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THE MICROBIOME AND GASTRIC CANCER: AN UPDATE

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Abstract: *Helicobacter pylori* is a bacterium that colonises the human stomach and is the major risk factor for several gastroduodenal diseases, including gastric cancer. Currently, we know that the stomach harbours a complex microbial community, but the impact of the gastric microbiota in the promotion of health or disease is not fully understood. The present article is a review of the most relevant literature published between April 2019 and March 2020 exploring the role of the microbiota in gastric diseases. During this period, the great majority of the publications focused on the modulation of the gastric microbiota by *Helicobacter pylori* infection, and on the relationships between the gastric and gut microbiotas and gastric tumorigenesis.

Keywords: Gastric microbiota, Gut microbiota, *Helicobacter pylori,* Gastric cancer, Gastric precancerous lesions.

INTRODUCTION

Helicobacter pylori is the best known member of the complex microbial community that colonises the human stomach. In recent years, and driven by the availability of next-generation sequencing technologies, there has been a rising interest in the study of the gastric microbiota and how changes in its structure and functions contribute to disease, in particular to gastric cancer (GC) development. In this context, numerous reviews have been published in the period contemplated by this article[1-7]. A systematic review by Rajilic-Stojanovic et al[8] providing the most comprehensive overview of the gastric microbiota in health and disease, concluded that *H. pylori* remains the central factor in gastric disease, but no major gastric microbiota profiles could be related to specific gastric conditions, mostly because of lack of consistent results between different studies. This review also highlighted the need for a better understanding of host-microbial interactions as a means to comprehend the influence of the gastric microbiota on gastric disease[8].

HELICOBACTER PYLORI INFECTION AND MODULATION OF THE GASTRIC MICROBIOTA

H. pylori infection is the best-known gastric coloniser and the most common cause of gastritis worldwide. In a study aiming to characterise the gastric microbiome of *H. pylori*-negative gastritis, Gantuya et al[9] analysed 11 subjects with *H. pylori*-negative gastritis, 40 with *H. pylori*-positive gastritis and 24 *H. pylori*-negative and gastritis-negative controls. Compared to sub-

jects with *H. pylori*-negative gastritis and to controls, *H. pylori*-positive patients had lower microbial diversity. The microbial structure was significantly different between *H. pylori*-positive and *H. pylori*-negative gastritis, but not between the latter and controls. While *Helicobacter* was the only distinguishing genus in *H. pylori*-positive gastritis, *Streptococcus*, *Haemophilus*, *Fusobacterium*, *Veillonella*, and *Prevotella* were identified as distinctive in *H. pylori*-negative gastritis. After multivariate analysis, *Streptococcus* and *Haemophilus* remained the strongest biomarkers to explain *H. pylori*-negative gastritis.

To advance our understanding of the effects of *H. pylori* eradication therapy on the gastric and also on the intestinal microbiota, Guo et al[10] conducted a prospective population-based study, where 16S rRNA gene deep sequencing was conducted in paired gastric biopsies and stool samples from uninfected (n=58), successfully *H. pylori*-treated (n=57) and failed *H. pylori*-treated (n=49) subjects. Richness and diversity increased significantly after successful *H. pylori* eradication (i.e., 10-day treatment with omeprazole, tetracycline, metronidazole and bismuth citrate), reaching comparable levels to those in *H. pylori*-negative subjects. The composition of the gastric microbiota significantly changed, with 18 bacterial taxa associated with *H. pylori* eradication. Gastric dysbiotic changes that were associated with gastric precancerous lesions were reversible with *H. pylori* eradication. In contrast, diversity of the gut microbiota was not modified by *H. pylori* eradication. Increased abundance of *Bifidobacterium* and *Clostridiales* and downregulation of drug-resistance mechanisms, were observed in the gut microbiota of successfully *H. pylori*-treated individuals.

