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REVIEW

Epidemiology of stomach cancer

Milena Ilic, Irena Ilic

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Abstract

Despite a decline in incidence and mortality during the last decades, stomach cancer is one of the main health challenges worldwide. According to the GLOBOCAN 2020 estimates, stomach cancer caused approximately 800000 deaths (accounting for 7.7% of all cancer deaths), and ranks as the fourth leading cause of cancer deaths in both genders combined. About 1.1 million new cases of stomach cancer were diagnosed in 2020 (accounting for 5.6% of all cancer cases). About 75% of all new cases and all deaths from stomach cancer are reported in Asia. Stomach cancer is one of the most lethal malignant tumors, with a five-year survival rate of around 20%. There are some well-established risk factors for stomach cancer: *Helicobacter pylori* infection, dietary factors, tobacco, obesity, and radiation. To date, the most important way of preventing stomach cancer is reduced exposure to risk factors, as well as screening and early detection. Further research on risk factors can help identify various opportunities for more effective prevention. Screening programs for stomach cancer have been implemented in a few countries, either as a national or opportunistic screening of high-risk individuals only. Generally, due to its high aggressiveness and heterogeneity, stomach cancer still remains a severe global health problem.

Key Words: Stomach cancer; Epidemiology; Incidence; Mortality; Survival; Predictive factors; Prevention

Core Tip: Despite the decline in incidence and mortality during the last decades, stomach cancer is one of the main health challenges worldwide. According to the GLOBOCAN 2020 estimates, stomach cancer caused approximately 800000 deaths, and ranks as the fourth leading cause of deaths from cancer in both genders combined. Around 1.1 million new stomach cancer cases were diagnosed in 2020. There are some well-established risk factors for stomach cancer: *Helicobacter pylori* infection, dietary factors, tobacco, obesity, and radiation. To date, the most important way of preventing stomach cancer is reduced exposure to risk factors, as well as screening and early detection.

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INTRODUCTION

Stomach cancer was the fifth most common malignant tumor in the world in 2020 with approximately 1.1 million new cases, and is the fourth leading cause of cancer death, with around 800000 deaths[1,2]. Over 85% of stomach cancer cases are registered in countries with high and very high Human Developing Index (590000 and 360000 cases, respectively)[1]. The highest number of cases of stomach cancer (almost 820000 new cases and 580000 deaths) was registered in Asia (mainly in China)[1,2-4]. The estimated five-year survival rate is lower than 20%[2,5-8].

Worldwide, stomach cancer incidence and mortality correlate with increasing age and are relatively rare in persons of both gender younger than 45 years[2,4,7]. The frequency of stomach cancer in men is approximately double that in women[1-3]. In men, stomach cancer was the most commonly diagnosed cancer in 2020 in seven countries (all countries were in Asia: Iran, Afghanistan, Turkmenistan, Uzbekistan, Tajikistan, Kyrgyzstan, and Bhutan) and the leading cause of death from cancer in ten countries (Iran, Afghanistan, Tajikistan, Kyrgyzstan, and Bhutan in Asia, Mali and Cape Verde in Africa, Colombia and Peru in South America, and Costa Rica in Central America)[1,2]. Although stomach cancer was not the most diagnosed cancer in women in any country, stomach cancer was the leading cause of death from cancer among females in three countries (Tajikistan, Bhutan, and Peru)[1,2]. The incidence and mortality rates from stomach cancer were generally low in Northern America and Northern Europe in 2020 and equivalent to rates registered across most of the African regions[1,2].

In the first half of the 20th century, gastric cancer was the leading cause of death from malignant tumors in the United States and Europe[9,10]. Over the past decades, the incidence and mortality due to stomach cancer have substantially declined in many countries[1,2].

Stomach cancer is a multifactorial disease[9-12], including both lifestyle and environmental risk factors *Helicobacter pylori* (*H. pylori*) infection, low socioeconomic status, dietary factors, such as high intake of salty and smoked food and low consumption of fruits and vegetables, fiber intake, in addition to tobacco smoking, alcohol use, low physical activity, obesity, radiation, gastroesophageal reflux disease, positive family history and inherited predisposition. However, the etiology of stomach cancer has not yet been sufficiently elucidated.

Topographically, stomach cancer is classified into two subsites: cardia stomach cancer (arising from the upper stomach) and noncardia stomach cancer (arising from the other parts of the stomach), which differ in epidemiologic patterns and etiology[13]. The majority of all stomach cancers (approximately 90%) are adenocarcinomas, while other types (including lymphoma, sarcoma, neuroendocrine tumors) are rare[12,14]. Two major histologic types of stomach cancer adenocarcinomas are diffuse and intestinal, which differ in epidemiological peculiarities, such as age at diagnosis, gender ratio, *etc.*[15,16].

Despite the strong declining trends in incidence and mortality, stomach cancer remains an important part of the global burden of cancer. Many of the risk factors remain insufficiently understood and need to be the focus of further research in order to achieve more specific, targeted prevention measures.

INCIDENCE

Worldwide, there is a considerable geographic variation in stomach cancer incidence. Stomach cancer incidence rates in 2020 were highest in Eastern Asia (22.4 per 100000 people), followed by Central and Eastern Europe (11.3 per 100000 people), and South America, Polynesia and Western Asia (equally about 8.6 per 100000 people) (Figure 1A)[2]. The lowest rate (3.3 per 100000 people) was registered in Southern Africa.

More than three quarters (75.3%; 819944) of all stomach cancer cases are residents of Asia[2]. Most (86.7%; 944591 cases) stomach cancer cases were residents of more developed regions. The least number of stomach cancer cases was recorded in Micronesia/Polynesia.

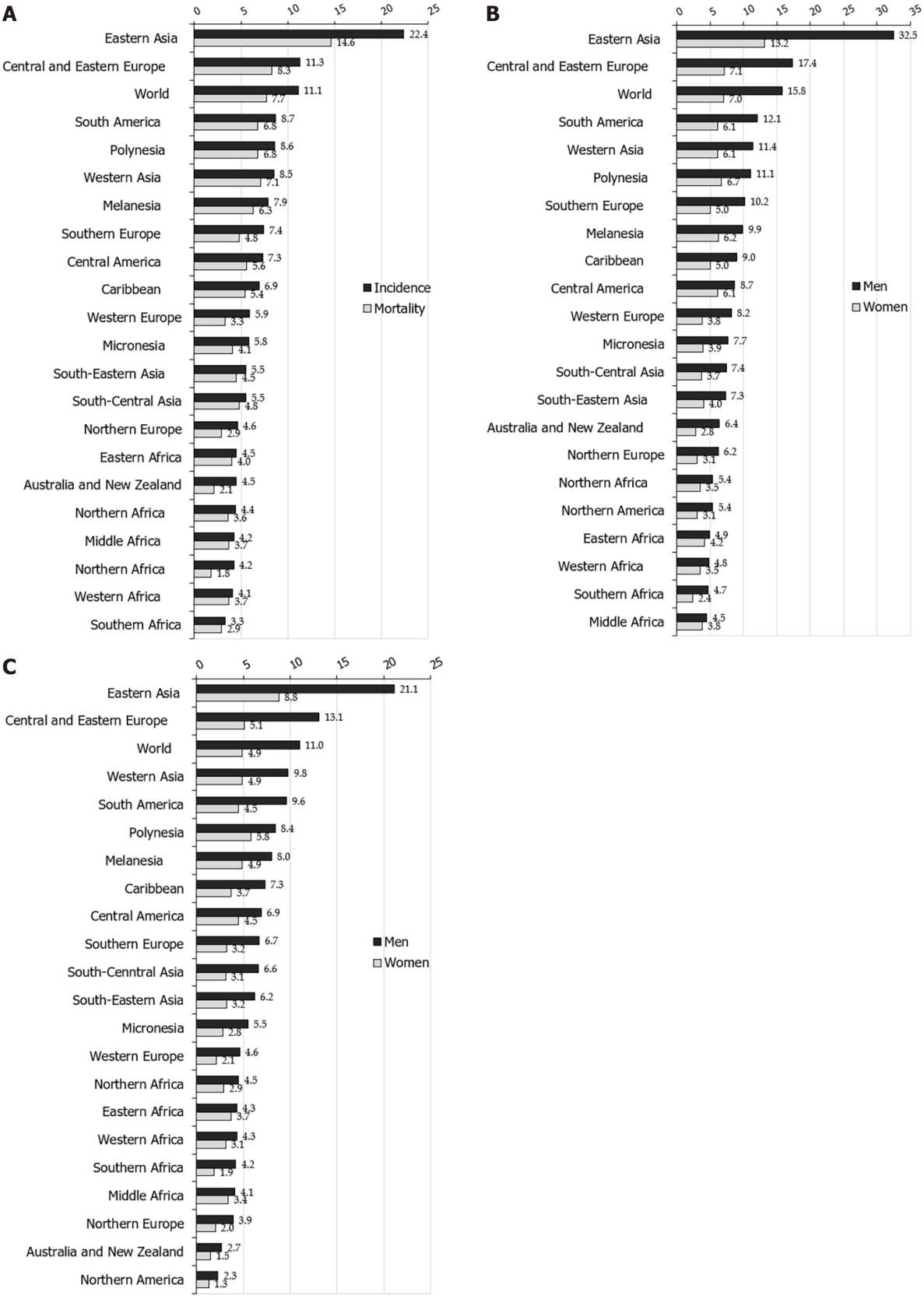

Figure 1 Stomach cancer incidence and mortality, by regions. A: Stomach cancer incidence and mortality; B: Stomach cancer incidence in men and women; C: Stomach cancer mortality in men and women. GLOBOCAN 2020 estimates[2]: Age Standardized Rate (using World standard population, per 100000).

Notable variations in the incidence of stomach cancer in 2020, as well as for mortality, exist around the world (Figures 1-4)[2]. The highest incidence rates were recorded in countries of eastern Asia (Mongolia, Japan, Republic of Korea), while the highest death rates were observed in countries of western Asia (Tajikistan, Kyrgyzstan, Iran). The lowest incidence and mortality rates of stomach cancer

were recorded in Northern America and Northern Europe, Australia/New Zealand and some African countries. Patterns in females were broadly similar to those observed in males, but large differences were observed between sexes and throughout different countries/regions (Figures 1-4)[2]. Globally, the incidence rate of stomach cancer in males was 15.8 per 100000 in 2020, and in females 7.0 per 100000 (Figure 1B)[2]. The gastric cancer incidence rates were about 2 to 3 times higher in males than in females (ranging from 32.5 per 100000 in Eastern Asia to 4.5 per 100000 in Middle Africa for men, and in women ranging from 13.2 in Eastern Asia to 2.4 in Southern Africa) (Figure 1B)[2]. By countries, the differences were fifty-fold: the incidence rates of gastric cancer in men ranged from 48.1 per 100000 in Japan to 1.0 per 100000 in Mozambique in 2020 (Figure 2A). Also, similar differences were observed by regions: the highest incidence rates were reported in Eastern Asia (Japan: 48.1, Mongolia: 47.2, Republic of Korea: 39.7), while the lowest rates were recorded in South Africa (Mozambique: 1.0, Lesotho: 2.1). The incidence rates of stomach cancer in women ranged from 20.7 per 100000 inhabitants in Mongolia (followed by Tajikistan: 18.7, Republic of Korea: 17.6 and Japan: 17.3) to about 0.5 in Indonesia and Mozambique in 2020 (Figure 2B).

However, the distribution of stomach cancer did not have a clear geographical pattern: namely, even though the highest risk populations in the world are in Asian countries (*e.g.* Japan, Mongolia, Republic of Korea), some other countries in Asia register relatively low rates (such as Sri Lanka, Indonesia, Thailand) (Figure 2A and B)[2]. On the other hand, in some low-risk populations, there are some high-risk groups for stomach cancer, such as Koreans and Japanese who live in the United States[17,18].

Also, rates varied across races. Stomach cancer incidence in men in the United States was highest in blacks, followed by Asians/Pacific Islanders, Hispanics, and American Indian/Alaska natives[7]. In women, the highest rates were registered in Hispanics, followed by blacks and Asian/Pacific Islanders, and American Indian/Alaska natives. In the United States, for both sexes, the lowest rates were recorded in whites.

Also, incidence and mortality of gastric cancer in all indigenous groups exceeded the frequency among their non-indigenous counterparts: the highest gastric cancer rates were registered in Indigenous Siberians, Mapuche in Chile and among Alaskan Inuit[19]. Additionally, increasing incidence trends were observed in some indigenous groups, especially in Inuit residing in the circumpolar region and in Maori in New Zealand.

Although differences in stomach cancer incidence in different parts of the world are still not fully clear, most of the variation in stomach cancer incidence worldwide is due to variations in exposure to environmental or lifestyle related risk factors[20-22]. Additionally, migrant studies[23] and secular trends of gastric cancer rates also indicate that environmental factors have an important role in the etiology of gastric cancer[24]. The most important established risk factor for gastric cancer is infection with *H. pylori*[25]. Internationally, variations in *H. pylori* infection prevalence show similarities with variations in stomach cancer prevalence; in developing countries, *H. pylori* infection prevalence in adults is 76% *vs* 58% in developed countries[26]. The prevalence was estimated to be 77.6% in South Africa, 55.8% in China, 52.2% in Mexico, 24.6% in Australia and 22.1% in Denmark[27]. In the United States of America, the prevalence in non-Hispanic blacks was 53%, in Mexican Americans was 62%, but was 26% among non-Hispanic whites[28]. In part, the geographical variation of *H. pylori* infection rates correlate with the frequency of stomach cancer across populations. On the other hand, certain highly infected populations (*e.g.* in Africa and South Asia), unlike the East Asian countries, do not have a high incidence of stomach cancer, which can be explained, at least in part, by the differences in prevalence of genotypes of *H. pylori* (in East Asian the vacA m1 genotype is predominant, whereas the m^2 genotype predominated in Africa, South Asia, and Europe)[29].

Additionally, several other environmental factors are also considered as contributors to gastric cancer occurrence[21,22,30]. Differences between sexes and international variations could likely be due to tobacco smoking[31]. Based on the Global Burden of Disease study[22], the drop in burden of stomach cancer was associated with improved Socio-demographic Index, then to high-sodium diet in both genders combined, as well as to smoking in males, in particular in east Asian populations.

In addition, some research points to the role of aging[24] and hereditary and genetic factors[6] in stomach cancer burden. Incidence differences by sex have never been fully explained, but some theories have suggested a protective role of female sex-specific hormones[15,32]. A higher stomach cancer incidence in males than in females may be due to differences in the incidence of different subtypes of adenocarcinoma according to histology (intestinal or diffuse) and location (proximal or distal)[12,13,33]. Diffuse adenocarcinoma is more common in younger and female patients, whereas intestinal adenocarcinoma is more common in males and the elderly[15]. Intestinal adenocarcinoma dominates high-risk areas and is considered responsible for much of the international variation in incidence. The observed differences in stomach cancer incidence worldwide could be due to diagnostic capacity and changes in the quality of registries, where coverage, completeness and accuracy vary by country[34].

MORTALITY

Nearly three quarters of stomach cancer deaths (74.8%; 575206 deaths) were registered in Asia[2]. Most

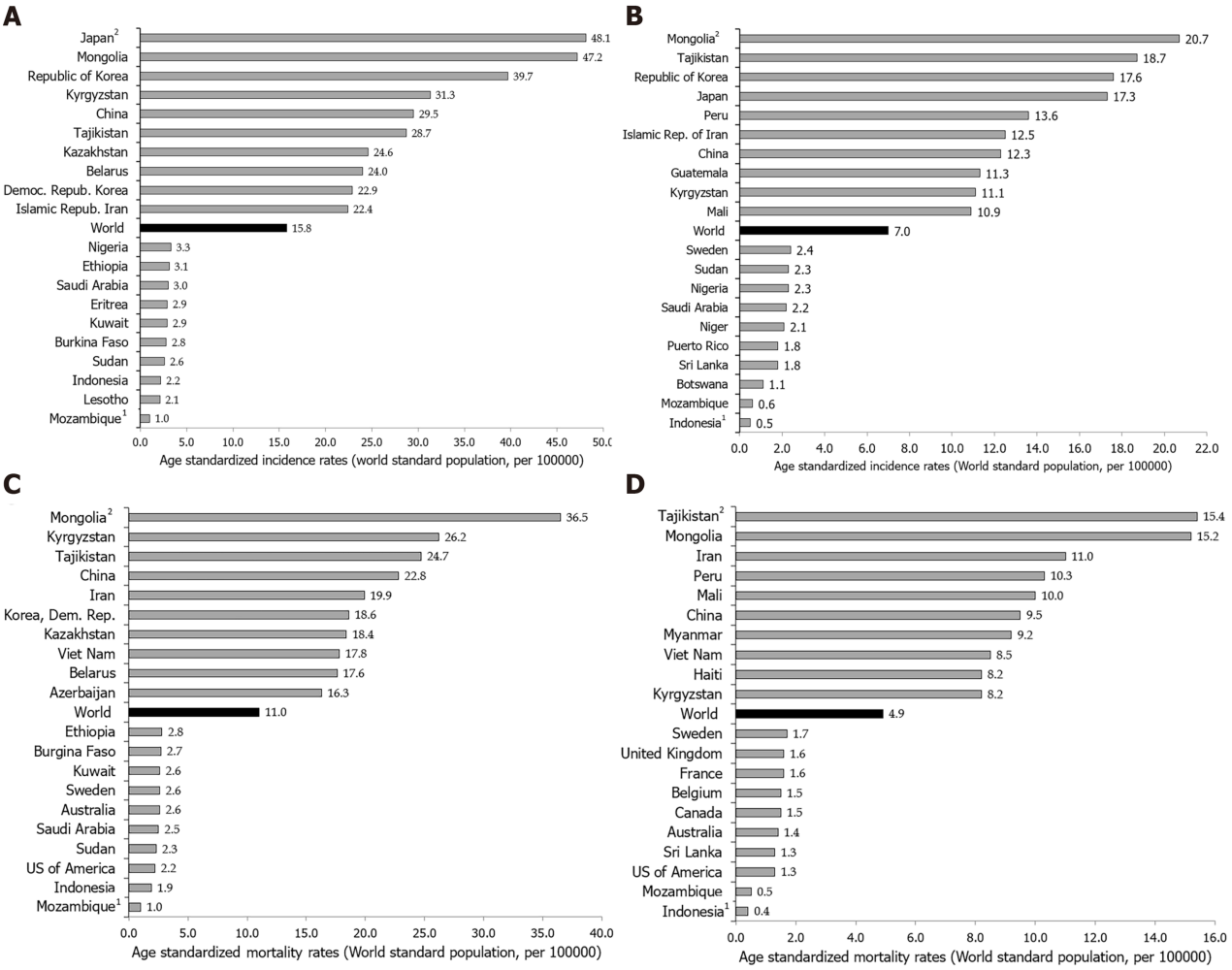

Figure 2 Stomach cancer incidence and mortality, by countries. A: Stomach cancer incidence in men; B: Stomach cancer incidence in women; C: Stomach cancer mortality in men; D: Stomach cancer mortality in women. GLOBOCAN 2020 estimates[2]. [1]Country with the lowest rates; [2]Country with the highest rates.

(83.7%, 643609 deaths) of those who died due to stomach cancer were residents of more developed regions. The least number of deaths were recorded in Micronesia/Polynesia.

Stomach cancer mortality varies greatly across populations and regions. Mortality rates for stomach cancer in 2020 in both genders were highest in the Eastern Asia region (14.6 per 100000 people), followed by South America, Polynesia, Western Asia and Central and Eastern Europe (equally about 8.5 per 100000 people) (Figure 1A)[2]. The lowest mortality rates (about 2.0 per 100000 people) were registered in Northern Africa and Australia. The differences in mortality rates were thirty-fold between the population with the highest rate (Mongolia - 24.6), and the one with the lowest rate (Mozambique - 0.7).

Stomach cancer mortality by gender shows significant geographic variations[1,2,6-8]. Globally, the mortality rate of stomach cancer in males in 2020 was 11.0 per 100000, and in females 4.9 per 100000 (Figure 1C)[2]. The region with the highest mortality rates due to stomach cancer in 2020 in both genders was Eastern Asia (21.3 and 8.8 per 100000, respectively) (Figure 1C)[2]. The lowest rates of stomach cancer mortality in both sexes were in North America (2.3 and 1.3 per 100000, respectively). In men, the risk of dying from stomach cancer was highest in Mongolia (36.5), followed by Kyrgyzstan, Tajikistan and China (approximately 25.0 per 100000) (Figure 2C). By contrast, the risk of death from stomach cancer was lowest in men in Mozambique (1.0) and Indonesia (1.9). Women living in Tajikistan and Mongolia had the greatest risk (approximately 15.0 per 100000) of death from stomach cancer, while the risk for women in Indonesia and Mozambique was lowest (less than 1.0 per 100000) (Figure 2D).

Gastric cancer mortality rates begin to rise in middle-aged persons, with the highest rates observed in the elderly (aged 75 years and older) age group for both males and females (Figure 4B).

Stomach cancer mortality showed apparent geographical variability. Generally, the large differences in mortality rates are between developing and developed countries. Considering developed countries, this mortality pattern could be explained by increased hygiene standards, dissemination of food refrigeration, better preservation of food, high intake of fresh fruits and vegetables and eradication of *H. pylori*[11,22,35]. In the second decade of the 21st century in Japan, mortality due to stomach cancer

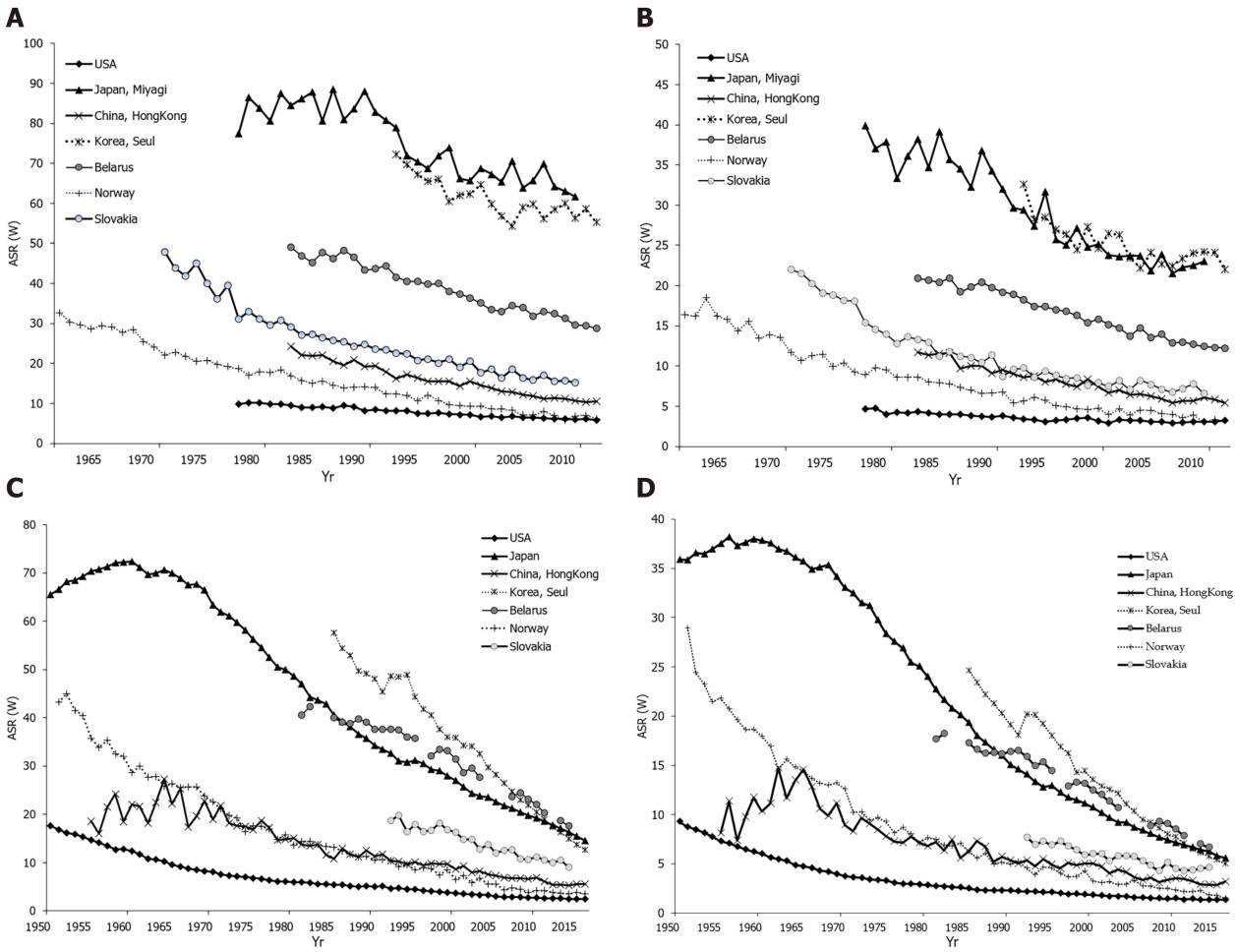

Figure 3 Stomach cancer incidence and mortality trends. A: Stomach cancer incidence trends among men in selected countries; B: Stomach cancer incidence trends among women in selected countries; C: Stomach cancer mortality trends among men in selected countries; D: Stomach cancer mortality trends among women in selected countries. GLOBOCAN 2020 estimates[2]. Age Standardized Rate (using World standard population, per 100000).

reached the levels of Western countries (Figure 4B), which could be attributed to the introduction of gastric cancer screening and to changes in lifestyle, such as the reduction in salt use and an increase in the consumption of fresh fruits and vegetables, improvement in food storage, smoking reduction and prevention of infection with *H. pylori*[22,36,37]. However, reasons for the significant international variations in stomach cancer mortality rates are not fully clear. Diffuse adenocarcinoma is more common in females, while intestinal adenocarcinoma is dominant in males, this subtype being responsible for most of the international variations[15].

There is a wide variation in the relative contribution of cardia and noncardia cancers to the overall number of stomach cancer cases, with a higher proportion of cardia cancers in countries with lower stomach cancer incidence and mortality rates (such as the United States, Canada and Denmark)[38]. In males in Europe, the proportion of cardia and noncardia stomach cancers ranged between 11.6% (Belarus) and 72.0% (Finland), with higher proportions observed in Northern Europe and lower proportions in Eastern Europe. Among other countries worldwide, the proportion of cardia stomach cancers ranged between 5.8% (Republic of Korea) and 64.8% (Iran). In females, a similar geographic pattern was observed, although rates were lower: in Europe, the proportion of cardia and noncardia stomach cancers ranged between 10.6% (Italy) and 44.5% (United Kingdom), while worldwide it ranged from 4.3% (Republic of Korea) to 31.5% (Australia).

Low incidence rates of stomach cancer, which notably became a rare diagnosis among the white United States population, are attributed to the "unplanned triumph" of prevention, which involves a decreased *H. pylori* prevalence and improved food storage and preservation[9,39].

Cancer mortality data are influenced by data on incidence, as well as the success of treatment. Although the World Health Organization estimates present detailed and high-quality information on the incidence and mortality of stomach cancer recorded by cancer registries (regional or national) around the world, these estimates should be interpreted with considerable caution, due to the limited quality and coverage of cancer data worldwide, especially in low- and middle-income countries, due to issues of local data quality, registry coverage, and analytical capacity[2,40,41]. The effect of the coronavirus disease 2019 pandemic on cancer burden is not yet clear, particularly taking into consid-

eration the geographical variations and evolution of the pandemic across countries (because of the lockdown, possible delays in cancer diagnoses, *etc.*)[2].

The differences in availability of improvements in stomach cancer diagnosis and treatment may have had some role in the observed variations in mortality rates worldwide, but this contribution remains open to further quantification[42]. Screening programs and early detection of stomach cancer which have been implemented in Japan[43] and in Korea[44] can partly explain the differences in mortality rates. Also, in Japan, advancements were made in the surgical treatment of early disease, resulting in a better survival rate compared to other countries[45]. However, stomach cancer survival remains unacceptably low in most areas of the world[46,47].

The high prevalence of *H. pylori* infection is widely recognized as the key contributor to high rates of stomach cancer mortality[48]. There is abundant evidence that exposure to other risk factors (tobacco, diet, alcohol use, *etc.*) may have contributed to the apparent international differences in mortality rates of gastric cancer[49-51]. Also, disparities in socio-economic status could have an influence on stomach cancer mortality rates, mediated by varying exposures to infection, environmental factors, as well as barriers in accessing medical care[22,52].

TEMPORAL TRENDS

Declining gastric cancer incidence rates are the dominant epidemiological pattern globally[1,2]. Figures 3A and B show data, for males and females, on stomach cancer incidence secular trends for selected populations. In both sexes, the underlying pattern was a rapid decline in incidence rates over the whole considered time period, regardless of the background stomach cancer risk. There were two exceptions to this pattern. The first exception was seen in the Japanese population (Miyagi prefecture) where, particularly in males, very high rates were observed until the 1990s, and then declined but remained high. The second exception was for the United States population where over the entire time period the rates were constantly very low. The exact reason for the decrease in the incidence of gastric cancer in the last few decades is not completely known, but it most likely includes improvements in diet, food storage and declining prevalence of infection with *H. pylori* due to a general improvement in sanitation and increased use of antibiotics[53]. Eradication of *H. pylori* can be achieved with antibiotic therapy; but, the treatment of asymptomatic carriers is not practical because many countries have a very high infection burden (*e.g.*, over 75% of adult persons living in sub-Saharan Africa have *H. pylori* infection) and reinfection is relatively easy[54,55]. Figure 3C and D represent secular trends for stomach cancer mortality, for males and females, in selected countries over the period 1961 to 2016[2]. Also, downward trends for stomach cancer mortality rates show a very similar pattern as well as incidence trends. In men, the steep decreasing trends for stomach cancer mortality were observed in all selected countries continuously over the observed period. Two exceptions to the mortality pattern were seen. The first exception was for Slovakia where, particularly in women, the rates showed a slower downward trend up to the 2000s, with a flattening of the mortality trend from the 2010s onwards. The second exception was for the United States population where mortality rates remained constantly very low over the entire time period. Stomach cancer mortality in both women and men has shown a significant declining trend in most developed countries over the past 50 years[1,2]. A similar trend, although starting later, has been seen in some countries in Asia, such as Japan and China[37,50]. Factors that led to a decline in mortality involve increased availability of fresh fruits and vegetables, reduced use of salt, reduced incidence of *H. pylori* infection due to improved hygiene and use of antibiotics, and the implementation of screening programs[56,57].

By age, incidence and mortality patterns for stomach cancer in women were broadly similar to those in men, regardless of the background stomach cancer risk being high or low (Figure 4). In selected countries, stomach cancer was predominantly a disease of the elderly, and almost 90% of all cases were diagnosed after the age of 55 years (Figure 4A). For both sexes, stomach cancer mortality continuously increases with age, and is two times higher in those older than 70 years (Figure 4B).

The favorable trend of stomach cancer incidence in developed countries could largely be attributed to a decrease in *H. pylori* prevalence: this is reflected by the "birth cohort effect" where in some countries (including Korea, Japan, the United States) rates of *H. pylori* have been declining in younger generations [24,34,38]. Intestinal adenocarcinoma dominates high-risk areas and is considered responsible for much of the variation in incidence. Recent studies indicate an increase in gastric cancer incidence (cardia and noncardia stomach cancers combined) in persons under the age of 50 in both low- and high-risk countries (such as the United Kingdom, the United States, Canada, Belarus, Chile)[58]. The increasing prevalence of autoimmune gastritis and dysbiosis of the stomach microbiome could have contributed to the increase in stomach cancer incidence among younger generations[59].

In both men and women, trends in the prevalence of cigarette smoking are related to trends in incidence and mortality of stomach cancer with a lag of roughly several decades[22,60]. Besides, stomach cancer mortality trends were minimally influenced by changes in the coding of this disease in the second half of the twentieth century[61].

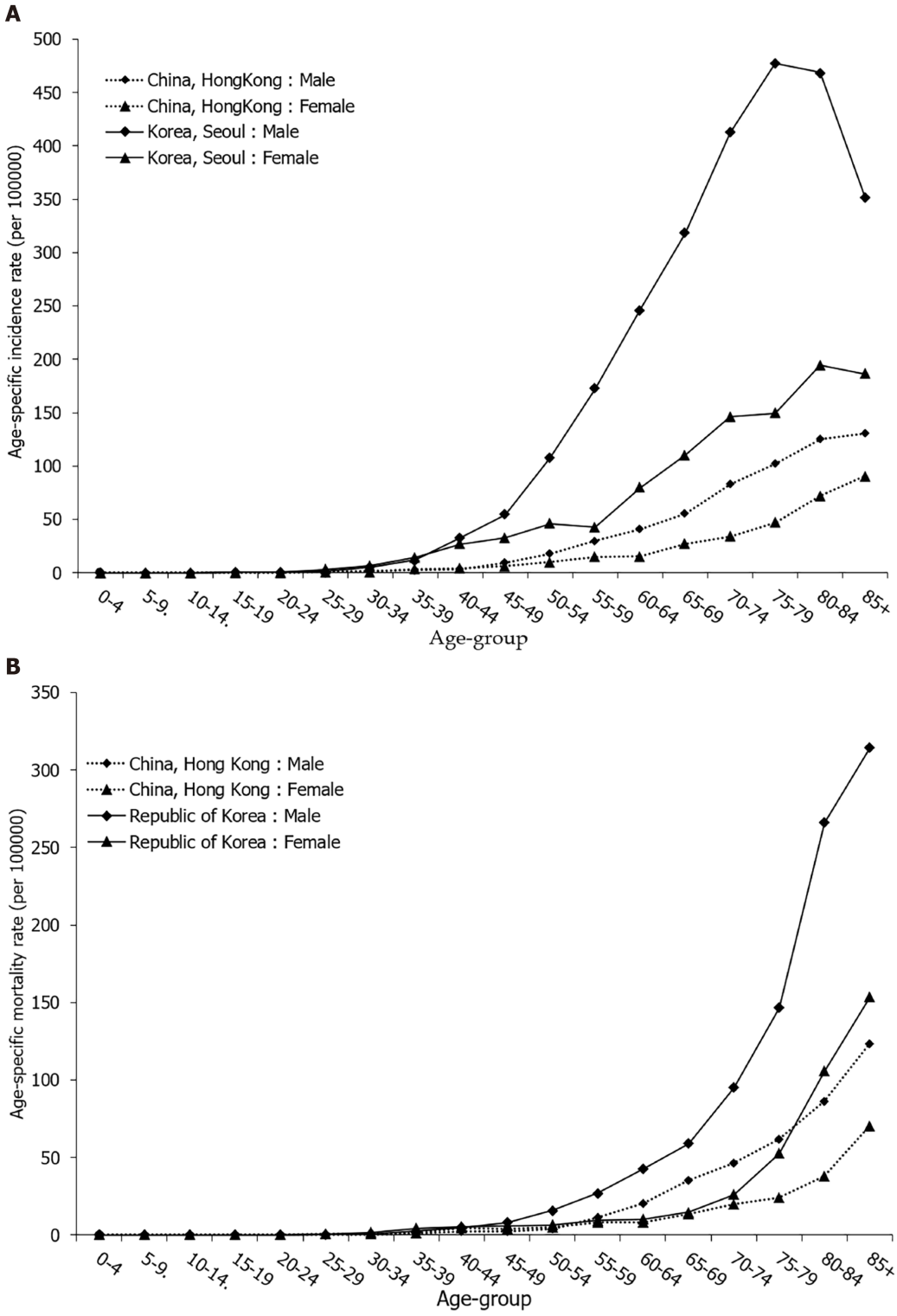

Figure 4 Stomach cancer incidence and mortality trends by age and sex. A: Stomach cancer incidence trends by age and sex in selected countries in 2012; B: Stomach cancer mortality trends by age and sex in selected countries in 2016. GLOBOCAN 2020 estimates[2].

SURVIVAL

In general, survival for patients with stomach cancer is poor[5,62]. In addition, there is global variation in stomach cancer survival[34,47]. Worldwide, with the exception of Japan and Korea, most areas have an overall 5-year relative survival of stomach cancer of about 20%-30%[63]. A 5-year relative stomach cancer survival rate of about 20% is observed in Western developed countries and in developing countries according to an international comparison of data from population based cancer registries[64-66]. In contrast to North America and Europe[66], stomach cancer survival is higher in East Asia: *e.g.*, 5-year survival rate is 67% in Korea and 69% in Japan[67,68], followed by Jordan (56%) and Costa Rica

(46%) in 2010-2014[69]. Also, a notable increase in stomach cancer survival was seen in China (from 30.2% to 35.9%) in recent years[69]. These differences are in part explained by the early stomach cancer detection due to the screening programs implemented in East Asia[63]. The relatively high overall survival for stomach cancer in Japan is the result of a high proportion of patients being diagnosed in the early stage of the disease: in 1995-2000, 53% of stomach cancers were diagnosed at an early stage in Japan[70] in contrast to about 27% in the United States[71]. Differences in tumor biology and stomach cancer subtype (with East/Central Asia and Eastern Europe having a larger proportion of noncardia stomach cancers than North America and Western Europe) may also contribute to survival differences [8,46,72,73]. Worldwide, the proportion of cardia stomach cancers, indicating worse prognosis, ranged from 6% in South Korea to 72% in Finland for men, and for women it ranged from 4% in South Korea to 52% in Serbia[38]. Similarly, cases with the intestinal subtype had a higher survival rate than patients with diffuse tumors[70]. Many factors influence the survival of stomach cancer, including the type of cancer, stage at diagnosis, age, sex, race, overall health, and lifestyle[74-76]. Generally, due to high aggressiveness and heterogeneity, stomach cancer still remains a severe global health problem[47,77].

ETIOLOGY AND RISK FACTORS

Stomach cancer is a multifactorial disease. The notable international variation, time trends, and the migratory effect on stomach cancer frequency suggest that environmental and lifestyle factors are very important in the development of this disease.

