etiological therapy[1]. Effective etiological treatment is paramount for recompensation and involves sustained alcohol abstinence in cases of alcohol-induced cirrhosis, sustained viral suppression in hepatitis B-related cirrhosis, and viral elimination in hepatitis C-related cirrhosis. However, research on the recompensation of cirrhosis due to other causes remains scarce.

The transjugular intrahepatic portosystemic shunt (TIPS) procedure reduces the portal pressure gradient (PPG) and is primarily utilized to manage various complications of portal hypertension in cirrhotic patients, such as esophagogastric variceal bleeding and refractory ascites. Thus, TIPS may promote recompensation in patients with decompensated cirrhosis. The study by Gao *et al*[2] was pioneering in investigating the impact of TIPS on recompensation across different etiologies of decompensated cirrhosis. Their findings indicated that approximately one-third of the patients achieved cirrhosis recompensation following TIPS and identified a postoperative PPG of less than 12 mmHg as an independent predictor of cirrhosis recompensation. Although a reduction in PPG is associated with a decreased risk of variceal rebleeding, the risk of hepatic encephalopathy post-TIPS escalates with the magnitude of PPG reduction[3], potentially impeding recompensation. Consequently, employing small-diameter (6-mm) stents may be an effective strategy to mitigate portal hypertension and minimize the risk of postoperative hepatic encephalopathy, warranting further investigation. Additionally, the findings of this study necessitate validation in a larger, prospective cohort due to its limited sample size and retrospective nature. Furthermore, including only TIPS patients limits the study; comparing the recompensation frequency between TIPS and non-TIPS groups could elucidate the procedure's facilitative role in cirrhosis recompensation. Future research should elucidate how TIPS influences recompensation across various cirrhosis etiologies, with a focus on developing tailored treatment strategies to enhance patient outcomes.

## FOOTNOTES

**Author contributions:** Jin YN wrote the letter; Zhang W revised the letter.

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