The interactions between *H. pylori* and the gastric microbiota and the extent to which they impact gastric pathophysiology and modulate disease have been addressed by Noto et al[11]. They demonstrated that *H. pylori* infection induces gastric dysbiosis in a cagA-dependent manner in a Mongolian gerbil model, decreasing the diversity of the gastric microbiota and altering the composition of the microbial community as carcinogenesis progresses. Mongolian gerbils were infected with the carcinogenic cagA+ *H. pylori* strain 7.13 or a 7.13 cagA isogenic mutant to investigate the role of CagA in gastric dysbiosis. Further, as iron deficiency has been shown to increase *H. pylori*-induced inflammation and subsequent gastric adenocarcinoma development, gerbils challenged with sterile brucella broth or wild-type cagA+ carcinogenic *H. pylori* strain 7.13, were maintained on iron-depleted or iron-replete diets. Infection with the wild-type *H. pylori* strain 7.13 led to increased gastric inflammation and injury (i.e., dysplasia and adenocarcinoma) compared to the cagA isogenic mutant. However, both *H. pylori* strains were able to significantly decrease αdiversity and led to significant differences in the composition of the gastric microbiota of challenged gerbils compared to non-challenged controls. Infection with *H. pylori* resulted in enrichment of Proteobacteria including *Enterobacteriaceae* with concomitant decrease of *Porphyromonadaceae*, *Akkermansia*, *Bacteroides*, and *Lachnospiraceae*. When the gastric microbiota of gerbils with comparable *H. pylori* colonisation burden was analysed, the composition in gerbils infected with *H. pylori* strain 7.13 was also significantly different to that of gerbils infected with the cagA isogenic mutant, highlighting the relevance of CagA in the modulation of the composition of the *H. pylori*-associated gastric microbiota. Iron deficiency in this model led to increased inflammation in *H. pylori*-infected gerbils, of which 93% showed gastric dysplasia and adenocarcinoma. Despite this marked gastric injury, no significant changes were observed in the gastric microbiota in *H. pylori*-infected gerbils maintained on iron-depleted compared to iron-replete diets. Importantly, gastric inflammation and injury *per se* correlated with significant changes in diversity and composition of the gastric microbiota as *H. pylori*-infected gerbils with normal gastric mucosa or gastritis alone significantly differed from gerbils with gastric dysplasia or adenocarcinoma. Increased abundance of *Lactobacillus* and *Enterobacteriaceae* were the main changes associated with malignant lesions.

GASTRIC MICROBIOTA IN GASTRIC CANCER AND PRECANCEROUS LESIONS

It is now clear that the gastric microbiota in GC patients significantly differs from that of patients without cancer[12,13]. In a study in Korean patients that investigated the gastric microbiota of 268 GC cases and 288 controls undergoing health-screening exams, Gunathilake et al[14] showed decreased microbial diversity in GC cases compared to controls. The gastric micro-

biota composition of GC patients was significantly different from that of the controls, with significantly higher relative abundances of *H. pylori*, *Propionibacterium acnes*, and *Prevotella copri* and lower relative abundance of *Lactococcus lactis* in the gastric microbiota of GC patients than in the gastric microbiota of controls. In multivariate analysis, adjusted for age, family history of GC, exercise, education, occupation, income, and total energy intake, patients with the highest relative abundances of *H. pylori* and *P. acnes* and those that carried *P. copri* had the highest GC risk, whereas those carrying *L. lactis* had lowest GC risk.

The changes in the composition and structure of the gastric microbial communities in the different histological stages that occur along the gastric carcinogenesis pathway, remain incompletely clarified. Gantuya et al[15] characterised the gastric microbiota in Mongolian patients with normal gastric mucosa (n=20), gastritis (n=20), atrophy (n=40), intestinal metaplasia (n=40), and GC (n=48). In the majority of samples *Helicobacter* was the dominant genus, but in accordance with previous studies[13,16], the mean relative abundance decreased from gastritis, to atrophy, intestinal metaplasia, and GC. *Enterococcus* and *Lactobacillus* were identified as two dominant genera in the microbiota of GC patients, with the highest relative abundance in this patient group. Principal component analyses (PCA) could separate each patient group into different clusters, when considering all cases and also when cases with dominant genera or *H. pylori*-positive were excluded. Patients with normal mucosa, intestinal metaplasia and GC presented the highest microbial diversity, whereas patients with gastritis and atrophy had lowest microbial diversity, but after excluding *H. pylori*-positive cases and cases with dominant genera, significant differences persisted only in intestinal metaplasia and GC. When the authors performed Linear discriminant analysis Effect Size (LEfSe) analysis, *Enterococcus*, *Lactobacillus*, *Glutamicibacter*, *Paeniglutamicibacter*, *Escherichia*, *Carnobacterium, Helicobacter,* and *Pseudomonas*, were the genera that best explained the differences between cancer and non-cancerous cases, and the latter two genera enriched in the gastric microbiota of non-cancerous cases.