In 1994, the International Agency for Research on Cancer has classified *H. pylori* infection as a carcinogen in humans due to the evidence which links *H. pylori* infection and risk of gastric cancer[48]. *H. pylori* as a carcinogen most likely acts indirectly, by causing gastritis, which is a precursor to stomach atrophy, metaplasia, and dysplasia. While the risk of stomach cancer correlated with the duration of *H. pylori* infection, no association was found for the histologic subtype of stomach cancer (intestinal or diffuse), or sex. Based on a meta-analysis of cohort studies, the risk of stomach cancer in people with *H. pylori* infection was 2.36[78]. Chronic or recurrent *H. pylori* infection is a major cause of stomach cancer; the relative risk is estimated to be 2.7-3.8 for cancer of cardia, and 1.1-11.1 for noncardia stomach cancer [20]. *H. pylori* infection is attributed to 592000 (63.4%) of all cases of stomach cancer globally[26]. *H. pylori* is a stomach colonizing bacterium; how *H. pylori* is transmitted has not been elucidated definitely, but the person-to-person pathway is most likely a contact pathway[48]. *H. pylori* infection is acquired in childhood, and population prevalence is associated with socioeconomic status[79]. High prevalence of *H. pylori* infection, and little international variation, suggest that other factors are important in the etiology of gastric cancer[53]. The main risk factors for noncardia stomach cancer are *H. pylori* infection, tobacco smoking and dietary factors, while gastroesophageal reflux disease, obesity and possibly tobacco smoking play an important role in the development of cardia gastric cancer[6,9-13,22].

The main cofactors responsible for the development of stomach cancer are smoking and diet[30,31,80-82]. After adjusting for alcohol intake or the presence of chronic *H. pylori* infection in the stomach, an independent association with smoking was confirmed[83,84]. Over 45 case-control studies and 27 cohort studies confirmed the association of tobacco with stomach cancer, with the average relative risk being RR = 1.5-2.0[85]. One recent meta-analysis of prospective observational studies suggests that the summary relative risk was higher in men (1.63) than in women (1.30)[31]. The risk of stomach cancer increases significantly with cigarette smoking (40% for smokers and 82% for heavy smokers) and alcohol consumption[86]. It is estimated that in developing countries the gastric cancer risk attributable to smoking is 11% in men and 4% in women, while in developed countries the risk is 17% in men and 11% in women[85,87].

While some authors believe that diet has no role in the etiology of gastric cancer, the American Cancer Society states that smoked foods, salted fish and meat, and pickled vegetables represent risk factors for gastric cancer[88]. Some bacteria, like *H. pylori*, can convert nitrates and nitrites (commonly found in meat products) to substances which are shown to cause gastric cancer in animals[48,89]. It is also known that adherence to the Mediterranean diet is significantly inversely correlated with gastric cancer[89,90].

The correlation between salt intake (high in salt, smoked foods, salted fish and meat) and stomach cancer risk has been indicated in several epidemiological studies[91-94]. Sodium chloride is known to increase gastrocarcinogenesis using N-methyl-N-nitro-N-nitrosoguanidine in a rat experiment[95], as well as in a human study[96]. The mucin layer which covers and protects the stomach epithelium is damaged by high doses of salt, which also cause high osmotic pressure that further damages epithelial cells. Prolonged damage to the mucous membrane leads to chronic atrophic gastritis and intestinal metaplasia, which are precursors for stomach cancer.

Higher consumption of fruits and vegetables has been associated with a lower risk of malignant tumors in a number of epidemiological studies (over 200 case-control and cohort studies)[97,98], while results are particularly numerous and consistent for stomach cancer[99]. The intake of fresh fruits and vegetables, which contain antioxidant vitamins (*e.g.* vitamins A and C), reduces gastric cancer risk. In a cohort of 900000 adults (404576 men and 495477 women) who were not diagnosed with malignancy at

the time of enrollment, 57145 people died after 16 years, with the highest weight subjects having a higher mortality rate from malignant tumors in general: men had a 52% higher rate, and women a 62% higher rate, compared to people with normal body weight[100]. Higher mortality was found for esophageal, colon, liver, gallbladder, pancreatic and kidney cancer, but also for non-Hodgkin's lymphoma and multiple myeloma.

Patients with gastroesophageal reflux disease (GERD), especially with the long-standing forms, had a significantly increased risk for cardia stomach cancer and the majority of studies noted a 2-4-fold increase in risk[10,101,102], although not all[103]. One of the explanations for the association between GERD and cardia gastric cancer is that GERD may cause metaplasia with potential progression to adenocarcinoma[104]. On the other hand, a lack of association between GERD and noncardia stomach cancer might be explained, at least in part, by the association with atrophic gastritis which might be associated with a decrease in gastric acid secretion and lower risk of GERD[105].

Of the demographic factors, socio-economic status, older age and male gender play an important role [9-11]. Socio-economic deprivation, within any population, is consistently linked with increased gastric cancer risk[106,107]. The risk of developing stomach cancer increases with age[7,34]. Stomach cancer rarely develops before the age of 40, more than 80% of stomach cancers occur between 60 and 80 years of age. Stomach cancer affects men more than women[1-3]. The consistency of risk difference by sex has never been adequately explained although possible explanations included differences in environmental exposures and lifestyle factors, as well as the theory regarding the potentially protective role of female sex-specific hormones[108]. According to the results of studies conducted in the United States, stomach cancer occurs more often in African Americans compared to the white population[7]. Possible reasons for this increased risk include socioeconomic factors, prevalence of *H. pylori* infection, cigarette smoking, and obesity[7,22,109,110].

It is estimated that around 10% of stomach cancer cases aggregate in families, and only 1%-3% are hereditary[10,13,111]. A positive family history of stomach cancer in a first-degree relative is a risk factor for stomach cancer, but the magnitude of risk varies with different ethnic groups and geographic regions, ranging from 2 to 10[112,113]. Although familial aggregation could be a risk factor because of shared genetic factors, the influence of shared environment cannot be ruled out, *e.g.*, passage of *H. pylori* infection from parents to children, the same dietary factors, *etc.* Although migrant studies indicate a significant reduction in the risk of gastric cancer in Japanese immigrants, the results of many studies point out that exposure to environmental factors in childhood is important for determining gastric cancer risk[48,53,79]. Namely, migrant studies show that exposure in childhood is important in stomach cancer etiology: *e.g.* infection with *H. pylori* often occurs before the age of 10, *i.e.* often before the migration, also children born in the immigrant country are likely to acquire the infection from family members who have migrated from their native country[114].

Gastric cancer risk is increased in many genetic disorders, such as hereditary diffuse gastric cancer, Peutz-Jeghers syndrome, and familial adenomatous polyposis[10,112]. Persons who have mutations or deletion in genes such as *p53*, *BRCA2*, *MSH2*, and *MLH1*, have an increased risk of stomach cancer[10, 111].

Some studies found a link between stomach cancer and antioxidant use, non-steroidal anti-inflammatory drugs, statins, physical activity, and radiation[6,9-13]. Some researchers have suggested a correlation between the excess or deficit of iodine, goiter, and stomach cancer, as well as a decrease in stomach cancer mortality after performing effective iodine prophylaxis[115].

Other potential risk factors in relation to stomach cancer include poor oral hygiene and tooth loss [116], hookah and opium use[117], Epstein-Barr virus infection[118], and consumption of pickled vegetables[119], but the results are not convincing, at least not yet. In addition, in many persons with stomach cancer there is no one specific stomach cancer risk factor.

PREVENTION

During the past century, Western developed countries experienced a major reduction in stomach cancer incidence and mortality, without the introduction of specific primary and secondary prevention measures. Generally, favorable trends in the frequency of stomach cancer are thought to be an important part a consequence of changes such as the reduction in the use of salt and an increase in the consumption of fruit and fresh vegetables due to improvements in food storage (refrigerators, freezers). This phenomenon has been dubbed the "unplanned triumph" of prevention[9,39].

Primary and secondary prevention strategies are the focus of stomach cancer prevention.

Primary prevention measures involve improvements in environment and lifestyle habits such as tobacco control/smoking cessation, reducing salt intake, increasing fruit and vegetable intake, developing other healthy behaviors (such as Mediterranean diet, higher intake of fiber, physical activity), *H. pylori* eradication, other medications (intake of non-steroidal anti-inflammatory drugs, statins), refraining from high alcoholic beverages, sanitation and hygiene improvements. The WHO has set a global goal of reducing the intake of salt to less than 5 g (2000 mg of sodium) per person per day by the year 2025[120]. A meta-analysis of randomized trials (all trials were performed in areas with a high

incidence of stomach cancer, mostly in Asia), in a total of 6695 participants followed from 4 to 10 years showed that the risk of stomach cancer can be reduced by 35% with the treatment of *H. pylori*[121]. In addition to endoscopic and histological surveillance, the American and European guidelines recommend eradication of *H. pylori* in all persons who have atrophy and/or intestinal metaplasia and all persons who are first-degree relatives of stomach cancer patients[122,123]. According to the Asian Pacific Gastric Cancer Consensus, population-based screening and treatment of *H. pylori* infection is recommended in regions which have an annual stomach cancer incidence of more than 20/100000[124]. Eradication of *H. pylori* can be achieved with antibiotic therapy; but, the treatment of asymptomatic carriers is not practical as many countries have a very high infection burden (*e.g.*, over 75% of adult persons living in sub-Saharan Africa have *H. pylori* infection) and reinfection is relatively easy[54].

Japan has had a national endoscopic surveillance program since the early 1970s because of the high stomach cancer risk[125]. It is recommended that all people older than 40 years undergo screening with a double-contrast barium X-ray radiography and endoscopy every year[126]. A study in China demonstrated that a preventive intervention which included eradication of *H. pylori*, nutritional supplements, and screening (with double-contrast radiography and endoscopy) resulted in a 49% reduction in relative risk for overall mortality in a high-risk group of individuals[127].

Upper gastrointestinal endoscopy is the gold standard for stomach cancer diagnosis and due to its high detection rate it is used for stomach cancer screening in high-risk areas (such as Japan, Korea, Venezuela and other areas), but the available evidence shows that endoscopic surveillance of premalignant gastric lesions showed conflicting results[128]. Besides, the procedure is expensive, unpleasant for the patient and carries a risk of hemorrhage and perforation[129,130].

Stomach cancer screening might be possible *via* the detection of potential markers of gastric atrophy (a stomach cancer precursor lesion)[125,131,132], including serum pepsinogens, serum ghrelin, *H. pylori* serum antibodies, gastrin-17, or antigastric parietal cell antibodies, but the results are not convincing, at least not yet.

CONCLUSION

Worldwide, stomach cancer incidence and mortality have declined significantly during the past five decades. However, stomach cancer remains a global health problem as the fifth leading cancer and fourth most common cause of cancer-related deaths in the world. Further illumination of risk factors can help identify various opportunities for prevention. Primary and secondary prevention strategies with more effectiveness are needed in order to reduce stomach cancer incidence and mortality, particularly in populations with a high burden of stomach cancer.

FOOTNOTES

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REVIEW

Antibiotics, gut microbiota, and irritable bowel syndrome: What are the relations?

Zarina Mamieva, Elena Poluektova, Valery Svistushkin, Vasily Sobolev, Oleg Shifrin, Francisco Guarner, Vladimir Ivashkin

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Abstract

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder in which recurrent abdominal pain is associated with defecation or a change in bowel habits (constipation, diarrhea, or both), and it is often accompanied by symptoms of abdominal bloating and distension. IBS is an important health care issue because it negatively affects the quality of life of patients and places a considerable financial burden on health care systems. Despite extensive research, the etiology and underlying pathophysiology of IBS remain incompletely understood. Proposed mechanisms involved in its pathogenesis include increased intestinal permeability, changes in the immune system, visceral hypersensitivity, impaired gut motility, and emotional disorders. Recently, accumulating evidence has highlighted the important role of the gut microbiota in the development of IBS. Microbial dysbiosis within the gut is thought to contribute to all aspects of its multifactorial pathogenesis. The last few decades have also seen an increasing interest in the impact of antibiotics on the gut microbiota. Moreover, antibiotics have been suggested to play a role in the development of IBS. Extensive research has established that antibacterial therapy induces remarkable shifts in the bacterial community composition that are quite similar to those observed in IBS. This suggestion is further supported by data from cohort and case-control studies, indicating that antibiotic treatment is associated with an increased risk of IBS. This paper summarizes the main findings on this issue and contributes to a deeper

understanding of the link between antibiotic use and the development of IBS.

Key Words: Gut microbiota; Irritable bowel syndrome; Antibiotics; Intestinal barrier; Gut motility; Gut sensitivity

Core Tip: Irritable bowel syndrome (IBS) is among the most common gastrointestinal disorders; however, its etiology and underlying pathophysiology have yet to be fully elucidated. The present review focuses on the existing evidence on the pathogenic role of the gut microbiota in the development of IBS. Moreover, it provides a comprehensive review on the magnitude of changes in the gut microbiota in response to antibiotics. The paper contributes to a deeper understanding of the link between antibiotic use and the development of IBS.

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INTRODUCTION

Recent advances in culture-independent techniques have greatly expanded our understanding of the human gut microbiota and its functionalities. It is becoming increasingly recognized that gut bacteria play a pivotal role in host homeostasis and are involved in the progression and development of numerous human diseases.

The gut microbiota is established early in life, remains relatively stable thereafter, and is subject to shaping by environmental and host factors (*e.g.*, age, diet, lifestyle, and medications)[1,2]. With regard to the environment, antibiotics have been reported to play a particularly important role in the modulation of the gut microbial community. However, most studies in this area were undertaken 30 to 40 years ago and relied on culture-based techniques. Global antibiotic use has grown 66% since 2000 and continues to grow at a high rate[3,4]. This fact, along with rapid technological advancements for culture-independent analysis, has reinforced the need to take a fresh look at antibiotic-induced changes in the human gut microbiota and clinical consequences of antibiotic intervention. Several studies have reported that antibiotic treatment is associated with an increased risk of irritable bowel syndrome (IBS)[5-8].

IBS is a common gastrointestinal disorder affecting 10%–15% of the population in Europe and North America[9]. This condition negatively affects the quality of life of patients and imposes a significant socioeconomic burden[10]. Over the past few decades, the gut microbiota has emerged as a potential factor that contributes to the pathophysiology of IBS[11,12]. Microbial dysbiosis within the gut has been implicated in intestinal barrier dysfunction, visceral hypersensitivity, impaired gastrointestinal motility, and altered immune response[13-17]. Moreover, various studies have consistently shown the efficacy of microbiota-directed therapies, including prebiotics, probiotics, nonabsorbable antibiotics, dietary changes, and fecal microbial transplantation, in alleviating IBS symptoms[18].

In this paper, we provide a brief overview of the human gut microbiota and its impact on host homeostasis. We highlight what is currently known regarding the role of gut bacteria in the pathophysiology of IBS. Furthermore, we provide an overview of the most up-to-date literature about the impact of antibiotics on gut microbiota composition and discuss a possible link between antibiotic use and the development of IBS. Finally, we identify knowledge gaps and uncertainties that must be filled to orient future research in this area.

GUT MICROBIOTA AND ITS ROLE IN HOST HOMEOSTASIS

The human gut microbiota is a community of microorganisms that inhabit the gastrointestinal tract and is composed of approximately 10^{14} bacterial cells[19,20]. In healthy adults, more than 90% of gut bacteria belong to four dominant phyla, namely, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, whereas other phyla are far less abundant[21,22].

Currently, the gut microbiota is considered an indispensable "organ" within the body with distinct metabolic and immune functions (Table 1). Most of its effects are mediated through metabolites.

Table 1 Gut microbiota functions

Bacterial phylum	Key representatives	Functions
Firmicutes	Members of the genera *Enterococcus, Ruminococcus, Clostridium, Lactobacillus, Faecalibacterium, Roseburia,* and *Eubacterium*	Metabolism of amino acids[23,24], carbohydrates[25], bile acids, and their salts[22]. Lipid metabolism and cholesterol synthesis[25]. Synthesis of vitamins K2, B1, B2, B6, B7, B9, and B12[26]. Maintenance of a proper immune response[28,29] and intestinal epithelial barrier integrity[31,32]. Protection against enteric pathogens[33]
Bacteroidetes	Members of the genera *Bacteroides* and *Prevotella*	Metabolism of amino acids[24], carbohydrates[25,141], bile acids, and their salts[22,142]. Synthesis of vitamin K2[27]. Regulation of appetite[143]. Maintenance of a proper immune response[28-29] and intestinal epithelial barrier integrity[31]. Protection against enteric pathogens[33]
Actinobacteria	Members of the genera *Bifidobacterium* and *Corynebacterium*	Metabolism of bile acids and their salts[22]. Synthesis of vitamins K2, B1, B2, B6, B7, B9, and B12[26]. Protection against enteric pathogens[33]
Proteobacteria	Members of the genera *Desulfovibrio, Escherichia,* and *Shigella*	Metabolism of amino acids[144]

Thus, some of the most important roles of the gut microbiota include metabolism of dietary compounds[23-25], synthesis of vitamins[26,27], regulation of the immune response[28-30], maintenance of intestinal epithelial barrier integrity[25,31,32], and protection against enteric pathogens[33].

MODERN CONCEPT OF IBS: EVOLVING ROLE OF GUT MICROBIOME

Despite extensive research, the etiology and underlying pathophysiology of IBS remain incompletely understood. Proposed mechanisms involved in its pathogenesis include visceral hypersensitivity, impaired gut motility[13,34], increased intestinal permeability[34-36], emotional disorders[11,37], and changes in the immune system[34,37,38].

Over the past decade, there has been an increasing amount of literature on the role of the gut microbiota in the pathogenesis of IBS. The concept of the "microbiota-gut-brain" axis has been proposed [14-17], supporting the crucial role of microbial dysbiosis in the development of IBS symptoms. It is thought that, in genetically predisposed individuals, environmental factors alter the composition of the gut microbiota, leading to disruption of intestinal epithelial barrier integrity[13]. Once the intestinal barrier is breached, bacteria interact with the immune system of the host, provoke a series of immune reactions, and lead to low-grade mucosal inflammation in the gut wall. Collectively, these changes result in sensitivity and motility abnormalities, emotional disorders, and the development of IBS symptoms (abdominal pain, bloating, and alterations in bowel habits)[35]. Interestingly, the gut microbiota not only initiates such a pathological cascade in IBS but also contributes to all aspects of its multifactorial pathogenesis through the release of metabolites[11,12]. These provisions will be discussed below.

Microbiota and motility/sensitivity abnormalities

The enteroendocrine system modulates gut motor and sensory functions through the secretion of neuropeptides and neurotransmitters[39].

Bacterial metabolites are able to stimulate the production of several neuropeptides, including neuropeptide Y, peptide YY, glucagon-like peptide-1 (GLP-1)[40], cholecystokinin, and substance P (Figure 1)[15,41].

For instance, short-chain fatty acids (SCFAs), secondary bile acids, and indole, which are produced by members of the genera *Clostridium, Bacteroides,* and *Ruminococcus*[23,25], stimulate intestinal L-cells to secrete GLP-1[42]. GLP-1 reduces postprandial motility in the upper gastrointestinal tract (antrum, duodenum, and jejunum) and increases colonic transit[43,44]. A study conducted by Li *et al*[45] reported decreased serum GLP-1 levels and reduced mucosal expression of GLP-1 receptors in patients with constipation-predominant IBS (IBS-C). The authors suggested that lower GLP-1 levels lead to the loss of its prokinetic effects in the colon, resulting in constipation and abdominal pain. In a rat model of bowel dysfunction, administration of the GLP-1 receptor agonist exendin-4 alleviated stress-induced defecation and visceral pain sensitivity[46,47]. Clinical interventions in patients with IBS demonstrated that the synthetic GLP-1 analog ROSE-010 reduced abdominal pain and increased colonic transit[45,48]. The underlying molecular mechanisms responsible for the amelioration of symptoms remain unknown. The authors suggest that modulation of enteric neuronal function and tight junction expression, as well as the activation of serotonergic pathways in the colon, may play a role.

Secondary bile acids and SCFAs, which are mainly produced by *Eubacterium, Bacteroides*, and *Clostridium* (clusters IV, XI, XIII, and XIVa)[22], promote serotonin synthesis from colonic enterochromaffin cells[49]. Serotonin is an important neurotransmitter that, among its other functions, regulates gastrointestinal motility[50]. Serum serotonin levels were found to be increased in those with diarrhea-

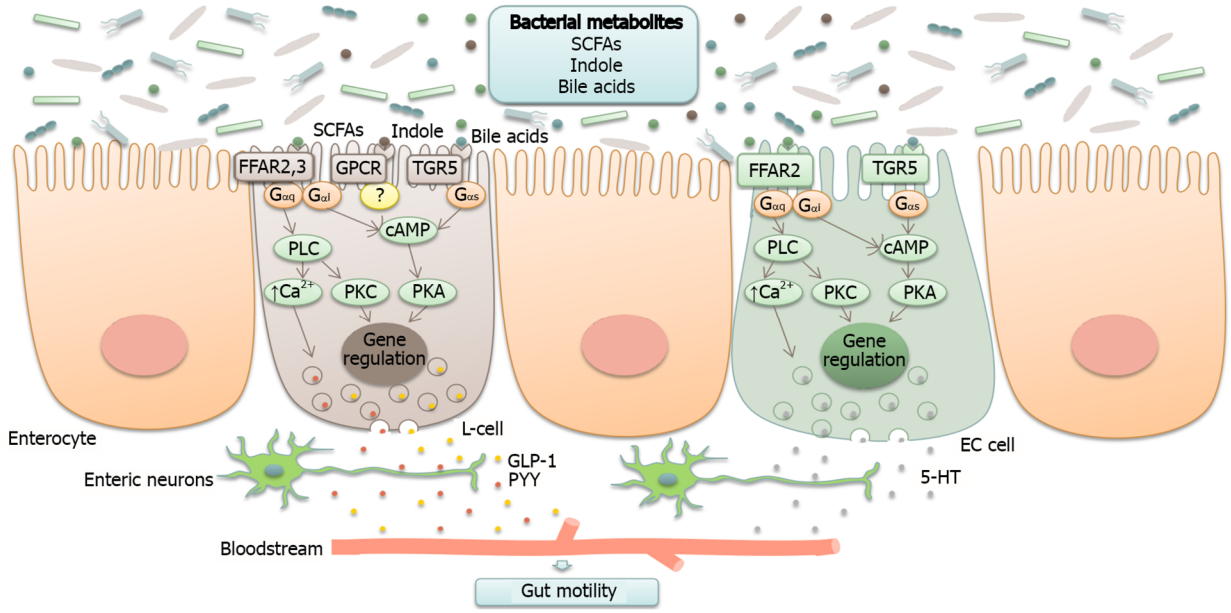

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Figure 1 Neurotransmitter modulation by gut microbiota (schematic illustration). Bacterial metabolites, such as short-chain fatty acids (SCFAs), secondary bile acids, and indole, are able to stimulate the production of neurotransmitters, including glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and serotonin (5-HT). They act through G-protein coupled receptors (GPCRs) FFAR2, FFAR3, and TGR5 that are coupled to different types of G proteins ($G_{\alpha s}$, $G_{\alpha q}$, and $G_{\alpha i}$) and activate different pathways known to regulate gene expression and promote exocytosis by raising intracellular Ca^{2+} levels. SCFAs are recognized by FFAR2 and FFAR3. Enteroendocrine L-cells express both of these proteins, whereas enterochromaffin (EC) cells have been reported to express FFAR2. Bile acids are recognized by TGR5 receptors expressed in L-cells and EC cells. The sensing of indole remains elusive, although it is thought to act through GPCR. $G_{\alpha s}$ stimulates adenylate cyclase and elevates cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA). $G_{\alpha i}$ inhibits the cAMP pathway. $G_{\alpha q}$ stimulates phospholipase C (PLC), resulting in the generation of diacylglycerol (DAG) and inositol triphosphate (IP_3), which activate protein kinase C (PKC) and induce intracellular Ca^{2+} release[23,138-140]. SCFA: Short-chain fatty acids; GLP-1: Glucagon-like peptide-1; PYY: Peptide YY.

predominant IBS (IBS-D) and reduced in those with IBS-C[34].

The serotonin system represents a potential therapeutic target in IBS. The effects of serotonin are mediated through 5-HT receptors located on the surface of distinct cell types. Fourteen different serotonin receptor subtypes have been identified and classified into seven groups (5-HT_{1-7}), with 5-HT_3 and 5-HT_4 being the most investigated receptors in the intestine. Both receptor subtypes are expressed on neurons within the myenteric and submucosal plexuses of the enteric nervous system, intrinsic and extrinsic sensory neurons, interstitial cells of Cajal, enterocytes, and enterochromaffin cells[51]. 5-HT_3 receptors are involved in the contraction of intestinal smooth muscle and in gut-brain communication through vagal afferent fibers[52]. Activation of 5-HT_4 receptors induces neuronal release of acetylcholine and accelerates the peristaltic reflex[53]. 5-HT_3 receptor antagonists have been shown to improve abdominal pain and global IBS symptoms in patients with nonconstipated and IBS-D[54,55]. 5-HT_4 agonists have been shown to relieve overall and individual symptoms (abdominal pain/discomfort, stool frequency, stool consistency, and straining during defecation) in patients with IBS-C[56-58]. However, cardiovascular side effects were seen with these drugs, and they were either withdrawn from the market (cisapride) or approved for a limited population (tegaserod). Therefore, new safe and well-tolerated 5-HT_4 agonists are under development[59,60].

A number of animal studies have shown the prominent role of the gut microbiota in visceral hypersensitivity[41]. For example, colonization of germ-free rats with the gut microbiota from patients with IBS reduced the pain threshold to colorectal distension[42]. Furthermore, the beneficial effects of probiotic strains (*e.g.*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, and *Bifidobacterium longum*) in alleviating visceral sensitivity have been documented[61-63].

Thus, the microbiota influences the main pathogenetic factors of IBS (*i.e.*, motility and sensitivity) both directly and through microbial metabolites.

Microbiota as a regulator of stress and emotional responses

The physiological response to stress is mediated through the hypothalamic-pituitary-adrenal (HPA) axis [64]. Activation of this axis results in the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. CRH acts on the anterior pituitary and induces the production of adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete cortisol.

Different types of stressors are known to contribute to the development, maintenance, and exacerbation of IBS symptoms[11]. The results of multiple studies suggest that there is HPA axis dysregulation in IBS. For instance, patients with IBS were found to have excess levels of ACTH in the plasma and cortisol in the serum in response to CRH infusion[65].

Growing evidence indicates that the gut microbiota is involved in the regulation of HPA axis activity. It has been shown that colonization with beneficial microorganisms in early life is of great importance for the normal development of this axis[66]. Moreover, alterations in the gut microbiota may influence the release of ghrelin and galanin, which are endocrine peptides contributing to the stress response through modulation of CRH, ACTH, and glucocorticoid secretion[40,67].

Dysfunction of the HPA axis, along with alterations in neurotransmitter metabolism, appear to be crucial factors in the development of psychiatric disorders, such as anxiety and depression[68,69]. A recent meta-analysis of 27 studies have reported elevated levels of anxiety and depression in patients with IBS as compared to those in healthy controls[70]. Comorbid emotional disorders lead to persistence of symptoms, drive patients to seek medical care, and contribute to poor outcomes[11].

A growing body of literature supports the association between microbial dysbiosis and the development of anxiety and depression. For instance, certain species within the *Lactobacillaceae* and *Bifidobacteriaceae* families are known to produce gamma-aminobutyric acid (GABA). GABA is the main inhibitory neurotransmitter of the central nervous system, playing an important role in the pathogenesis of mood disorders[49,71]. Interestingly, a specific type of GABA receptor (GABA-b) is localized on submucosal and myenteric neurons of the enteric nervous system[72] and is thought to be involved in the modulation of gut motility and sensitivity[37]. Furthermore, members of the genera *Bacillus* and *Escherichia* have been found to produce other neurotransmitters affecting mood and behavior, such as dopamine, serotonin, and norepinephrine[15,73]. In recent studies, germ-free mice have been widely used as a tool for assessing the role of intestinal microbes in brain function and behavior. Studies on germ-free and specific pathogen-free mice indicate that intestinal microbes can cause imbalances of the HPA axis, resulting in an anxiety-like behavioral phenotype[74]. Fecal microbiota transplantation studies have indicated the rodent-to-rodent and human-to-rodent transfer of anxiety-like behaviors[75,76]. Moreover, animal studies have shown that transplantation of the microbiota from depressed patients to rodents is able to induce depression-like behavior. The authors linked microbiota-induced depression in mice to alterations in the cAMP-response element binding protein (CREB) signaling pathway in the olfactory bulb[77] and alterations in carbohydrate and amino acid metabolism[78].

However, despite the data obtained, further research is needed to investigate the difference in emotional disorder levels in patients with postinfectious and other forms of IBS.

Microbiota and host immunity

Recently, considerable literature has grown around the theme of immune system activation in IBS. For instance, an increased number of mast cells located in close proximity to enteric nerve fibers have been found in colonic biopsies from patients with IBS and have been associated with the severity of symptoms[11,38,79]. Mast cells are thought to be key players in intestinal mucosal inflammation[79]. Their degranulation causes the release of inflammatory mediators (histamine, serotonin, and proteases), resulting in lymphocyte activation and cytokine imbalance[80]. Patients with IBS were found to have higher levels of proinflammatory interleukin (IL)-6, IL-8, IL-1β, and tumor necrosis factor-α (TNF-α) and lower levels of anti-inflammatory IL-10 in both serum and the intestinal mucosa[81,82]. These changes result in altered pain thresholds and visceral hypersensitivity[38,83]. In addition, mast cell degranulation has been shown to reduce the expression of tight junction proteins, probably through tryptase release[13]. Apart from mast cells, increased numbers of eosinophils and intraepithelial lymphocytes have been observed in colonic biopsies from patients with IBS[11,79].

Gut bacteria play an important role in the modulation of the immune response. For example, butyrate produced by members of the phylum *Firmicutes*[25] induces the differentiation of regulatory T cells[29,84], thereby preventing an excessive immune response and autoimmunity[22,85]. Furthermore, *Lactobacilli* spp. metabolize dietary tryptophan into indole-3-aldehyde, which acts as an aryl hydrocarbon receptor (AHR) ligand[85]. AHR is a ligand-activated transcription factor that is expressed by immune cells and regulates the number of intraepithelial lymphocytes and IL-22 production[86]. Probiotic strains, such as *Lactobacillus rhamnosus*, *Lactobacillus casei,* and *Bifidobacterium breve,* were shown to induce IL-4 and IL-10 production, whereas *L. reuteri* and *L. plantarum* were found to downregulate the expression of TNF-α[87,88].

The importance of the interaction between the gut microbiota and host immune system in IBS is highlighted by a number of studies in patients with postinfectious IBS, indicating activation of the gastrointestinal immune system after acute gastroenteritis[89,90]. Moreover, animal studies have shown that stress-induced changes in the gut microbiota are associated with altered immune response and increased susceptibility to enteric pathogens[91,92].

Microbiota and intestinal barrier integrity

Intestinal epithelial barrier integrity is of great importance for gut homeostasis, as it prevents the translocation of luminal antigens to the mucosa, thus averting the development of low-grade mucosal inflammation in the gut wall (Figure 2).

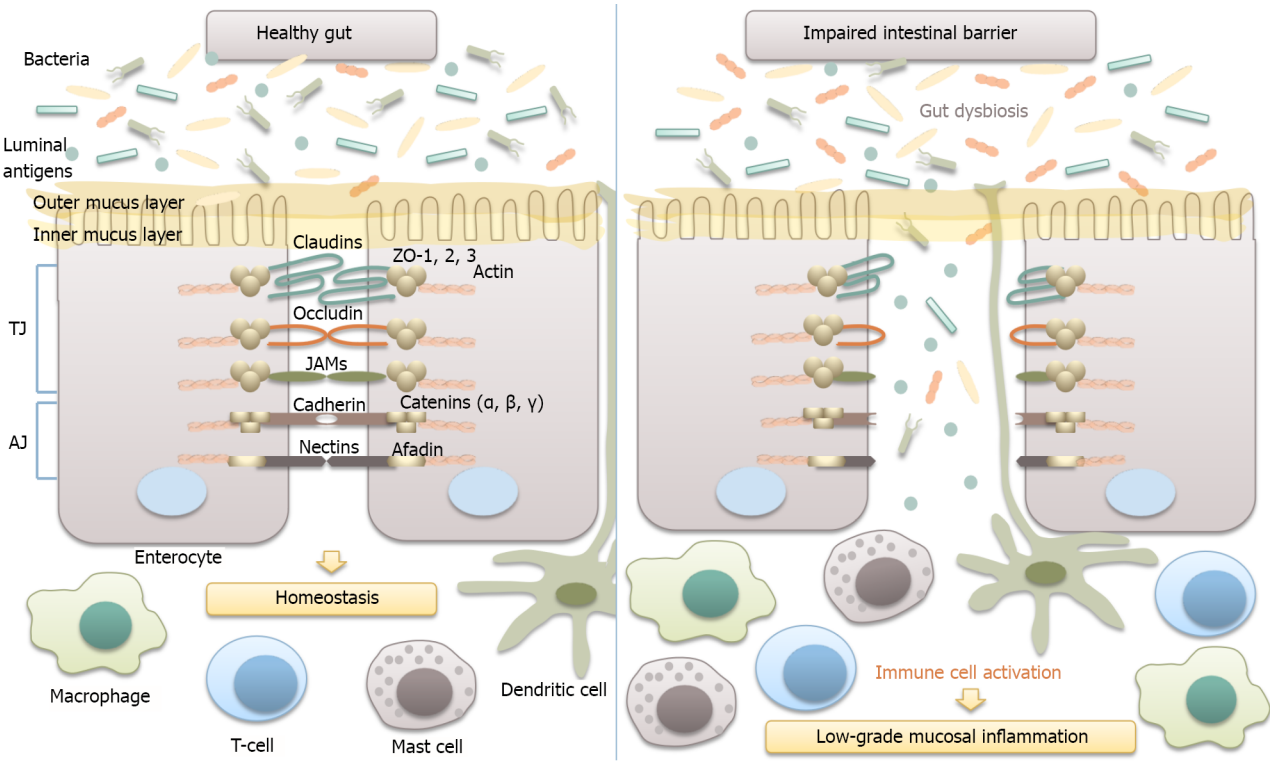

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Figure 2 Microbiota and intestinal barrier integrity. The intestinal barrier plays an essential role in maintaining host homeostasis. It is mainly composed of the mucus layer, the epithelial layer, and the underlying lamina propria. Intestinal epithelial cells are tightly attached to each other by junctional complexes. Tight junctions (TJs) are composed of several proteins, including occludin, claudins, zonula occludens (ZOs), and junctional adhesion molecules (JAMs), which interact with each other, as well as with the cytoskeleton. The adherence junction is composed of the nectin-afadin system and the E-cadherin-catenin system. Intestinal epithelial barrier integrity prevents the translocation of bacteria and luminal antigens to the mucosa, thus averting their interaction with the host immune system and the development of low-grade mucosal inflammation in the gut wall. TJ: Tight junctions; AJ: Adherence junction; JAM: Junctional adhesion molecules.

An increased density of epithelial gaps has been shown by electron microscopy in gut biopsies of patients with IBS[93]. Furthermore, histological examination of colonic biopsies revealed decreased expression of tight junction proteins, such as occludin; claudins 1, 3, and 5; and zonula occludens-1[13,36,82,93]. Increased serum levels of anti-flagellin antibodies in patients with IBS further support the substantial role of intestinal permeability in the pathogenesis of IBS[94].

The gut microbiota is an important determinant of intestinal epithelial barrier integrity. In particular, certain gut bacteria, such as *Bacteroides thetaiotaomicron, Faecalibacterium prausnitzii,* and *Ruminococcus* spp., were shown to affect the mucus layer thickness and composition[1,22,31]. Moreover, SCFAs, which are produced predominantly by members of the genera *Eubacterium, Clostridium, Ruminococcus,* and *Faecalibacterium,* have been demonstrated to augment the expression of claudins 3 and 4 and occludin. Polyamines (putrescine, spermidine, and spermine), which are produced by certain species within the *Clostridium, Enterococcus, Streptococcus,* and *Lactobacillus* genera, have been shown to stimulate the production of E-cadherin and zonula occludens-1[95]. There is also evidence that probiotic strains of *Bifidobacterium* and *Lactobacillus* promote intestinal barrier function and prevent bacterial translocation [32,96].

Most likely, the preservation of the optimal composition of the microbiota (*e.g.*, a sufficient number of SCFA producers) may serve as a factor preventing the development of IBS.

GUT MICROBIAL COMPOSITION IN PATIENTS WITH IBS

A considerable amount of literature has been published on the compositional changes of the gut microbiota in patients with IBS. Although data from these studies are inconsistent and even conflicting, some common features can be found (Table 2). The discrepancy in findings is possibly due to differences in the population studied (*e.g.*, age, lifestyle, initial microbiota composition, prior antibiotic and/or probiotic use, and diagnostic criteria for IBS) and methodological issues, such as study design and methods for microbiota assessment and data analysis.

The majority of authors report decreased microbial diversity in patients with IBS[97-101]. Furthermore, a substantial number of studies have shown a lower abundance of butyrate-producing

Table 2 Compositional changes in gut microbiota in patients with irritable bowel syndrome (common threads)

Ref.	Subjects	Method	Specimen	Diversity	*Faecalibacterium*	*Enterobacteriaceae*	*Bifidobacterium*	*Lactobacillus*
Dior *et al* [145], 2016	IBS-D (*n* = 16), IBS-C (*n* =15), Controls (*n* = 15)	Real-time PCR	Stool	No data	–	↑ in IBS-D (*Escherichia*)	↑ in IBS-C	–
Ringel-Kulka *et al* [108], 2016	IBS (*n* = 56), Controls (*n* = 20)	16S rRNA	Stool	No data	–	–	–	↑
Maharshak *et al*[102], 2018	IBS-D (*n* = 23), Controls (*n* = 24)	16S rRNA	Stool	↓[1]	↓	↑ (*unclassified genus*)	–	–
			Colonic biopsy	–[1]	–	–	–	↑
Gobert *et al* [146], 2016	IBS-C (*n* = 33), Controls (*n* = 58)	16S rRNA	Stool	No data	–	↑	↓	–
Shukla *et al* [105], 2015	IBS (*n* = 47), Controls (*n* = 30)	16S rRNA; real-time PCR	Stool	No data	–	–	↓	–
Su *et al* [107], 2018	IBS-D (*n* = 40), Controls (*n* = 20)	16S rRNA; real-time PCR	Stool	No data	–	–	↓	↓
Zhuang *et al* [109], 2018	IBS-D (*n* = 30), Controls (*n* = 13)	16S rRNA	Stool	–[2]	–	–	–	↓
Zhong *et al* [147], 2019	IBS-D (*n* = 20), Controls (*n* = 16)	FISH	Colonic biopsy	No data	–	↑ (*E. coli*)	↓	–
Jeffery *et al* [100], 2020	IBS (*n* = 80), Controls (*n* = 65)	16S rRNA, shotgun sequencing	Stool	↓[2]	–	–	–	–
Rangel *et al* [148], 2015	IBS (*n* = 33), Controls (*n* = 16)	Microarray analysis	Stool	↓[2]	↓ (*F. prausnitzii*)	–	–	–
			Colonic biopsy	–[2]	–	–	–	–

[1]Rarefaction analysis.

