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Helicobacter pylori Infection, the Gastric Microbiome and Gastric Cancer

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Abstract

After a long period during which the stomach was considered as an organ where microorganisms could not thrive, *Helicobacter pylori* was isolated *in vitro* from gastric biopsies, revolutionising the fields of Microbiology and Gastroenterology. Since then, and with the introduction of high-throughput sequencing technologies that allowed deep characterization of microbial communities, a growing body of knowledge has shown that the stomach contains a diverse microbial community, which is different from that of the oral cavity and of the intestine. Gastric cancer is a heterogeneous disease that is the end result of a cascade of events arising in a small fraction of patients colonized with *H. pylori.* In addition to *H. pylori* infection and to multiple host and environmental factors that influence disease development, alterations to the composition and function of the normal gastric microbiome, also known as dysbiosis, may also contribute to malignancy. Chronic inflammation of the mucosa in response to *H. pylori* may alter the gastric environment, paving the way to the growth of a dysbiotic gastric bacterial community. This dysbiotic microbiome may promote the development of gastric cancer by sustaining inflammation and/or inducing genotoxicity. This chapter summarizes what is known about the gastric microbiome in the context of *H. pylori*-associated gastric cancer, introducing the emerging dimension of the microbiome into the pathogenesis of this highly incident and deadly disease.

Keywords

Helicobacter pylori · Gastric microbiome · Gastric microbiota · Gastric cancer · Microbial dysbiosis

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1 Introduction

The human body is inhabited in its different niches by a vast collection of microbes, generally known as the microbiota. These microorganisms, their genetic information, as well as the information of the niche in which they interact, are usually referred to as the microbiome (Cho and Blaser 2012). Currently, the term microbiome is also used to refer to the microorganisms themselves, i.e. the microbiota (Knight et al. 2017). The number of microbial cells was commonly thought to outnumber the quantity of human cells by a ten-fold ratio, but recent assessments propose a 1:1 ratio as a better estimate (Sender et al. 2016).

Bacteria constitute so far the best explored component of the microbiome. Progress in this research area had been hampered by the fact that only a very small fraction of the microbial species can be cultured *in vitro*. The advent of high-throughput sequencing technologies, together with the emergence of large international and interdisciplinary projects, have strongly contributed to expand our understanding of the microbiome structure and functions (Turnbaugh et al. 2007; Qin et al. 2010; Arnold et al. 2016).

It is currently accepted that the microbiome plays a major role in the maintenance of the normal physiology and health of the host, being involved in a wide variety of metabolic functions and participating in the normal maturation of the immune system (Gilbert et al. 2018). The composition of the normal microbiome varies between individuals and is influenced by local conditions inherent to the anatomic site, host genetics, diet, and antibiotic consumption (Lloyd-Price et al. 2017; Gilbert et al. 2018). Disruption of the balance that exists between the microbiome and the host, called dysbiosis, may promote numerous diseases, including cancer (Gilbert et al. 2018). For example, members of the gut microbiome such as *Fusobacterium nucleatum*, *Escherichia coli,* and *Bacteroides fragilis*, have been found enriched in colorectal cancer (Goodwin et al. 2011; Ahn et al. 2013; Bonnet et al. 2014). Nevertheless, and although the exact mechanisms linking microbial dysbiosis and cancer are still largely unknown, it can be anticipated that bacterial metabolites and toxins, as well as inflammation triggered by the microbiome contribute to the promotion of cancer. Here, we discuss in detail the current knowledge on the human gastric microbiome in the context of health and disease, and provide insights into the potential impact of microbial dysbiosis in the development of *H. pylori*-associated gastric cancer, by revisiting Correa's hypothesis of gastric carcinogenesis (Correa 1992).

2 Gastric Cancer

Gastric cancer is the fifth most incident cancer worldwide, with almost 1 million new cases per year (Ferlay et al. 2015). Gastric cancer is also the third cause of cancer-related death worldwide, with about 750,000 deaths estimated to having occurred in 2012. The incidence and mortality of gastric cancer show wide geographic variation, with East Asian countries registering the highest rates (Ferlay et al. 2015). Gastric cancer is a heterogeneous disease in what concerns morphology, genetics, and context. Histologically, gastric cancer heterogeneity is reflected by the diversity in classifications. The most commonly used histological classification systems are the one of the World Health Organization, comprising five main types – tubular, papillary, mucinous, poorly cohesive, and rare histological variants – and Lauren's, comprising two main types – diffuse and intestinal (Fenoglio-Preiser et al. 2010; Lauren 1965). Lauren's classification remains the most widely used and each cancer type has distinct epidemiologic and pathophysiological characteristics (Carneiro 1997; Spoto et al. 2018). Gastric cancer of the diffuse type occurs more frequently in females and at earlier ages, and is characterised by isolated or small groups of neoplastic cells that do not form glandular structures. In contrast, gastric cancer of the intestinal type is more prevalent at advanced ages, mainly in males, and is characterized by the presence of glandular structures and a higher to moderate degree of cell differentiation (Lauren 1965;