In the prospective population-based study mentioned above[10], which took place in Linqu, a rural county in the Chinese Shandong province that shows one of the highest GC mortality rates worldwide, nine bacterial taxa were found to be decreased in subjects presenting with gastric precancerous lesions (i.e., chronic atrophic gastritis, intestinal metaplasia and dysplasia), including *Alloprevotella*, *Fusobacterium*, *Neisseria*, *Porphyromonas*, *Prevotella*, *Rothia*, and *Veillonella*. Furthermore, co-exclusion interactions were identified between *Helicobacter* and *Fusobacterium*, *Neisseria*, *Prevotella*, *Veillonella*, and *Rothia*, and they occurred only in advanced gastric lesion patients, but not in normal/superficial gastritis patients. These findings are in accordance with others that reported *Neisseria* and *Prevotella* as depleted in GC[13,17].

Park et al[18] performed gastric microbiota network analysis in Korean patients that were classified as: A) *H. pylori*-negative and no atrophy (n=48); B) *H. pylori*-positive and no atrophy (n=14); C) *H. pylori*-positive and atrophy with intestinal metaplasia (n=12); and D) *H. pylori*-negative and atrophy with intestinal metaplasia (n=9). Based on co-occurring taxa, bacteria were classified into 18 different modules. Two highly similar modules (denoted as pink and brown) were positively correlated with more advanced stages in the gastric carcinogenesis cascade (i.e., higher ABCD groups). Both modules positively correlated with age and negatively correlated with pepsinogen I, and the brown module was also inversely correlated with *H. pylori* infection. The blue module, which was highly dissimilar to the pink and brown modules and included *H. pylori*, was inversely correlated with higher histopathological stages of carcinogenesis. In *H. pylori*-positive patients (groups B and C), the abundance of *H. pylori* decreased in patients presenting with atrophy and intestinal metaplasia (group C), whereas non-*H. pylori* bacteria from the pink and brown modules were identified in both groups with intestinal metaplasia (groups C and D). Taxa identified in the pink and brown modules included nitrosating/nitrate-reducing bacteria and bacteria with genes encoding type IV secretion system proteins, such as *Acidobacteriaceae*, *Burkholderiaceae*, *Neisseriaceae*, *Pasteurellaceae*, *Veillonellaceae*, *Bartonellaceae*, *Brucellaceae*, *Rhizobiales*, *Pseudomonadaceae*, *Sphingomonadaceae*, *Staphylococcaceae*, and *Xanthomonadaceae.* These results suggest that bacteria other than *H. pylori* are involved in gastric carcinogenesis.

Caguazango and Pazos[19], investigating the role of gastric microbiota in carcinogenesis according to gastric topography (antrum *vs.* body) in two Colombian populations with varying degrees of GC risk, also suggest that carcinogenesis could be related to the gastric microbiota

accompanying *H. pylori*. As shown by other scholars[20], the prevalence of *H. pylori* infection was similar in these two populations, with comparable *H. pylori* relative abundance in both anatomical sites in individuals from both Túquerres (high-risk population from the Andes mountainous range) and Tumaco (low-risk population from the Pacific coast). *H. pylori* presence significantly decreased the diversity of the gastric microbiota in the antrum but not in the body of these subjects. Importantly, statistically significant differences were found in the *H. pylori*-associated gastric microbiota according to population and diagnosis (non-atrophic gastritis vs intestinal metaplasia). In Tumaco, Bacilli dominated the body while Betaproteobacteria dominated the antrum. In Túquerres, Gammaproteobacteria dominated the body while Epsilonproteobacteria dominated the antrum. This led to two distinctive clusters in these populations, one defined by the presence of *Streptococcaceae* and *Pseudomonadaceae* and a second cluster characterised by the presence of *Helicobacteraceae*. Interestingly, no significant differences were found in the composition of the gastric microbiota according to topography in neither *H. pylori*-positive nor *H. pylori*-negative individuals from both populations. These results suggest that *H. pylori* is the main risk factor for gastric carcinogenesis and distinct dysbiosis in high- and low-risk populations, and that these dysbiotic changes in composition, but not diversity, are similar in the gastric antrum and body.