[2]Shannon diversity index.

↓: Decreased abundance; ↑: Increased abundance; –: No significant differences found; IBS: Irritable bowel syndrome; IBS-D: Diarrhea-predominant irritable bowel syndrome; IBS-C: Constipation-predominant irritable bowel syndrome; FISH: Fluorescence *in situ* hybridization; *E. coli*: *Escherichia coli*; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

bacteria from the genus *Faecalibacterium*, mainly *F. prausnitzii*,[97,98,102,103] as well as an increase in the abundance of the *Enterobacteriaceae* family, including pathogens such as *Escherichia coli* and *Enterobacter* spp.[98,104-106]. Moreover, patients with IBS were found to have a reduced prevalence of *Bifidobacterium*, providing a range of beneficial properties to the host[98,103,104,106,107]. Significant differences in *Lactobacillus* numbers were also observed between patients with IBS and healthy controls, but the findings of different studies were not consistent. Some authors reported an increased amount of *Lactobacillus*[98,99,102,108], while others documented a decrease in the abundance of this commensal[103,104,106,107,109].

Overall, there seems to be some evidence to indicate that patients with IBS have decreased numbers of bacteria contributing to the maintenance of host homeostasis and proper immune response, as well as increased numbers of microbes with proinflammatory properties.

ANTIBIOTICS, GUT MICROBIOTA, AND IBS

Effects of antibiotics on gut microbiota composition

The discovery of antibiotics in the early 20th century was a great milestone in the history of medicine, as

it changed the natural course of most infectious diseases and saved countless lives[110,111]. However, a growing number of studies have shown that inappropriate use of antibiotics promotes the development of antibiotic resistance[112,113]. Furthermore, accumulating evidence indicates that antibiotic exposure in early life increases the risk of obesity and autoimmune and allergic diseases[114-117].

During the past four decades, there has been an increasing interest in the impact of antibiotics on the composition of the gut microbiota. A substantial number of studies in this area were conducted in the 1980s and 1990s and relied on culture-based techniques. However, researchers indicate that up to 80% of gut bacteria are nonculturable[118]. Therefore, the focus has shifted to culture-independent approaches mainly based on 16S rRNA gene sequence analysis.

Extensive research has established that antibiotic treatment induces a dramatic loss of diversity and remarkable shifts in community composition (Table 3), with the time of recovery varying substantially [119-123].

The inconsistency in the results of various studies can be attributed to substantial heterogeneity in sample characteristics (age, ethnicity, diet, *etc.*) and study methodology. Furthermore, antibiotic characteristics, such as their class, pharmacokinetics (absorption and excretion), range of action, and dosing regimen, have been shown to shape the response of the gut microbiota to antibiotic perturbation[124]. For instance, vancomycin is poorly absorbed when administered orally, resulting in high fecal concentrations. Therefore, it significantly alters the composition of the gut microbiota by increasing pathogenic *Proteobacteria*, such as *Klebsiella*, *Escherichia*, and *Shigella*, and decreasing members of the *Bacteroidetes* phylum[122]. Lipophilic antibiotics (*e.g.*, lincosamides and macrolides) are eliminated mainly by biliary excretion and therefore cause profound changes in the intestinal microbiota[125]. For example, treatment with clindamycin resulted in a reduction in microbial diversity and a decrease in *Roseburia, Lachospira, Coprococcus, Dorea,* and *Ruminococcus*. Changes in microbial composition were observed throughout 12 mo after clindamycin exposure[121]. In a recent study conducted by Haak *et al*[123], it was shown that treatment with broad-spectrum antibiotics (ciprofloxacin, vancomycin, and metronidazole) promotes the growth of *Streptococcus* and *Lactobacillus*. Furthermore, the authors found reduced numbers of anaerobes producing SCFAs, such as *Bacteroides*, *Subdoligranulum*, and *Faecalibacterium*. Interestingly, a return toward baseline was observed between 8 and 31 mo, but the composition of the microbiota often remained changed from its initial state.

There is some evidence that antibiotics can indirectly affect the composition of the gut microbiota. This is due to interdependence among different microbial taxa, as they have a variety of shared metabolic pathways[124,126]. Thus, the loss or reduction of certain taxa affects the growth of other members of the community. As an example, vancomycin treatment reduces the number of Gram-negative commensals, although this drug selectively targets Gram-positive bacteria[127].

In a recent systematic review, Zimmerman *et al*[128] summarized data from 129 studies on the effect of antibiotics on the composition of the gut microbiota. The authors concluded that the majority of antibiotics (amoxicillin, amoxicillin/clavulanate, cephalosporins, lipopolyglycopeptides, macrolides, ketolides, clindamycin, tigecycline, quinolones, and fosfomycin) increase the abundance of *Enterobacteriaceae,* mainly *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. These bacteria contain molecules that directly enhance the inflammatory response of the host and may play a significant role in the alteration of bile acid metabolism[129]. Moreover, expansion of bacteria belonging to the *Enterobacteriaceae* family was associated with inflammatory bowel diseases, both in animal models and in humans [130,131]. Zimmerman *et al*[128] reported that amoxicillin, piperacillin, ticarcillin, cephalosporins (except fifth generation cephalosporins), carbapenems, and lipoglycopeptides facilitate the overgrowth of *Enterococcus,* while treatment with macrolides and doxycycline results in decreased numbers of these bacteria. It has conclusively been shown that piperacillin, ticarcillin, carbapenems, macrolides, clindamycin, and quinolones markedly reduce the abundance of anaerobic bacteria. Finally, the authors documented that the most long-lasting changes in the community structure are caused by ciprofloxacin (1 year), clindamycin (2 years), and clarithromycin plus metronidazole (4 years).

Another negative effect of antibiotic treatment is the loss of colonization resistance. Depletion of beneficial gut commensals, such as *Lachnospiraceae, Ruminococcaceae,* and *Clostridium scindens*, as well as changes in their metabolic activity promote overgrowth of *Clostridium difficile, Enterococcus*, and other pathogens[33,124].

Antibiotics as a risk factor for IBS

Data from large cohort and case-control studies indicate that antibiotics are a risk factor for functional gastrointestinal disorders and IBS in particular. A retrospective study on more than 26000 patients showed that exposure to macrolides and tetracyclines may be associated with the development of IBS [5]. Similarly, a prospective case-control study found that antibiotic treatment of nongastrointestinal infections was associated with the development of IBS [odds ratio (OR) = 2.30; 95% confidence interval (CI): 1.22-4.33; P = 0.01] and other functional gastrointestinal disorders (OR = 1.90; 95%CI: 1.21-2.98; P = 0.005)[6]. A longitudinal study by Krogsgaard *et al*[7] also identified that the use of antibiotics was a predictor for IBS (OR = 1.8; 95%CI: 1.0-3.2). Additionally, a recent meta-analysis showed that the use of antibiotics for infectious enteritis was associated with an increased risk of IBS (OR = 1.69; 95%CI: 1.20-2.37)[8].

Table 3 Effects of antibiotics on gut microbiota composition (based on culture-independent approaches)

Ref.	Method	Antibiotic	Dosing regimen	Diversity	Compositional changes
Pallav *et al* [136], 2014	Pyrosequencing	Amoxicillin	250 mg 3 times daily for 7 d	–[1,2]	↑ *Escherichia, Shigella*
Kabbani *et al* [137], 2017	16S rRNA	Amoxicillin-Clavulanate	875/125 mg twice daily for 7 d	↓[1,3]	↑ *Escherichia, Parabacteroides, Enterobacter* ↓ *Roseburia*
Burdet *et al* [120], 2019	16S rRNA	Ceftriaxone	1 g once daily for 3 d	↓[1,4]	↓ *Firmicutes, Actinobacteria, Bacteroidetes*
Raymond *et al* [135], 2016	Shotgun sequencing	Cefprozil	500 mg twice daily for 7 d	↓[5]	↑*Flavonifractor, Lachnoclostridium, Parabacteroides*, ↓*Bifidobacteriaceae, Coriobacteriaceae, Eubacteriaceae, Oxalobacteraceae, Pasteurellaceae, Veillonellaceae*
Rashid *et al* [121], 2015	Pyrosequencing	Ciprofloxacin	500 mg twice daily for 10 d	↓[1]	↑ *Bacteroides*↓ *Faecalibacterium, Alistipes,* unculturable *Ruminococcaceae*
		Clindamycin	150 mg 4 times daily for 10 d	↓[1]	↓ *Roseburia, Lachospira, Coprococcus, Dorea, Ruminococcus*
Isaac *et al*[122], 2017	16S rRNA	Vancomycin	250 mg *per os* 4 times daily for 2 wk	↓[1,4]	↑ *Escherichia, Shigella, Klebsiella,* ↓ *Bacteroidetes, Faecalibacterium, Ruminococcus*

[1]OTU analysis.
[2]Rarefaction analysis.
[3]Chao1 index.
[4]Shannon index.
[5]Simpson index.

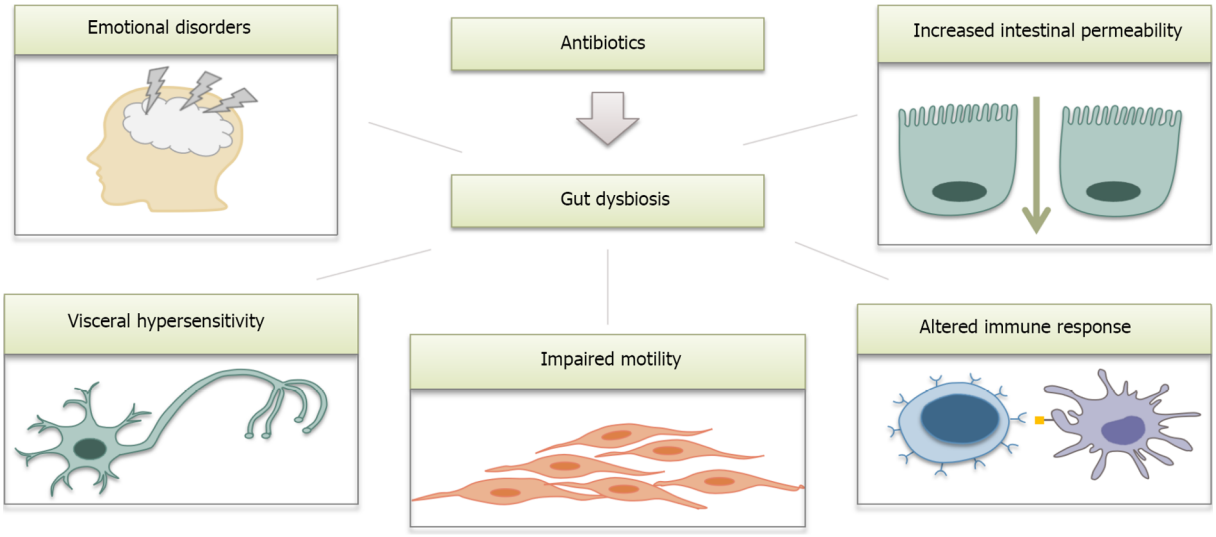
However, nonabsorbable antibiotics can be used to treat IBS. In a double-blind, randomized, placebo-controlled study, treatment with neomycin resulted in a 35% improvement in composite scores of IBS symptoms, compared with only 11% for placebo ($P < 0.05$)[132]. Nonetheless, the use of this antibiotic is limited by the risk for *C.difficile* infection and systemic adverse events. A recent meta-analysis of four studies and 1803 patients showed that rifaximin was more effective than placebo in the overall improvement of IBS symptoms (OR = 1.19; 95%CI: 1.08-1.32 and OR = 1.36; 95%CI: 1.18–1.58, respectively, $P < 0.05$ for both). There was no difference in adverse events between rifaximin and placebo[133]. Due to its safety, rifaximin was approved by the Food and Drug Administration for the treatment of IBS-D.

Similarities in gut microbiota between patients with IBS and those after antibiotic exposure

Analysis of data on changes in the gut microbiota in patients with IBS and those after antibiotic exposure uncovers some common features and trends. For instance, decreased microbial diversity[97-99,121,128] and a reduction in the abundance of *Faecalibacterium,* particularly *F. prausnitzii*[97,98,102,121,122], have been observed in both cases. *F. prausnitzii* is one of the most abundant bacterial species in the gut, exhibiting anti-inflammatory effects through inhibition of IL-8 production, promotion of IL-10 secretion, and upregulation of regulatory T cells[134]. Moreover, patients with IBS were shown to have reduced numbers of *Bifidobacterium*[98,103,104,106,107]. Likewise, several studies have reported a decreased abundance of these commensals after antibiotic exposure[121,128,135]. Most members of the genus *Bifidobacterium* are known to exert beneficial effects on host health, including competitive exclusion of enteric pathogens, metabolism of dietary compounds, and regulation of the immune response[22,26,33]. Furthermore, both IBS and antibiotic exposure are characterized by overgrowth of *Enterobacteriaceae*[98,104,106,136,137]. The *Enterobacteriaceae* family includes pathogenic bacteria (*e.g., Escherichia, Shigella, Klebsiella,* and *Enterobacter)* with proinflammatory properties that may contribute to low-grade inflammation in the gut wall[98].

CONCLUSION

There is clear and consistent evidence from a variety of studies that patients with IBS have altered composition of the gut microbiota and that these alterations are related to the generation of gastrointestinal symptoms. However, studies comparing fecal microbiota in patients with IBS and healthy controls produced variable findings. To date, there is still no consensus on distinct microbiome signatures in IBS. Although some common threads reviewed here were found, prospective large-scale studies need to be carried out to shed light on this issue. Independent analysis of the gut microbiota and its metabolites will help to develop novel microbiota-based treatment strategies that target the



DOI: 10.3748/wjg.v28.i12.1204

Figure 3 Possible link between antibiotic use and the development of irritable bowel syndrome (schematic illustration). Antibiotics cause profound changes in the gut microbiota and therefore contribute to all mechanisms involved in the pathogenesis of irritable bowel syndrome.

underlying pathophysiology of IBS rather than focusing on symptom alleviation.

A number of recent studies have addressed the effects of antibiotics on gut microbiota composition, and these effects were found to be quite similar to those observed in IBS. We suggest that the Rome V criteria could provide a new definition of postantibiotic IBS. As major disruptors of the gut microbiota, antibiotics seem to contribute to all aspects of IBS pathogenesis (Figure 3). However, further research in this area is definitely warranted.

FOOTNOTES

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MINIREVIEWS

Emerging role of colorectal mucus in gastroenterology diagnostics

Hesam Ahmadi Nooredinvand, Andrew Poullis

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Abstract

Colonoscopy is currently the gold standard for diagnosis of inflammatory bowel disease (IBD) and colorectal cancer (CRC). This has the obvious drawback of being invasive as well as carrying a small risk. The most widely used non-invasive approaches include the use of faecal calprotectin in the case of IBD and fecal immunochemical test in the case of CRC. However, the necessity of stool collection limits their acceptability for some patients. Over the recent years, there has been emerging data looking at the role of non-invasively obtained colorectal mucus as a screening and diagnostic tool in IBD and CRC. It has been shown that the mucus rich material obtained by self-sampling of anal surface following defecation, can be used to measure various biomarkers that can aid in diagnosis of these conditions.

Key Words: Colorectal mucus; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Colorectal cancer; Faecal calprotectin

Core Tip: We now know that non-invasively collected colorectal mucus contains diagnostically informative cells that can be analysed to look for various biomarkers. The presence of some of these biomarkers have the potential role in diagnosis of inflammatory bowel disease and colorectal cancer. This is an exciting field that we believe is worth exploring further.

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INTRODUCTION

The current gold standard for the diagnosis of inflammatory bowel disease (IBD) and colorectal cancer (CRC) is ileocolonoscopy. This however is time consuming, expensive and carries a small risk. Unnecessary colonoscopies could be avoided if reliable non-invasive tests were available to diagnose these conditions.

Non-invasive approaches to measurement of inflammation include serum biomarkers such as C-reactive protein (CRP) although this is not bowel specific. Stool calprotectin is bowel specific and is the best studied test[1] however necessity of collecting stool makes this unpopular with some patients[2-4].

In adults, a faecal calprotectin of > 50 μg/g has a sensitivity and specificity of 93% and 94% respectively for distinguishing between irritable bowel syndrome (IBS) and IBD[1]. While faecal calprotectin has an established position in helping to guide the need for ileocolonoscopy in those with bowel symptoms and the follow up of established IBD, it is not in itself a specific diagnostic test.

For CRC, fecal immunochemical test (FIT) is the most widely used non-invasive screening tool with a sensitivity and specificity of 79% and 94% respectively[5]. Similar to faecal calprotectin, an abnormal FIT prompts further investigations and assessment but is not a diagnostic test.

EMERGING ROLE OF COLORECTAL MUCUS

Over the recent years there has been emerging data looking at the use of non-invasive colorectal mucus sampling as a screening and diagnostic tool for IBD as well as CRC.

Colorectal mucus acts as an interface between colonic mucosa and gut content and is a recipient of cells released from the mucosal surface[6]. Some of these cells embedded in the colorectal mucus are excreted with faeces. This cell containing colorectal mucus can therefore be used for specific cell and biomarker detection[7-9]. There is increasing understanding of the complex roles of the colorectal mucus. The mucus itself is a dilute, aqueous and viscoelastic secretion with specific proteins, the mucins, being the major component[10]. The role, composition, physiology and pathophysiology are outside the scope of this review and have recently been reviewed elsewhere[11]. This review will focus on the emerging role of colorectal mucus in diagnostics.

Colorectal mucus can be obtained during proctoscopy[7,8,12] however this has the obvious drawback of being invasive and therefore unfavorable as a screening tool. Up until recently there was no reliable non-invasive method for colorectal mucus sampling. Over the past few years, a novel technique has been developed based on self-sampling of mucus-rich material from the anal surface immediately following defecation.

The use of colorectal mucus as a source of biomarkers has been evaluated in several studies. In the setting of IBD diagnostics and monitoring and CRC diagnostics colorectal mucus has been shown to be a novel and useful medium.

The first study assessing non-invasively collected colorectal mucus compared 141 patients (58 patients with IBD, 50 patients with IBS and 33 healthy volunteers). The study participants were instructed to swab the external anal area immediately following defecation and samples were collected for cytological and Mucin 2 (MUC2) analysis. This was the first study ever to describe non-invasively collected cytology demonstrating large numbers of preserved inflammatory cells in IBD. Significant differences in MUC2 levels were identified in IBD *vs* non-IBD groups raising the possibility that colorectal mucus was a useful diagnostic medium[9].

In a follow on study the performance of several biomarkers including calprotectin, eosinophil-derived neurotoxin (EDN) and protein S100A12 was evaluated in active IBD[13]. EDN is a major secretory protein of eosinophils and elevated levels of EDN have previously been detected in faeces of IBD patients[8]. S100A12 is another granulocyte protein and it has previously been proposed that measurement of the level of S100A12 in the faeces can help differentiate IBD from IBS[14,15].

The authors found that the medium concentration of all of these biomarkers were significantly higher in IBD patients compared to inflammation free individuals with calprotectin and EDN being more sensitive than S100A12 in detecting patients with IBD. Using a combined test taking into account the result of calprotectin and EDN (designated as CALEDN) resulted in sensitivity and specificity of 91% and 89% respectively in detecting patients with IBD (Figure 1). The concentration of all of these biomarkers was significantly higher in ulcerative colitis (UC) patients than those with Crohn's disease (CD) with the most pronounced difference seen for EDN. Over a follow up period of 30 d following treatment there was a steady decrease of all biomarker levels among patients who demonstrated clinical improvement however correlation was easier to detect in UC patients[13].

Further to the description of inflammatory cells in the colorectal mucus of IBD patients an additional follow on cytology study demonstrated that true diagnostic cytology could be carried out on non-invasively collected colorectal mucus. Using cytology and immunocytochemistry a number of different cell types were identified (neutrophils, plasma cells and erythrophagocytes). Blinded cytological analysis enabled an accurate diagnosis to be made in 61.8% of cases[16].

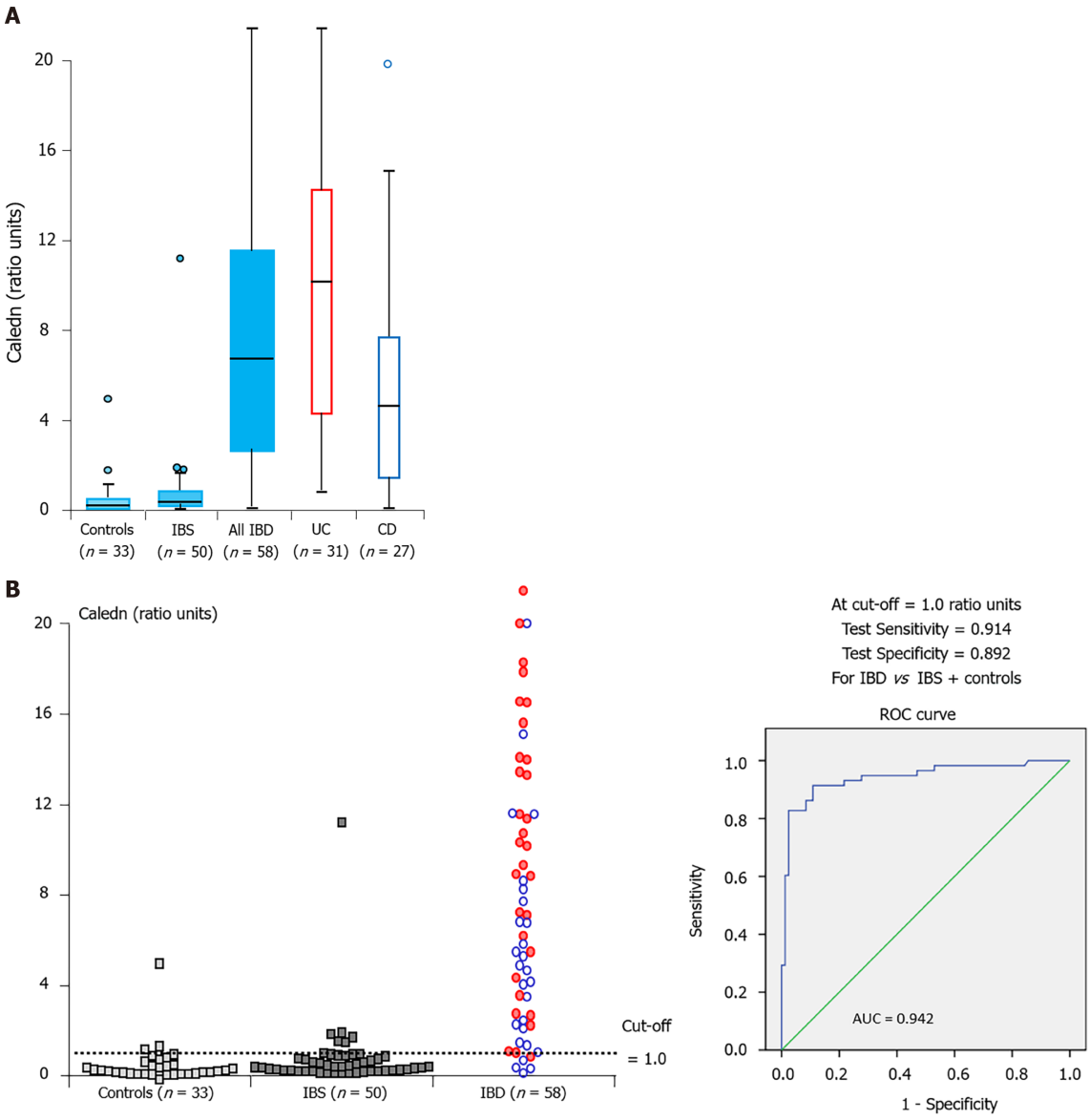

Figure 1 Box and whisker plot and individual result distributions and receiver operating characteristic curve for the combined CALEDN test at stage 1 of the study. In the panel within the inflammatory bowel disease group, blue and red circles correspond to Crohn's disease and ulcerative colitis cases, respectively. AUC: Area under the curve; IBS: Irritable bowel syndrome; CD: Crohn's disease; UC: Ulcerative colitis. Citation: Loktionov A, Chhaya V, Bandaletova T, Poullis A. Inflammatory bowel disease detection and monitoring by measuring biomarkers in non-invasively collected colorectal mucus. *J Gastroenterol Hepatol* 2017; 32: 992-1002. Copyright© The Authors 2017. Published by John Wiley and Sons. A: Box and whisker plot; B: Receiver operating characteristic curve.

Given the success of this new medium in the diagnosis and monitoring of IBD attention has turned to CRC diagnostics. A pilot study was performed looking at whether various biomarkers detected in the colorectal mucus can be reliably used to aid detection of CRC. The diagnostic performance of 24 biomarkers were evaluated. 17 CRC and 35 healthy controls were used to assess these biomarkers. Quantification of haemoglobin, tissue inhibitor of metalloproteinase 1 (TIMP1), M2-pyruvate kinase (M2-PK), peptidyl arginine deiminase-4 (PADI4), CRP, matrix metalloproteinase 9 (MMP9), epidermal growth factor receptor (EGFR), EDN and calprotectin all showed good diagnostic potential[17].

A larger follow on study with 35 healthy volunteers, 62 CRC-free symptomatic patients and 40 CRC patients was conducted to assess the utility of these biomarkers collected in colorectal mucus[18].

The sensitivity and specificity of each of these biomarkers was analyzed in two different scenarios. For assessment of these markers in bowel cancer screening (BCS) comparison was made between the CRC group and healthy controls "screening" arm. A "triage" arm to the study was set up to reflect non-BCS clinical practice and a comparison between CRC cases and symptomatic CRC free patients. Hemoglobin was the best performer with overall sensitivity of 80% and specificity of 94% and 85.5% in the 'screening' and the 'triage' group respectively. These values are comparable to those reported for CRC screening by FIT test[19]. All other biomarkers had a lower sensitivity and specificity especially in the triage group. Of note proximal CRC was associated with higher numbers of false-negative results.

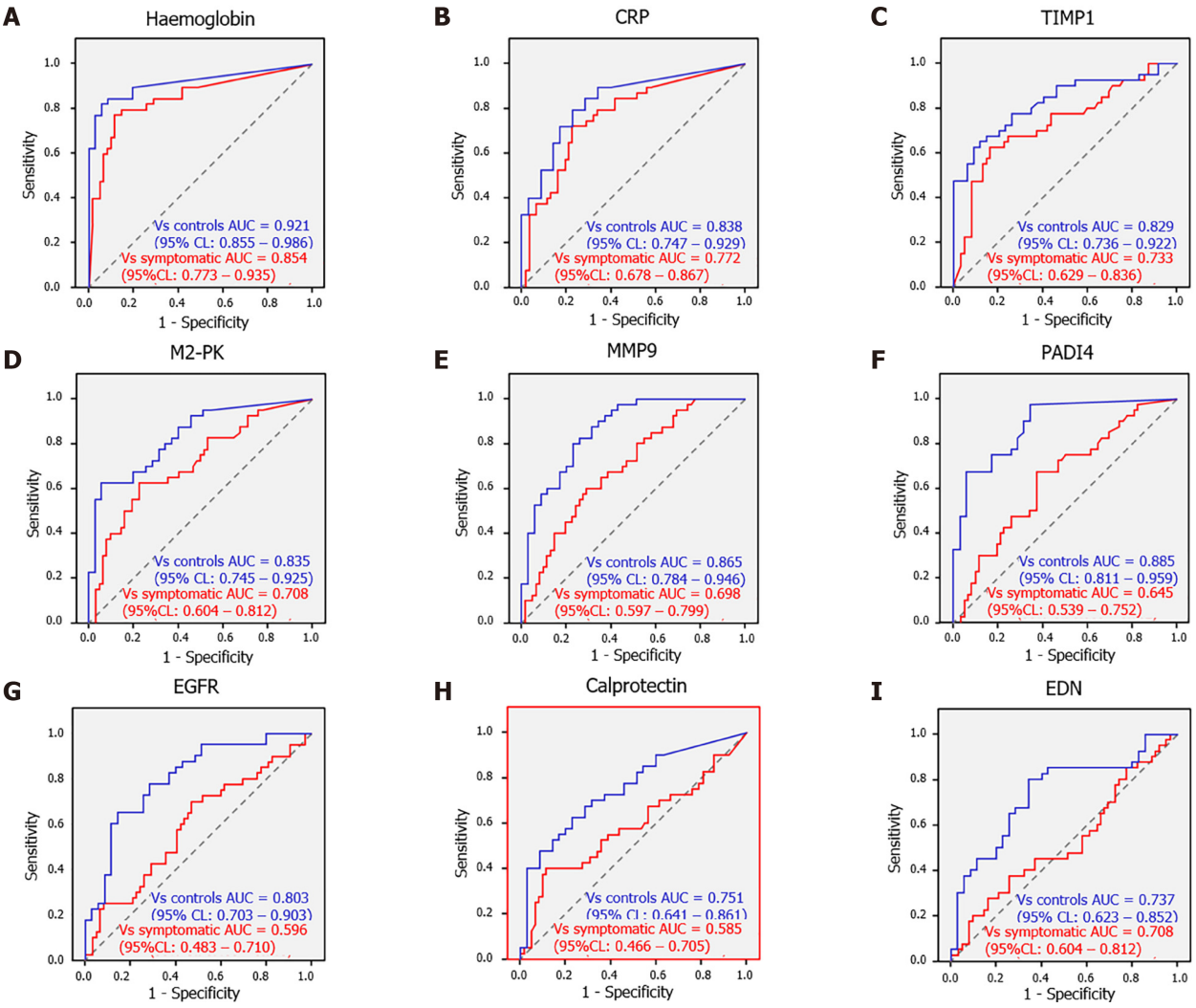

Figure 2 Receiver operating characteristic curves for 'screening' (blue) and 'triage' (red) settings for haemoglobin, C-reactive protein, tissue inhibitor of metalloproteinase 1, M2-pyruvate kinase, matrix metalloproteinase 9, peptidyl arginine deiminase-4, epidermal growth factor receptor, calprotectin and eosinophil-derived neurotoxin. A: Haemoglobin; B: CRP; C: TIMP1; D: M2-PK; E: MMP9; F: PADI4; G: EGFR; H: calprotectin; I: EDN. Citation: Loktionov A, Soubieres A, Bandaletova T, Francis N, Allison J, Sturt J, Mathur J, Poullis A. Biomarker measurement in non-invasively sampled colorectal mucus as a novel approach to colorectal cancer detection: screening and triage implications. *Br J Cancer* 2020; 123: 252-260. Copyright© The Authors 2020. Published by John Wiley and Sons.

EGFR, calprotectin and EDN were worst performing and could not be recommended as reliable diagnostic markers (Table 1 and Figure 2).

Through the use of a questionnaire we found that the method of colorectal mucus sampling was generally very well accepted by the patients arguing that non-invasive testing of colorectal samples for haemoglobin can present an attractive alternative to FIT. The average time taken for carrying out the sampling was about six minutes. Given the immunochemical colorectal mucus sample testing for haemoglobin differs very little from faecal sample testing and the cost being similar to that of FIT, this alternative may boost participation rates.

CONCLUSION

These studies demonstrate that diagnostically informative cells can be obtained from colorectal mucus which can be non-invasively obtained by simply swabbing the external anal area immediately following defecation. The proposed method of colorectal mucus sample collection eliminates the necessity of stool collection and appeared to be very well accepted by the study participants with the vast majority of samples being suitable for biomarker and cytological analysis.

Future work however needs to focus on direct head-to-head comparison between stool calprotectin and colorectal mucus calprotectin in the case of IBD and colorectal mucus haemoglobin concentration and FIT test for colonic cancer screening.

Table 1 Comparison of tested colorectal mucus biomarker performance for colorectal cancer detection versus groups of asymptomatic control subject and patients with abdominal symptoms (based upon receiver operating characteristic curve analysis)

Biomarker	Optimal cut-off level	Sensitivity (%)	AUC *vs* Sympt. Pat-s (95%CI)	Specificity *vs* Sympt. Pat-s (%)	AUC *vs* Control (95%CI)	Specificity *vs* Control (%)	Median biomarker level (CRC)	Median biomarker level (Sympt. Pat-s)	Median biomarker level (Control)
Haemoglobin	109.27 ng/mL	80.00	0.85 (0.77-0.93)	88.55	0.92 (0.85-0.99)	94.29	1708.74 ng/mL[a,b]	0.00 ng/mL[a,c]	0.00 ng/mL[b,c]
CRP	8.90 ng/mL	72.50	0.77 (0.68-0.87)	75.81	0.84 (0.75-0.93)	80.00	22.09 ng/mL[d,e]	1.41 ng/mL[d]	0.00 ng/mL[e]
TIMP1	3.25 ng/mL	67.50	0.73 (0.63-0.84)	75.81	0.83 (0.74-0.92)	85.71	8.26 ng/mL[f,g]	1.42 ng/mL[f,h]	0.71 ng/mL[g,h]
M2-PK	9.00 U/mL	62.50	0.71 (0.60-0.81)	77.42	0.83 (0.74-0.92)	91.43	11.98 U/mL[i,j]	2.97 U/mL[i,k]	0.57 U/mL[j,k]
MMP9	10.38 ng/mL	65.00	0.70 (0.60-0.80)	64.52	0.86 (0.78-0.95)	82.86	20.93 ng/mL[l,m]	6.37 ng/mL[l,n]	0.44 ng/mL[m,n]
PADI4	1.16 ng/mL	67.50	0.64 (0.54-0.75)	62.90	0.88 (0.81-0.96)	94.29	1.51 ng/mL[o,p]	0.88 ng/mL[o,q]	0.00 ng/mL[p,q]
EGFR	305.52 pg/mL	60.00	0.60 (0.48-0.71)	58.06	0.80 (0.70-0.90)	88.57	342.72 pg/mL[r]	187.01 pg/mL[s]	67.72 pg/mL[r,s]
Calprotectin	3.38 μg/mL	57.50	0.58 (0.47-0.70)	56.45	0.75 (0.64-0.86)	80.00	4.01 μg/mL[t]	2.94 μg/mL[u]	0.59 μg/mL[t,u]
EDN	12.83 ng/mL	45.00	0.52 (0.40-0.64)	62.90	0.74 (0.62-0.85)	88.57	8.04 ng/mL[v]	10.10 ng/mL[w]	2.85 ng/mL[v,w]

P value for CM biomarker level comparisons between study groups.
[a,b,d,e,g,j,m,p,q,r]$P < 0.00001$.
[c]$P = 0.01878$.
[f]$P = 0.00008$.
[h]$P = 0.0264$.
[i]$P = 0.00040$.
[k]$P = 0.01352$.
[l]$P = 0.00078$.
[n]$P = 0.00288$.
[o]$P = 0.01428$.
[s,t]$P = 0.00020$.
[u]$P = 0.00042$.
[v]$P = 0.00044$.
[w]$P = 0.00064$.
CRP: C-reactive protein; TIMP1: Tissue inhibitor of metalloproteinase 1; M2-PK: M2-pyruvate kinase; MMP9: Matrix metalloproteinase 9; PADI4: Peptidyl arginine deiminase-4; EGFR: Epidermal growth factor receptor; EDN: Eosinophil-derived neurotoxin; AUC: Area under the curve.

The non-invasive collection of colorectal mucus is a novel and exciting area of gastrointestinal diagnostics and may dramatically change our approach to the investigation and diagnosis of colorectal diseases.

FOOTNOTES

Author contributions: Nooredinvand HA drafted the manuscript; Poullis A proofread and revised the manuscript.

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MINIREVIEWS

Similarities, differences, and possible interactions between hepatitis E and hepatitis C viruses: Relevance for research and clinical practice

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Abstract

Hepatitis E virus (HEV) and hepatitis C virus (HCV) are both RNA viruses with a tropism for liver parenchyma but are also capable of extrahepatic manifestations. Hepatitis E is usually a viral acute fecal-oral transmitted and self-limiting disease presenting with malaise, jaundice, nausea and vomiting. Rarely, HEV causes a chronic infection in immunocompromised persons and severe fulminant hepatitis in pregnant women. Parenteral HCV infection is typically asymptomatic for decades until chronic complications, such as cirrhosis and cancer, occur. Despite being two very different viruses in terms of phylogenetic and clinical presentations, HEV and HCV show many similarities regarding possible transmission through organ transplantation and blood transfusion, pathogenesis (production of antinuclear antibodies and cryoglobulins) and response to treatment with some direct-acting antiviral drugs. Although both HEV and HCV are well studied individually, there is a lack of knowledge about coinfection and its consequences. The aim of this review is to analyze current literature by evaluating original articles and case reports and to hypothesize some interactions that can be useful for research and clinical practice.

Key Words: Hepatitis C virus; Hepatitis E virus; Co-infection; Genomic variability; Extrahepatic diseases; Vaccine

Core Tip: Hepatitis E virus (HEV) and Hepatitis C virus (HCV) are both RNA viruses characterized by greater variability than DNA viruses and mainly infect the liver. Despite these similarities, the two viruses have different species barriers and disease progression. Coinfection with particular HCV and HEV types could aggravate hepatic and/or extrahepatic diseases, taking into account virus–host interactions between the two viruses during viral replication.

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INTRODUCTION

Viral hepatitis is a global public health problem, affecting more than 325 million people globally. In countries with poor health care standards, coinfection among hepatotropic viruses is possible due to multiple risk factors. This condition increases morbidity and mortality rates in infected patients[1]. Hepatitis E virus (HEV) could influence hepatic or extrahepatic symptoms in patients with chronic hepatitis C virus (HCV) infection[2,3]. Both the prevalence and spreading of HEV and HCV infections worldwide reflect different routes of transmission and high genomic variability[4,5], however coinfections or superinfections with the two viruses in the same individuals may occur, though a paucity of data exist in this respect.

A summary of virological and pathogenic characteristics of both viruses discussed through the text of this review are reported in the Table 1.