Carneiro 1997; Van Cutsem et al. 2016). The sequence of histological changes that culminate in intestinal type gastric cancer is better characterized than the one leading to diffuse type cancer, despite both types being associated with chronic gastritis as a consequence of *H. pylori* infection. Intestinal type gastric cancer is the result of a long, multifactorial and multistep process, which starts with *H. pylori* chronic gastritis, followed by atrophic gastritis, intestinal metaplasia, dysplasia, and cancer (Correa et al. 1975; Correa 1992).

Gastric cancer heterogeneity is also manifested at the molecular level (Ottini et al. 2006). Comprehensive analyses of gastric cancer tissues from large cohorts of patients recently emphasized the complexity of this disease and led to the proposal of different molecular classifications (Lei et al. 2013; Cancer Genome Atlas Research 2014; Cristescu et al. 2015). For example, the Cancer Genome Atlas research network classification proposed four main gastric cancer types (Cancer Genome Atlas Research 2014): chromosomally unstable tumours, which have marked aneuploidy, frequent mutations in *TP53*, amplification of receptor tyrosine kinases and *RAS*; microsatellite unstable tumours, which are characterised by *MLH1* promoter hypermethylation and a high mutational rate of genes including *TP53*, *KRAS*, *ARID1A*, *PIK3CA,* and *PTEN*; genomically stable tumours that have mutations in *CDH1*, encoding E-cadherin, *ARID1A* and *RHOA*; and Epstein-Barr virus-positive tumours, that show recurrent *PI3KCA* and *ARID1A*, but very rare *TP53* mutations, *CDKN2A* promoter hypermethylation, and amplification of *JAK2*, and of PD-L1- and PD-L2-encoding genes.

It is important to acknowledge that the great majority of gastric cancers occur in a sporadic setting, with about 10% of the cases having familial clustering, and 1–3% occurring in a hereditary setting (Oliveira et al. 2015; Van Cutsem et al. 2016). Hereditary diffuse gastric cancer (HDGC) is the most common and best-studied hereditary gastric cancer syndrome, where about 40% of the affected families have germline mutations in the *CDH1* gene, encoding the cell-cell adhesion protein E-cadherin (Oliveira et al. 2015). The other two syndromes, gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) and familial intestinal gastric cancer (FIGC), are very rare, and while in the former germline point mutations in the *APC* promoter have been identified (Worthley et al. 2012; Li et al. 2016), in the latter no aetiological genetic alterations are known (Oliveira et al. 2015). The exact extent to which *H. pylori* infection and/or the microbiome of the stomach may contribute to the different molecular profiles and contexts of gastric cancer, however, remains largely unexplored.

3 *H. pylori* Infection and Gastric Cancer

H. pylori is considered as the major risk factor for the development of gastric cancer, being categorized as a class I carcinogen by the International Agency for Research on Cancer (IARC 1994). It has been estimated that at least 90% of all non-cardia gastric cancers worldwide are attributable to *H. pylori* (Plummer et al. 2015). The estimated worldwide prevalence of *H. pylori* is 44.3%, with considerable variation according to the geographic region (Zamani et al. 2018). There is a major geographic overlap between *H. pylori* prevalence and gastric cancer incidence, and in general countries with highest cancer incidence have high infection rates (Ferlay et al. 2015; Zamani et al. 2018). Since the initial collection of epidemiological and functional data that provided grounds for the classification of *H. pylori* as a class I carcinogen, numerous studies have been published demonstrating the causal relationship between chronic *H. pylori* infection and gastric cancer (IARC 2011). The magnitude of the risk of gastric cancer associated with *H. pylori* infection has now been estimated in different populations, and varies with the type of assay used to detect the infection, being about three-fold if serology is used (Helicobacter and Cancer Collaborative Group 2001) and reaching over 20-fold when more sensitive assays are used (Gonzalez et al. 2012). As an additional piece of evidence that links *H. pylori* infection and gastric cancer, the eradication of the infection has an

impact in reducing the incidence of this malignancy (Ford et al. 2015).