Gastric cardia adenocarcinoma (GCA) has increased dramatically in developed countries, where it has been clearly associated with an increased incidence of gastroesophageal reflux disease and Barret's oesophagus. In the East, however, these associations have been less clear. To elucidate this, Yan et al[21] investigated the incidence of gastric cardia inflammation and the changes in the gastric microbiota associated with this inflammation, in a Chinese population from the Chaoshan region, a known high-risk region for GCA. Among the 1117 individuals studied, the proportions of chronic inflammation, dysplasia, and GCA were 27.3%, 10.0%, and 62.7%, respectively. The phyla Proteobacteria, Firmicutes, Bacteroides, Actinobacteria, and Fusobacteria dominated the gastric cardia in these subjects, of which 6.8% were bacterial taxa typically found in the oral cavity. Importantly, diversity of the gastric microbiota did not change according to the severity of gastric cardia inflammation, but the composition was significantly different in mild, moderate and severe inflammation. Increased relative abundance of *H. pylori* was the main factor associated with progression of gastric cardia inflammation and dysbiosis. This was further confirmed with immunohistochemistry and immunofluorescence as *H. pylori* colonisation density was positively correlated with the degree of gastric cardia inflammation. Importantly, other 63 bacterial taxa, some of which have been previously associated with non-cardia GC[12,13,22,23], including *Lactobacillus*, *Leptotrichia*, *Fusobacterium*, and *Clostridium*, were also shown to be increased in subjects presenting with high inflammation, while species frequently found in the oral microbiota, including *Prevotella*, *Porphyromonas* and *Halomonas*, were found to be decreased in these subjects.

The contribution of gastric microbiota for the development and perpetuation of precancerous gastric lesions in the absence of *H. pylori,* has been addressed by Sung et al[24]. The authors compared the gastric microbiota of *H. pylori*-positive patients who received eradication therapy with that of patients that received placebo, before the intervention and after one year of follow-up. After *H. pylori* eradication, the structure of the gastric microbiota was different and there was a significant increase in microbial diversity, with increased abundances of the genera *Pseudomonas*, *Massilia*, and *Cryocola*. Eradication treatment reduced the abundance of *Helicobacter*, but also of *Haemophilus*, *Actinobacillus* and *Neisseria*. One year after *H. pylori* eradication there was an alteration in microbial ecology, with reduction in co-occurrence networks and the emergence of a cluster of bacteria including *Peptostreptococcus*, *Parvimonas*, *Fusobacterium*, *Haemophilus*, *Neisseria*, *Gemella*, *Granulicatella*, *Rothia*, *Streptococcus,* and *Porphyromonas*, while no major changes were registered in the placebo group. LEfSe analysis identified *Acinetobacter*, *Ralstonia*, *Actinobacillus*, and *Erwinia* enriched and *Sphingomonas* and *Roseburia* depleted in patients with persistent inflammation after *H. pylori* eradication. In patients with emergence of atrophy one year after *H. pylori* eradication treatment, *Granulicatella*, *Streptococcus*, *Rothia* and *Leptotrichia* were enriched. Additionally, patients that had intestinal metaplasia that persisted or progressed after *H. pylori* eradication, had enrichment of *Pseudomonas*, *Peptostreptococcus*, *Halomonas* and *Parvimonas*. The gastric microbial community in these patients was enriched in functional features involved in amino acid metabolism and inositol phosphate metabolism.

In addition to bacteria other than *H. pylori* having a role in progression along the Correa cascade, diet seems to be implicated in gastric carcinogenesis *via* modulation of the gastric microbiome. Arita and Inagaki-Ohara[25] aimed to unravel the mechanisms by which a fat diet induces gastric microbiota changes *via* leptin, a hormone that has been associated with GC progression. For that, they used male C57BL/6 J, leptin receptor (Lepr)-mutated db/db, and gastrointestinal epithelium-specific Lepr conditional knockout (T3 b-Lepr cKO) mice that were fed a high fat diet (HFD; 60% calories from fat) or control diet (CD; 10% calories from fat) for up to 20 wks. HFD led to a significant increase of Firmicutes in C57BL/6 J mice, which accounted for 95% of bacterial taxa by 20 wk. Importantly, this increase was associated with a significant increase of Lactobacillales (mainly *Lactobacillus reuteri*) but not Bifidobacteriales. Changes in the large intestine microbiota followed a similar pattern of the gastric microbiota, however, these changes were less pronounced. The gastric microbiota from HDF-fed mice was further transplanted into recipient C57BL/6 J mice to determine its impact on the development of intestinal metaplasia. Recipient mice of gastric microbiota from HDF-fed mice exhibited changes in the gastric mucosa compatible with intestinal metaplasia, while mice transplanted with gastric microbiota from CD-fed mice showed no changes. Further antibiotic treatment decreased the expression of intestinal metaplasia markers, however, it failed to completely suppress these markers. Lepr mutated db/db (lack systemic Lepr) and T3 b-Lepr cKO mice (lack Lepr specifically in the gastrointestinal tract) were then used to investigate Lepr impact on gastrointestinal microbiota. Importantly, lack of Lepr in the gastrointestinal tract was found to protect against gastrointestinal dysbiosis and HFD-induced intestinal metaplasia, suggesting that gastric Lepr-mediated signalling might impact gastric carcinogenesis.