EPIDEMIOLOGY

HEV affects around 20 million people worldwide, and the infection is distributed in both developing and industrialized countries[1]. This enteric non-enveloped virus, belonging to the *Hepeviridae* family, *Orthohepevirus* genus, is classified into eight genotypes and 24 subtypes. HEV1 and HEV2 infect only humans in resource limited settings, such as Asia, Mexico, and sub-Saharan and Central Africa[6]. HEV3, emerging in Europe as a sporadic infection, and HEV4 infect both humans and animals. HEV4 shows a high prevalence in Asia[7]. In 2014, HEV5 and HEV6 were isolated from wild boars, while HEV7 (originally infecting dromedaries) was isolated from a human case for the first time[8,9]. Lastly, HEV8 was detected in Bactrian camels[10]. Of note, the nomenclature system of this virus is constantly changing due to frequent identification of novel strains in various animal species[11]. The main routes of transmission are fecal-oral and zoonotic (*i.e.*, undercooked meat or close contact with animals). In industrialized countries, transmission is related to travelers returning from endemic areas and to blood transfusion or organ transplantation[12,13]. Human-to-human transmission was also described in men having sex with men[14], as well as HEV can infect newborns by vertical transmission[15]. Sero-prevalence studies identified specific risk categories, such as veterinarians, forestry workers, butchers and hunters, occurring as sporadic cases of infection[16].

HCV is also very widely disseminated throughout the world. Indeed, approximately 71 million people worldwide are infected by HCV, an enveloped virus belonging to the *Flaviviridae* family and *Hepacivirus* genus. In 2018, Borgia and colleagues identified the eighth genotype in patients from India [17]. The distributions of the genotypes and 86 subtypes are related to risk factors and geography across the world. In developing countries, HCV1 and HCV2 with high subtype diversity are prevalent. HCV3 is predominant in Europe, North America and Southeast Asia. In the Middle East and Central Africa, HCV4 is endemic, while HCV5 was found exclusively in South Africa[18]. HCV6 is present essentially in Japan and nearby areas. HCV7 is responsible for less than 1% of cases of HCV hepatitis. In industrialized countries, the most prevalent subtypes are HCV1a, 1b, 2c, 3a, and 4a[19,20]. HCV1b and 2c are mainly transmitted by blood transfusion and infect older population groups, whereas HCV1a, 3a and 4a are prevalent in intravenous drug users[21,22]. Low standards for healthcare procedures have allowed HCV spreading among patients in hemodialysis units[23]. After 1992, blood screening controlled the spread of this infection. Sexual and mother-to-infant (6%) transmissions increased in subjects coinfected with human immunodeficiency virus (HIV), while breastfeeding does not significantly increase the risk of transmission from mother to baby[24].

Table 1 Similarities, differences and potential interactions across the major points of hepatitis E virus and hepatitis C virus infections

Categories	Similarity	Difference	Interaction
Epidemiology	High prevalence in developing countries[1]	HEV infects humans and animals[6,7]	Co-infections or superinfections[2,3]
Genetic variability	RNA viruses (*quasispecies*), genotype classification, recombination events[4,5]	Replication rate, HEV has non-enveloped or quasi-enveloped virions [12,26]	None
Pathogenesis	Disease progression in immunocompromised patients[32,54]	Microbiota alteration, hepatic severity [23,33,83,84]	HEV could influence hepatic or extrahepatic symptoms in patients with chronic HCV infection [2,3]
Treatment	Choice of therapy[35,50]	PEG-IFN-α and RBV are still the therapies of choice for HEV[35]	DAA therapy can be effective against both viruses[50,53]
Prevention	Public health measures[58]	Vaccine availability[60,62]	Improvement of screening policies[35,50]

DAA: Directly acting antivirals; PEG-IFN: Pegylated interferon; RBV: Ribavirin; HEV: Hepatitis E virus; HCV: Hepatitis C virus.

GENETIC VARIABILITY

RNA viruses have high genetic plasticity, and they can rapidly generate a drug-resistant viral population or evade the host system under pressure. The key of this variability is the polymerase without proofreading activity[25]. During viral replication with a mutation rate ranging from 10^{-6} to 10^{-4} substitutions *per* nucleotide, the virus produces hundreds of progeny (*quasispecies*), which differ by one or a few nucleotides in the genomic sequence. The fitness of *quasispecies* reflects Darwinian evolution and natural selection allows the spread of a better adapted viral population[26]. HEV and HCV are both positive-sense single-stranded (ss) RNA viruses, even if the organization and length of the genome are different.

The HEV genome (7.2 kb) contains three open reading frames (ORFs) between the 5′UTR- and 3′-UTR (polyA-tract) regions. ORF1 encodes enzymes, including RNA-dependent RNA polymerase (RdRp) and non-structural proteins. ORF2 and ORF3 encode for capsid protein and a multifunctional phosphoprotein, respectively. ORF4 is directly involved during replication[27]. By contrast, the HCV genome (9.6 kb), containing one ORF between the 5′-3′UTRs, encodes three structural (C, core) proteins, envelope glycoproteins 1 and 2 (E1 and E2), and finally seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. In particular, NS5B encodes the polymerase enzyme[28].

HEV genetic characteristics make it suitable for infecting humans and animals through various transmission routes, since it is maintained in the environment[12]. Virions are present in two different forms, non-enveloped excreted in the feces of humans or animals and quasi-enveloped coming from blood. Quasi-enveloped virions bind cells in a less effective way, showing minor infectivity[12]. The high similarity among HEV3 and HEV4 strains isolated from humans and animals demonstrated that adaptation is not necessary for infection. On the other hand, HEV1 does not have zoonotic reservoirs, as experimentally, intra-species transmission failed to infect the progeny in pig, rat or goat. Species barriers of HEV1 appear to be related to genetic elements carried on the ORF1 non-structural protein[11]. As far as HCV is concerned, the barrier between species may be responsible for the unique targeting of humans by this virus. However, endemic circulation in an area of the world where human, ape and monkey populations overlap and the discovery of viruses closely related to HCV in animals suggested a zoonotic origin[29].

Recombination events increased the genetic variability for both HCV and HEV viruses. Among HEV genotypes, as well as fragment of human genes and HEV strains, recombination is possible. In particular, two insertions of the ORF1 hypervariable domain on the human RPS17 gene (ribosomal protein S17) increased replication in hepatoma cells[27]. Likewise, during HCV superinfection, recombination events (inter-genotype or inter-subtype), using different breakpoints within the viral genome were identified. The first circulating form was HCV2k/1b with a mapped breakpoint in the NS2 gene. At present, seven inter-genotypes (2k/1b, 21/6p, 2b/1b, 2/5, 2b/6w, 3a/1b, and 2a/1a) and three inter-subtypes (1b/1a, 1a/1c, and 4a/4d) recombinant forms (RFs) are known[30].

PATHOGENESIS AND NATURAL HISTORY

The incubation period of HEV infection ranges from 2 to 10 wk[31]. HEV determines acute hepatitis with a very low incidence (1%-4%), varying severities, which resolves in 2–3 mo[32]. One of the most serious outcomes is fulminant hepatitis (FH), which is characterized by hepatic parenchyma necrosis, renal failure or coma[33,34]. The wide spectrum of clinical illness could indeed be related to the infecting genotype[31]. In 2015, Smith and Simmonds reviewed published papers for causal association

between FH and genomic variability[34]. The correlation appears to be related to epidemiological factors, namely, restricted geographical areas and time span of collected isolates[34]. The majority of people acquiring infection do not have severe consequences. However, HEV1 and HEV2 are the principal genotypes related to severe disease and mortality and HEV1 was the principal responsible for outbreaks in some countries of Asia and Africa between 1987 and 2015[15]. Several studies reported FH to be related to specific nucleotide substitutions in the HEV1, HEV3 and HEV4 genomes. For instance, the U3148 and C5907 substitutions in HEV3 and HEV4 strains were significantly associated with FH [27]. However, HEV3 hardly progresses to acute liver failure[35]. Extra-hepatic manifestations such as membranoproliferative glomerulonephritis and cryoglobulinemia are not rare in HEV infected patients [13] and it was suggested that in severely immunocompromised patients HEV could be implicated in development of hepatic cancer[36]. Also, common neurological disorders in the course of HEV infection were found such as nerve root, plexus disorders and meningoencephalitis[37,38].

HEV can also cause chronic infection, lasting a year or more in immunosuppressed individuals[32], which has only been observed for HEV3 and HEV4[39]. Comparison of HEV3 isolates between blood donors and patients with hepatitis showed just one polymorphism difference (leucine to phenylalanine ORF2 substitution) in sequences from the first category. Anyway, there is no evidence of pathogenesis related with substitutions occurring in virus genomes[33]. Interestingly, the fast progression to liver fibrosis has been associated with slow *quasispecies* diversification during one year of chronic infection [40].

In contrast to HEV, HCV frequently (50%-80%) causes chronic hepatitis, which is associated with liver cirrhosis, steatosis and hepatocellular carcinoma (HCC)[40]. The variability of genotypes/subtypes was associated with pathogenetic significance. HCV1b hypervariable region 1 (HVR1) of E2 protein displays significantly higher genetic variability than HCV3. HCV3 establishes hepatic chronic infection in less cases compared to other HCV types, particularly HCV1b. The hypervariable E2 region of HCV1b displays low evolutionary dynamics during the course of infection, generating few viral variants, which could provide a fitness advantage under immune system and therapy pressures[41], while the lower variability of HCV3 results in a lower chance to establish chronic infection[41]. On the other hand, HCV3 core protein expression is able to induce more intracellular lipid accumulation causing steatosis more than other genotypes[40]. Indeed, HCV3 infection is associated with steatosis more frequently than HCV1. Some amino acid substitutions in HCV3 core proteins upregulate the sterol regulatory element binding protein-1 (SREBP-1), inducing intracellular lipid accumulation[41].

TREATMENT

Usually, acute HEV infection does not require antiviral therapy[35]. Ribavirin (RBV) monotherapy may be considered in cases of severe acute hepatitis or chronic infection in solid-organ transplant recipients. PEGylated-interferon-a (PEG-IFN-α) was effectively administered to patients after liver transplant or hemodialysis[35], although IFN can cause several side effects[34]. RBV therapy with or without PEG-IFN-a is contraindicated during pregnancy[15]. Sustained virological response (SVR) is achieved only in 78% of chronic patients treated with ribavirin for a median period of three months, probably because of viral mutants[35]. Deep sequencing detected the Y1320H, K1383N and G1634R polymerase substitutions on HEV3 isolates from patients who relapsed or failed RBV therapy[35,42]. Clearly, RBV increases viral heterogeneity, leading to the emergence of different viral populations[35].

PEG-IFN-α and RBV were the standard of care to treat HCV until 2011. Direct-acting antiviral (DAA) drugs quickly changed the landscape of infection, as patients achieved a high SVR rate (95%-99%). Five pan-drug combinations are available right now to treat HCV: Sofosbuvir (SOF), sofosbuvir/velpatasvir (SOF/VEL), sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX), glecaprevir/pibrentasvir (GLE/PIB) and grazoprevir/elbasvir (GZR/EBR)[43]. DAA drugs determined direct pressure on the viral genome, producing *quasispecies* with resistance associated substitutions (RASs) on NS3/4A, NS5A and NS5B target regions escaping therapy[44]. Several RASs on all target regions after treatment with first-/second-generation and IFN-free regimens in specific HCV types were reported[44-46]. Additionally, natural polymorphisms carried on specific subtypes can confer resistance to NS5A inhibitors. In the last EASL guidelines, experts recommended to detect resistance on NS5A (from 24 to 93 amino acid positions) for subtypes 1l, 4r, 3b, 3g, 6u, and 6v prior to first-line treatment[43]. Indeed, patients who failed therapy displayed NS5A RAS at baseline in the same rate of virological failure[47]. The HCV RFs have been reported in few cases around the world, thus pathogenesis and therapy efficacy are not well characterized. Two patients infected by RF 2b/1b achieved viral clearance with an interferon-free regimen[48]. In contrast, a patient infected by the same RF failed two different interferon-free regimens[49].

Of note, new DAA therapies for HCV had an indirect effect on HEV in coinfected patients. SOF is approved for the treatment of chronic HCV infection but can also inhibit HEV replication *in vitro* (especially if co-administered with RBV) and could be an interesting treatment option in coinfected individuals[50], but clinical universal efficacy has not yet been demonstrated[35]. A SOF based DAA regimen excludes occult HCV or HEV infection in patients who received a liver or renal transplant[51]

and successful treatment was reported in some cases of HEV/HCV coinfection. Biliotti and colleagues reported viral clearance of HCV3 and HEV3 in one infected patient after therapy with SOF plus RBV [52]. In a subject infected through liver transplantation, the combination of SOF, daclatasvir (DCV) and RBV led both to HCV-RNA undetectability 6 wk after the initiation of therapy and to HEV-RNA undetectability at 12 wk after initiation of therapy[53]. In another immunosuppressed patient affected by both HCV and HEV infections, SOF in combination with DCV reduced HCV-RNA to undetectable levels after 4 wk of treatment but did not have a significant effect on serum HEV-RNA levels[54]. Lastly, one patient treated for 12 wk with SOF/DCV/RBV and tenofovir cleared HCV and HEV without risk of HBV reactivation[55]. In clinical practice, detection of HCV and potential HEV genome substitutions may be useful to predict treatment failure[25,44].

In 2016, a new molecular mechanism against HCV and HEV was proposed by Wang and colleagues [56]. INF-γ and TNF-α play essential roles in infections by intracellular agents and show a synergistic effect in experimentally transfected cells with HCV or HEV by activating NF-kB signaling. Antiviral activity is related to innate immune responses. Cooperation between INF-γ and TNF-α, activating signaling cascades, protects against HCV and HEV infection[56].

PREVENTION

Prevention of infections is possible through public health measures and screening policies. In endemic areas for HEV, it is important to wash hands frequently, drink bottled water and eat fruits and vegetables washed with safe water[57]. In areas with low endemicity and zoonotic transmission, simple hygiene measures and cooking meat well done can be fundamental to reduce transmission[30]. HCV and HEV may share the same route of transmission, and blood transfusion and organ transplantation can be dangerous for recipient patients and their immunosuppressed status[57,58]. Tests to detect anti-HCV antibodies are standardized. Additionally, HCV core antigen and molecular assay are used to identify patients with ongoing viral infection[43]. On the other hand, a HEV diagnosis needs a combination of an antibody test and molecular assay due to the specificity of the assay being suboptimal and anti-HEV IgM not being a really robust marker[35].

However, vaccines are the best protection against viral infections. HEV genotypes represent one single serotype, with a serological cross-reactivity, thus one vaccine should protect against all types, despite genetic heterogeneity[30]. In China, a vaccine based on the ORF2 protein had high efficiency in a large human population and has been licensed, but is not available elsewhere at this moment[59]. However, mutations on the ORF changed the structure of the ORF2 protein, reducing the protective efficacy of the vaccine. For preventive purposes, naturally attenuated viral variants carrying substitutions in the polymerase region could be used in the future[27]. Very recently, Chen and colleagues evaluated the safety and efficacy of immunization with an accelerated HEV239 vaccine (Hecolin®). Protective antibodies, produced within 21 d, can be useful during an ongoing HEV outbreak or for travelers and humanitarian workers moving to endemic areas in a short time[60]. At present, HEV Vaccine Working Group by the WHO's Strategic Advisory Group of Experts (SAGE) considered the use of Hecolin® for the general populations residing in endemic areas during outbreaks as quickly as possible. However, due to the lack of data about immunogenicity and safety, the Working Group did not recommend the routine use of this vaccine for specific risk groups, such as pregnant women, patients with chronic liver disease and immunocompromised persons[1]. In the next future, human and animal vaccinations should be associated, considering the One Health concept, for preventing transmission and improving public health[57].

In contrast to HEV vaccine, the HCV vaccine is still under development since there are several limitations, such as easy culture systems not being available, animal models for testing, and viral genetic diversity (genotypes, subtypes and *quasispecies*). The extraordinary variability of HCV determines several opportunities to select, within and between infected individuals, viral variants escaping the immune response[61]. In 2017, University of Oxford in collaboration with other industries developed a candidate vaccine using the entire HCV NS3-5B protein. At present, the vaccine is in phase 1 (EudraCT Number 2016-000983-41) to assess the safety and effectiveness of the immune response against the virus in healthy volunteers. The estimated completion of the study is August 2022[62]. Eradication of HCV by 2030 is the goal of the World Health Organization, and the organization must consider improvements in screening policies and hope for an effective vaccine.

MAJOR CAUSES AND EFFECTS OF COINFECTIONS

A schematic overview of HEV/HCV possible interactions is reported in Figure 1.

Epidemiological considerations

Co-infections or superinfections of HEV with HCV may be due to a common parenteral route of

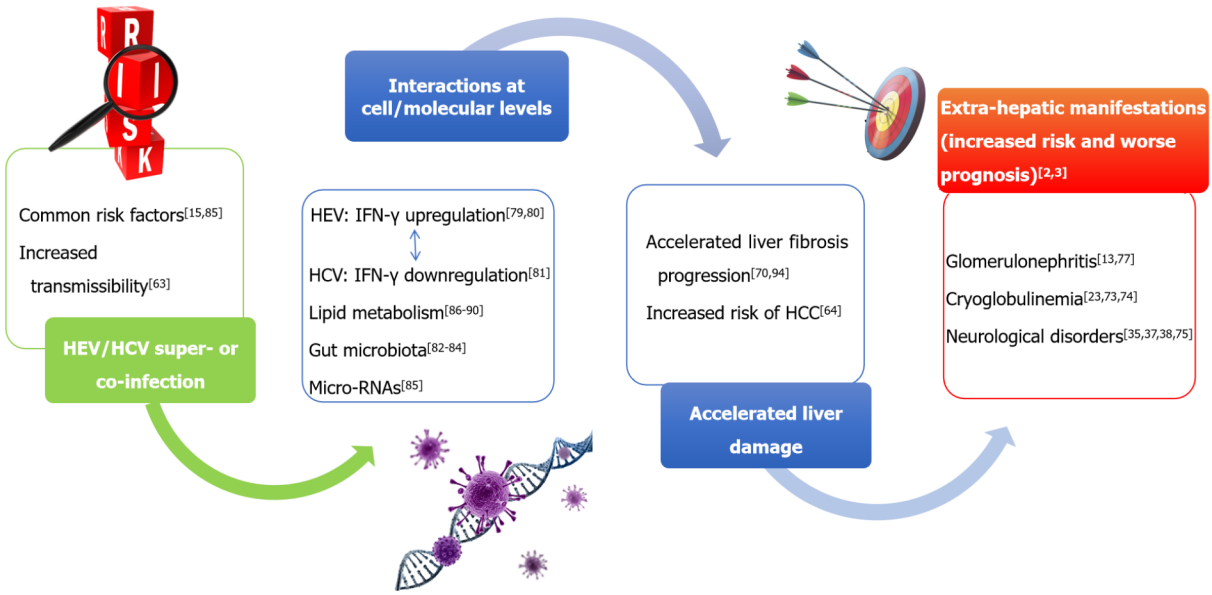

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Figure 1 Possible interactions at cell-molecular level of hepatitis E virus and hepatitis C virus infecting the same individual. IFN: Interferon; HEV: Hepatitis E virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

transmission. Moreover, it was hypothesized that alteration of the intestinal mucosa associated with chronic liver damage due to HCV facilitates HEV translocation from the gut to the liver of patients infected through the oral route[63]. There is a lack of studies investigating the prevalence of possible coinfections with HCV and HEV. At present, it is impossible to provide reliable estimates of the actual prevalence, since information came from few studies and case reports. Future studies, including adequately large sample size, should be planned to estimate the actual prevalence of coinfections. Moreover, the main limitation of the epidemiological surveys conducted so far is that only antibody tests were used[58,64]. Detection of anti-HEV immunoglobulins is related to specificity and sensitivity of commercial kits, among which discordant results were reported in the literature[65]. In 2016, Norder and coworkers[66] evaluated the performance of five commercial assays to determine IgM and IgG levels against HEV. IgM titer was detected by a sensitive HEV IgM/HEV IgG test after the onset of symptoms, providing concordant results in 99% samples from patients with suspected HEV infection. By contrast, recomWell™ HEV IgG/IgM (Mikrogen Diagnostik, Neuried, Germany) and DS-EIA-ANTI-HEV-G/M™ (DSI Srl, Milan, Italy) tests were found to be less specific. In conclusion, investigating the actual rate of coinfections and the effect of both viruses on liver disease progression would require more accurate serological assays and more studies using direct detection of HCV and HEV RNA by molecular tests.

Clinical considerations

Hepatic damage: Infections due to HEV and HCV, even if occurring at different times, can lead to a worse clinical course[58,67]. In fact, serum IgG directed against HEV were associated with a faster evolution towards more severe degrees of fibrosis in patients with chronic HCV infection[58]. Coexistence of the two viruses appeared to be associated with accelerated progression of liver damage as evidenced by the reduced number of platelets, increased transaminases and prolonged prothrombin times observed in patients with chronic HCV hepatitis with HEV exposure during their lifetime (IgG-positive) when compared to HCV mono-infected patients[68]. It is possible that HEV infection in patients infected by HCV with a significant degree of liver fibrosis, accelerates liver damage to such an extent that liver decompensation and death may occur more frequently[69]. These considerations point to the importance of treating HCV and preventing HEV superinfection (either primary prevention or vaccine strategies) in patients affected by chronic HCV infection, a situation which may be particularly frequent or problematic in resource-limited settings. In patients with HCV related HCC, HEV seroprevalence was 11% (compared to 6% in the healthy population), while it reached 42% in patients who underwent liver transplantation for chronic HCV infection[67]. In 2005, Elhendawy and coauthors reported HCV/HEV coinfections in 71.4% of chronic hepatitis patients and in 96.1% of cirrhotic patients with or without HCC, suggesting a possible relationship between the two viruses on progression of liver disease[67]. Recently, the prevalence of HEV infection among adults with chronic liver disease, from 2011 and 2018, was evaluated and anti-HEV IgG positivity was found in 8.6% of HCV chronic positive patients, with a high prevalence in the oldest individuals compared to young age groups[68]. Also, possible effects of HEV infection in increasing the risk of liver cancer over HCV-induced

subclinical liver injury[70] further emphasizes the importance of treatment and preventative strategies for these two viruses to reduce overlap in the same individuals.

Extra-hepatic diseases: Since both viruses may be responsible for extra-hepatic diseases, several studies described these manifestations and correlated them with genetic features[38]. Importantly, HCV does not infect only hepatic cells, and the virus has been found in peripheral blood mononuclear cells, T cells, and monocytes, as well as in B cells and macrophages of colonic tissue. HCV replicates within carotid plaques induce arterial inflammation, probably through the pro-inflammatory cytokine interleukin 1β regardless of viral type[71]. The extrahepatic infection, demonstrated by cell lines producing HCV2a virions, could explain the late relapses observed in clinical trials[72]. Both acute and chronic hepatitis E infections are associated with antinuclear antibodies and cryoglobulinemia in the serum of patients that is similar to untreated HCV infection. The cryoglobulin concentration correlates with the viral load rather than with the degree of inflammation[73]. Serum cryoglobulins in the serum of patients affected by HCV infection are associated with a worse degree of steatosis and fibrosis, and it is not known if the same can happen in HEV infection[74]. Likewise, the risk of evolution to lymphoproliferative diseases associated with HEV cryoglobulinemia with or without HCV cryoglobulinemia is unknown. Furthermore, insulin resistance and metabolic syndrome have already been related to HCV infection, as well as HEV infection recently, which can contribute to the progression of fibrosis in patients with chronic liver disease[3]. As far as HEV is concerned, the neurological disease Guillain-Barre syndrome did not appear to be genotype specific[38], but HEV1 was associated with neurological injury[35], as well as HCV[75]. Moreover, HEV1 and HEV3 were found to be responsible for acute pancreatitis, which has already been described for major hepatitis viruses, in a large number of reports or case control studies[39]. In 2012, a causal link between HEV3 and renal injury was reported[76]. Additionally, mechanisms inducing glomerular disease were found to be similar to those induced by HCV[77]. HCV increased the risk of chronic kidney disease, inducing glomerular injury through the high viral load related to HCV1 or HCV2[23].

Virological and pathogenetic considerations

It is known that HEV inhibits production of type I IFNs[78], while it induces upregulation of IFN-γ by natural killer (NK) or natural killer T lymphocytes[79,80]. The core and some non-structural proteins of HCV (NS3, NS5A and NS5B) were demonstrated to alter the function of dendritic cells (DCs) in vitro, resulting in impaired CD4+ and CD8+ T-cell responses to the virus. Also, patients with chronic HCV infection have reduced interleukin-12 and IFN-γ levels compared to those who cleared the virus[81]. Therefore, at least in principle, HEV could counteract chronicity of HCV through IFN-γ upregulation, but interactions between the two viruses *via* cytokine cross-talk may be complex and not well demonstrated or easy to predict.

Interestingly, liver health is related to the composition of gut microbiota. This is influenced by enteric virome, with whom is in continuous and dynamic equilibrium, and by viruses chronically infecting host tissues[82]. The number of studies on the gut-liver axis and hepatitis infections is presently very low, but microbiota alteration is related to liver disease. HCV-positive people had lower bacterial diversity (*i.e.*, less *Clostridium* and more *Streptococcus* and *Lactobacillus* species) compared with non-infected people[83]. Exacerbation of HEV infection was negatively related to high *Lactobacillaceae* levels[84]. The relationship between gut dysbiosis and viral hepatitis needs to be further investigated, but clearly unfavorable shift in gut microbiota composition driven by the two hepatic viruses may correlate with increase of inflammation and a worse liver stiffness[83,84].

Lastly, at molecular level, microRNAs (miRNAs) play a pivotal role in the progression of liver diseases[82]. The roles of the miRNAs are still under study, but it was already speculated that miR-628-3p, miR-194, miR-151-3p, miR-512-3p, miR-335 and miR-590 are potentially involved in HEV/HCV coinfection[85].

Studies in animal models highlighted the ability of HCV to determine changes in the expression of genes that regulate the lipid metabolism[86]. The role of statins in inhibiting viral replication was subsequently proven[87]. Interestingly, not all statins show an inhibitory effect on HCV replication, suggesting an anti-viral mechanism independent from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase[88]. However, the capability of fluvastatin in lowering HCV RNA in people with chronic hepatitis C appears to be modest, variable, and often fleeting[89]. In contrast, patients treated with statins who are chronically infected with HEV show significantly higher viral loads than chronically infected patients without statin administration and this underlines the possible impact of lipid metabolism on HEV replication[90], while treatment with proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, such as alirocumab, determines a poor antiviral activity against HEV. These observations led to the hypothesis that the antiviral activity of these molecules is related to their ability to determine an increase in intracellular cholesterol, which is greater for statins than for PCSK9 inhibitors[90]. Possible indirect interactions between the two viruses through their influence on lipid metabolism merit determination.

Table 2 Research perspectives for the next future

No.	Research perspectives for the next future
1	*In vitro* and *in vivo* studies to define pathogenic interactions during HEV/HCV coinfection. Cell lines model could explain interactions between viral proteins and cellular pathway responsible for liver fibrosis progression, liver steatosis and insulin resistance encountered in patients
2	Studies to understand relationships among immune phenomena (autoantibodies, cryoglobulins and autoimmune diseases) in patients infected by both viruses, and their correlated in terms of T- and B-cell responses and human leukocyte antigen type
3	Clinical trials to test safety and effectiveness of DAA in co-infected patients and new therapies. At present, data on DAA treatment is limited to *in vitro* studies or very few case reports
4	*In vitro* studies to evaluate genetic interactions between HEV and HCV during viral replication. Permissive cell lines, infected simultaneously by the two viruses, could show whether there is an interference or synergy between them during viral progeny production

DAA: Directly acting antivirals; HEV: Hepatitis E virus; HCV: Hepatitis C virus.

Special populations

The interactions between the two viruses could be even promoted by immune-suppression induced by HIV, which may facilitate HEV transmission[91]. High prevalence of IgG anti-HEV antibodies (> 15%) was found in people living with HIV (PLWH) affected by HCV chronic infection, in particular if CD4+ T-cell count was below 350 cells/mm^3[92]. In endemic rural areas, HEV/HCV coinfection also occurred frequently among pregnant women, inducing a significant worsening of biochemical liver indices than women with negative HCV serology[2]. HCV pathogenesis during pregnancy is poorly understood, and it was related to preterm delivery, placental abruption, and low birth weight in a large cohort of infected women[93]. HEV replicates in the human placenta, among pregnant women, the fatality rate being around 20% and up to 30% in the third trimester. HEV infection determines fulminant hepatic failure, membrane rupture and spontaneous abortions[27].

CONCLUSION

Since HEV/HCV coinfection is a novel topic, several clinical and research questions remain summarized in Table 2.

As previously discussed, seroprevalence studies demonstrated that the lifetime risk of HEV infection in patients affected by chronic HCV hepatitis is not rare. Although the prevalence of HEV/HCV coinfection is not known, it is reasonable to speculate that in resource limited settings where HEV is a frequent cause of acute hepatitis, superinfections with this virus in patients with chronic HCV infection is quite frequent[94], and the consequences in terms of worsening liver damage and liver decompensation merit to be further investigated. By contrast, since HEV infection is a much rarer cause of chronic liver disease than HCV, chronic co-infections with both viruses are less frequently observed unless in immune-compromised individuals.

Immune phenomena are described for both viruses, and physicians should be aware that patients with autoantibodies and cryoglobulins could be tested for both acute and chronic HEV or HCV infection. However, to the best of our knowledge, no one has described immune alterations in patients affected by HEV/HCV coinfection. We propose, given the relative rarity of the infection, that physicians (who diagnose coinfection) also screen for immune phenomena.

Some DAA drugs, such as SOF, are active against both HEV and HCV *in vitro*, but a regime with SOF and DCV failed to clear HEV RNA in a coinfected patient who did not tolerate ribavirin[54]. Our limited knowledge is based on too few cases being described[52,53,55], and it is not possible to get definitive conclusions on the use of DDA drugs in coinfected patients. It is desirable that researchers focus on *in vitro* studies to better define possible pathogenetic interactions determined by the two viruses. People at risk of HEV or HCV infection (such as transfused or transplanted patients) should be screened regularly to identify coinfected patients. Also, PLWH should be screened for HEV in cases of unexpected elevations of liver enzymes, with or without HCV co-infection.

HEV and HCV are both RNA viruses characterized by greater variability than DNA viruses and mainly infect the liver. Despite these similarities, the two viruses have different species barriers and disease progression. However, coinfection in endemic areas can be a serious public health problem, especially for immunosuppressed individuals or pregnant women. The evolutionary behavior of RNA viruses is responsible for its pathogenesis and antiviral success in infected hosts, as well as vaccine design[26]. Coinfection with particular HCV and HEV types could aggravate hepatic and/or extrahepatic diseases, taking into account viruses–host interaction and the possible genetic interaction between the two viruses during viral replication. At present, the prevention of infections is mainly related to screening policies and public health measures.

FOOTNOTES

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Spinal anesthesia alleviates dextran sodium sulfate-induced colitis by modulating the gut microbiota

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) is a chronic disease with recurrent intestinal inflammation. Although the exact etiology of IBD remains unknown, the accepted hypothesis of the pathogenesis to date is that abnormal immune responses to the gut microbiota are caused by environmental factors. The role of the gut microbiota, particularly the bidirectional interaction between the brain and gut microbiota, has gradually attracted more attention.

AIM

To investigate the potential effect of spinal anesthesia on dextran sodium sulfate (DSS)-induced colitis mice and to detect whether alterations in the gut microbiota would be crucial for IBD.

METHODS

A DSS-induced colitis mice model was established. Spinal anesthesia was administered on colitis mice in combination with the methods of cohousing and fecal microbiota transplantation (FMT) to explore the role of spinal anesthesia in IBD and identify the potential mechanisms involved.

RESULTS

We demonstrated that spinal anesthesia had protective effects against DSS-induced colitis by alleviating clinical symptoms, including reduced body weight loss, decreased disease activity index score, improved intestinal permeability and colonic morphology, decreased inflammatory response, and enhanced intestinal barrier functions. Moreover, spinal anesthesia significantly increased the abundance of *Bacteroidetes*, which was suppressed in the gut microbiota of colitis mice. Interestingly, cohousing with spinal anesthetic mice and FMT from spinal anesthetic mice can also alleviate DSS-induced colitis by upregulating the abundance of *Bacteroidetes*. We further showed that spinal anesthesia can reduce the increase in noradrenaline levels induced by DSS, which might affect the gut microbiota.

CONCLUSION

These data suggest that microbiota dysbiosis may contribute to IBD and provide evidence supporting the protective effects of spinal anesthesia on IBD by modulating the gut microbiota, which highlights a novel approach for the treatment of IBD.

Key Words: Spinal anesthesia; Inflammatory bowel disease; Gut microbiota; Intestinal barrier; Intestinal inflammation; Intestinal immune

Core Tip: Inflammatory bowel disease (IBD) is a chronic inflammation with rising trends, but the pathogenesis is still not well understood. The effects of the gut microbiota, particularly the bi-directional interaction between brain and gut microbiota, have gradually attracted increasing attention. In the present study, we found that spinal anesthesia, a regional sympathetic block, alleviated the intestinal inflammation, maintained immunological function, and improved intestinal barrier function by modulating the gut microbiota. And reducing the increase of noradrenaline level in dextran sodium sulfate-treated mice by spinal anesthesia could be one of the mechanisms. The study highlights a novel approach for the treatment of IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract that includes Crohn's disease (CD) and ulcerative colitis (UC). Over the past decades, the incidence of IBD has rapidly increased globally with consistently rising trends, particularly in Asian countries[1,2]. However, the pathogenesis of IBD is still not well understood. Multiple factors, such as environmental factors, the host's genetics, immune responses, and the intestinal microbiome, might participate in the progression of the disease[3]. Currently, the available clinical treatments for IBD include corticosteroids, salicylates, and biologics. However, as a life-long disease, IBD still lacks a radical cure due to its multiple mechanisms.

With the development of genomics, people have gained a better understanding of a "forgotten organ", the gut microbiota. The imbalance of the gut microbiota damages intestinal epithelial barrier function and the immune response, which are related to the occurrence and progression of IBD[4,5]. Intestinal dysbiosis, which means a compositional imbalance of commensal bacteria, is the central characteristic of the gut microbiota in IBD[6]. Probiotics can effectively improve intestinal symptoms and suppress inflammation in IBD[7,8]. More importantly, recent studies have shown that intestinal dysbiosis can be regulated through a central system called the "brain-gut axis"[9]. The brain-gut axis is a bidirectional communication network that links the central nervous system and the gastrointestinal tract. The central nervous system communicates with intestinal targets, such as the muscle layer, gut mucosa, permeability, mucus secretion, immunity, and enteric microbiota, when is activated in response to environmental factors, such as pain and stress[10].

Neuraxial anesthesia includes spinal, epidural, and combined spinal-epidural anesthesia to maintain regional sympathetic blocks. The postganglionic sympathetic neurons, which are located in the celiac, superior mesenteric, and inferior mesenteric ganglia, interact with the intestines at the serosal surface

and innervate the vascular beds as well as the central nervous system. Neuraxial anesthesia has been shown to exert a positive effect on intestinal microvascular perfusion[11,12]. In an animal model of sepsis, thoracic epidural anesthesia was demonstrated to ameliorate perfusion deficits in the muscularis and mucosal layer of the gut[13]. However, whether neuraxial anesthesia affects intestinal inflammation in IBD is still unclear.

In this study, a dextran sodium sulfate (DSS)-induced colitis mouse model was established to explore the role of spinal anesthesia in IBD and identify the potential mechanisms involved. Moreover, cohousing and fecal microbiota transplantation (FMT) were used to help mice from separate lines share microbes across coached individuals[14,15]. This may open up an opportunity to regulate the distribution of the gut microbiota and prevent IBD.

MATERIALS AND METHODS

Animals

Male C57BL/6 WT mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). All animal procedures were conducted in accordance with guidelines for laboratory animal care after approval by the Laboratory Animals Ethics Committee of Zhejiang University. These mice were housed in the standard animal care facility for 1 wk with free access to food and water at the Laboratory Animal Center of Zhejiang University with conventional housing circumstances of a 12 h/12 h light/dark cycle and 22 °C. All animal experiments were completed at the Laboratory Animal Center of Zhejiang University. Efforts were made to minimize any discomfort or pain, and the minimum number of animals was used.

Acute colitis was induced by administration of 2% DSS (MW: 36000-50000 Da, Sigma–Aldrich) in drinking water for 1 wk in male C57BL/6 mice (6-8 wk old). Mice were randomly divided into four groups with 10 mice per group: Untreated normal controls, mice receiving DSS + spinal saline (saline, subarachnoid administration), mice receiving DSS + spinal lidocaine (2% lidocaine, subarachnoid administration), or those receiving DSS + 5-aminosalicylic acid (5-ASA) [200 mg/kg body weight (BW) in saline, *i.g.*]. After cessation of DSS exposure and lidocaine or 5-ASA treatment, the mice were given water *ad libitum* for an additional 1 wk (n = 10). The weight, food intake, and disease activity index (DAI) were recorded every day, and at the end of the experiments, the mice were sacrificed. At autopsy, the colon was rinsed with saline and excised. After the length of the colon was measured, it was cut and fixed in 10% formalin before paraffin embedding as previously described[16]. In addition to protein extracts, the colon was snap-frozen immediately in liquid nitrogen and stored at -70 °C before the preparation of soluble extracts[16].

Hematoxylin and eosin staining

For paraffin-embedded tissue, colon sections cut at 3 μm were stained with hematoxylin and eosin (HE). The inflammatory index was measured by the histological score, which is the sum of scores of four individual inflammatory parameters: Ulceration (0 or 1), inflammation severity (0, 1, 2, or 3), inflammation area involved (0, 1, 2, 3, or 4), and hyperplasia and dysplasia (0, 1, 2, or 3) as detailed previously [16].

Transmission electron microscopy

The tissues from the colon were fixed with 2.5% glutaraldehyde overnight at 4 °C and then postfixed with 1% osmium tetraoxide for 2 h. After three rinses with phosphate buffer solution (PBS), the tissues were then rinsed with distilled water, followed by a graded ethanol dehydration series ending with propylene oxide. The tissues were embedded in resin after infiltration in a mixture of one-half propylene oxide and one-half resin. Sections (120 nm) were cut and stained with 4% uranyl acetate for 20 min and with 0.5% lead citrate for 5 min. Microvilli in the colon were observed by transmission electron microscopy (Philips Tecnai 10, Holland) at the Center of Cryo-Electron Microscopy at Zhejiang University.