Although the association between *H. pylori* and gastric cancer is extensively recognized, the majority of the infected patients do not develop this malignancy, which arguments in favour of the multifactorial nature of this disease. Host genetic susceptibility, namely polymorphisms in genes that are involved in the inflammatory response to *H. pylori* infection have been associated with the risk of gastric cancer. Among the best studied are those that encode interleukin (IL)-1β, IL-1 receptor antagonist, tumour necrosis factor (TNF)-α pro-inflammatory cytokines and the anti-inflammatory IL-10. Genetic variation in the promoters or in non-coding regions of these genes are associated with increased risk for the development of gastric cancer (El-Omar et al. 2001; Machado et al. 2003; Persson et al. 2011). Remarkably, in genetically susceptible hosts, infection with more virulent *H. pylori* strains markedly enhances gastric cancer risk (Figueiredo et al. 2002).

Cigarette smoking, alcohol intake, and salt consumption are recognized environmental factors that influence the risk of gastric cancer. Indeed, ever and current smokers have higher risk to develop gastric cancer compared with never smokers, and among current smokers the risk increases with number of cigarettes per day (Praud et al. 2018). Heavy and very heavy alcohol drinkers have higher risks for developing gastric cancer in comparison with abstainers, and these associations are independent of the *H. pylori* infection status (Rota et al. 2017). Dietary salt intake is also associated with gastric cancer risk, the risk being gradually increased for higher consumption levels (D'Elia et al. 2012). Accordingly, in an animal model of infection, a diet with high salt content accelerated the development of gastric cancer, in particular in animals infected with *cagA*-positive *H. pylori* strains (Gaddy et al. 2013). On the other hand, the consumption of fruit and white vegetables, which are rich sources of vitamin C, are inversely associated with gastric cancer risk (Fang et al. 2015).

Adding to the influence of host and environmental factors in gastric cancer, the genetic diversity of *H. pylori*, and in particular variation in virulence genes associated with the pathogenicity of strains, also impact gastric cancer risk (Ferreira et al. 2014). CagA is the best-documented *H. pylori* virulence factor influencing gastric cancer. CagA is encoded by a pathogenicity island that is present in about 60–70% of *H. pylori* strains worldwide. The same pathogenicity island also encodes a type IV secretion system, which functions as a molecular syringe and allows CagA to be delivered into the host cells (Backert et al. 2015). Once in the host cell cytoplasm, CagA can be phosphorylated by host kinases within EPIYA motifs. Both phosphorylated and non-phosphorylated CagA are capable of activating signalling pathways that influence host responses, including inflammation, proliferation, and cell polarity (Backert et al. 2010). CagA phosphorylation, however, appears to be important in gastric cancer development, as transgenic mice expressing wild-type CagA, but not phosphorylation-resistant CagA, develop gastric tumours (Ohnishi et al. 2008). Patients who are infected with *H. pylori cagA*-positive strains, and with strains with CagA harbouring higher number of phosphorylation motifs, are associated with increased risk for gastric premalignant lesions and for gastric cancer (Ferreira et al. 2014). Additionally, CagA influences host disease progression, and infection with *H. pylori cagA*-positive strains increases the risk of progression of preneoplastic lesions (Plummer et al. 2007; Gonzalez et al. 2011). Variation in other *H. pylori* virulence factors, such as the VacA toxin, has also been associated with gastric precancerous lesions and cancer (Gonzalez et al. 2011; Ferreira et al. 2014). This and other virulence factors of *H. pylori* and their relationship with disease are discussed in Chap. 3 of this

volume. Additionally, the molecular mechanisms that underlie *H. pylori*-mediated malignant transformation are discussed in Chap. 8.