GASTRIC MICROBIOTA IN THE TUMOUR MICROENVIRONMENT

The stomach of GC patients comprises cancerous and non-cancerous tissues, each with distinct microenvironments. Chen et al[26] investigated the gastric microbiota in the tumour microenvironment by comparing cancerous and non-cancerous matched samples from Chinese GC patients. As expected, *H. pylori* presence was low in these subjects with *H. pylori* sequencing-positive cases accounting for 29%, with decreased *H. pylori* relative abundance in cancerous tissue compared to non-cancerous tissue. The cancerous tissue was dominated by Proteobacteria, followed by Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, and Fusobacteria. When compared to cancerous tissue, the gastric microbiota in non-cancerous tissue was characterised by decreased abundance of Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, and Fusobacteria, but increased abundance of Proteobacteria. A distinct and active micro-ecological system was found in cancerous tissue. Diversity of the gastric microbiota in cancerous tissue was increased compared to non-cancerous tissue, particularly in patients aged over 60 years old, a finding possibly associated with immunosenescence and elevated gastric pH due to atrophy in these subjects. The interaction network also displayed greater complexity in cancerous compared with non-cancerous tissue, showing denser co-occurrence interactions between *Streptococcus*, *Peptostreptococcus*, *Fusobacterium*, *Dialister*, and *Prevotella*. Further, nucleotide metabolism, energy metabolism, carbohydrate metabolism and enzymes involved in denitrification, were all enriched in cancerous tissue. Composition of the gastric microbiota also differed between non-cancerous and cancerous tissues, which was confirmed by ANOSIM and visualised using principal coordinate analysis (PCoA) and non-metric multidimensional scaling (nMDS) plots. Further LEfSe analyses showed enrichment of 33 bacterial taxa in tumour tissue, including *Streptococcus*, *Peptostreptococcus*, *Prevotella*, *Acinetobacter*, *Bacillus*, *Selenomonas*, *Lachnoanaerobaculum*, and *Sphingomonas*, while 16 taxa were enriched in non-cancerous tissue, including *Serratia*, *Helicobacter*, *Niveispirillum* and *Lactococcus*. In addition, DESeq2 identified *Fusobacterium* enrichment in the tumours while *Helicobacter* and *Lactobacillus* were found to be enriched in non-cancerous tissue. Interestingly, *H. pylori* infection significantly modified the gastric microbiota in non-cancerous tissue but not in tumour tissue, with increased alpha diversity and enrichment of 15 other genera, including *Serratia*, *Lactobacillus*, and *Streptococcus* found in non-cancerous tissue of *H. pylori*-positive individuals.

Rodriguez et al[27] used whole exome sequencing data from nine types of solid cancers from The Cancer Genome Atlas (TCGA) to derive their microbiota profiles. The study included 85 GC cases, with paired tumour and adjacent normal tissue. Higher bacterial richness (number of species per sample) was identified in tumours in comparison with adjacent normal tissues. Although there were 11 taxa, including 8 genera, differentially abundant between tumour and normal tissues, after false discovery rate correction, only *H. pylori* remained statistically significantly more abundant in adjacent normal than in tumour tissues. EBV reads were detected in 25 tumours and 25 adjacent normal tissues, the proportions of reads detected being significantly higher in the former than in the latter. However, the authors failed to identify bacterial reads in 16.5% of the gastric tissues, and in cases where bacteria were identified, the average microbial read per sample was 360 in tumours and 107 in adjacent normal tissues, which highlights limitations in sensitivity of deriving microbiome information from the whole exome sequencing. In fact, it has been shown that in tissue samples that contain low abundance of bacterial DNA and high proportions of host DNA, metagenomics has decreased sensitivity in detecting very low and low abundant species[28].

It is now known that the microbiota influences the tumour immune microenvironment[29-31]. Driven by this evidence and by their previous findings that regulatory T cells (Tregs) and plasmacytoid dendritic cells (pDCs) together contribute to tumour immunosuppression in the context of GC[32], Ling et al[33] analysed the relationships between Tregs and pDCs and the gastric microbiota in tumour, peritumoural and normal tissues from the same GC patients. They performed immunohistochemistry using antibodies against blood dendritic cell antigen-2 (BDCA2) and forkhead box protein 3 (Foxp3), respectively, as markers of pDCs and Tregs. In comparison with normal tissues, BDCA2+pDCs and Foxp3+Tregs were significantly more abundant in peritumoural and tumour areas than in normal tissues. Diversity indices were significantly higher in peritumoural areas than in normal or tumour tissues. Correlations between the gastric microbiota and immunosuppressive cells in the tumour microenvironment included *Stenotrophomonas*, *Acinetobacter*, *Gemella*, and *Neisseria* positively correlated and *Comamonas* and *Brevibacterium* negatively correlated with BDCA2+pDCs; and *Selenomonas*, *Campylobacter*, *Massilia*, and *Dialister* positively correlated and *Rhizobium*, *Gaiella*, *Cupriavidus*, *Faecalibacterium*, and *Dolosigranulum* negatively correlated with Foxp3+ Tregs. The authors suggest that the interactions between bacteria of the gastric microbiota and the immunosuppressive cells may contribute to dysfunction of the immune system in the gastric tumour microenvironment.