Immunohistochemistry

Immunohistochemistry (IHC) of colon sections from mice was performed using formalin-fixed paraffin-embedded tissue as described previously[17]. Sections were stained using primary antibody against CD4 (1:1000; HuaBio, ER1706-80) and appropriate horseradish peroxidase-conjugated secondary antibody (1:1000; MXB, KIT-5006). Images were captured under a light microscope (Olympus BX41, Shanghai, China). Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, United States) was used to analyze the staining intensity. The semiquantitative results of IHC were based on the average value from three mice per group. Three separate slides from each mouse were analyzed. Five microscopic fields at 100 × magnification were randomly selected, and the integral optical density of the protein of interest was calculated.

Immunoblotting

Colon tissues from each group were homogenized in RIPA buffer (Beyotime, P0013B) with 1 × protease inhibitor cocktail (Beyotime, P1010). The supernatant was collected by centrifugation at 13000 × g for 10 min, and the protein concentration was detected with a bicinchoninic acid protein assay kit (Beyotime, P0012S). An aliquot of 50 μg protein from each sample was separated using SDS-PAGE, transferred to a nitrocellulose membrane, and then blocked with 5% nonfat milk in PBS (pH 7.4). The membranes were incubated with a primary antibody against claudin-1 (1:5000, Proteintech, 130501-1-AP), occludin (1:500, Huabio, ET1701-76), or β-actin (1:5000, ABcam, ab8227) at 4 °C overnight. Blots were incubated in a secondary antibody against rabbit or mouse IgG (1:5000, CST, 7071, and 7072) for 2 h at room temperature and then subjected to chemiluminescent detection using ChemiDoc (BioRad). Digital images were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, United States).

Alcian blue/periodic acid-Schiff staining

The slices of colon tissues were immersed in xylene twice for dewaxing. After the gradient in water, the slices were incubated in Alcian blue reagent for 20 min and distilled water for 3 min. The sample was immersed in 0.5%-1% periodic acid solution and oxidized for 5 min, and it was washed in distilled water at a rapid rate. After applying Schiff reagent staining for 30-60 min (at 37 °C boxes), the slices were washed 2-3 times with sulfate water and distilled water twice. After redying the cell nucleus with hematoxylin solution, the samples were dehydrated with 95% alcohol and 100% alcohol for 1 min and xylene. After sealing with neutral gum, the samples were observed under a microscope.

Real-time quantitative polymerase chain reaction

Total RNA from different groups was extracted using a TRIzol RNA Kit (TransGen Biotech) based on the manufacturer's protocol. Subsequently, cDNA synthesis and antisense RNA amplification were performed using HiScript II Q Select RT SuperMix (Vazyme). Polymerase chain reaction (PCR) was conducted on a StepOne Real-Time PCR System (ABI StepOnePlus, Applied Biosystems; Thermo Fisher Scientific, Inc.) using Hieff UNICON qPCR SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.). The primers were synthesized by BioSun Biotechnology. The following primers were used: Interleukin *(IL)-1β* forward, 5′-AGTTGACGGACCCCAAAAG-3′ and reverse, 5′-TTGAAGCTGGATGCTCTCAT-3′; *IL-6* forward, 5′-AGTCCTTCCTACCCCAATTTCC-3′ and reverse, 5′- GGTCTTGGTCCTTAGCCACT-3′; inducible nitric oxide synthase (*iNOS*) forward, 5′- CTCACCTACTTCCTGGACATTAC-3′ and reverse, 5′- CAATCTCTGCCTATCCGTCTC-3′; *β-actin* forward, 5′- CCACCATGTACCCAGGCATT-3′ and reverse, 5′-AGGGTGTAAAACGCAGCTCA-3′. Each assay was performed in triplicate. Fold changes were calculated after normalizing the change in expression of β-actin using the $2^{-\Delta\Delta cycle}$ threshold method.

Enzyme-linked immunosorbent assay

Serum IL-1β, IL-6, tumor necrosis factor-alpha (TNF-α), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) concentrations in plasma were measured using an RSGELISA kit (Affymetrix, United States) following the manufacturer's instructions.

Analysis of CD4⁺ T cells and regulatory T cells

Alterations in $CD4^+$ T cell and regulatory T cell (Treg) populations in the intestine were analyzed by staining cells with specific antibodies using flow cytometry performed on a BD FACS S ORP ARIA II (BD Biosciences, Mountain View, CA, United States) according to a method previously described with slight modifications.

Briefly, after DSS and lidocaine treatment, about 1×10^6 cells were collected, and 10 μL of PerCP-Cy5.5-conjugated monoclonal mouse anti-human CD4 antibody and 10 μL of PE-conjugated monoclonal mouse anti-human CD25 antibody (BD Biosciences) were added after washing once with PBS (pH 7.4) (Gibco; Thermo Fisher Scientific, Waltham, MA, United States). To exclude the amount of nonspecific binding, 10 μL of PerCP-Cy5.5-conjugated and 10 μL of PE-conjugated mouse IgG1κ isotype control (BD Biosciences) were used and evaluated as blank controls. After incubation in the dark at 37 °C for 20 min, the cells were washed with PBS and resuspended in diluted Foxp3 buffer A (BD Biosciences), followed by incubation in the dark at room temperature for 10 min. Then, the cells were washed once with PBS, resuspended in 0.15 mL of Foxp3 buffer C composed of 49 parts of Foxp3 buffer A and one part of Foxp3 buffer B (BD Biosciences), and incubated in the dark at room temperature for 30 min. After incubation, the cells were washed once with PBS, and 10 μL of Alexa Fluor 488-conjugated mouse anti-mouse Foxp3 antibody or 10 μL of Alexa Fluor 488-conjugated mouse IgG1 isotype control was added, followed by incubation for 30 min in the dark at 37 °C. The cells were suspended in 0.4 mL staining buffer [0.4% (v/v) formaldehyde neutral buffer solution in PBS (pH 7.4)]. After washing once with PBS, the cells were analyzed by flow cytometry, and the data were further analyzed using FlowJo software (BD Biosciences). $CD4^+$ T cells in the lymphocyte fraction were gated, and the percentages of $CD4^+$ T and Tregs in the $CD4^+$ T cells were calculated.

Intestinal permeability testing

Each group of mice were fasted for 8 h before being sacrificed. After 3 h of administration with FITC-dextran (MW: 4K, R-FD-001, XINQIAO Biotechnology, China, 400 mg/kg in PBS), 300 μL of heart blood was harvested, and plasma was separated. Different concentrations of standard products were prepared with mixed mouse plasma without FITC-dextran gastric filling, PBS, and FITC-dextran solutions. The intracellular fluorescence was measured with a microplate reader at an excitation wavelength of 492 nm and an emission wavelength of 525 nm (DTX880, Beckman Coulter, United States). The FITC-dextran content was calculated according to the standard curve. The fluorescence intensity of FITC-dextran in the blood of mice was positively correlated with the permeability of their intestines.

Gut microbial community sequencing

At the end of the experiment, overnight fasted mice were sacrificed. Fecal samples were collected from the mice, and gut microbiota DNA was extracted. The V3-V4 region of 16S rDNA was amplified with universal primers. The PCR products were then quantified by electrophoresis on a 1.5% agarose gel followed by cDNA purification with the QIAquick Gel Extraction kit (Qiagen). Sequencing and data analysis were subsequently performed on an Illumina HiSeq platform by Novogene (Beijing, China). Briefly, after the raw sequences were identified by their unique barcodes, Ribosomal Database Project Classifier 2.8 was used to perform the assignment of all sequences at 50% confidence. Operational Taxonomic Units (OTUs) present in 50% or more of the colon content samples were identified as core OTUs. PLS-DA of core OTUs was performed using Simca-P version 12 (Umetrics), and to visualize and cluster the bacterial community into different groups, a heatmap was generated using Multi-Experiment Viewer software. Community diversity was measured by the Shannon-Weiner biodiversity index (Shannon index), alpha diversity index (Ace index), and Chao1 richness estimator (Chao 1 index).

Cohousing

For cohousing experiments, siblings (male C57BL/6 mice, 4-12 wk, n = 12) were divided into four groups consisting of a group of WT mice (n = 3), a noncohousing DSS group (n = 3), a cohousing DSS group (n = 3), and a cohousing lidocaine + DSS group (n = 3). The DSS group and lidocaine + DSS group were treated as described above. The cohousing DSS group and the cohousing lidocaine group were transferred to fresh cages to start the cohousing experiments in the same experimental room. The mice remained together for 2 wk with free access to water and chow diet.

FMT

Bacterial strains that were previously isolated from lidocaine mice were mixed and resuspended in PBS. The mice were exposed to DSS for 7 d and then randomly separated into either an FMT group or a DSS group. The FMT and DSS groups were gavaged with fecal microbiota (0.3 mL) or PBS (0.3 mL). After observation for 30 min, all mice were housed separately.

Measurement of serum noradrenaline level

A method for the determination of noradrenaline was established by high-performance liquid chromatography-tandem mass spectrometry. The noradrenaline standard was detected and analyzed by gradient. After blood samples were collected, the serum was separated and frozen at -80 °C. After thawing, methanol was added, and then acetonitrile was added after the internal standard solution was vortex centrifuged again. After stratification, the upper organic phase was placed in another tube, and 10 μL of the organic phase was taken for determination. Chromatographic conditions are as follows: Agilent Zorbax sb-c8 column (2.1 mm × 100 mm, 1.8 μm); mobile phase, water (containing 0.1% formic acid, 2 mmol/L ammonium acetate): Acetonitrile (containing 0.1% formic acid) = 55:45; flow rate, 0.4 mL/min; column temperature, 30 °C; injection volume, 2 μL; autosampler temperature, 10 °C. For mass spectrometry, the ionization mode was electrospray ionization, multireaction ion detection MRM was used in the positive ion mode, the capillary voltage was 3.5 kV, the temperature of the dryer was 350 °C, the flow rate of the dryer was 5 L/min, the atomization gas was 60 psi, the sheath gas temperature was 350 °C, and the sheath gas flow rate was 11 L/min.

Statistical analysis

Experiments were performed in triplicate and repeated at least three times. Data are presented as the mean ± SD or SEM from independent experiments. Statistical analyses involved one-way ANOVA and two-way ANOVA performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, United States) or SPSS 22 (IBM Corp., Armonk, NY, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Spinal anesthesia alleviates DSS-induced colitis

Acute colitis was induced in C57BL/6 mice by the administration of 2% DSS in drinking water for 1 wk. To test whether spinal anesthesia could relieve colitis, colitis mice were given lidocaine (subarachnoid administration), saline (vehicle, subarachnoid administration), and 5-ASA (positive control, *i.g.*) daily until the termination of the experiment after 7 d. The experimental protocol is shown in Figure 1A. The DSS-treated colitis mice exhibited an increasing DAI level with dramatic body weight loss, colon shortening, rectal bleeding, and stool consistency reduction. In contrast, colitis mice treated with lidocaine or 5-ASA showed obvious improvements in body weight (Figure 1B), DAI score (Figure 1C), and colon length (Figure 1D). H&E staining images of colonic tissues of the DSS-treated mice showed obvious intestinal injury, including the loss of crypts and discontinuous brush borders and large lumens. However, these damage signs were noticeably ameliorated by lidocaine treatment, which appeared to protect the colonic mucosal structure by limiting multifocal inflammation (Figures 1E and 1F). Furthermore, SEM showed that the microvilli of colitis mice became shorter and uneven, and the arrangement was disordered. Interestingly, the lidocaine group had more microvilli than the saline group (Figure 1G). Taken together, these results suggested that spinal anesthesia with lidocaine relieved the symptoms of colitis and colonic epithelial injury in a DSS-induced IBD mouse model.

Spinal anesthesia relieves intestinal inflammation activated by DSS

To better understand the alleviation of colitis by spinal anesthesia, the inflammatory response was determined by detecting proinflammatory cytokines and infiltration of immune cells in the colon. As expected, in colitis mice, enzyme-linked immunosorbent assay (ELISA) analysis showed that the levels of IL-1β, IL-6, and TNF-α in plasma were dramatically higher than those in the control mice (Figures 2A, 2B and 2C). The levels of these cytokines were lower in the 5-ASA group than in the saline + DSS group. Interestingly, spinal anesthesia with lidocaine also significantly suppressed the expression of IL-1β, IL-6, and TNF-α in plasma of DSS-induced mice (Figures 2A, 2B and 2C). Meanwhile, the transcriptional levels of cytokines were detected by real-time quantitative PCR (qRT-PCR), and the results revealed that spinal anesthesia also decreased the mRNA levels of *IL-1β*, *IL-6*, and *iNOS* in DSS-induced mice (Figures 2D, 2E and 2F). CD4+/CD25+/FoxP3+ Tregs and CTLA4 cells play an important role in maintaining the healthy intestinal immune state. We then focused on those immune cells. IHC analysis showed that DSS treatment dramatically increased the area of infiltration of CD4+ T cells in the colon, whereas spinal anesthesia or treatment with 5-ASA significantly inhibited the production of CD4+ T cells (Figures 2G and 2H). Importantly, flow cytometry assays showed that spinal anesthesia led to an increase in Tregs (Figure 2I), thereby inducing a change in the proportion of T lymphocytes. Similar results were also obtained when we determined the level of CTLA4 in serum. The lidocaine + DSS group had a higher serum CTLA4 level than the saline + DSS group (Figure 2J). Together, these data suggested that spinal anesthesia may downregulate proinflammatory cytokines and upregulate the levels of CTL4 and CD4+/CD25+/Foxp3+ Tregs to alleviate the inflammatory activity in DSS-induced mice.

Spinal anesthesia protects intestinal barrier function

To examine intestinal barrier function, we first detected intestinal permeability. After spinal anesthesia, the concentration of FITC-dextran in the serum of colitis mice decreased significantly, which indicated the strengthening of intestinal physical barrier function (Figure 3A). Intercellular tight junctions (TJs), whose integrity is a key determinant of paracellular permeability, are important for maintaining epithelial barrier function. Western blot analysis showed that claudin-1 and occludin, two representative TJ proteins, were significantly lower in the DSS groups than in the control group (Figure 3B). However, spinal anesthesia with lidocaine reversed the levels of claudin-1 and occludin in the positive control 5-ASA group. Goblet cells secrete gel-forming mucins to lubricate and protect the intestine. Alcian blue/periodic acid-Schiff staining showed that there was a clear trend of decreasing neutral (Figures 3C and 3D) and acidic (Figures 3E and 3F) mucins after DSS treatment. Spinal anesthesia with lidocaine resulted in a significant increase in mucins. Together, these results showed that spinal anesthesia with lidocaine can protect intestinal barrier function in the DSS-induced IBD mouse model.

Spinal anesthesia changes the intestinal microflora in the IBD mouse model

A wealth of information has been generated regarding the altered microbiota composition, or dysbiosis, at different taxonomic levels that manifests in patients with IBD and animal models of IBD[18,19]. The composition of the fecal microbiota was determined by analysis of -30000 paired reads per mouse of the -250 bp v3-v4 region of the 16S rRNA gene by Illumina MiSeq sequencing (Figures 4A and 4B). The PCoA analysis revealed that specific bacterial alterations occurred during colitis. Ace index varied between the saline + DSS group and the lidocaine + DSS group (Figure 4C). Interestingly, spinal anesthesia significantly rescued the abundance of *Bacteroidetes*, which was suppressed by DSS (Figures 4D and 4E). Other types of bacteria, including *Verrucomicrobia* and *Proteobacteria*, were also affected by spinal anesthesia, although the difference did not reach statistical significance. Together, these data demonstrated that spinal anesthesia with lidocaine can alter the intestinal flora in the IBD mouse model.

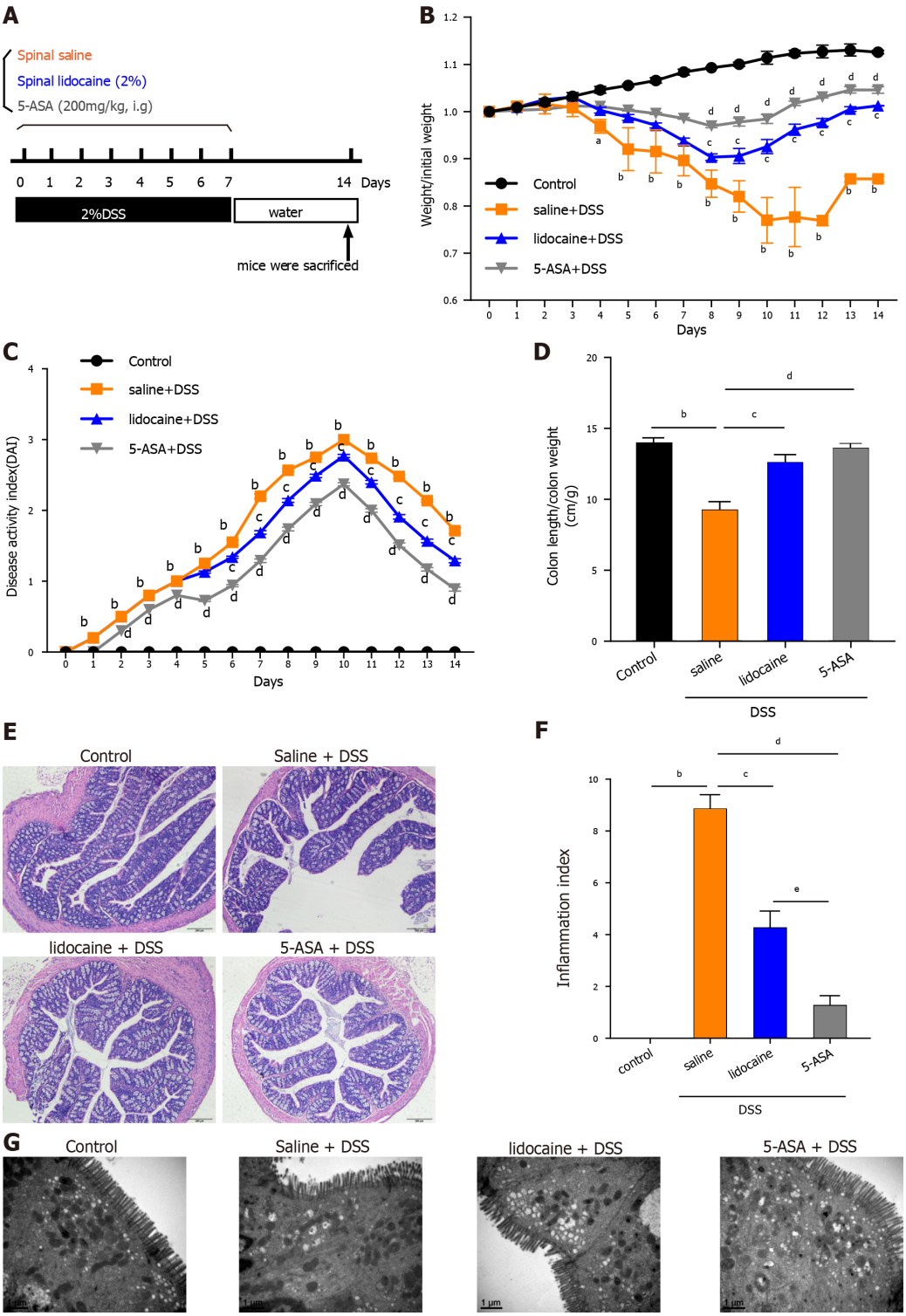


Figure 1 Spinal anesthesia alleviates dextran sodium sulfate-induced colitis. A: Diagram of experimental design; B: Body weight; C: Disease activity index. Each group was evaluated daily (n = 10); D: Colon length of each group measured at the end of the experiment (n = 10); E: Representative images of hematoxylin and eosin staining; F: Inflammation index quantitation; G: Transmission electron microscopy. Data are presented as the mean ± SEM of three independent experiments. P-values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$, control *vs* saline + DSS; $^{b}P < 0.01$, control *vs* saline + DSS; $^{c}P < 0.01$, saline + DSS *vs* lidocaine + DSS; $^{d}P < 0.01$, saline + DSS *vs* 5-ASA + DSS; $^{e}P < 0.05$, lidocaine + DSS *vs* 5-ASA + DSS. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

Cohousing spinal anesthesia and DSS mice have alleviated DSS-induced colitis

To certify whether the gut microbiota contributes to relieving colitis by spinal anesthesia, DSS mice and spinal anesthesia with lidocaine mice were cohoused (Figure 5A). Using microbiota sequencing analysis, we compared the gut microbiota in the cohousing and noncohousing groups. Figure 5B reveals that cohousing altered the relative abundance of intestinal flora. There was no significant difference in various measures, including the Chao1 index, Shannon index, and Ace index (Figure 5C). However,

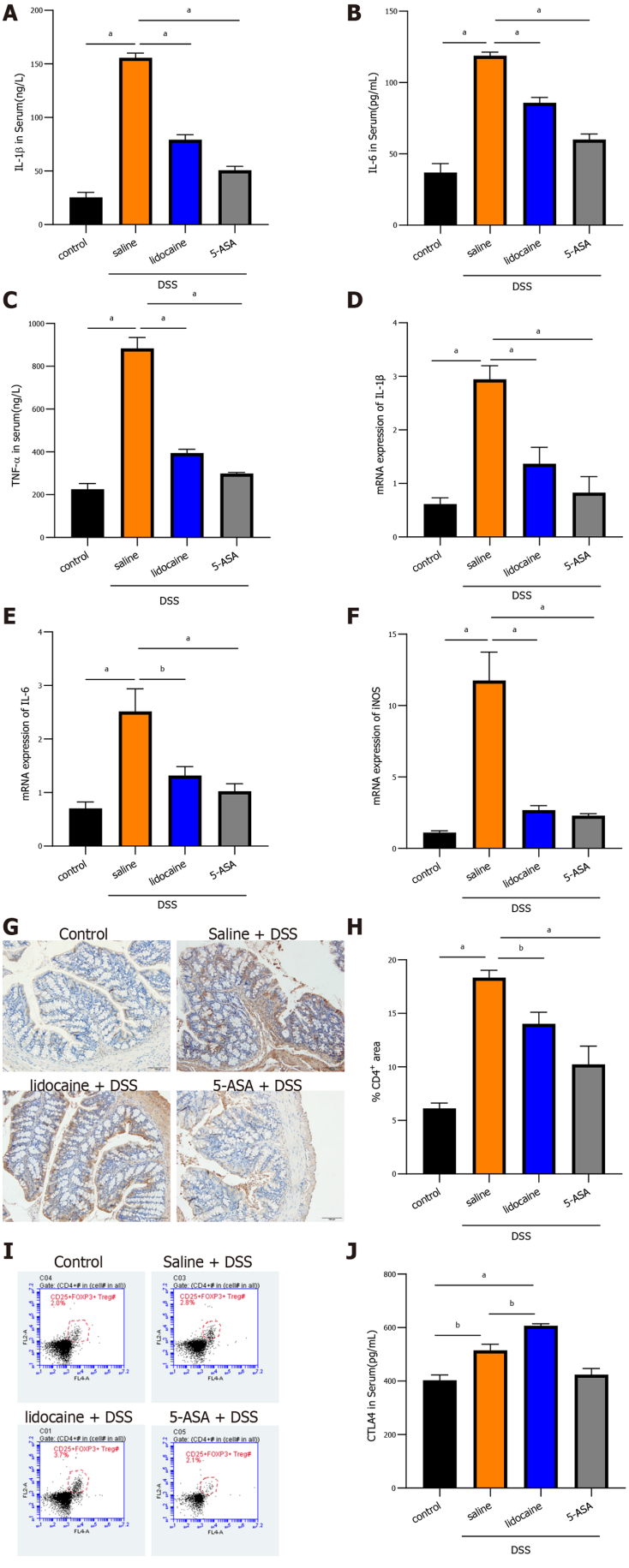


Figure 2 Spinal anesthesia relieves intestinal inflammation activated by dextran sodium sulfate. A-C: Serum levels of interleukin (IL)-1β, IL-6, and

tumor necrosis factor-alpha detected by enzyme-linked immunosorbent assay (ELISA); D-F: The mRNA expression of *IL-1β*, *IL-6*, and inducible nitric oxide synthase detected by real-time quantitative polymerase chain reaction; G: Expression of CD4 detected using immunohistochemistry; H: Quantitation of the immunohistochemistry result; I: Proportion of CD4+/CD25+/Foxp3+ Tregs analyzed by flow cytometry; J: Serum level of cytotoxic T-lymphocyte-associated protein determined by ELISA; Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. [a]*P* < 0.05, [b]*P* < 0.01. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate; IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; iNOS: Inducible nitric oxide synthase.

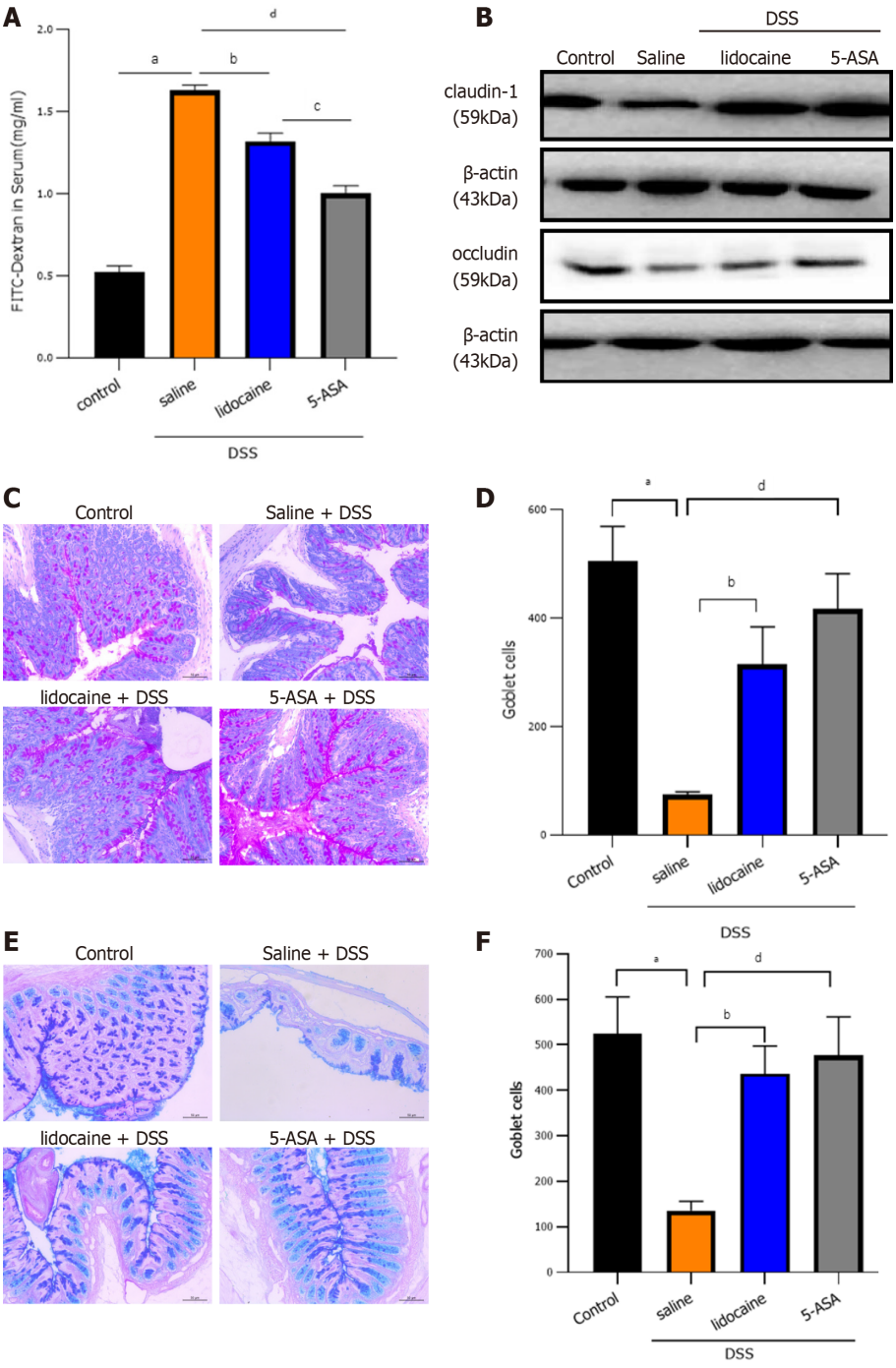
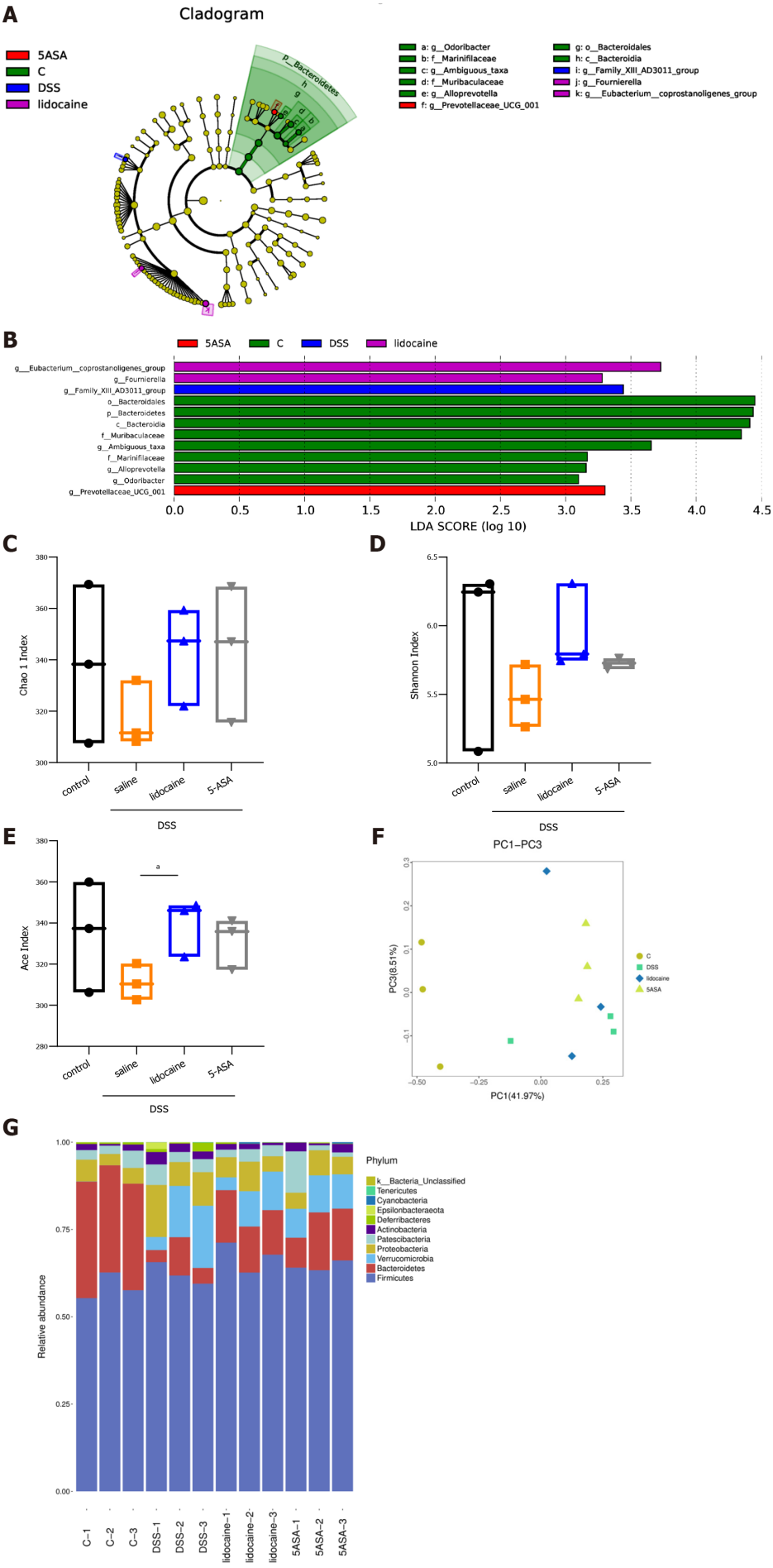


Figure 3 Spinal anesthesia with lidocaine protects the intestinal barrier. A: Intestinal permeability tested by FITC-dextran assay; B: Western blot analysis showed the expression of claudin-1 and occludin as key tight junction proteins in each group; C: Mucins in tissue sections observed by Alcian blue staining; D: Quantitation of the Alcian blue staining result; E: Mucins in tissue sections observed by periodic acid-Schiff staining. F: Quantitation of the periodic acid-Schiff staining result. Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. [a]*P* < 0.01, control *vs* saline + DSS; [b]*P* < 0.01, saline + DSS vs lidocaine + DSS; [c]*P* < 0.01; lidocaine + DSS *vs* 5-ASA + DSS; [d]*P* < 0.01, saline + DSS *vs* 5-ASA + DSS. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.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b: f__Marinifilaceae
c: g__Ambiguous_taxa
d: f__Muribaculaceae
e: g__Alloprevotella
f: g__Prevotellaceae_UCG_001
g: o__Bacteroidales
h: c__Bacteroidia
i: g__Family_XIII_AD3011_group
j: g__Fournierella
k: g__Eubacterium__coprostanoligenes_group
B
5ASA
C
DSS
lidocaine
g__Eubacterium__coprostanoligenes_group
g__Fournierella
g__Family_XIII_AD3011_group
o__Bacteroidales
p__Bacteroidetes
c__Bacteroidia
f__Muribaculaceae
g__Ambiguous_taxa
f__Marinifilaceae
g__Alloprevotella
g__Odoribacter
g__Prevotellaceae_UCG_001
LDA SCORE (log 10)
C
Chao 1 Index
control
saline
lidocaine
5-ASA
DSS
D
Shannon Index
E
Ace Index
F
PC1–PC3
PC3(8.51%)
PC1(41.97%)
G
Phylum
k__Bacteria_Unclassified
Tenericutes
Cyanobacteria
Epsilonbacteraeota
Deferribacteres
Actinobacteria
Patescibacteria
Proteobacteria
Verrucomicrobia
Bacteroidetes
Firmicutes
Relative abundance
C–1
C–2
C–3
DSS–1
DSS–2
DSS–3
lidocaine–1
lidocaine–2
lidocaine–3
5ASA–1
5ASA–2
5ASA–3

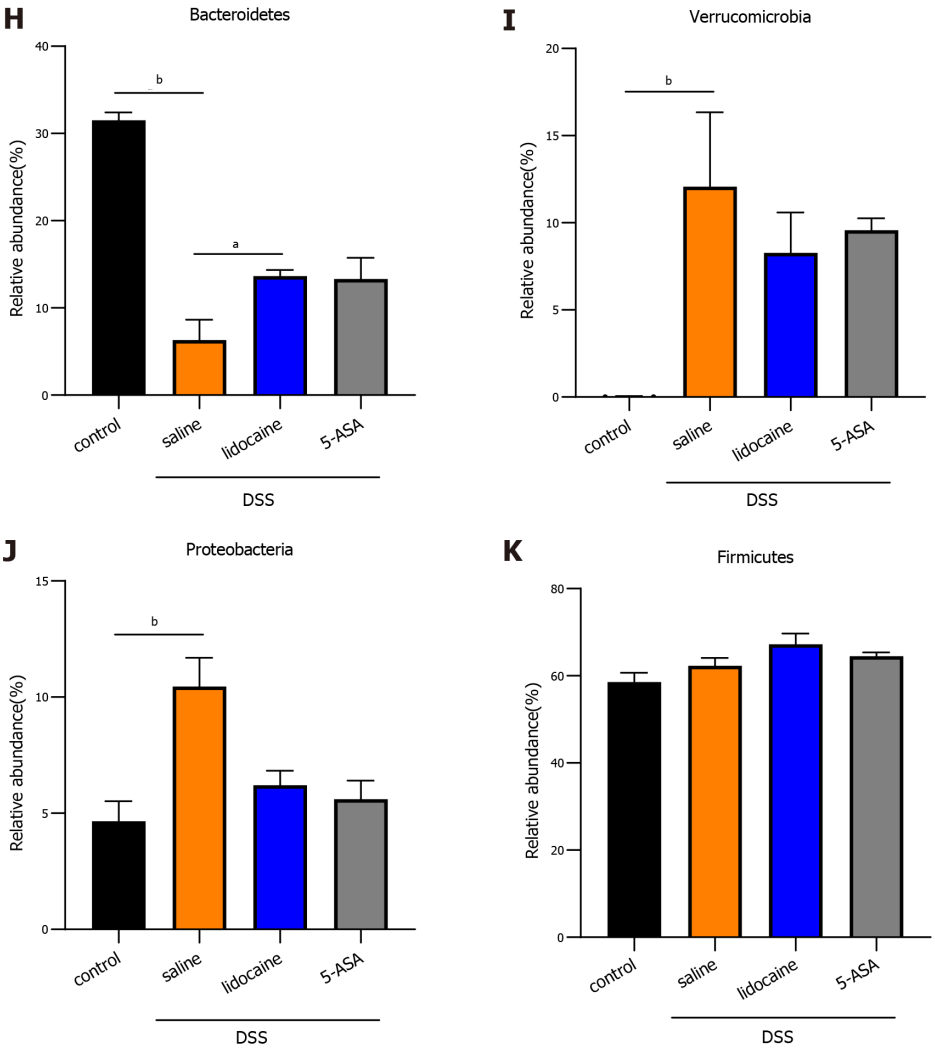

Figure 4 Spinal anesthesia changes the intestinal microflora. A: Cladograms (family level) of the intestinal microflora in different groups; B: Differentiating taxa (family level) of the intestinal microflora in different groups; C: Chao1 index (richness); D: Shannon index (diversity); E: Ace index; F: PCoA plots revealing specific bacterial alterations in different groups; G: Relative abundance plots showing community variation in various groups; H: Abundance of *Bacteroidetes* in different groups; I: Abundance of *Verrucomicrobia* in different groups; J: Abundance of *Proteobacteria* in different groups; K: Abundance of *Firmicutes* in different groups. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as the mean ± SEM, and statistical significance was assessed using *t* tests. n = 3 mice per group. *P* values were calculated using one-way ANOVA between different groups. $^{a}P <$ 0.01, saline + DSS *vs* lidocaine + DSS; $^{b}P < 0.01$, control *vs* saline + DSS. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

cohousing significantly increased the abundance of *Bacteroidetes* (Figure 5D), which was consistent with the change affected by spinal anesthesia. Meanwhile, cohousing rescued the shortened colon and intestinal permeability in DSS-induced mice (Figures 5E and 5F), and the body weight and DAI of the cohousing spinal anesthesia and DSS mice were better than those of the DSS mice (Figures 5G and 5H).