4 The Gastric Microbiota, Is There More Than *H. pylori*?

For many years, the human stomach was assumed to be sterile, given its high acidic pH, gastric peristalsis, and the presence of digestive enzymes, among other protective and antimicrobial factors (Martinsen et al. 2005). With the discovery and isolation of *H. pylori* (Warren and Marshall 1983) this dogma was broken, and more recently the idea that the stomach harbours a complex bacterial community became accepted. Initial analyses of the bacteria present in the stomach relied on microbiological cultures. These have identified *Firmicutes* as the most common phylum, followed by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, and genera that were most commonly isolated included *Streptococcus*, *Lactobacillus*, *Bacteroides*, *Staphylococcus*, *Veillonella*, *Corynebacterium, Clostridium,* and *Neisseria* (Stockbruegger 1985; Thorens et al. 1996; Adamsson et al. 1999; Mowat et al. 2000; Zilberstein et al. 2007). This type of approach, however, yielded an incomplete and biased landscape of the gastric microbiota, since most of the bacteria are difficult to culture or are uncultivable. The development of culture-independent methods revealed that the human gastric ecosystem has a more diverse and complex microbiota than initially anticipated (Monstein et al. 2000; Bik et al. 2006; Andersson et al. 2008; Li et al. 2009; Delgado et al. 2013; Schulz et al. 2018).

The bacterial community of the normal stomach has not been extensively characterised, probably due to difficulties in recruiting normal individuals for upper endoscopy. A 16S rRNA gene cloning and sequencing-based approach was undertaken to analyse the gastric microbial communities of five individuals with normal gastric mucosa and five patients with non-*H. pylori* and non-NSAID (non-steroidal anti-inflammatory drug) (NHNN) gastritis, all Chinese from Hong-Kong (Li et al. 2009). *Firmicutes* and *Proteobacteria* were the most represented phyla, and while in the normal stomach the *Proteobacteria* was the most abundant, in the NHNN gastritis the most abundant phylum was the *Firmicutes*. The five most common genera were *Streptococcus*, *Prevotella*, *Neisseria*, *Haemophilus*, and *Porphyromonas*; together, *Streptococcus* and *Prevotella* represented over 40% of all sequences.

Following studies exposed the diversity and the inter-individual variability of the gastric microbiota derived from the analysis of populations from distinct origins, but also from different sample types, and using various technical approaches. Overall, the most common gastric bacteria can be assigned to five major phyla – *Proteobacteria*, *Firmicutes, Bacteroidetes*, *Actinobacteria* and *Fusobacteria*, and the two most prominent genera of the non-*H. pylori* infected stomach are *Streptococcus* and *Prevotella* (Bik et al. 2006; Andersson et al. 2008; Li et al. 2009; Delgado et al. 2013). A more recent study that included 20 Caucasians from the UK with a normal stomach, without evidence of *H. pylori* infection, concurred that the bacterial family *Prevotellaceae* was the most abundant (23%), followed by *Streptococcaceae* (10%). In fact, the microbiota of these stomachs had the highest levels of microbial diversity and bacterial richness in comparison with other groups of patients infected with *H. pylori* (Parsons et al. 2017).

According to the great majority of reports, when *H. pylori* is present, this bacterium is the most abundant microbial component, representing between 40% to over 95% of the gastric microbiota (Bik et al. 2006; Andersson et al. 2008; Li et al. 2017; Klymiuk et al. 2017; Schulz et al. 2018; Ferreira et al. 2018; Parsons et al. 2017). In addition to finding *H. pylori* as the most abundant bacterium in the stomach of patients who test positive for *H. pylori*, it has been shown that the microbiota of *H. pylori*-positive subjects has lower diversity than that of *H. pylori*-negative subjects (Bik et al. 2006; Andersson et al. 2008; Schulz et al. 2018). Our analysis of the gastric microbiota of 81 chronic

gastritis cases from Portugal that were 99% *H. pylori*-positive, revealed that as *H. pylori* abundance increases, there is a significant decrease in diversity (data not shown). Accordingly, a study that evaluated the gastric microbiota before and after *H. pylori* eradication treatment showed that the eradication of *H. pylori* resulted in an increase in bacterial diversity (Li et al. 2017).

The influence of *H. pylori* on the composition and dynamics of the gastric microbiota is still not fully understood. Difficulties may in part relate to the differences in methods to diagnose *H. pylori* infection and various studies using sequencing-based methods have demonstrated that *H. pylori* could be detected at low levels in samples of subjects that were diagnosed as *H. pylori*-negative by conventional methods (histopathology, rapid urease test, serology, and PCR) (Bik et al. 2006; Maldonado-Contreras et al. 2011; Delgado et al. 2013; Thorell et al. 2017).