INTESTINAL MICROBIOTA IN GASTRIC CANCER

It is plausible that gastric disease is associated not only with gastric but also gut dysbiosis (Figure 1). The gut microbiota could not only be involved in gastric carcinogenesis *via* immunomodulation, but it could also influence chemotherapy efficacy in GC patients. Qi et al[34] demonstrated the presence of gut dysbiosis in GC patients and correlated dysbiotic changes with peripheral cellular immunity. Venn diagram analyses revealed 35 unique OTUs in healthy subjects (n=88) and 240 unique OTUs in GC patients (n=116). Two enterotypes were identified in this population; enterotype 1 was dominated by *Bacteroides* and enterotype 2 was dominated by *Prevotella_9*. However, no significant difference in enterotype distribution was found between GC patients and controls. Increased richness, decreased butyrate-producing bacteria and enrichment of 12 genera (mainly *Lactobacillus*, *Escherichia*, and *Klebsiella*), was observed in the gut of GC patients when compared to healthy subjects. Importantly, random forest analysis showed that the combination of *Lactobacillus*, *Tyzzerella_3*, *Veillonella*, *Streptococcus*, and *Lachnospira* was sufficient to distinguish GC patients from healthy subjects. Further, CD3+ T cell count was positively correlated with the relative abundance of *Lactobacillus* and *Streptococcus* while CD3+ T cells, CD4+ T cells, and NK cells were correlated with *Lachnospiraceae*.

Zhou and Yang[35] evaluated the impact of gastrointestinal hormones on inflammation and the intestinal microbiota of Chinese patients presenting with GC. The levels of serum gastrin-17, pepsinogen II, IL-6 and IL-17 were elevated in GC patients and correlated with disease severity. After FOLFOX4 chemotherapy (i.e., oxaliplatin/5-fluorouracil/leucovorin), gastrin-17