Transplantation of fecal microbiota from lidocaine-treated mice alleviates DSS-induced colitis

FMT is generally accepted as a promising experimental treatment for patients suffering from gut dysbiosis. We were interested in determining whether transplantation of the intestinal flora from the spinal anesthesia group has an effect on DSS-induced colitis (Figure 6A). FMT also altered the relative abundance of intestinal flora (Figure 6B). Various measures, including the Chao1 index, Shannon index, and Ace index, varied between the cohousing group and the noncohousing group (Figure 6C). FMT significantly increased the abundance of *Bacteroidetes* and decreased the abundance of *Verrucomicrobia*, recovering the change in DSS-induced mice (Figure 6D). Similarly, FMT reduced colon swelling and relieved intestinal permeability (Figures 6E and 6F). The body weight and DAI of the DSS mice treated by FMT were better than those of the DSS mice (Figures 6G and 6H), which was consistent with changes by cohousing treatment. Taken together, these results suggested that the gut microbiota was implicated in relieving colitis by spinal anesthesia due to the key role that it plays in intestinal inflammation.

Spinal anesthesia decreases the noradrenaline level induced by DSS

Several studies[12,13] have indicated that the sympathetic system primarily exerts an inhibitory

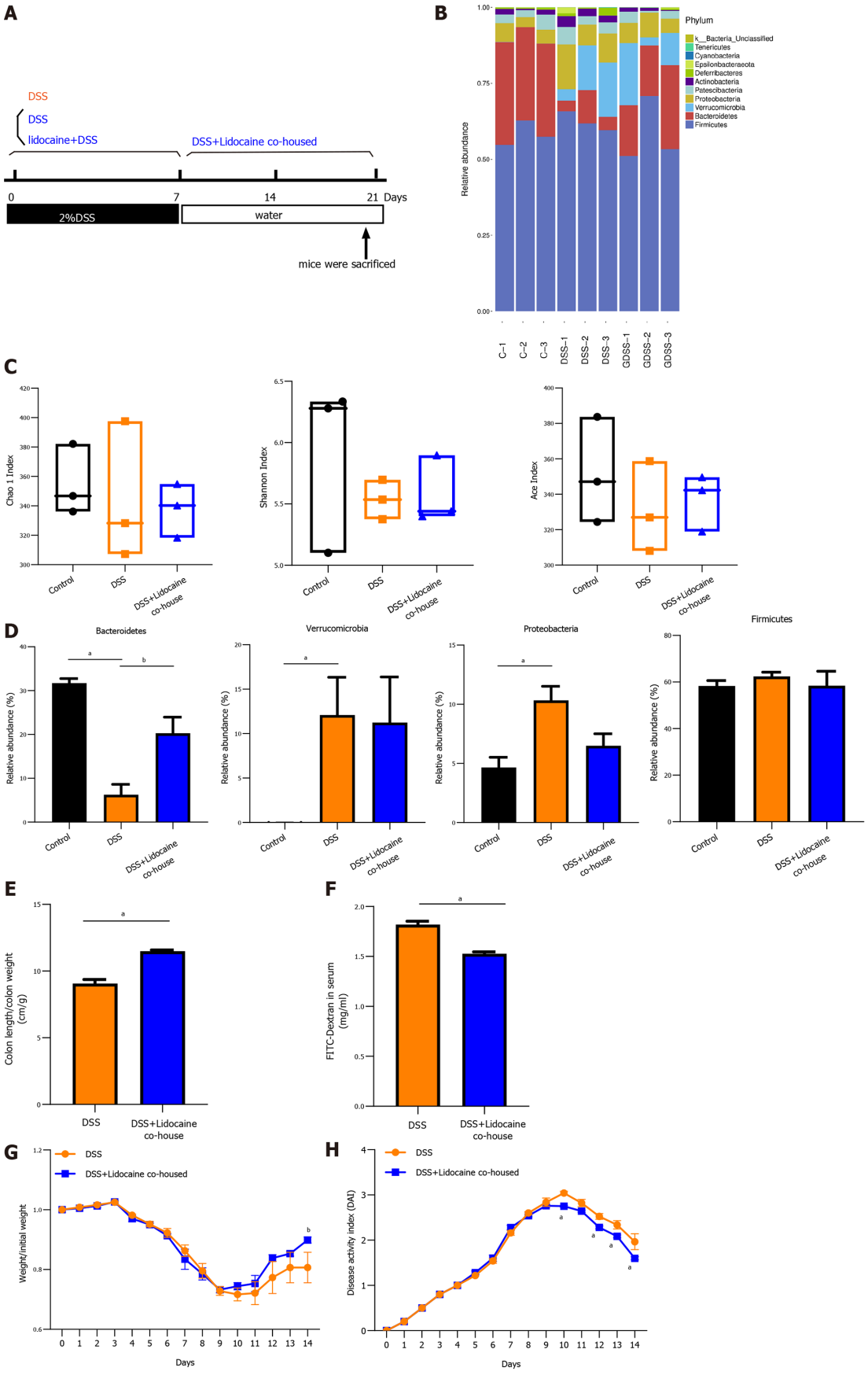

Figure 5 Cohousing alleviates dextran sodium sulfate-induced colitis in mice. C57BL/6 mice were divided into four groups: A normal control group (*n* = 3), a no-cohousing dextran sodium sulfate (DSS) group (*n* = 3), a cohousing DSS group (*n* = 3), and a cohousing lidocaine + DSS group (*n* = 3). The DSS group and lidocaine + DSS group were treated with DSS. The cohousing DSS group and the cohousing lidocaine group were transferred to fresh cages. A: Diagram of experimental design; B: Relative abundance plots showing community variation in various groups; C: Alpha diversity calculated using Chao1 index (richness),

Shannon index (diversity), and Ace index; D: Abundance of *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria*, and *Firmicutes* in different groups; E: Colon length; F: Intestinal permeability compared between cohousing DSS mice (n = 6) and no-cohousing DSS mice (n = 6); G: Body weight; H: Disease activity index compared between cohousing DSS mice (n = 6) and no-cohousing DSS mice (n = 6). Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$, $^{b}P < 0.01$. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as the mean ± SEM, and statistical significance was assessed using *t* tests. n = 3 mice per group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

influence on the gut by the release of noradrenaline. To verify that spinal anesthesia modulates the gut microbiota *via* noradrenaline, we used liquid chromatography to detect the level of noradrenaline in our model (Figure 7A). The results indicated that the level of noradrenaline was increased in the saline + DSS group compared with the control group (Figures 7A and 7B). Spinal anesthesia with lidocaine decreased the noradrenaline level in DSS-induced mice. The altered level of noradrenaline could be one of the mechanisms underlying the effect of spinal anesthesia on the gut microbiota.

DISCUSSION

The pathogenesis of IBD is not fully understood, and changes in intestinal microbes, impairment of the epithelial barrier, and the chronic dysregulated immune response in the gastrointestinal tract are strongly implicated in the development of IBD[20,21]. In particular, increasing evidence from animal and human studies has shown that gut microbes are key factors contributing to IBD by acting on different parts of the gut-brain-microbiota axis[22]. Therefore, our study explored the possibility of improving the clinical outcomes of IBD by acting on this axis. Interestingly, we found that IBD mice treated by lidocaine spinal injection, which is a regional sympathetic block, showed a significant amelioration of symptoms and intestinal permeability. Moreover, this treatment increased the abundance of specific genera, such as *Bacteroidetes*, and regulated gut immunity. Here, we, for the first time, directly highlighted the therapeutic effects of spinal anesthesia on DSS-induced colitis mice.

Neuraxial anesthesia (*i.e.*, spinal, epidural, and combined spinal-epidural techniques) is widely used to induce analgesia for lower extremities and lower abdominal surgery. In addition, the specific beneficial effect of neuraxial anesthesia attributed to sympathetic nerve blockade, which increases gastrointestinal track microvascular perfusion and function, has been observed in animal and clinical studies[23,24]. Recently, several reports have shown that spinal or epidural anesthesia may improve the ischemic and insufficient oxygenation stage of organs[11,12]. In the present study, we found that spinal anesthesia with lidocaine not only relieved intestinal permeability but also increased the diversity of the gut microbiota and changed the composition of microbiota species in colitis mice, which suggests that spinal anesthesia affects part of the gut-brain-microbiota axis to alleviate colitis in DSS-induced mice.

Microbial predominance includes four different phyla, *Bacteroidetes*, *Prodominace*, *Firmicutes,* and *Verrucomycrobia*[25], in healthy people, and an alteration of the intestinal microbiota occurs in IBD patients. Prior studies have noted that polysaccharides, a component of *Bacteroidetes,* can prevent intestinal inflammatory disease, and *Akkermansia* exerts beneficial effects on colitis[26]. In the DSS model, we found that the abundance of *Proteobacteria* and *Verrucomycrobia* increased, but the abundance of *Bacteroidetes* decreased, which might lead to abnormal intestinal immune responses and intestinal imbalance. After spinal anesthesia, we identified that the abundance of *Bacteroidetes* with health-promoting functions significantly increased and the abundance of *Proteobacteria* and *Verrucomycrobia* decreased. Furthermore, our cohousing and FMT experiments revealed that the microbiota of spinal anesthesia mice transferred to DSS colitis mice can effectively relieve disease. These results confirmed that altered gut microbiota caused IBD and that *Bactericides* and *Verrucomycrobia* species are critical in keeping the gut microbiota healthy.

Extensive studies have found that behavioral disorders, such as anxiety, stress, or depression, change the composition of the intestinal flora and influence the recurrence of CD[27,28]. The sympathetic nerve innervated to the gut plays a critical role in affecting the composition of the gut microbiota and maintaining immune homeostasis *via* the hypothalamus-pituitary-adrenocortical axis mainly by secreting catecholamine[9,29,30]. In the stress model, the sympathetic system primarily exerts an inhibitory influence on the gut, decreasing intestinal motor function and secretion *via* the release of neurotransmitters, such as noradrenaline[31]. Noradrenaline, one of the main catecholamines, can influence the microbiota in the gut, leading to the altered release of cytokines and bacterial molecules. Noradrenaline can also increase the growth of several bacteria in nutrient-deficient environments, including *Campylobacter jejuni*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Salmonella enterica spp*[32]. Catecholamines were found to facilitate the removal of iron from human lactoferrin and transferrin in a dose- and time-dependent manner, which also correlated with bacterial growth[33]. In our study, the level of noradrenaline was increased after DSS treatment, which may influence the microbiota present in the colitis gut. Moreover, spinal anesthesia with lidocaine recovered the level of noradrenaline. This could be one of the mechanisms underlying the effect of spinal anesthesia on the gut

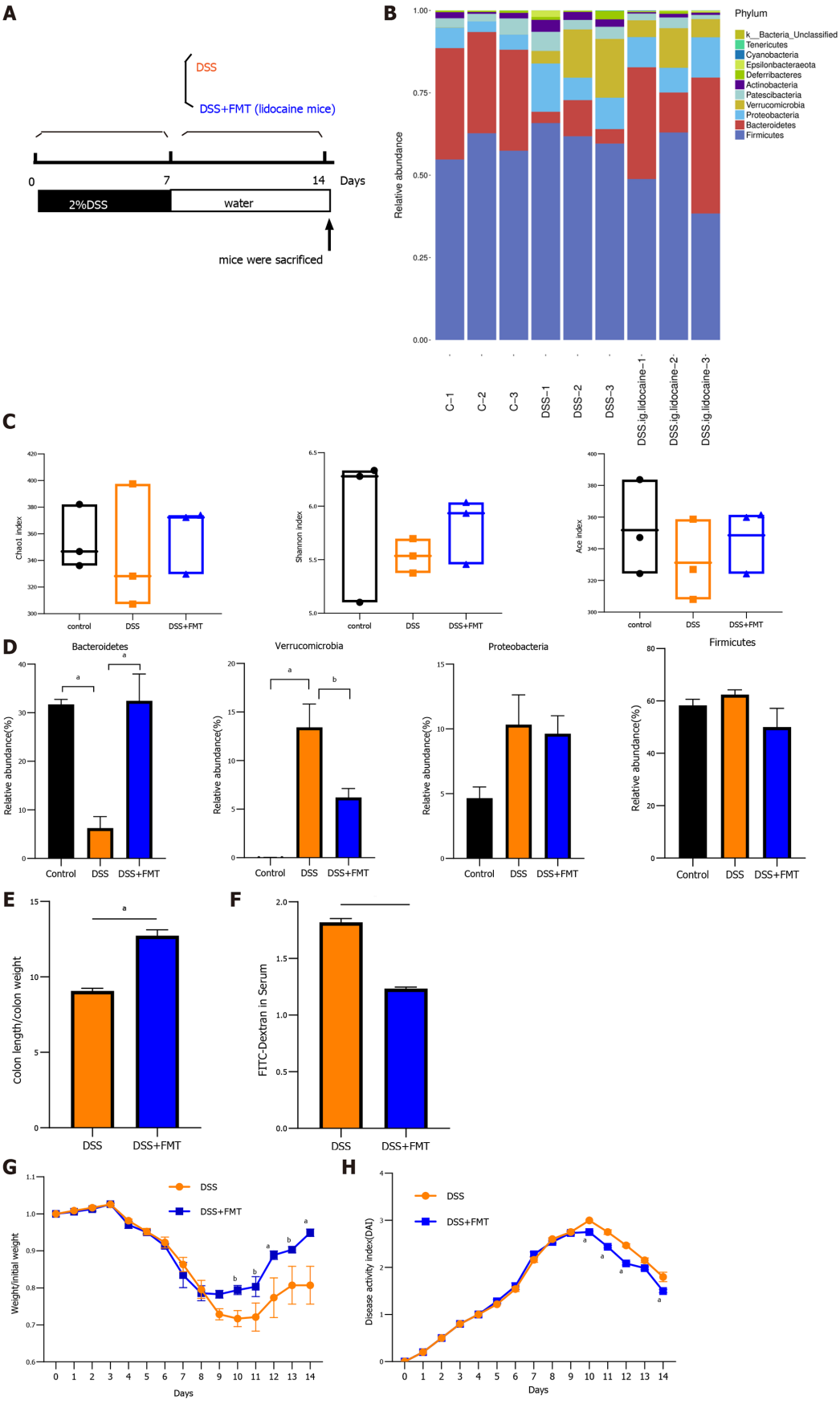

Figure 6 Transplantation of fecal microbiota from lidocaine-treated mice alleviates dextran sodium sulfate-induced colitis. C57BL/6 mice were exposed to dextran sodium sulfate (DSS) for 7 d and then randomly separated into either a fecal microbiota transplantation (FMT) group or a DSS group. The FMT and DSS groups were gavaged with fecal microbiota (from lidocaine mice) and phosphate-buffered saline, respectively. A: Diagram of experimental design; B: Relative abundance plots showing community variation in various groups; C: Alpha diversity calculated using Chao1 index (richness), Shannon index (diversity), and

Ace index ; D: Abundance of *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria*, and *Firmicutes* in different groups; E: Colon length compared between DSS mice (n = 6) and FMT + DSS mice (n = 6); F: Permeability compared between DSS mice (n = 6) and FMT + DSS mice (n = 6); G: Body weight compared between DSS mice (n = 6) and FMT + DSS mice (n = 6); H: Disease activity index compared between DSS mice (n = 6) and FMT + DSS mice (n = 6). Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$, $^{b}P < 0.01$. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as the mean ± SEM, and statistical significance was assessed using *t* tests, n = 3 mice per group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

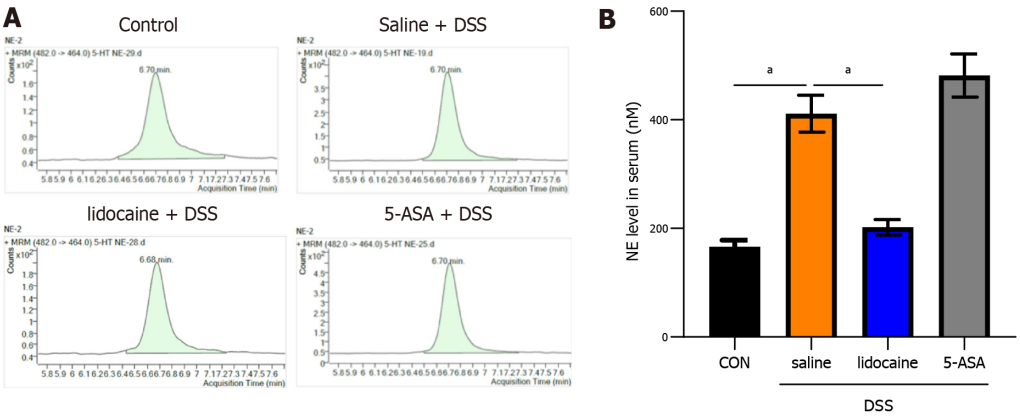

Figure 7 Spinal anesthesia decreases the noradrenaline level induced by dextran sodium sulfate. At the end of the experiment, according to Figure 1A, the serum of mice in each group was collected. A: Noradrenaline level measured by high performance liquid chromatography (HPLC); B: Quantitation of the HPLC result. Data are presented as the mean ± SEM of three independent experiments. *P* values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

microbiota.

It is evident that the sympathetic nervous system has the potential to serve as a therapeutic target for inflammatory disease. Increasing sympathetic tone has a deteriorating effect on IBD, including the development of intestinal inflammation and inhibition of immune defenses[34,35]. This is in line with the finding in the present study that thoracic sympathetic block through spinal anesthesia in colitis mice increased the expression of FoxP3+ Treg cells, which are a subset of CD4+ T lymphocytes and plays an important role in maintaining the immune response[36]. Moreover, the levels of caludin-1 and occludin, the main tight junction proteins of the intestinal epithelial barrier, were changed. All these results indicated that immune function and colon barrier function were impaired, which may be caused by increased noradrenaline levels and altered gut microbiota.

In recent years, diet and short-chain fatty acids have been recommended to replace or increase conventional IBD therapies[37]. Some peptides named food protein-derived bioactive peptides against intestinal inflammation have attracted increasing attention, but their mechanism and effect are under exploration[38]. Experts have said "as we enter a new era of patient-centered health care, treating the 'brain' is as important as the 'gut' for comprehensive, whole-person IBD management"[39]. However, it is difficult to verify the bidirectional relationship of the gut-brain axis, and here, we for the first time used spinal anesthesia to block thoracic nerve in IBD mice, and we luckily found that it was effective for IBD.

CONCLUSION

In conclusion, this study clearly revealed that spinal anesthesia inhibited the development of DSS-induced colitis in mice. We demonstrated that spinal anesthesia alleviated intestinal inflammation, maintained immunological function, and improved intestinal barrier function by modulating the gut microbiota. Reducing the increase in noradrenaline levels in DSS-treated mice by spinal anesthesia could be one of the mechanisms underlying the effect on the gut microbiota. The present study provided evidence supporting the protective effects of spinal anesthesia on IBD by modulating the gut microbiota, which highlights a novel approach for the treatment of IBD.

ARTICLE HIGHLIGHTS

Research background

Neuraxial anesthesia has been shown to exert a positive effect on intestinal microvascular perfusion. In an animal model of sepsis, thoracic epidural anesthesia was demonstrated to ameliorate perfusion deficits in the muscularis and mucosal layers of the gut. However, whether spinal aneathesia as a neuraxial anesthesia affects intestinal inflammation in inflammatory bowel disease (IBD) is still unclear.

Research motivation

The exact etiology of IBD remains unknown, and the imbalance of the gut microbiota is related to the occurrence and progression of IBD. The bidirectional between the brain and gut microbiota has gradually attracted more attention. Finding interventions on the brain-gut axis will be a new vision.

Research objectives

A dextran sodium sulfate (DSS)-induced colitis mouse model was established to explore the role of spinal anesthesia in IBD and to identify the potential mechanisms involved.

Research methods

A DSS-induced colitis mice model was established, and then we used spinal anesthesia on colitis mice to explore the role of spinal anesthesia in IBD and identify the potential mechanisms involved. Moreover, cohousing and fecal microbiota transplantation were used to help mice from separate lines share microbes across cocaged individuals.

Research results

This study clearly revealed that spinal anesthesia inhibited the development of DSS-induced colitis in mice. We demonstrated that spinal anesthesia alleviated intestinal inflammation, maintained immunological function, and improved intestinal barrier function by modulating the gut microbiota. Reducing the increase in noradrenaline levels in DSS-treated mice by spinal anesthesia could be one of the mechanisms underlying the effect on the gut microbiota.

Research conclusions

The study implied a positive effect of spinal anesthesia in relieving intestinal inflammation, protecting intestinal barrier function, and regulating the intestinal microflora in an IBD mouse model. Decreasing the noradrenaline level would be a possible mechanism of spinal anesthesia.

Research perspectives

The present study provided evidence supporting the protective effects of spinal anesthesia on IBD by modulating gut microbiota, which highlights a novel approach for the treatment of IBD.

FOOTNOTES

Author contributions: Hong Y and Zhao J contributed equally to this work; Xin Y, Hong Y, and Chen YR designed the research study; Huang ZH and Chen YR performed the research; Zhao J and Hou LD contributed analytic tools; Shen B and Zhao J analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

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Institutional animal care and use committee statement: All animal procedures were conducted in accordance with guidelines for laboratory animal care after approval by the Laboratory Animals Ethics Committee of Zhejiang University.

Conflict-of-interest statement: Xin Y, Chen YR, and Zhao J have received research funding from Zhejiang Provincial Natural Science Foundation. And Hong Y has received research funding from Zhejiang Science and Technology Department and Zhejiant Health Commission.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at [xinxin_yu@zju.edu.cn].

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Basic Study

Microbiologic risk factors of recurrent choledocholithiasis post-endoscopic sphincterotomy

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Abstract

BACKGROUND

Choledocholithiasis is a severe disorder that affects a significant portion of the world's population. Treatment using endoscopic sphincterotomy (EST) has become widespread; however, recurrence post-EST is relatively common. The bile microbiome has a profound influence on the recurrence of choledocholithiasis in patients after EST; however, the key pathogens and their functions in the biliary tract remain unclear.

AIM

To investigate the biliary microbial characteristics of patients with recurrent choledocholithiasis post-EST, using next-generation sequencing.

METHODS

This cohort study included 43 patients, who presented with choledocholithiasis at the Guangdong Second Provincial General Hospital between May and June 2020. The patients had undergone EST or endoscopic papillary balloon dilation and

were followed up for over a year. They were divided into either the stable or recurrent groups. We collected bile samples and extracted microbial DNA for analysis through next-generation sequencing. Resulting sequences were analyzed for core microbiome and statistical differences between the diagnosis groups; they were examined using the Kyoto Encyclopedia of Genes and Genomes pathway hierarchy level using analysis of variance. Correlation between the key genera and metabolic pathways in bile, were analyzed using Pearson's correlation test.

RESULTS

The results revealed distinct clustering of biliary microbiota in recurrent choledocholithiasis. Higher relative abundances (RAs) of *Fusobacterium* and *Neisseria* (56.61% ± 14.81% *vs* 3.47% ± 1.10%, 8.95% ± 3.42% *vs* 0.69% ± 0.32%, respectively) and the absence of *Lactobacillus* were observed in the bile of patients with recurrent disease, compared to that in stable patients. Construction of a microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia*, and an antagonistic relationship among *Lactobacillales, Fusobacteriales*, and *Clostridiales*. Functional prediction of biliary microbiome revealed that the loss of transcription and metabolic abilities may lead to recurrent choledocholithiasis. Furthermore, the prediction model based on the RA of *Lactobacillales* in the bile was effective in identifying the risk of recurrent choledocholithiasis ($P = 0.03$).

CONCLUSION

We demonstrated differences in the bile microbiome of patients with recurrent choledocholithiasis compared to that in patients with stable disease, thereby adding to the current knowledge on its microbiologic etiology.

Key Words: Choledocholithiasis; Biliary tract; Microbiome; Endoscopic sphincterotomy; Recurrence; *Lactobacillus*

Core Tip: Treatment of choledocholithiasis by endoscopic sphincterotomy (EST) has become widespread, but recurrence post-EST is relatively common. In this study, we analyzed the bile microbiome of patients with recurrent choledocholithiasis. Increase in Fusobacterium and Neisseria, and the absence of Lactobacillus in bile were the key microbiologic features of recurrent choledocholithiasis. Bile microbiome imbalance might cause poor metabolism of carbohydrates and amino acids and increased glycan biosynthesis in the biliary tract, leading to disease recurrence. The microbiological features in bile could be an effective predictor for choledocholithiasis recurrence post-EST. The findings of our study will help develop new prevention strategies for post-surgery recurrence of choledocholithiasis.

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INTRODUCTION

Cholelithiasis is a common and socially significant health problem worldwide, occurring in approximately 5%-22% of adults, with synchronous common bile duct stone (CBDS) in 20% of these patients[1-4]. In western countries, it is one of the leading gastrointestinal conditions that results in hospitalization [4]. Cholelithiasis with CBDS can lead to biliary obstruction, secondary cholangitis, and obstructive jaundice, endangering lives in some severe cases and often requiring surgical interventions[5]. The introduction of endoscopic treatment started a new era in the treatment of choledocholithiasis[6-9], and management by endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) has become widespread, replacing open laparoscopic cholecystectomy or open common bile duct exploration with choledochoscopy[10,11].

However, long-term surveys have revealed up to 39% recurrence of choledocholithiasis post-EST, and life-long follow-ups are still needed after surgery[12-14]. Recurrent choledocholithiasis post-EST involves complicated factors, including infections and biliary anatomical abnormalities[15,16]. The elimination of certain pathogens in the bile duct can significantly reduce the recurrence rate[17,18].

Therefore, further investigations into the microbiological etiology and underlying mechanisms of recurrent choledocholithiasis post-EST are crucial for its prediction and prevention in clinical practice.

Complex microbiomes in the biliary system have been observed using next-generation sequencing (NGS)[19]. In these systems, the microbiota metabolize and secrete cholesterol and bile acids; their dysfunction may cause pathophysiological defects and result in stone formation[20,21]. Unlike primary stones, secondary stones in recurrent choledocholithiasis predominately consist of more cholesterol than calcium bilirubinate[22], and their microbiological etiology remains unclear.

In this study, we investigated the microbiological etiology of recurrent choledocholithiasis using NGS to find the key pathogens associated with recurrence post-EST and their metabolic characteristics in disease relapse.

MATERIALS AND METHODS

Study participants and sample collection

Ethical compliance: Consecutive recruitment of eligible patients was carried out in the Department of Endoscopy at Guangdong Second Provincial General Hospital from May to June 2020. All experimental protocols were approved by this hospital's ethics committee (project 2019-QNJJ-14-02). The study design complied with all relevant ethical regulations in accordance with the Declaration of Helsinki and Belgian Privacy Commission. Written consent was obtained from all patients in the study.

Study cohort: In this study, we included 43 choledocholithiasis participants diagnosed using computed tomography (CT) or magnetic resonance cholangiopancreatography (MRCP). All patients were assessed by experienced doctors, without risk of EST-related complications[10]. Patients with a history of malignant diseases, autoimmune diseases, diabetes, structure abnormality of the biliary tract, or any exposure to antibiotics within one month were excluded from the study. All patients accepted laparoscopic cholecystectomy (LC) treatment following an EST or endoscopic papillary balloon dilation (EPBD), during the same hospitalization episode. The components of the stones were recorded according to the method of Dosch[23]. All patients received CT or MRCP examinations one week after the treatment to ensure the complete removal of stone in the biliary tract. All participants underwent at least one-year follow-up with transabdominal ultrasonography every three months, and CT or MRCP was performed once recurrence of choledocholithiasis was indicated through clinical presentations or imaging examinations. Patients were divided into stable and recurrent groups according to their disease evaluation at the end of the follow-up period.

Bile sample collection: Bile samples were collected during endoscopic treatment. The ERCP was performed to confirm the diagnosis of choledocholithiasis, followed by the EST or EPBD treatment, and the bile sample was collected through suction during the treatment. The bile samples were immediately transported to the laboratory and stored at -80 °C until extraction.

Microbiome DNA extraction and 16S rRNA gene amplicon sequencing

Bile sample (3 mL) was centrifuged at 16000 × *g* at 4 °C for 10 min, and the pellet was washed twice with phosphate-buffered saline before DNA extraction. Microbiome DNA was then extracted using the QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The *16S rRNA* gene obtained from each bile sample was amplified by targeting the V3-V4 hypervariable regions using the following primers: 341F 5′-CCTACGGGNGGCWGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ using the UCP Multiplex PCR Kit (Qiagen)[24]. The amplicon library was prepared using the QIAseq Ultralow Input Library Kit (Qiagen). An Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, United States) and Qubit dsDNA HS Assay Kit (Invitrogen Life Technologies, Carlsbad, CA, United States) were used to validate the library pooling. Paired-end sequencing was conducted using the MiSeq platform (Illumina, San Diego, CA, United States) with MiSeq Reagent Kit version V3 (Illumina).

Microbiome sequence curation and analysis

Trimming and quality filtering of the data were performed using the CLC Genomic Workbench version 20.0, with the Microbial Genomics Module (Qiagen). Sequences were matched to the Greengenes database version 13.5.

The amplicon sequencing, and the taxonomic and statistical analyses were performed using Calypso version 8.84[25]. Alpha diversity was determined based on Fisher's alpha index, which was assessed using the analysis of variance test. Microbial diversity was visualized using the canonical correspondence analysis based on the prognosis groups. Key taxonomic discovery analysis related to prognosis was performed using linear discriminant analysis effect size (LEfSe) at the genus level[26]. The relative abundance (RA) measurements of the genera, with biomarker significance were compared using the Wilcoxon rank test.

The core microbiome was identified as described by Ainsworth[27]. Network analysis was performed to identify the co-occurring and exclusive bacteria using Calypso[25]. Genera and orders of the bile microbiome were represented as nodes, taxa RAs as node size, and edges as positive and negative associations. Networks were generated based on the associations between both genera and orders using Pearson's correlation; nodes were colored based on their association with different prognosis groups. Only relationships with statistical significance ($P < 0.05$) were visualized in the network.

Metagenome prediction of the bile microbiome was performed using the amplicon sequencing approach in the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[28]. The statistical differences between the diagnosis groups were examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway hierarchy level using analysis of variance. Correlation analysis was carried out between the key genera and metabolic pathways in bile using Pearson's correlation test. Survival analysis of the identical microbiological risk factors was carried out using Kaplan-Meier analysis.

Statistical analyses

Except for those analyzed using Calypso, data were analyzed using GraphPad Prism v7.00 software (GraphPad, La Jolla, CA, United States). All analyses in the study were statistically significant at $P < 0.05$, and P values were adjusted using false discovery rate, Bonferroni, or area under curve correction.

RESULTS

Clinical features and prognosis of patients with choledocholithiasis one-year post-EST

Forty-three choledocholithiasis patients, who underwent LC following EST were recruited in this study and received a one-year follow-up survey. Thirteen patients had co-occurrence of cholelithiasis; the baseline clinical characteristics of the 43 choledocholithiasis patients were shown in Table 1. The stone components were recorded according to the methods of Dosch[23]; they were classified as brown pigmented stones, black pigmented stones, cholesterol stones, and mixed stones. Four recurrent cases without other complications were observed using routine ultrasonography as well as CT during the follow-up period. No significant differences were found in the clinical features or the stone components between patients with and without recurrent choledocholithiasis.

Bile microbiome characteristics in patients with choledocholithiasis

A total of 702 unique operational taxonomic units were identified in the bile of all patients with choledocholithiasis, indicating the diversity of the microbiome in bile (Figure 1). *Streptococcus* and an unclassified genus of *Enterobacteriaceae* were the most dominant genera; they were detected in the bile of 28 and 29 patients, respectively. The average RAs of *Streptococcus* and *Fusobacterium* in bile were 13.59% and 19.91%, respectively.

Key microorganisms in bile of patients with recurrent choledocholithiasis

The bile microbial structure in patients with recurrent choledocholithiasis was different from that in patients without recurrence, with lower alpha diversity ($P = 0.41$) and distinct beta diversity ($P = 0.03$; Figure 2).

LEfSe biomarker discovery analysis identified *Fusobacteriales* and *Neisseriales* as biomarkers in the recurrent group and *Lactobacillales* in the stable group at the order level (Figure 3A). The RAs of *Fusobacteriales* (56.61% ± 14.81% *vs* 3.47% ± 1.10%) and *Neisseriales* (8.95% ± 3.42% *vs* 0.69% ± 0.32%) were higher in patients with recurrent choledocholithiasis than that in stable patients post-EST ($P < 0.05$), while the RA of *Lactobacillales* was significantly lower in the recurrent group (1.48% ± 1.28%) than that in the stable group (25.04% ± 4.76%; $P < 0.05$; Figure 3B).

Bile microbiological ecosystem analyses in patients with choledocholithiasis with different prognoses post-EST

Core microbiome analyses showed that *Streptococcus, Prevotella, Fusobacterium*, an unclassified genus of *Enterobacteriaceae*, and an unclassified genus of *Clostridiaceae* were the shared core genera in both the stable and the recurrent group. *Veillonella*, *Oribacterium*, *Neisseria*, *Leptotrichia*, and *Campylobacter* were the specific core genera in the recurrent group, while *Enterococcus*, *Clostridium*, and an unclassified genus of *Aeromonadaceae* were the unique genera in the stable group (Table 2). Construction of a microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia* (Figure 4A).

Additionally, *Lactobacillales*, *Fusobacteriales*, *Enterobacteriales*, *Clostridiales*, and *Bacteroidales* were the shared core orders in both the stable and the recurrent group. *Pasteurellales*, *Neisseriales*, and *Campylobacterales* were the unique core orders in the recurrent group, while *Pseudomonadales*, *Burkholderiales, Bacillales, Aeromonadales*, and *Actinomycetales* were the unique orders in the stable group. Co-occurrence network analyses suggested mutual enhancement among the key recurrence-related pathogens in bile

Table 1 Clinical characteristics of choledocholithiasis patients

	Stable (*n* = 39)	Relapse (*n* = 4)	*P* value
Age (yr) (range)	47 (38-64)	44 (38-46)	0.142
Sex			
Male (cases) (%)	24 (61.54)	3 (75.00)	0.626
Female (cases) (%)	15 (38.46)	1 (25.00)	
History of smoking (cases) (%)	10 (25.64)	1 (25.00)	0.978
Comorbidities			
Type 2 diabetes mellitus	4 (10.26)	1 (25.00)	0.381
Hypertension	6 (15.38)	1 (25.00)	0.620
Hyperlipoidemia	13 (33.33)	2 (50.00)	0.505
Accompanied diagnosis			
Cholelithiasis (cases) (%)	11 (28.21)	2 (50.00)	0.366
Acute cholangitis (cases) (%)	4 (10.26)	1 (25.00)	0.381
Pancreatitis (cases) (%)	1 (2.56)	0 (0.00)	-
Serum biochemical indexes			
ALT (U/L)	161.50 ± 159.11	67.75 ± 75.61	0.210
AST (U/L)	128.51 ± 151.74	34.75 ± 38.20	0.063
Total Bilirubin (μmol/L)	92.19 ± 82.97	24.23 ± 23.50	0.057
Direct Bilirubin (μmol/L)	84.92 ± 91.99	18.73 ± 23.69	0. 060
Amylase (U/L)	118.69 ± 192.30	77.50 ± 30.39	0.544
Follow-up time (d)	369.80 ± 2.67	372.00 ± 4.00	0.101
Recurrent time from EST (d)	-	208.80 ± 87.97	-
Stone components			
Brown pigment (cases) (%)	29 (74.36)	2 (50.00)	0.303
Black pigment (cases) (%)	8 (20.51)	1 (25.00)	
Cholesterol (cases) (%)	0 (0.00)	0 (0.00)	
Mixed component (cases) (%)	2 (5.13)	1 (25.00)	

EST: Endoscopic sphincterotomy.

and antagonistic relationships among *Lactobacillales*, *Fusobacteriales*, and *Clostridiales* in the ecosystem, indicating the role of probiotics in the prevention of recurrence (Figure 4B).

Functional characteristics of bile microbiome in patients with choledocholithiasis with different prognoses post-EST

The metabolites from microorganisms are the key pathogenic factors for the host; therefore, the characteristics of the metabolic pathways in bile were analyzed for deeper insight into the microbiologic etiology of recurrent choledocholithiasis post-EST. Comparative analyses of microbiological functions were carried out at the 2nd hierarchy level of the KEGG pathway. In the stable group, the bile microorganisms were active in the transcription and metabolism related to the nervous system, infectious diseases, biosynthesis of carbohydrates and amino acids; while, in the recurrent group, the microbes were active in translation, replication and repair, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, genetic information processing, energy metabolism, and biosynthesis of secondary metabolites (Figure 5).

Furthermore, correlations between the key genera in the two groups and the different metabolic pathways were analyzed to identify the influence of certain microbes on the host (Figure 6). In the bile ecosystem of the patients with recurrent disease, *Fusobacterium* and *Campylobacter* had positive correlations with the metabolism of amino acids, replication and repair, and translation ($P < 0.05$), while the unclassified genus of *Enterobacteriaceae* had a negative correlation with all the discrepant metabolic

Table 2 Core microbiome in bile of choledocholithiasis patients with different prognosis

Core microbiome	Type	Group	Recurrent. Occ	Stable. Occ
Clostridium	Unique	Stable	0	0.44
Enterococcus	Unique		0	0.44
Unclassified genus of *Aeromonadaceae*	Unique		0.25	0.49
Fusobacterium	Core	Recurrent&Stable	1	0.41
Prevotella	Core		0.5	0.56
Streptococcus	Core		0.5	0.67
Unclassified genus of *Clostridiaceae*	Core		0.75	0.49
Unclassified genus of *Enterobacteriaceae*	Core		0.5	0.69
Campylobacter	Unique	Recurrent	0.5	0.18
Leptotrichia	Unique		0.75	0.28
Neisseria	Unique		0.75	0.31
Oribacterium	Unique		0.5	0.18
Veillonella	Unique		0.5	0.38

pathways between the two groups ($P < 0.05$). *Leptotrichia* had a positive correlation with all the discrepant metabolic pathways in the bile of the stable group ($P < 0.05$). Correlation analyses indicated that in bile of the recurrent group, increased *Fusobacterium* could alter the metabolism of amino acids, replication and repair, and translation functions, leading to the formation of secondary bile stones.

Microbiologic risk factor analysis of the recurrent group post-EST

Fusobacteriales and *Neisseriales* were identified as the bile biomarkers in the recurrent group and *Lactobacillales* in the stable group. Kaplan-Meier analysis was carried out to confirm whether these biomarkers can be used as independent predictive factors for recurrence post-EST (Figure 7). The statistical results revealed that patients with *Lactobacillales* in the bile were at a lower risk of recurrence post-EST ($P = 0.03$) than patients, who lacked this order in their bile.