The majority of reports show no major alterations on the pattern of distribution of phyla between *H. pylori*-positive and *H. pylori*-negative patients (Bik et al. 2006; Maldonado-Contreras et al. 2011; Schulz et al. 2018). Using the PhyloChip microarray, Maldonado-Contreras *et al.* reported a similar representation of the four dominant phyla between *H. pylori*-infected and -uninfected rural Amerindians (Maldonado-Contreras et al. 2011). In regression analyses, authors were able to identify an association between *H. pylori* positivity and decreased relative abundance of *Actinobacteria*, *Bacteroidetes,* and *Firmicutes*. These results are sustained by our data on Portuguese patients with chronic gastritis, in which we found an inverse correlation between the relative abundance of *H. pylori* and non-*Helicobacter* α- and β-*Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Ferreira et al. 2018).

Experimental infections of the rhesus macaque model were used to assess the impact of *H. pylori* challenge upon the pre-existing gastric microbiota (Martin et al. 2013). Data showed that although *Helicobacter* became dominant in challenged animals, the removal of the *Helicobacter* reads from the libraries did not significantly alter the relative abundance of taxa between challenged and unchallenged animals. Nevertheless, the impact of *H. pylori* on relatively rare taxa was not determined. In contrast, in a mouse model of infection, challenge of animals with *H. pylori* significantly and consistently affected the abundance of several species, suggesting that *H. pylori* influences the gastric microbiota composition at lower taxonomic levels (Kienesberger et al. 2016).

It has been a matter of debate whether bacteria found in the stomach represent transient swallowed bacteria or active members of a resident microbiota colonizing the gastric mucosa. Comparisons of the microbial communities along different sites of the gastrointestinal (GI) tract have shown that the gastric microbiota is different from that at other sites. Although some proximity with the microbiota of the oral cavity and throat exists, the stomach microbial communities cluster together (Andersson et al. 2008; Stearns et al. 2011; Delgado et al. 2013). Recent data aiming to evaluate the metabolically active microbial communities in different regions of the GI tract found that the transient luminal microbiota present in gastric juice is closely related with that of saliva and of duodenal aspirates and significantly different from that of gastric biopsies, supporting the idea that the stomach has a local mucosa-associated microbiota (Schulz et al. 2018).

5 The Gastric Microbiota in Gastric Carcinogenesis

While *H. pylori* is recognized as being fundamental in gastric carcinogenesis, the role of non-*H. pylori* microbiota has not yet been established. The majority of the publications so far included low number of patients and/or had limitations in sensitivity and depth of coverage, which in general did not allow producing statistically based conclusions. One of the first DNA-based descriptions of the gastric bacterial community in patients with gastric cancer, used terminal restriction fragment length polymorphism (T-RFLP) in combination with 16S rRNA

gene cloning and sequencing to characterize 10 patients with gastric cancer and five *H. pylori*-negative dyspeptics with normal gastric mucosa (Dicksved et al. 2009). A complex bacterial community dominated by different species of *Streptococcus*, *Lactobacillus*, *Veilonella* and *Prevotella*, and with low abundance of *H. pylori* was reported in the stomach of cancer patients.

A study of 15 patients from Mexico with non-atrophic gastritis, intestinal metaplasia, or gastric cancer, using the PhyloChip, showed a gastric microbiota profile separation between non-atrophic gastritis and gastric cancer based on the presence/absence of taxa. This analysis could neither separate non-atrophic gastritis and intestinal metaplasia, nor metaplasia and cancer (Aviles-Jimenez et al. 2014). Taxa with differences in abundance between non-atrophic gastritis and gastric cancer were identified, with significant decreases in the abundance of *Porphyromonas*, *Neisseria* and bacteria from the *TM7* phylum, and increases in the abundance of *Lactobacillus* and *Lachnospiraceae* observed in gastric cancer. Diversity, as measured by bacterial richness, was statistically significantly decreased from non-atrophic gastritis to gastric cancer. In contrast, a survey of the metabolic active bacteria of the stomach of 12 gastric cancer and 20 functional dyspepsia patients of Chinese ethnicity from Singapore and Malaysia, detected an increase in species richness and in phylogenetic diversity in cancer (Castano-Rodriguez et al. 2017). An earlier study of 10 chronic gastritis, 10 intestinal metaplasia and 11 gastric cancer patients from Korea, also suggested an increase in bacterial diversity from gastritis to cancer, but without supporting statistical analysis (Eun et al. 2014). Still, the majority of publications so far report a decrease in bacteria diversity and richness from non-atrophic gastritis to gastric cancer (Aviles-Jimenez et al. 2014; Li et al. 2017; Coker et al. 2018; Ferreira et al. 2018).