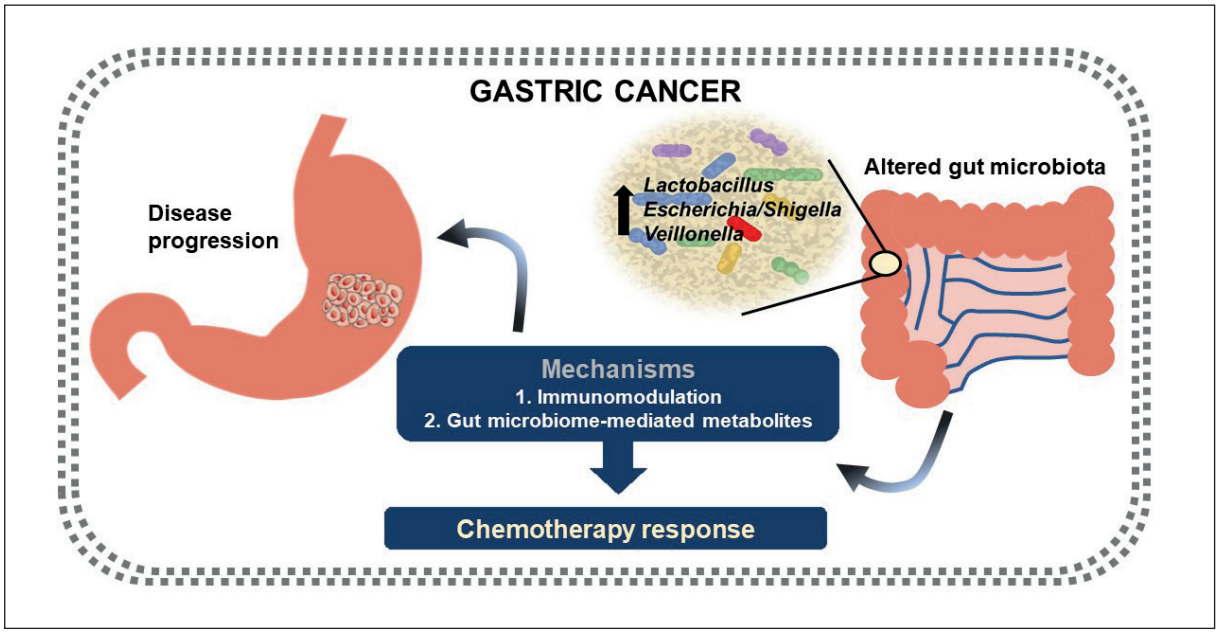

Figure 1. The relationship between the gut microbiota and gastric cancer. The gut microbiota is altered in gastric cancer (GC) patients, showing significant differences in both diversity and composition when compared to healthy individuals. Bacterial taxa recently shown to be increased in the gut of GC patients include *Lactobacillus, Escherichia/Shigella* and *Veillonella.* The altered gut microbiota could impact the progression of GC via immunomodulation (i.e. immunosuppression and/or tumour immune evasion) and the production of gut microbiome-mediated metabolites, such as bile acids and N-nitroso compounds. In addition, these same mechanisms are believed to shape the host response to chemotherapy.

and pepsinogen II remained constant, however, pepsinogen I increased, and IL-6 levels decreased in these patients. The intestinal microbiota was then investigated using faecal samples and standard culture-based identification methods. *Bifidobacterium*, *Lactobacillus* and Bacilli were less abundant in the gut of GC patients compared to controls, and their levels were restored after FOLFOX4 chemotherapy. *Escherichia coli*, *Staphylococcus*, *Enterococcus* and *Peptostreptococcus* were more abundant in the gut of GC patients compared to controls, and their levels decreased after FOLFOX4 chemotherapy. Interestingly, gastrin-17 was negatively correlated to the relative abundance of *Bifidobacterium* and *Lactobacillus* in the gut of GC patients.

To explore the changes in the gut microbiota of patients with GC in the perioperative period, Liang et al[36] analysed faecal samples from GC patients before and after radical distal gastrectomy (n=6). Surgery had limited effect on diversity; however, the composition of the gut microbiota was significantly impacted, showing increased relative abundance of *Akkermansia*, *Escherichia/Shigella*, *Lactobacillus* and *Dialister*. Further, when the gut microbiota of GC patients (n=20) was compared to those of healthy controls (n=22), higher relative abundance of *Escherichia/Shigella*, *Veillonella*, and *Clostridium XVIII* and lower abundance of *Bacteroides*, were identified. Interestingly, the genus *Helicobacter* showed low abundance (<1%) in faecal samples from both GC patients and controls.

Mechanistic support for the role of the gut microbiome in gastric carcinogenesis has been provided by Yu et al[37], using a rat model of chemically induced GC. Gastric carcinogenesis was induced in male Wistar specific pathogen-free (SPF) rats using a combination of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), sodium salicylate, irregular fasting, and ranitidine, which led to increased richness (ACE and Chao indices) but decreased diversity (Shannon index) of the gut microbiota. The composition of the gut microbiota also differed between normal and disease states (non-atrophic gastritis, chronic atrophic gastritis, precancerous lesions and GC). Rats presenting with precancerous lesions had the highest ratio of Firmicutes/

Bacteroidetes while rats presenting with GC showed high abundance of *Lactobacillus*, *Bifidobacterium* and *Escherichia-Shigella* and low abundance of *Lachnospiraceae* and *Ruminococcaceae* in their guts, partially supporting previous findings in human studies[34-36].

MICROBIOME-RELATED BIOMARKERS IN GASTRIC CANCER

The microbiome that exists at different anatomic locations can be associated with different cancer types[13,38-40]. Poore et al[41] demonstrated that unique microbial DNA and RNA signatures are present in the tumours and also in the blood of patients with different cancer types. Microbiome profiles were derived from whole-genome and whole-transcriptome sequencing data of TCGA from 33 different types of cancer. The authors, then, trained machine learning (ML) models to identify microbiome signatures and showed that the models were strong in discriminating between the great majority of cancer types. In GC, ML could also discriminate between tumour and normal tissue, and between stage I and stage IV tumours. Ecological validation of bacterial reads showed that *Fusobacterium* was more abundant in primary GI tumours, including GC, than in solid tissue normal samples, and in blood-derived normal samples. In primary gastric tumours and adjacent normal tissue no differences were detected in *H. pylori* abundance, consistent with previous investigations in TCGA[42]. Ecological validation of viral reads showed EBV enrichment in EBV-infected tumours in comparison with the chromosomal instability (CIN), microsatellite unstable (MSI), and genome stable (GS) GC molecular subtypes. ML models based on blood microbial DNA (mbDNA) signatures could discriminate between cancer types, and continued predictive when used in circumstances in which circulating tumour DNA (ctDNA) assays fail, namely in patients with stage Ia–IIc tumours and tumours without detectable genomic alterations. In addition to the limitations already identified above in the study of Rodriguez et al[27], and despite the authors mitigated bioinformatically potential effects of contamination, it is known that TCGA samples have not been prepared for the purpose of microbiome characterisation, and thus additional layers of contamination may be present, biasing the findings. Nevertheless, these findings open new possibilities for the design of microbiome-based cancer diagnostic tools.