DISCUSSION

It was assumed that the biliary system is sterile in healthy people; however, an increasing amount of NGS-supported evidence shows that bile supports a complex and abundant microbiome in healthy individuals[19,29]. The frequently identified microorganisms using traditional culture techniques are *Enterococcus*, *Klebsiella*, and *Pseudomonas*; these bacteria are active in reducing the bile acid pool and regulating bile acid metabolism[30-33]. However, the contribution of microbes to the biliary system is still unclear. NGS techniques revealed that the most common inhabitants of the biliary tract are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Synergistetes*, and candidate phylum *Saccharibacteria* (*TM7*)[34]. Some of these microorganisms regulate the hydrolysis of bile acids to constituent components, cleavage of exogenous aromatic rings, deconjugation of bile acid complexes by hydrolytic enzymes, and the formation of free bile acids[35]. The disturbance of the microbiologic ecosystems in bile may lead to dysfunctional bile acid metabolism, resulting in a series of bile duct diseases[20,21]; however, the most disease-specific pathogens and their unique functions remain unknown.

Similar to the results from previous studies[29,34,36], this study revealed that the biliary tract was composed of a diversity of bacteria, and the majority of microorganisms in the bile were *Streptococcus*, *Prevotella*, *Fusobacterium*, *Enterococcus*, *Veillonella*, and *Clostridium*. *Lactobacillus* and *Lactococcus* were reported as the major genera in bile[36]; however, these two genera could only be detected in 11 patients in this study. These differences could be attributed to the differences in study designs; we included only patients with severe choledocholithiasis, who needed surgical intervention, for the analysis of microbial risk factors for disease recurrence. Another factor could be the difference in bile sampling; we chose the endoscopic route over open surgery for the collection of bile.

Endoscopic treatment such as EST can provide definitive relief to choledocholithiasis; however, the formation of gallstones will not stop unless the etiologic factors are eliminated[12,13,37]. Among all the risk factors for choledocholithiasis recurrence, only biliary infections are correctable; microbiological treatment is the most potential therapy against the recurrence of choledocholithiasis[5,15,16,38,39].

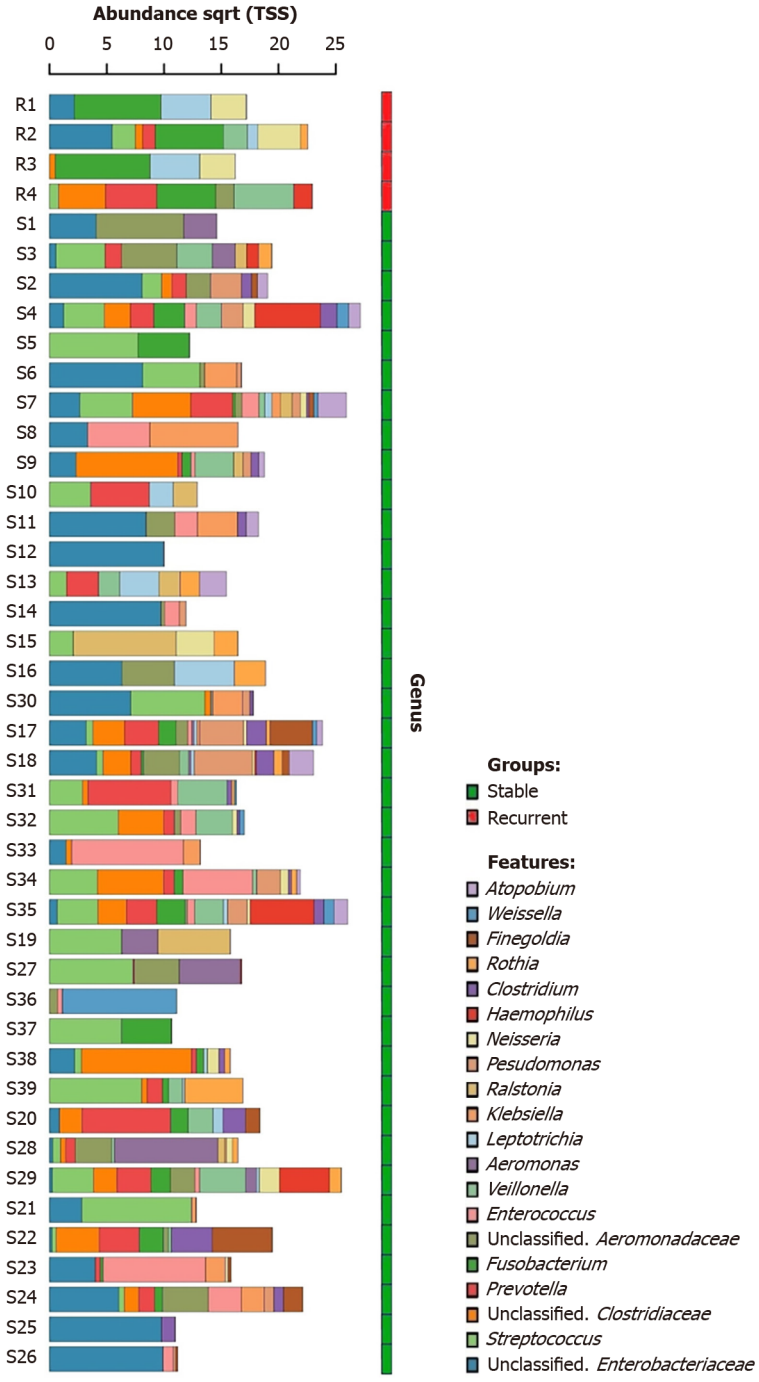

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Figure 1 Dominant bacterial genera in the bile of choledocholithiasis patients. The top 20 dominant bacterial genera in bile are shown in the bar chart, the bile microbiome of recurrent choledocholithiasis patients post-endoscopic sphincterotomy are in the red group and choledocholithiasis patients without recurrence post-EST are in the green group. The genera were listed from the bottom to the top according to their relative abundance in bile samples.

Therefore, investigation into the biliary microbiology characteristics of recurrent choledocholithiasis is crucial to both etiology and prevention studies. To the best of our knowledge, this is the first pilot study to investigate the microbiological risk factors in recurrent choledocholithiasis post-EST. Increased *Fusobacterium* and *Neisseria* were recurrence-related biomarkers in the bile microbiome. Furthermore, we discovered the antagonistic potentials of *Lactobacillus* and an unclassified genus of *Enterobacteriales* against *Fusobacterium* and *Neisseria*, indicating the potential use of probiotics in the prevention of recurrence post-EST.

Bacteria in bile play an active role in gallstone formation[35]. *Escherichia coli* and *Klebsiella* in bile can produce hydrolytic enzymes such as β-glucuronidase, phospholipase A[40], and conjugated bile acid hydrolase; in addition, they can cause deconjugation of bilirubin diglucuronide and precipitation of calcium bilirubinate, which ultimately leads to biliary stone formation[41,42]. We identified *Clostridium* as one of the key microorganisms in the bile microbiome, which, according to Leung *et al*[43], is a more

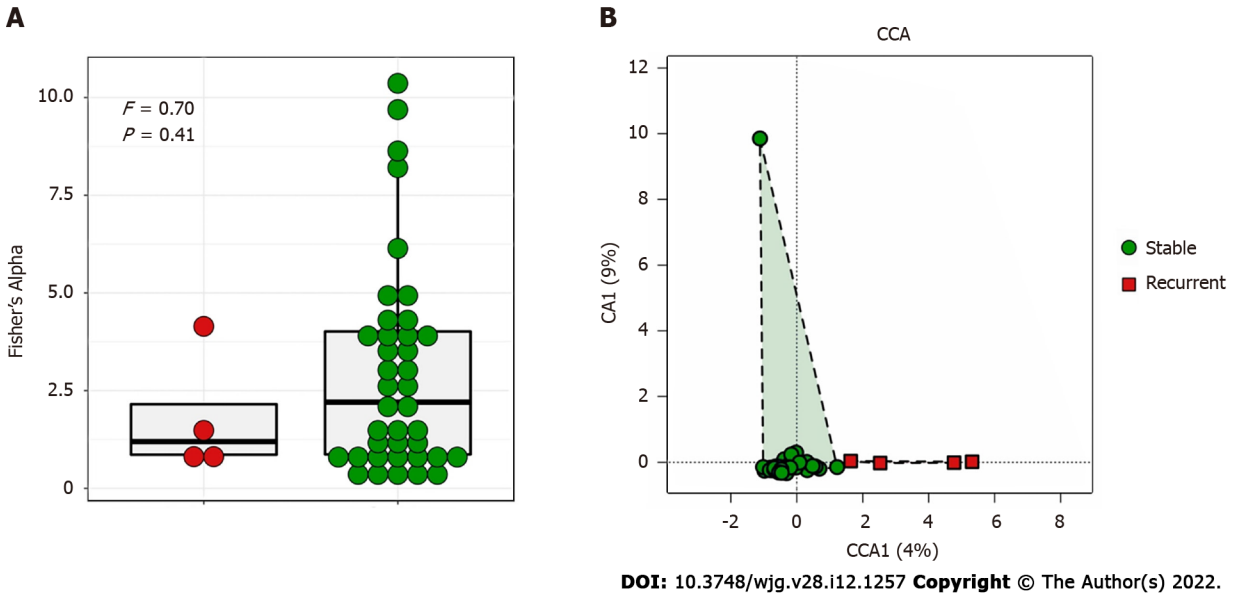

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Figure 2 Diversity analysis of bile microbiome of choledocholithiasis patients. A: Comparison of alpha diversity of bile microbiome by the Fisher's Alpha Index between stable (green) and recurrent (red) choledocholithiasis patients post-endoscopic sphincterotomy (EST); B: Comparison of beta diversity of bile microbiome using canonical correspondence analysis between stable (green) and recurrent (red) choledocholithiasis patients post-EST.

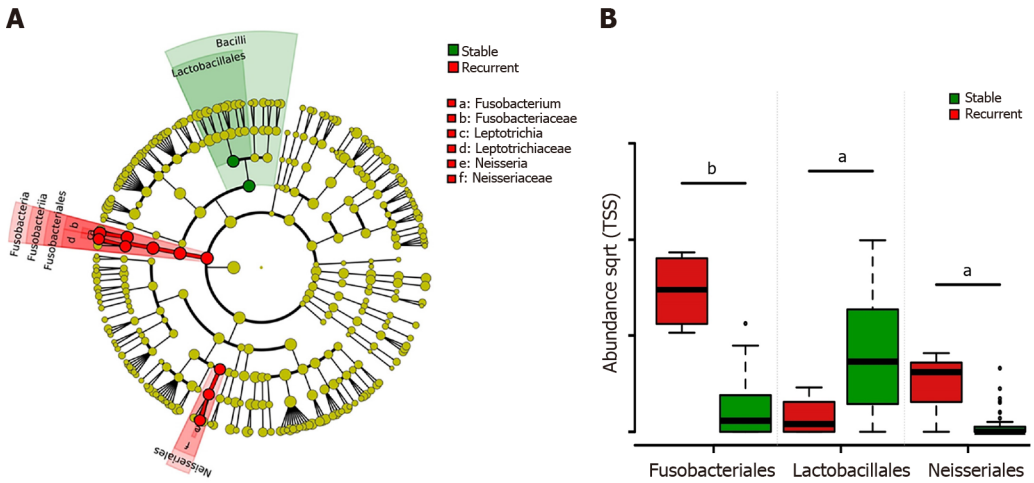

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Figure 3 LEfSe analysis of group-specific microbes in choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. A: Colored cladogram showing microbiota with biomarker significance in choledocholithiasis patients with different prognosis post-endoscopic sphincterotomy (EST) (red for biomarkers in recurrent patients and green for biomarkers in patients without recurrence post EST); B: Relative abundance comparison of microbes with biomarker significance in choledocholithiasis patients with different prognosis post-EST. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.001$.

important microorganism in the deconjugation of bilirubin diglucuronide than *E. coli*, because it exhibits a 34-fold higher β-glucuronidase enzyme activity in the biliary tract. A lack of *Lactobacillus* in the bile could be a probable risk factor for choledocholithiasis, because *Lactobacillus* in bile can absorb cholesterol and reduce total serum cholesterol[44,45]. The core microbiome pattern in the bile of patients with choledocholithiasis in this study offers a more comprehensive understanding of the influence of the bile microbiome on biliary stone formation.

Furthermore, functional analysis indicated that the loss of transcription and metabolic abilities, and increased function of translation, replication and repair, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, genetic information processing, energy metabolism, and biosynthesis of other secondary metabolites could lead to recurrent choledocholithiasis. Most of these microbiologic functions were caused by the increased abundance of *Fusobacterium* and *Leptotrichia* and the loss of an unclassified genus of *Enterobacteriales*. However, little is known about the specific health-related functions of the metabolites of these microbes in the bile, and these important metabolic pathways require further research.

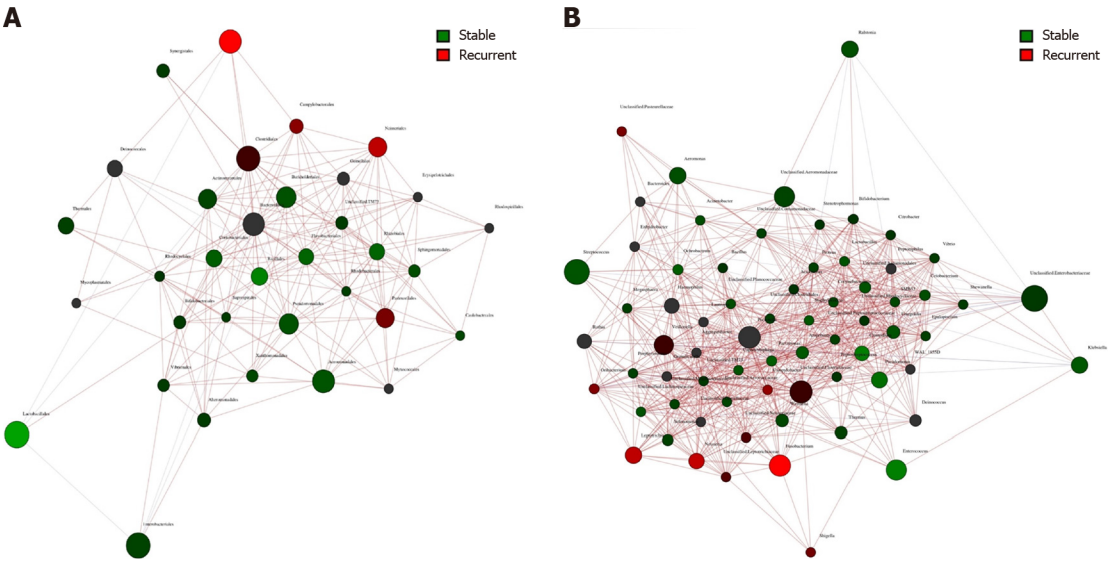

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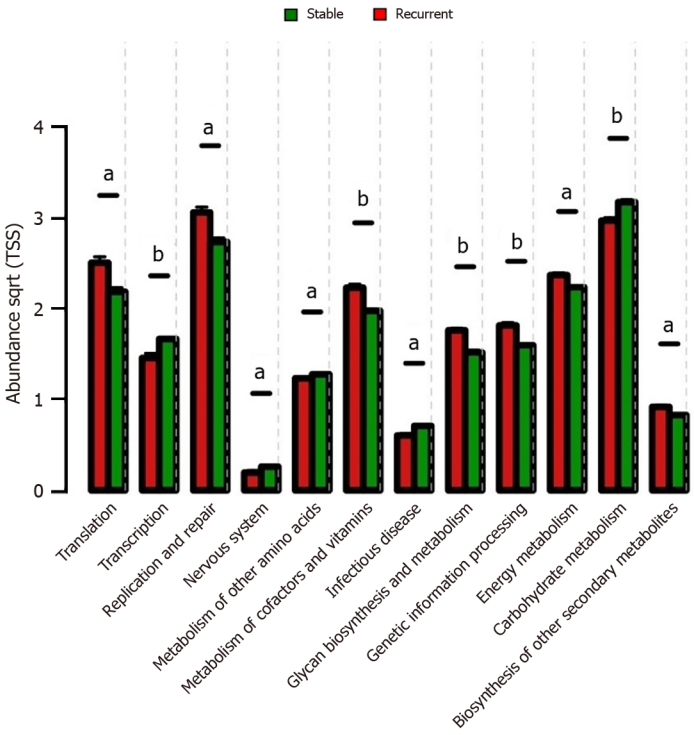
Figure 4 Co-occurrence network analysis of bile microbiome of choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. A: Co-occurrence and disease-specific bacterial interactions at the order level. Order was presented as nodes (stable group specific order in green and recurrent group specific order in red), order abundance was presented as node size, and edges were represented based on their association tested using Pearson's correlation (positive inter-node correlations in blue, negative inter-node correlations in red); B: Co-occurrence and disease-specific bacterial interactions at the genus level. Genus was presented as nodes (stable group specific genus in green and recurrent group specific genus in red), genus abundance was presented as node size, and edges were represented based on their association tested using Pearson's correlation (positive inter-node correlations in blue, negative inter-node correlations in red).



DOI: 10.3748/wjg.v28.i12.1257

Figure 5 Comparison of microbial function prediction of bile microbiome of choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. Functional analysis was performed at the 2nd hierarchy level of the Kyoto Encyclopedia of Genes and Genomes pathways in the bile microbiome of choledocholithiasis patients. Wilcoxon test was applied to the comparison of each category of microbial function; those with significant differences are shown in the bar chart. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$.

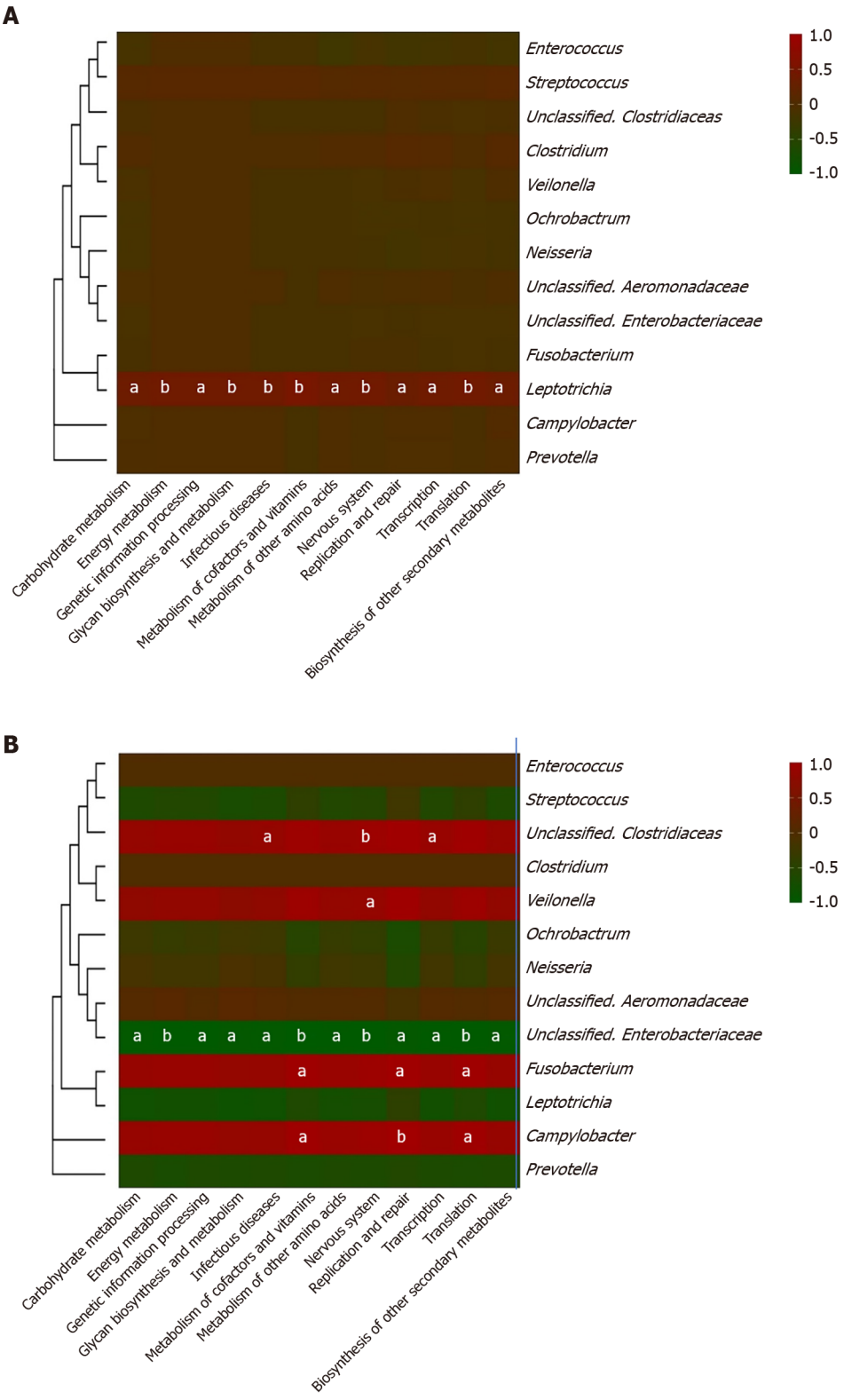


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Figure 6 Heatmap of correlation between the core microbiome and key metabolic pathway in choledocholithiasis patients. Thirteen core genera of bile microbiome and their correlations with the twelve discrepant metabolite pathways in different prognosis groups were analyzed using Pearson correlation analysis. The Pearson correlation coefficient between the genus and the metabolic pathway was calculated and shown in colored matrix; red represents a positive correlation, while green represents a negative correlation. A: Matrix heatmap shows the correlations between different genera and metabolite pathways in choledocholithiasis patients without recurrence post-endoscopic sphincterotomy (EST); B: Matrix heatmap shows the correlations between different genera and metabolite pathways in recurrent choledocholithiasis patients post-EST. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$.

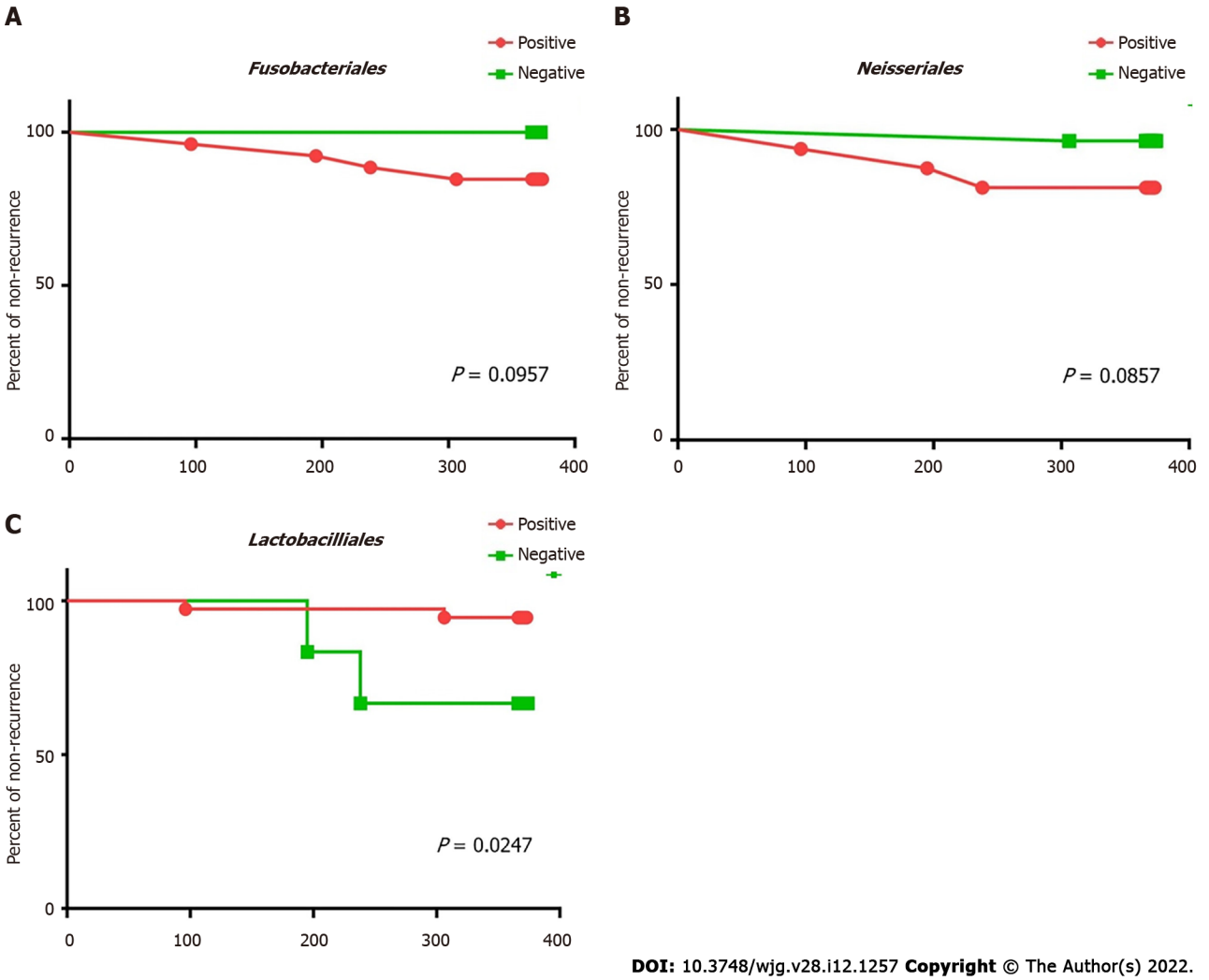

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Figure 7 Kaplan-Meier analysis of recurrent time post endoscopic sphincterotomy with different microbiologic risk factors. A: Kaplan-Meier analysis of recurrent time post-endoscopic sphincterotomy (EST) between choledocholithiasis patients with (red) and without (green) *Fusobacteriales* in bile; B: Kaplan-Meier analysis of recurrent time post-EST between choledocholithiasis patients with (red) and without (green) *Neisseriales* in bile; C: Kaplan-Meier analysis of recurrent time post-EST between choledocholithiasis patients with (red) and without (green) *Lactobacillales* in bile.

Certain microorganisms in bile could predict the time taken before disease recurrence post-EST, and this was evaluated. The existence of *Lactobacillales* is crucial for predicting recurrence time post-EST, because patients with *Lactobacillales* in their bile had a longer progression-free time post-EST than patients without *Lactobacillales*. Therefore, the examination of *Lactobacillales* existence in bile at the time of endoscopic examination could help doctors identify high-risk patients, who are likely to have early choledocholithiasis recurrence post-EST.

The limited number of early recurrent choledocholithiasis patients could have introduced a bias in statistical analysis and could have limited the generalizability of the prediction model in this study. Furthermore, the diagnosis of choledocholithiasis recurrence relied mainly on the imaging examinations; we could have missed some stones which were invisible in the CT, underestimating the rate of choledocholithiasis recurrence. The molecular mechanisms of microorganisms underlying the recurrence post-EST was based on the PICRUSt model. Verification experiments such as analyzing the correlation between the bile microbiome and the stone composition, and animal experiments to ascertain the preventive effects of *Lactobacillus* in choledocholithiasis recurrence are warranted.

CONCLUSION

The microbiological characteristics of bile from patients with recurrent choledocholithiasis post-EST indicated that an increase in *Fusobacterium* and *Neisseria* are potential biomarkers for the identification of high-risk patients in the first EST. It elucidated the role of microbial metabolites in the underlying etiology of choledocholithiasis. A co-occurrent network of the biliary bacterial community was constructed. Potential preventive therapy against recurrent choledocholithiasis through supple-

mentation with *Lactobacillus* and maintenance of the balance of the microbial systems could be promising. These findings could help doctors better understand the etiology of recurrent choledocholithiasis and develop better monitoring and treatment strategies against recurrence post-EST.

ARTICLE HIGHLIGHTS

Research background

Choledocholithiasis is a common and socially significant health problem worldwide, and endoscopic sphincterotomy (EST) has become widespread in treating choledocholithiasis; however, recurrence post-EST is relatively common. The bile microbiome has a profound influence on the recurrence of choledocholithiasis; however, the key pathogens and their functions are not fully elucidated.

Research motivation

To determine the microbiologic risk factors of recurrent choledocholithiasis post EST.

Research objectives

To investigate the biliary microbial characteristics of the recurrent choledocholithiasis post-EST, using next-generation sequencing.

Research methods

This cohort study included 43 choledocholithiasis patients who had undergone EST were followed up for over a year. They were divided into either the stable or recurrent groups and comparison of their bile microbiome was carried out through next-generation sequencing. Resulting sequences were analyzed for core microbiome and statistical differences between the microbiologic compositions and functions. Correlation between the key genera and metabolic pathways in bile, were analyzed using Pearson's correlation test.

Research results

The results revealed distinct clustering of biliary microbiota in recurrent choledocholithiasis, in which higher relative abundances (RAs) of *Fusobacterium* and *Neisseria* and the absence of *Lactobacillus* were observed in the bile of the recurrent patients. Microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia*, and an antagonistic relationship among *Lactobacillales, Fusobacteriales*, and *Clostridiales*. Functional analysis revealed that the loss of microbiologic transcription and metabolic abilities may lead to the choledocholithiasis recurrence. Furthermore, the prediction model based on the RA of *Lactobacillales* in the bile was effective in identifying the risk of recurrent choledocholithiasis.

Research conclusions

We concluded the microbiologic differences in the bile of recurrent choledocholithiasis patients post EST, thereby adding to the current knowledge on its microbiologic etiology.

Research perspectives

The findings of our study will help develop new prevention strategies for post-surgery recurrence of choledocholithiasis.

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FOOTNOTES

Author contributions: Li Y, Tan WH, Wu JC and Wu QP designed the research; Wu JC, Tan WH and Liang B recruited the clinical cohort, collected samples, and performed the follow-up surveys; Li Y, Huang ZX and Shang YY contributed to the amplicon sequencing; Li Y, Chen JH, Pang R and Xie XQ analyzed the data; Li Y, Wu JC, Huang ZX and Xue L wrote the paper; Zhang JM, Ding Y, Chen MT, Wang J, Tan WH and Wu QP performed critical revisions of the manuscript; Li Y, Tan WH and Wu JC contribute equally to the manuscript; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Study

Epidemiological, clinical, and histological presentation of celiac disease in Northwest China

Man Wang, Wen-Jie Kong, Yan Feng, Jia-Jie Lu, Wen-Jia Hui, Wei-Dong Liu, Zi-Qiong Li, Tian Shi, Mei Cui, Zhen-Zhu Sun, Feng Gao

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Abstract

BACKGROUND

Research on celiac disease (CD) in northwest China is still in its infancy. At present, large-sample data on the epidemiological, clinical, and pathological characteristics of CD are limited.

AIM

To investigate the epidemiological, clinical, and pathological characteristics of CD in northwest China.

METHODS

The clinical data of 2884 patients with gastrointestinal (GI) symptoms were retrospectively analyzed. Total immunoglobulin A (IgA) and anti-tissue transglutaminase (tTG) IgA levels were examined in all patients. Gastroscopy and colonoscopy were performed in patients with positive anti-tTG IgA and deficient total IgA levels. Atrophy of the duodenal and ileal villi was examined and histopathological examinations were performed. The modified Marsh–Oberhuber classification system was used to grade villous atrophy in the duodenum or distal ileum. The patients' *Helicobacter pylori* (*H. pylori*) infection status was compared in terms of clinical presentation and Marsh grade. Statistical analyses were performed using the t-test or chi-square test.

RESULTS

Among the 2884 patients, 73 were positive for serum anti-tTG IgA, and 50 were diagnosed with CD. The CD detection rate was significantly higher in Kazakhs

(4.39%) than in Uyghurs (2.19%), Huis (0.71%), and Hans (0.55%). The main symptoms of CD were chronic diarrhea, anorexia, anemia, fatigue, weight loss, sleep disorders, osteopenia, and osteoporosis. The body mass index of patients with CD was significantly lower than that of patients without CD. A total of 69 patients with positive serum anti-tTG IgA and two patients with deficient total IgA levels underwent GI endoscopy. Endoscopy revealed crypt hyperplasia and/or duodenal villous atrophy, mainly manifested as nodular mucosal atrophy, grooves, and fissures. The difference in *H. pylori* infection rates was not statistically significant between CD and non-CD patients but was significantly different among CD patients with different Marsh grades.

CONCLUSION

Among the patients with GI symptoms in northwestern China, the prevalence of CD was more in the Uyghur and Kazakh populations. *H. pylori* infection may be associated with CD severity.

Key Words: Celiac disease; Epidemiology; Gastrointestinal symptoms; Pathology; *Helicobacter pylori* infection

Core Tip: Celiac disease (CD) is an autoimmune disease caused by the ingestion of gluten in genetically susceptible individuals. The global prevalence of CD is approximately 1.4%. An increase in celiac-specific autoantibody levels can lead to varying degrees of damage to the small intestinal mucosa and consequently to various gastrointestinal and systemic symptoms. This study reports the epidemiological, clinical, and pathological characteristics of CD and its association with *Helicobacter pylori* infection and aims to provide useful information for the clinical diagnosis and treatment of CD.

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INTRODUCTION

Celiac disease (CD) is an autoimmune chronic inflammatory disorder of the small intestine caused by ingestion of gluten in genetically susceptible individuals. Intestinal mucosal gluten-reactive CD4+ T cells are involved in the pathogenesis of CD[1]. The presence of T-cells in the mucosa can cause varying degrees of damage to the small intestinal mucosa, leading to a variety of gastrointestinal (GI) and systemic symptoms[2]. Typical GI manifestations include abdominal pain, abdominal distension, and diarrhea, whereas non-GI manifestations include anemia, osteoporosis, herpetic dermatitis, and neurological symptoms[3,4]. Early epidemiological studies have suggested that CD is common in Caucasian populations, particularly in Europe and North America[5,6]. Several studies in other regions have shown similar CD prevalence rates in the Middle East, Asia, Southeast Asia, and Oceania (0.2%–1%)[7-9]. The global prevalence of CD is approximately 1.4%, which is gradually increasing[9,10]. The prevalence of CD among hospitalized patients has also been investigated. A report from Brazil found that the prevalence of CD was 1.9% among 1030 hospitalized patients[11]. The detection rate of CD was 4.48% in patients with irritable bowel syndrome[12].

There are scarce data on the prevalence of CD in Asia, while no data have been compiled for some countries. The prevalence of CD among asymptomatic adults in Japan is 0.05%, and no study has investigated the prevalence of CD in Japanese children[13]. The prevalence of CD in Indian children is approximately 1%[14]. In Central Asia, the prevalence of HLA-DQ alleles susceptible to CD is similiar to that in Europe; however, epidemiological and clinical studies are lacking[15]. The seropositivity for CD in the Chinese population is mainly concentrated in the northern region. A meta-analysis reported that the CD seroprevalence in the general population in China was 0.27%, whereas the CD seroprevalence in the high-risk population was 8.34%[16]. According to a study screening 118 Chinese children with chronic diarrhea, 14 patients were subsequently diagnosed with CD[17]. Research on CD in China is still in its infancy, with only a few cases reported[17,18]. However, the presence of CD susceptibility genes is not uncommon among the Chinese population, and it is believed that the actual number of CD cases in China may be much higher than the currently reported number of diagnosed cases[19].

Serum endomysium antibodies (EMAs) and antibodies against tissue transglutaminase (tTG) are commonly used serological tests for CD. Studies have shown that the sensitivity and specificity of anti-tTG immunoglobulin A (IgA) were 92.5% and 97.9%, respectively. Though EMA IgA testing is less sensitive, it is more specific than anti-tTG IgA, with sensitivity and specificity of 79.0% and 99.0%, respectively[20]. Anti-tTG IgA is the standard test used to screen for CD, while EMA IgA is widely used to confirm the diagnosis. Human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 genotyping can be used to exclude CD[21,22]; however, these are poor diagnostic tests because not all individuals with these genetic variations develop CD. Duodenal mucosal biopsy remains the gold standard for diagnosing CD, and characteristic changes include villous atrophy, crypt hyperplasia, and intraepithelial lymphocytosis. Therefore, specific serum antibody testing and endoscopic duodenal mucosal biopsy should be performed for patients with suspected CD[23,24].

The clinical presentation of CD is both complex and diverse. However, the diagnosis and treatment of CD is relatively simple. A strict gluten-free diet (GFD) is the most effective dietary intervention for disease control, but it has some limitations. Clinical trials of other non-dietary therapies are currently underway. However, owing to the lack of understanding of the disease, identification of high-risk populations for CD remains a challenge, which leads to high rates of missed diagnoses of early-stage CD, resulting in patients frequently developing serious complications.

Northwest China is a multiethnic region with ethnic groups such as Hans, Uyghurs, Huis, and Kazakhs. People living in this area have similar eating habits, with wheat being the staple food crop. In addition, this region is located in Central Asia and geographically close to Europe, where the incidence of CD is high. Genetic exchanges may have occurred between residents and travelers on the ancient Silk Road in this region. Therefore, many cases of CD may remain undiagnosed in this geographical area owing to insufficient knowledge of the disease. This study explored the prevalence, clinical manifestations, and pathological characteristics of CD in northwest China with the aim of improving clinician awareness of the disease, reducing the rates of missed diagnoses and misdiagnoses, and improving patients' quality of life.

MATERIALS AND METHODS

Patient and public involvement

This retrospective cross-sectional study was conducted in the Department of Gastroenterology of the People's Hospital of Xinjiang Uygur Autonomous Region. The study was approved by the hospital's institutional review board (IRB) (Register number: KY2021052611). All patients who underwent gastroduodenoscopy signed an informed consent form, and the IRB waived the requirement for informed consent for other clinical data. This study was conducted in adherence to STROBE guidelines.

Inclusion and exclusion criteria

The clinical data of 3147 patients, including adults and children, with GI symptoms, such as chronic diarrhea, abdominal pain, abdominal distension, constipation, vomiting, nausea, anorexia, heartburn, acid reflux, and burping, were collected from both inpatient and outpatient services between March 2016 and February 2021. All included patients agreed to undergo tests for CD and all relevant clinical data were kept confidential. To investigate the incidence of ileal villous atrophy and exclude diseases other than CD, anti-tTG IgA-positive patients were further examined using gastroduodenoscopy. Colonoscopy with ileal biopsy was not mandatory for the diagnosis of CD. The exclusion criteria were as follows: Physically healthy patients without GI symptoms; patients with digestive tract tumors or a history of other cancer types; patients with a history of cholecystectomy or gastric, duodenal, colon, or small intestinal surgery; and patients with liver cirrhosis, hepatitis, or acquired immunodeficiency syndrome.