Lee et al[43] investigated microbiome-associated metabolites as potential GC progression biomarkers in the gastric juice of patients. For that, they used liquid chromatography tandem mass spectrometry (LC-MS/MS) to quantitatively profile histamine, histidine, and various bile acids (BA) in the gastric juice of 20 chronic gastritis, 12 intestinal metaplasia and 28 GC patients. Significantly increased concentrations of histamine and histidine were found in the gastric juice of intestinal metaplasia patients in comparison with chronic gastritis patients, but no differences were observed in GC patients. The authors also identified higher ratios of deoxycholic acid (DCA)/cholic acid (CA) in the gastric juice of intestinal metaplasia and GC patients in comparison to that observed in chronic gastritis patients. Since DCA is a secondary BA converted from CA by few species of *Clostridium* in the GI tract, the authors suggest that the composition of the gut microbiome may influence gastric carcinogenesis and that these metabolites could be used as biomarkers of disease progression.

The identification of biomarkers to predict response to cancer immunotherapy has recently emerged, following the observation that not all patients respond to immune checkpoint blockade. For GC immunotherapy, the approved therapeutic agents so far are pembrolizumab and nivolumab monoclonal antibodies targeting programmed death-1 (PD-1). Pembrolizumab was approved by the Food and Drug Administration for patients with recurrent locally advanced or metastatic, GC or gastroesophageal junction cancer whose tumours express programmed cell death ligand 1 (PD-L1)[44], or for patients with unresectable or metastatic, microsatellite instability-high or mismatch repair deficient site-agnostic solid tumours[45]. Nivolumab was approved in Japan for treating GC refractory to standard chemotherapies irrespective of the PD-L1 status[46]. Sunakawas et al[47] report the protocol of the DELIVER trial, a multicentre, translational, observational study to examine efficacy and toxicity of nivolumab treatment in patients with advanced GC. Rather than addressing predictive factors related with the tumours, the trial will focus on the identification of host-related predictive biomarkers, including genetic polymorphisms and gene expression, and most interestingly, the gut microbiome

TABLE 1. CURRENT LIMITATIONS IN GASTRIC MICROBIOTA STUDIES.

- Limited study sample sizes
- Sample preparation not suitable for microbiota characterisation (e.g. TCGA samples with high host DNA contamination)
- Methodological differences that lead to biases:
  a) Culture-based methods vs next-generation sequencing
  b) High variability in the number and location of gastric biopsies
  c) Negative controls to correct for the issue of "kitomes" (i.e., contamination introduced by nucleic acids extraction kits) are not included in most studies
- Overrepresentation of some ethnic groups (i.e., Asian)
- No implementation of OLGA and/or OLGIM classifications to stratify patients presenting with gastric precancerous lesions

and blood metabolome. Indeed, it has been recently shown that the gut microbiome influences the response to cancer immunotherapy in patients with melanoma, and with several epithelial-derived cancers[29-31], but so far, the relationship between the gut microbe and the efficacy of anti-PD-1 treatment in GC is unknown. It is the expectation of the authors that the results of this trial will enable us to determine which patients with GC will benefit from nivolumab treatment.

CONCLUSIONS

Despite our growing understanding of the gastric microbiota, *H. pylori* remains a pivotal factor in gastric disease as a carcinogen and a major, if not the main, cause of gastric dysbiosis. Importantly, recent studies have focused on the role of both the gastric and gut microbiota in gastric carcinogenesis, highlighting the complexity of the microbial interactions that can occur in continuous body compartments with different microbial communities. Further studies assessing host-microbial interactions are required to help us comprehend the involvement of host genetics, the gastrointestinal microbiome and environmental factors such as diet, in gastric carcinogenesis. These future studies must avoid the limitations outlined in Table 1, which are commonly found in current gastric microbiota studies.

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Conflict of Interest

CF owns patent WO/2018/169423 on microbiome markers for gastric cancer. NCR declares no conflict of interests.

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