Chronic diarrhea was defined as diarrhea lasting for > 4 wk, or recurrent diarrhea with an intermittent period of 2–4 wk. Anemia was defined as hemoglobin (Hb) levels < 110 g/L in children aged 6 months to 6 years, < 120 g/L in children aged 6–14 years, < 130 g/L in adult men, and < 120 g/L in adult women. Bone mineral density was measured using dual energy X-ray absorptiometry, with T-scores of -2.5 to -1 defined as osteopenia and T-scores of ≤ -2.5 defined as osteoporosis. Weight loss was defined as an unexplained reduction of > 5% in initial body weight within 6 mo. Anxiety and depression were quantified using the Hamilton Anxiety Rating Scale and Hamilton Depression Rating Scale, respectively. General patient information including sex, age, race, body mass index (BMI), GI signs and symptoms, comorbidities, *Helicobacter pylori* (*H. pylori*) infection status, and GI endoscopy and pathology results were collected.

Serological tests

Approximately 3–5 mL of venous blood was drawn from each patient, centrifuged to separate the serum, aliquoted, and frozen at -70 °C until required. Serum total IgA was evaluated using the immunoturbidimetric method, with levels of < 0.82 g/L considered as absence of selective IgA. Anti-tTG IgA levels were measured in patients with normal total IgA levels using enzyme-linked

immunosorbent assays, with anti-tTG IgA levels > 20 CU defined as positive. Testing was conducted in accordance with the kit instructions, and the test kit was sourced from INOVA Diagnostics Inc. (United States). Patients positive for anti-tTG IgA and total IgA deficiency underwent GI endoscopy.

Endoscopic, histological assessments and H. pylori infection

GI endoscopy was performed using an Olympus endoscope (Olympus EVIS LUCERA CV290, Tokyo, Japan). The mucosa of the duodenal bulb, descending duodenum, and terminal ileum were observed using white-light endoscopy. Villous architecture was further observed by near-focus narrow-band imaging, the water immersion method, and indigo carmine staining. Pathological biopsies were performed on the duodenal bulb (two pathological tissue samples), descending duodenum (four pathological tissue samples), and terminal ileum (two pathological tissue samples). Two blinded pathologists made the histopathological diagnoses and graded villous atrophy in the duodenum or distal ileum according to the modified Marsh–Oberhuber classification system[25]. Disagreements in the classification and grading were resolved by consensus. CD was diagnosed when the biopsy result was classified as Marsh grade ≥ 2.

For histological diagnosis of *H. pylori* infection, biopsy specimens were obtained from the antrum, corpus, and angulus of the stomach. Hematoxylin-eosin and Giemsa staining was performed as appropriate. *H. pylori* infection was considered negative if *H. pylori* was absent in all biopsy sites and positive if *H. pylori* was present in at least one biopsy site. If the histological diagnosis of *H. pylori* infection was negative, but the urea breath test showed positive results, the patient was diagnosed with *H. pylori* infection.

Statistical analysis

The SPSS software (version 17.0) was used for all statistical analyses. Normally distributed continuous data were compared using the t-test and are presented as mean ± SD, whereas categorical data were compared using the chi-square or Fisher's exact test and are presented as numbers and percentages. Statistical significance was set at $P < 0.05$.

Data availability

The datasets used and/or analyzed during the study are available from the corresponding author upon reasonable request.

RESULTS

Epidemiological characteristics

Of the 3147 patients with GI symptoms, such as chronic diarrhea, abdominal pain, abdominal distension, and weight loss, 2884 met the inclusion criteria (Figure 1). The participants, with ages ranging from 2 to 96 years, were divided into categories according to age. The majority of subjects fell within the 40-59 years, and ≥ 60 years age groups (34.3% and 30.7%, respectively). There were 1531 men (53.1%) and 1353 women (46.9%). When patients were grouped by ethnicity, 1097 (38.0%) were Hans, 1048 (36.3%) were Uyghurs, 387 (13.5%) were Kazakhs, 283 (9.8%) were Hui, and 69 (2.4%) were of other ethnicities. Table 1 summarizes the incidence of CD based on ethnic group, sex, age group, and BMI, and the correlation analysis results for each variable. Among these factors, there were significant associations based on ethnicity ($P < 0.05$) and BMI ($P < 0.01$). In terms of ethnicity, CD incidence was lowest in Hans (0.55% in Hans, 2.19% in Uyghurs, 4.39% in Kazakhs, and 0.71% in Huis). Among the other ethnicities, one Mongolian and one Uzbek patient were diagnosed with CD; however, this was not analyzed further because of the small sample size. All participants were tested for total serum IgA and anti-tTG IgA levels. Overall, two IgA-deficient patients and 73 anti-tTG IgA-positive patients were identified. The rate of positive serum anti-tTG IgA level was 2.53%. A total of 71 patients underwent GI endoscopy: Two IgA-deficient patients and 69 anti-tTG IgA-positive patients. Pathological classification was performed according to the modified Marsh–Oberhuber classification (Table 2). Two patients with total IgA deficiency had Marsh grades of 0. Among the 69 patients, 10 had Marsh grade 0, 9 had Marsh grade 1, and 50 had Marsh grade ≥ 2. Patients with Marsh grades 0 and 1 were excluded, and 50 patients with Marsh grade ≥ 2 were eventually diagnosed with CD. The overall CD detection rate was 1.73%.

Clinical signs and symptoms

CD was more common in patients with a BMI ≤ 18.49 kg/m^2 (5.50%). No significant differences were noted in CD incidence when patients were evaluated based on age or sex. The incidence rates for abdominal pain in non-CD and CD patients were 50.7% and 54.0%, respectively, and abdominal distension were 49.4% and 58.0%, respectively. The rates of chronic diarrhea, anorexia, anemia, fatigue, weight loss, sleep disorders, osteopenia, and osteoporosis were significantly higher in patients with CD than in those without CD. No significant differences were noted in the incidence of constipation, vomiting and/or nausea, heartburn and/or acid reflux, belching, headache and/or dizziness, anxiety

Table 1 General information of included subjects

	Subjects, *n* (%)		Coeliac disease	Frequency	95%CI	*P* value
Ethnicity						
Han	1097	38.0	6	0.55	0.1-1.0	< 0.001
Uygur	1048	36.3	23	2.19	1.3-3.1	
Kazakh	387	13.5	17	4.39	2.3-6.4	
Hui	283	9.8	2	0.71	0.0-1.7	
Others	69	2.4	2	2.90	0.0-7.0	
Gender						
Male	1531	53.1	22	1.44	0.8-2.0	0.202
Female	1353	46.9	28	2.07	1.3-2.8	
Age						
0-19	283	9.8	3	1.06	0.0-2.3	0.135
20-39	727	25.2	16	2.20	1.1-3.3	
40-59	989	34.3	22	2.22	1.3-3.1	
≥ 60	885	30.7	9	1.02	0.4-1.7	
BMI						
≤ 18.49	159	5.5	14	8.81	4.4-13.3	< 0.001
18.5-23.99	1237	42.9	23	1.86	1.1-2.6	
24-27.99	1064	36.9	7	0.66	0.2-1.1	
≥ 28	424	14.7	6	1.42	0.3-2.5	
Total	2884	100.0	50	1.73	1.3-2.2	

Table 2 The modified Marsh–Oberhuber classification[24]

	Marsh 0	Marsh 1	Marsh 2	Marsh 3			Marsh 4[2]
				3a	3b	3c	
IEL count[1]	< 30/100	> 30/100	> 30/100	> 30/100	> 30/100	> 30/100	< 30/100
Crypt hyperplasia	-	-	+	+	+	+	-
Villous atrophy	-	-	-	Mild	Moderate	Total	Total
	Pre-infiltrative	Infiltrative	Infiltrative-hyperplastic	Flat destructive			Atrophic-hypoplastic

[1]Number of intraepithelial lymphocytes *per* 100 enterocytes.
[2]This category is principally included for historic purposes.
IEL: Intraepithelial lymphocytes.

and/or depression, or *H. pylori* infection between the CD and non-CD patients (Table 3).

Histological presentation

The main endoscopic manifestations of duodenal villous atrophy in patients with CD are nodular mucosal atrophy, grooves, and fissure-like lesions. Overall, 24 patients showed nodular mucosal atrophy, 29 showed grooves and fissure-like lesions, 4 showed mosaic signs, 12 showed scallop-like lesions, 9 showed wrinkle reduction or disappearance, and 15 showed multiple manifestations. Villous atrophy in the terminal ileum was observed in 10 patients with CD, whereas normal terminal ileal mucosa was observed in 40 patients. The histological findings of CD included total villous atrophy, increased intraepithelial lymphocytes, and crypt hyperplasia.

H. pylori infection

The *H. pylori* infection rates in CD and non-CD patients were 48.0% and 57.4%, respectively, and the

Table 3 General clinical symptoms and frequency of coeliac disease

Symptoms[1]	Subjects without coeliac disease (*n* = 2834), *n* (%)		Coeliac disease (*n* = 50), *n* (%)		*P* value	Total (*n* = 2884), *n* (%)		Frequency of coeliac disease among patients with symptoms (95%CI)	*P* value
Chronic diarrhea	258	9.1	21	42.0	< 0.001	279	9.7	7.53 (4.4-10.6)	< 0.001
Abdominal pain	1437	50.7	27	54.0	0.644	1464	50.8	1.84 (1.2-2.5)	
Abdominal distension	1400	49.4	29	58.0	0.228	1429	49.5	2.03 (1.3-2.8)	
Constipation	397	14.0	5	10.0	0.417	402	13.9	1.24 (0.2-2.3)	
Anorexia	640	22.6	20	40.0	0.004	660	22.9	3.03 (1.7-4.3)	
Vomit or/and nausea	802	28.3	18	36.0	0.231	820	28.4	2.20 (1.2-3.2)	
Heartburn or/and acid reflux	740	26.1	12	24.0	0.736	752	26.1	1.60 (0.7-2.5)	
Belch	776	27.4	13	26.0	0.828	789	27.4	1.65 (0.8-2.5)	
Headache or/and dizziness	677	23.9	14	28.0	0.500	691	24.0	2.03 (1.0-3.1)	
Anemia	416	14.7	20	40.0	< 0.001	436	15.1	4.59 (2.6-6.6)	
Fatigue	536	18.9	24	48.0	< 0.001	560	19.4	4.29 (2.6-6.0)	
Weight loss	873	30.8	24	48.0	0.009	897	31.1	2.68 (1.6-3.7)	
Osteopenia or osteoporosis	329	11.6	38	76.0	< 0.001	367	12.7	10.35 (7.2-13.5)	
Sleep disorder	771	27.2	23	46.0	0.003	794	27.5	2.90 (1.7-4.1)	
Anxiety and depression	808	28.5	17	34.0	0.395	825	28.6	2.06 (1.1-3.0)	
H. pylori infection	1627	57.4	24	48.0	0.182	1651	57.2	1.45 (0.9-2.0)	

[1]Note that patients had more than one symptom.

difference was not statistically significant. Abdominal pain was significantly more frequent in patients with CD without *H. pylori* infection than in those with *H. pylori* infection. Of the 50 patients diagnosed with CD, 17 were classified as having Marsh grade 2 and 33 as having Marsh grade 3. The rates of *H. pylori* infection were significantly different among the different Marsh grades (P = 0.032) (Table 4). Further pairwise comparisons showed significant differences in the detection rate of *H. pylori* between CD patients with Marsh grades 2 and 3b (P = 0.025). Patients with *H. pylori* infection were more commonly found to have Marsh grade 2, and more patients without *H. pylori* had Marsh grade 3b.

DISCUSSION

Currently, there is a paucity of clinical and epidemiological data on CD in China. To the best of our knowledge, to date, no large-sized sample data analysis of the pathological characteristics of patients with CD is available in the literature. Additionally, there have been no published reports on the relationship between CD and *H. pylori* infection. The prevalence of CD is high in Europe[9,26]. Northwest China connects Eurasia and lies on the ancient Silk Road. Historically, owing to the possibility of intermarriages between the populations of the two regions, there may have been transfer of CD susceptibility genes present in European populations to this region, leading to an increase in CD incidence. In addition to genetic susceptibility, wheat is the main food crop for this population. These factors may have contributed to the high detection rate of CD in northwest China. Northwest China is a multi-ethnic region in which ethnic groups such as Hans, Uyghurs, Huis, and Kazakhs live together. A previous study by Zhou *et al*[27] found a higher incidence of CD in Xinjiang and a higher detection rate of CD in Kazakhs than in Uyghurs and Hans[27]. Studies have found that HLA-DQ2 and HLA-DQ8 gene carrier rates are high in Kazakhs and Uyghurs[27,28]. Genetic susceptibility may be the reason for the difference in prevalence among different races.

Table 4 Clinical signs and symptoms, celiac grading according to the presence of *Helicobacter pylori* in celiac disease patients

Symptom, sign, associatedcondition or, test	HP (+) (*n* = 24)	HP (-) (*n* = 26)	OR (95%CI)	*P* value
Chronic diarrhea	12 (50.0)	9 (34.6)	1.89 (0.61-5.89)	0.271
Abdominal pain	17 (70.8)	10 (38.5)	3.89 (1.19-12.68)	0.022
Abdominal distension	15 (62.5)	14(53.8)	1.43 (0.46-4.42)	0.536
Constipation	4 (16.7)	1 (3.8)	5.0 (0.52-48.34)	0.182
Anorexia	8 (33.3)	12 (46.2)	0.58 (0.19-1.84)	0.355
Vomit or/and nausea	11 (20.8)	7 (50.0)	2.30 (0.71-7.45)	0.164
Heartburn or/and acid reflux	8 (33.3)	4 (15.4)	2.75 (0.70-10.74)	0.138
Belch	6 (25.0)	7 (26.9)	0.91 (0.26-3.21)	0.877
Headache or/and dizziness	5 (20.8)	9 (34.6)	0.50 (0.14-1.78)	0.278
Anemia	8 (33.3)	12 (46.2)	0.58 (0.19-1.84)	0.355
Fatigue	11 (45.8)	13 (50.0)	0.85 (0.28-2.57)	0.768
Weight loss	10 (41.7)	14 (53.8)	0.61 (0.20-1.88)	0.389
Osteopenia or osteoporosis	20 (83.3)	18 (69.2)	2.22 (0.57-8.65)	0.243
Sleep disorder	11 (45.8)	12 (46.2)	0.99 (0.32-3.01)	0.982
Anxiety or/and depression	6 (25.0)	11 (42.3)	0.46 (0.14-1.52)	0.197
Celiac grading, No. (%)				0.032
Marsh grade 2 (*n* = 17)	12 (50.0)	5 (19.2)	1.0 (ref)	
Marsh grade 3a (*n* = 16)	6 (25.0)	10 (38.5)	0.25 (0.06-1.07)	
Marsh grade 3b (*n* = 13)	3 (12.5)	10 (38.5)	0.13 (0.02-0.66)	
Marsh grade 3c (*n* = 4)	3 (12.5)	1 (3.8)	1.25 (0.10-15.11)	

Patients may have one or more associated symptoms or conditions.

In this study, 2884 patients with GI symptoms were screened for CD according to the global guidelines of the World Gastroenterology Organization[29]. Among them, 73 were positive for anti-tTG IgA and 50 were pathologically diagnosed with CD. CD can occur at any age, and the prevalence rate in women is 2–4 times higher than that in men[30,31]. In line with this, our study found a higher prevalence of CD in female patients than in male patients. The clinical manifestations of CD include delayed growth, malnutrition, chronic diarrhea, abdominal pain, and abdominal distension. Up to 17% of female patients may present with severe clinical manifestations during pregnancy or puerperium [32]. This study found that the main clinical manifestations of patients with CD in Xinjiang included chronic diarrhea, severe malnutrition, osteoporosis, anemia, fatigue, and decreased BMI. BMI is an important index for evaluating and predicting CD, and diarrhea is a typical symptom of CD. The immune response caused by gluten intake in susceptible populations leads to intestinal absorption dysfunction and osmotic diarrhea. In our study, 21 patients with CD-related diarrhea mainly presented with profuse watery and fatty diarrhea. Owing to the lack of knowledge and limited diagnostic criteria for CD, diarrhea often becomes chronic, making the disease more difficult to control. Therefore, most patients exhibit significant weight loss, accompanied by anemia, iron and vitamin D deficiency, and other forms of malnutrition. In Britain, individuals with suspected CD are screened to avoid complications associated with delayed CD diagnosis[24]. Therefore, CD screening should be performed in patients with GI symptoms in China, especially in those with anorexia and significant weight loss. Most patients with CD in Europe initially present with extra-intestinal manifestations and are missed because they are not tested for CD[33]. The European Society for Pediatric Gastroenterology Hepatology and Nutrition suggests that relatives of patients with CD or other autoimmune diseases should also be screened for the same conditions. Mass screening for CD is currently not recommended[3]. At present, there are no relevant guidelines for CD in the Chinese population; however, a strategy similar to that followed in Europe could be adopted.

CD is caused by gluten in susceptible subjects, however, its etiology is not fully understood. With the increasing prevalence of CD, researchers have begun to consider environmental risk factors that may trigger autoimmunity in the small intestine[34]. *H. pylori* is one of the most common chronic bacterial infections worldwide and can cause severe gastroduodenal diseases[35]. Both *H. pylori* infection and CD

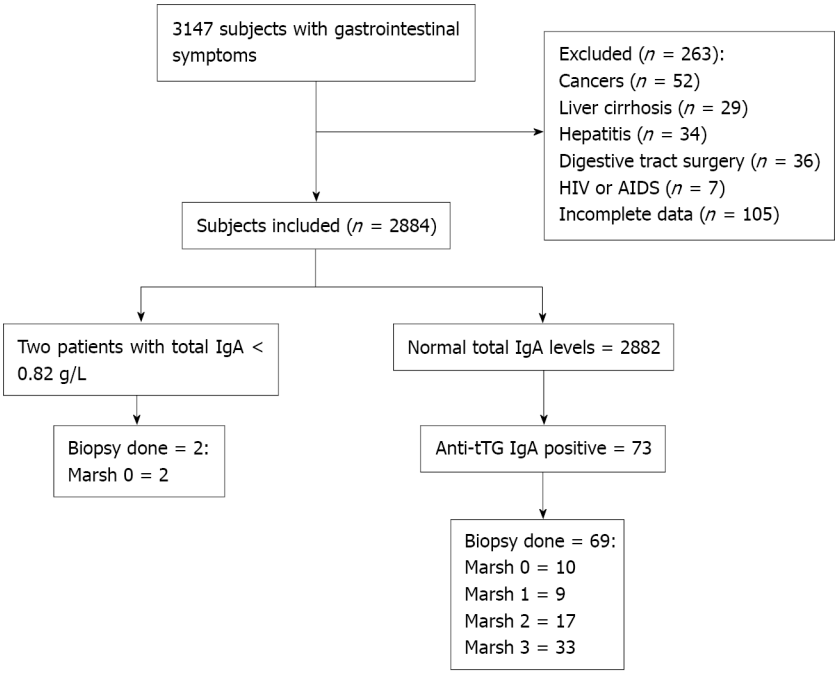

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Figure 1 Flow chart of the patient enrollment. IgA: Immunoglobulin A; HIV: Human immunodeficiency virus; AIDS: Acquired immunodeficiency syndrome.

involve systemic humoral and local inflammatory immune responses. Chronic gastric infections that can induce duodenal ulcers and affect the systemic immune response may trigger autoimmunity in the small intestine[36]. Whether *H. pylori* infection can prevent or induce CD remains debatable. Epidemiological studies have investigated the association between *H. pylori* infection and CD. However, these studies reported conflicting results[37-39]. The variability in the results may be due to the different prevalence of *H. pylori* infection in different populations and the identification of patients who have not yet demonstrated clinically significant CD. There are no reports on the relationship between CD and *H. pylori* infection in northwest China.

We evaluated the relationship between *H. pylori* infection and CD and found that *H. pylori*-positive CD patients demonstrated more severe mucosal damage than *H. pylori*-negative CD patients (Marsh grades 2 and 3) (P = 0.018). This finding is similar to that of Gungor *et al*[40]. However, it has been reported that in individuals without CD, *H. pylori* infection itself can cause duodenal mucosal damage [41]. In a study by Konturek *et al*[42], the prevalence of *H. pylori* infection was higher in patients with CD than in controls[42]. Previous studies have shown that *H. pylori* infection can prevent the development of CD[43,44]. This association may be related to the genetic factors of CD and/or *H. pylori*, virulence of *H. pylori*, and immunopathology involved. In addition to altering the acidity and content of gastric juice, *H. pylori* directly interacts with the immune system and increases intestinal permeability [45].

In patients with CD, biopsy usually shows villous atrophy, crypt hyperplasia, and inflammation. However, some serologically positive individuals can have normal intestinal mucosa, but many of these patients later develop CD, which is sometimes termed "latent CD"[46]. In these atypical cases, more than 95% of anti-tTG IgA-positive patients may be sensitive to glutenin[47]. Our study found that the grades of five patients who presented with Marsh grade 1 in 2016–2019 improved to Marsh grade 0 on gastroscopy after following a GFD for at least half a year. Therefore, we speculate that early initiation of a GFD can improve the condition of patients with anti-tTG IgA-positive "latent CD." The healing rates of patients often differ significantly, and the older the patient at the time of the first diagnosis, the slower the intestinal healing process and the higher the possibility of nonreactive CD. There is no relevant research on "latent CD" in China and more extensive screening and follow-up are necessary. Early diagnosis of CD can reduce the long-term and persistent damage caused by gluten to the intestinal tract and whole body, thus resulting in better patient prognosis. Common causes of CD-related deaths are intestinal non-Hodgkin's lymphoma and small-bowel cancers[48]. Refractory CD (RCD) is a major cause of poor prognosis. RCDs can be divided into types I (RCD I) and II (RCD II). The phenotype of intraepithelial lymphocytes (IELs) is abnormal in RCD II patients and normal in RCD I patients. Approximately 50%–60% of patients with RCD II develop EATL within 5 years after diagnosis[49]. Both are (pre)malignant complications of CD. Patients with RCD II and EATL often have more severe malnutrition due to intestinal malabsorption and hypermetabolism[50]. No patients with RCD or EATL were found in this study; however, Marsh grades were positively correlated with patient age. Therefore, patients with CD who have significant weight loss or are elderly should be screened for CD using GI

endoscopy.

Study limitations

To the best of our knowledge, this is the first study to comprehensively analyze the clinical and pathological characteristics of Chinese patients with CD and evaluate the association of CD with *H. pylori* infection. Our study not only bridges the gap in relevant research in the Chinese population but also provides reference values for the diagnosis and treatment of CD. However, this study has several limitations. The subjects were patients with GI symptoms in the hospital, which may have resulted in a selection bias. HLA-DQ2 and HLA-DQ8 genotypes were not identified in our study; therefore, further research on the relationship between these genotypes and the pathological types of CD is warranted.

CONCLUSION

Among people with GI symptoms in northwest China, the prevalence of CD is higher in the Uyghur and Kazak populations. Therefore, physicians should be aware of the risk of developing CD in regional populations. *H. pylori* infection may be related to CD severity, which warrants further study.

ARTICLE HIGHLIGHTS

Research background

Research on celiac disease (CD) in Northwest China is still in its infancy. At present, large sample data on the epidemiological, clinical, and pathological characteristics of CD are limited.

Research motivation

This study reports the epidemiological, clinical, and pathological characteristics of CD and its association with *Helicobacter pylori* (*H. pylori*) infection, and aims to provide useful information for clinical diagnosis and treatment of CD.

Research objectives

To investigate the epidemiological, clinical, and pathological characteristics of CD in northwest China.

Research methods

The clinical data of 2884 patients with gastrointestinal (GI) symptoms were retrospectively analyzed. Total immunoglobulin A and anti-tissue transglutaminase (tTG) immunoglobulin A (IgA) levels were examined for all patients. Gastroscopy and colonoscopy were performed in patients with positive anti-tTG IgA and deficient total IgA levels. Atrophy of the duodenal and ileal villi was examined, and histopathological examinations were performed. The modified Marsh–Oberhuber classification system was used to grade villous atrophy in the duodenum or distal ileum. Patient *H. pylori* infection status was compared in terms of clinical presentation and Marsh grade. Statistical analyses were performed using t-test or chi-square test.

Research results

The detection rate of CD was significantly higher in Kazakhs (4.39%) than in Uygurs (2.19%), Huis (0.71%), and Hans (0.55%). The main symptoms of CD were chronic diarrhea, anorexia, anemia, fatigue, weight loss, sleep disorders, osteopenia, and osteoporosis. The body mass index of CD patients was significantly lower than that of non-CD patients. Endoscopy revealed crypt hyperplasia and/or duodenal villous atrophy, which mainly manifested as nodular mucosal atrophy, grooves, and fissures. The difference in *H. pylori* infection rates was not statistically significant between CD and non-CD patients, but was significantly different among CD patients with different Marsh grades. Patients with *H. pylori* infection were more commonly found with Marsh grade 2 and more patients without *H. pylori* had Marsh grade 3b.

Research conclusions

Among people with GI symptoms in Northwest China, the prevalence of CD is higher in the Uygur and Kazak populations. Physicians should be aware of the risk of CD in the regional population. *H. pylori* infection may be related to the severity of CD, which warrants further study.

Research perspectives

H. pylori infection may be related to the severity of CD, which warrants further study.

FOOTNOTES

Author contributions: Wang M and Gao F designed the study; Wang M, Kong WJ, and Lu JJ acquired the data and drafted the article; Hui WJ and Liu WD analyzed and interpreted the data; Cui M and Sun ZZ made a pathological diagnosis; Feng Y, Li ZQ, Shi T and Gao F revised the article critically for important intellectual content; all the authors approved the version to be published.

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LETTER TO THE EDITOR

Near-infrared fluorescence imaging guided surgery in colorectal surgery

Sung Uk Bae

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Abstract

Near infrared fluorescence using indocyanine green is beneficial for visual assessment of blood vessels, blood flow, and tissue perfusion, sentinel lymph node biopsy, lymph node road mapping, identification of the vascular system round the major vessels, and the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions in colorectal surgery.

Key Words: Fluorescence; Enhanced reality; Anastomotic leak; Lymph node; Anastomosis

Core Tip: Near infrared fluorescence technique using indocyanine green can be used in estimation of intestinal vascularization to detect areas of poor perfusion for preventing anastomotic leakage, the visualization of sentinel lymphatic drainage and peritoneal metastases, and the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions in colorectal surgery. Additionally, this technique can be used in identifying suspected lymph nodes and preventing their incomplete dissection during lateral pelvic lymph node dissection and D3 Lymphadenectomy for rectal cancer and right-sided colon cancer, respectively, and in identification of the vascular system round the major vessels.

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TO THE EDITOR

Indocyanine green (ICG) emits an infrared signal when excited by laser light in situ, which can be detected with near infrared fluorescence (NIF) camera. NIF imaging uses laser technology to activate an intravenously delivered agent, ICG, which rapidly binds to plasma proteins. This allows ICG to remain predominantly in visual assessment of blood vessels, blood flow, and tissue perfusion, sentinel lymph node biopsy and lymph node road mapping[1,2].

In this issue of World Journal of World Journal of Gastroenterology, the review article by Zocola *et al*[3] highlights the role of NIF in colorectal surgery. They reviewed the literature regarding NIF for three main indications including the estimation of intestinal vascularization to detect areas of poor perfusion for preventing anastomotic leakage, the visualization of sentinel lymphatic drainage and peritoneal metastases, and the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions in colorectal surgery.

NIF in conjunction with ICG allows for visualization of the microcirculation before formation of the anastomosis, thereby allowing the surgeon to choose the point of transaction at an optimally perfused area (Figure 1). Zocola *et al*[3] intensively reviewed the role of NIF in the intraoperative bowel viability assessment to prevent anastomotic leaks. They divided the retrospective cohort study and prospective randomized controlled study and reviewed the effectiveness of NIF in reducing anastomotic leakage.

Regarding the role of NIF with ICG to detect metastatic lymph node, Zocola *et al*[3] reviewed studies on the identification of sentinel lymph node and mapping additional lymph nodes outside of the proposed resection margins to achieve radical lymphadenectomy for curative surgery (Figure 2). In addition to the studies mentioned by Zocola *et al*[3], I would like to mention some recent studies related to this issue. One of the issues related to radical lymphadenectomy in colorectal cancer is lateral pelvic node dissection (LPND), and recent introductions and data on this procedure using NIF have been reported. Kim *et al*[4] demonstrated a novel application of NIF using ICG during robotic total mesorectal excision (TME) with LPND to identify suspected lateral pelvic lymph nodes and prevent their incomplete dissection. They injected ICG at a dose of 2.5 mg around the tumor transanally before surgery and NIF imaging–guided robotic TME with lateral pelvic lymphadenectomy allowed the surgeon to identify lymph nodes and lymphatic flow of rectal cance. Zhou *et al*[5] compared patients who underwent TME and LPND with NIF technique (n = 12) and patients who received conventional TME and LPND without NIF-guided imaging (n = 30). They reported that the NIF group had significantly lower intraoperative blood loss (55.8 ± 37.5 mL *vs* 108.0 ± 52.7 mL, P = 0.003) and a significantly larger number of lateral pelvic nodes harvested (11.5 ± 5.9 *vs* 7.1 ± 4.8, P = 0.017), and lateral pelvic lymph nodes from two patients in the NIF group remained during LPND. Additionally, Park *et al*[6] and Bae *et al*[7] used NIF technique for colorectal cancer surgery is D3 Lymphadenectomy, especially in right-sided colon cancer. Park *et al*[6] injected ICG around the tumor for visualization of lymphatic flow and lymph nodes and demonstrated the numbers of apical lymph nodes (14 *vs* 7, P < 0.001) and total harvested lymph nodes (39 *vs* 30, P = 0.003) were significantly higher in the NIF group than in the conventional group.

When injected intravenously, ICG rapidly binds to plasma proteins and remains predominantly in the vasculature. Although there was no mention in the review article, NIF angiography can be used in identification of the vascular system (Figure 3). ICG can be easily injected into the blood circulation during surgery, when the blood vessels are exposed, to allow direct visual observation. Bae *et al*[8,9] included 11 patients who underwent a robotic TME with preservation of the left colic artery for rectal cancer using NIF technique. The optimal point of division was then chosen by the surgeon under NIF imaging that facilitated the identification of the left colic branch of the inferior mesenteric artery (IMA). In addition, NIF imaging was used for the identification of the collateral vessels (Arc of Riolan) around the inferior mesenteric vein in their study. The left colic artery branches mainly at the Griffith point (watershed), which is located in the splenic bend where the left branch of the middle colic and the ascending branch of the left colic join. This area is vulnerable to injury and ischemia during surgery due to poor blood supply. For this reason, great care must be taken not to interfere with the bifurcation of the left colic artery. Real-time identification of collateral vessels using NIF technology can help implement safe low ligation of the IMA while preventing damage to these vessels. For now, it remains a linear graded outcome that requires subjective interpretation of the demarcation point between sufficient and insufficient perfusion and perfusion is assessed is based on a subjective qualitative impression of the surgeon. Quantitative analysis of NIF images is desirable but not currently available in robotic or laparoscopic systems. Son *et al*[10] performed quantitative evaluation of colon perfusion patterns using NIF angiography to find the most reliable predictive factor of anastomotic complications after laparoscopic colorectal surgery. They found that the fluorescence slope, T1/2MAX, and time ratio were related with anastomotic complications and those complications were significantly correlated with the novel factor time ratio (> 0.6) as the most reliable predictor of perfusion and anastomotic complications. Recently, Han *et al*[11] compared the changes in perfusion status between high tie and low tie through quantitative evaluation of ICG using NIF technique. They demonstrated that T_max increased and Slope_max decreased significantly in the high tie group after IMA ligation, whereas the intensity of perfusion status (F_max), which indicates the intensity of perfusion, did not change according to the level of IMA ligation. They suggested that the speed of blood perfusion could be more delayed after

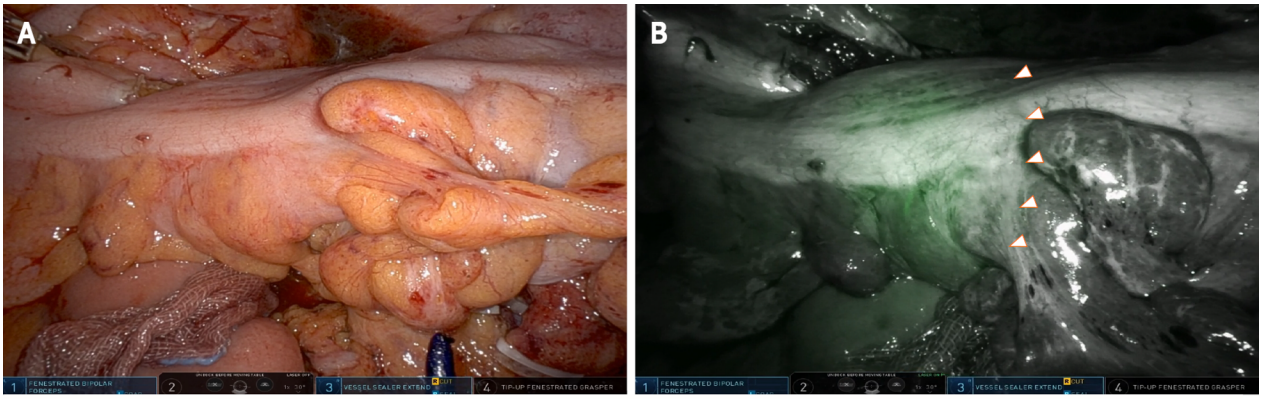

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Figure 1 Near infrared fluorescence in conjunction with indocyanine green allowing visualization of the microcirculation before development of the colorectal anastomosis. A: A white light image before visualizing the ischemic zone of the sigmoid colon using excited fluorescence; B: An intraoperative near infrared fluorescence image after visualizing the ischemic zone of the sigmoid colon using excited fluorescence.

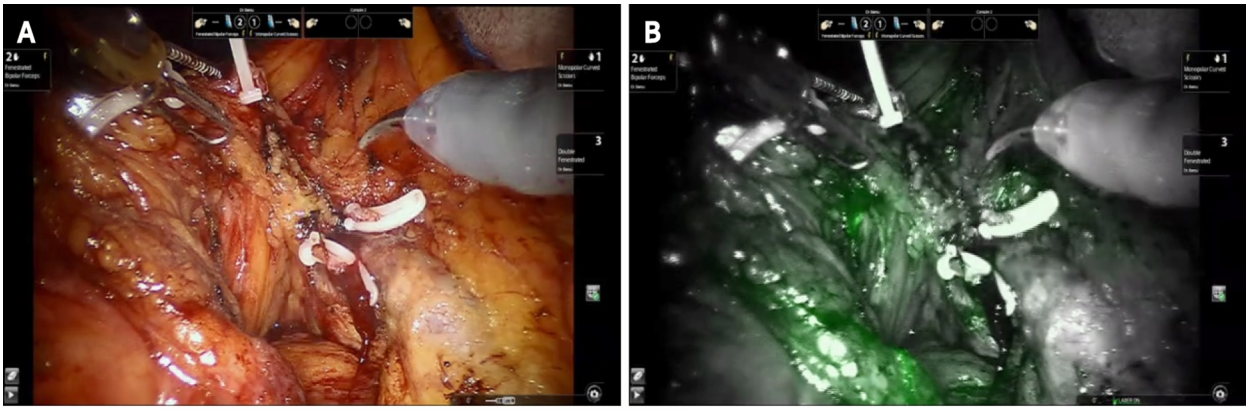

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Figure 2 Mapping of additional lymph nodes outside the proposed resection margins to achieve curative radical lymphadenectomy in robot-assisted right hemicolectomy. A: A white light image after D3 Lymphadenectomy around superior mesenteric vessels; B: A near infrared fluorescence image after visualizing the remained lymph nodes after lymphadenectomy using excited fluorescence.

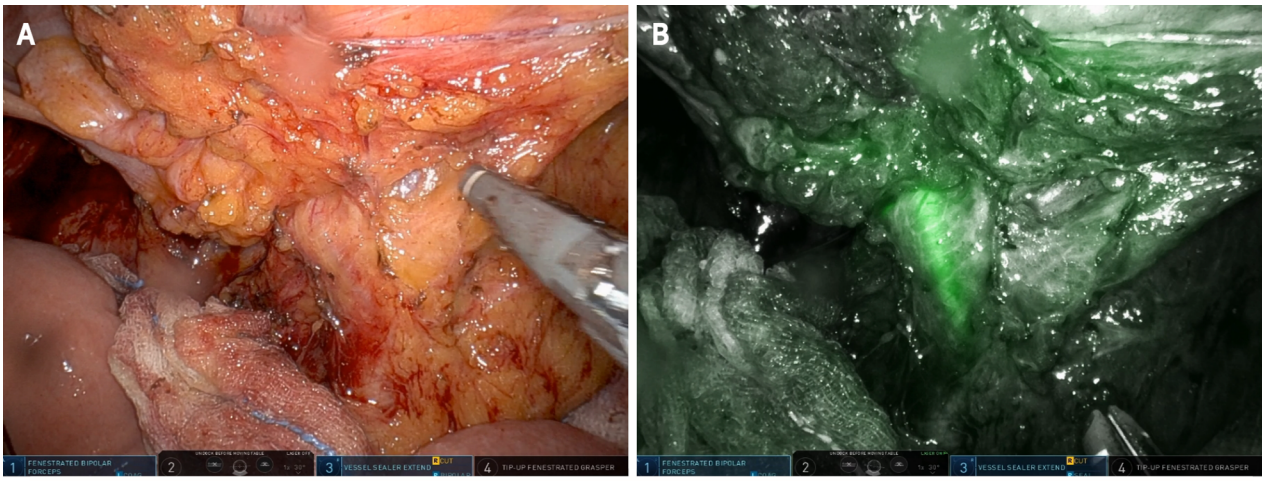

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Figure 3 Robot-assisted lymph node dissection around the inferior mesenteric artery with preservation of the left colic artery using near infrared fluorescence imaging. A: Dissection around the root of the inferior mesenteric artery (white light image); B: A near infrared fluorescence image visualizing the left colic artery using excited fluorescence.

high tie than low tie, but the intensity of perfusion was similar between high and low ligation of IMA. There are still a lot of questions and debates to be discussed, but we believe that the NIF technique will play an important role in improving the clinical and oncologic outcomes of colorectal surgery.

FOOTNOTES

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