The two most complete gastric microbiota studies in the gastric cancer field using 16S rRNA gene sequencing were published in the beginning of 2018 (Coker et al. 2018; Ferreira et al. 2018). Coker and colleagues studied the gastric mucosal microbiota in different histological stages of gastric carcinogenesis in 81 patients from Xi'an in China (Coker et al. 2018). The analysis of 21 superficial gastritis, 23 atrophic gastritis, 17 intestinal metaplasia, and 20 gastric cancer patients, demonstrated that the gastric microbiota of patients with intestinal metaplasia and with gastric cancer had significantly reduced microbial richness in comparison with that of superficial gastritis patients. Although no significant differences were found in microbiota profiles between superficial gastritis, atrophic gastritis and intestinal metaplasia, the microbiota of these stages were significantly different from that of the gastric cancer. The screen for differentially abundant taxa revealed 21 taxa enriched and 10 taxa depleted in gastric cancer in comparison with superficial gastritis, with increasing strengths of interactions among them along the progression of disease. Among the cancer-enriched bacteria were members of the human oral microbiome *Peptostreptococcus*, *Streptococcus*, *Parvimonas*, *Slackia*, and *Dialister*, which were the most significant in network interaction analysis. These bacteria were able to distinguish gastric cancer from superficial gastritis in receiver-operating characteristic (ROC) analysis. The authors validated their results in a Chinese Inner Mongolian cohort of patients (Coker et al. 2018).

Our own studies analysing 135 Portuguese patients, showed significant differences in the structure as well as in the composition of the gastric microbial communities between chronic gastritis and gastric cancer patients (Ferreira et al. 2018). Overall, patients with cancer had significantly decreased gastric microbial diversity, as assessed by the Shannon index, in comparison with patients with chronic gastritis. The gastric microbiota profiles of the two patient groups could be separated based on both the presence/absence and the relative abundance of taxa. In our series, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were identified as the five most abundant phyla in the stomach, in agreement with earlier descriptions (Bik et al. 2006; Aviles-Jimenez et al. 2014; Jo et al. 2016). Phyla ranked in the same relative abundance in the two patient

groups, with significantly increased abundance of non-*Helicobacter Proteobacteria*, *Actinobacteria* and *Firmicutes* and lower abundance of *Bacteroidetes* and *Fusobacteria* in the gastric cancer microbiota. While being the major genus in chronic gastritis with a mean relative abundance of 42% (varying from 0.01–95%), *Helicobacter* had a significant reduction in abundance in gastric cancer. In fact, and despite 87% of the gastric cancer patients were *H. pylori*-positive, the mean relative abundance of reads was just 6% (Ferreira et al. 2018). Actually, the gastric microbiota profiles of the two clinical settings could be distinguished based on *Helicobacter* abundance.

Overall, we have identified 29 microbial taxa, including 10 differentially abundant genera that best explain the differences between patient groups. Differential abundances in the great majority of these genera were further validated using quantitative polymerase chain reaction in the discovery cohort, and additionally confirmed in validation cohorts comprising patients from Portugal, China and Mexico (Ferreira et al. 2018). *Helicobacter*, *Neisseria*, *Prevotella*, and *Streptococcus* were enriched in the microbiota of chronic gastritis patients. *Streptococcus*, *Prevotella* and *Neisseria* are among the most abundant commensals of the oral cavity (Bik et al. 2010) and among the most frequently detected bacteria in the non-neoplastic stomach, having been cultured or identified in gastric juice and/or biopsies from *H. pylori*-positive and -negative gastritis and in the normal stomach (Thorens et al. 1996; Bik et al. 2006; Li et al. 2009; Delgado et al. 2013; Parsons et al. 2017; Schulz et al. 2018). Interestingly, in a comparison of the gastric microbiota of Colombian inhabitants from two regions with divergent gastric cancer risks, *Streptococcus* and *Neisseria* were identified only in individuals from the low risk, but not in those from the high risk gastric cancer region (Yang et al. 2016).

Genera that were enriched in the gastric cancer microbiota, and significantly more prevalent in patients with gastric cancer than in patients with chronic gastritis, were *Achromobacter*, *Citrobacter*, *Lactobacillus*, *Clostridium*, *Rhodococcus,* and *Phyllobacterium* (Ferreira et al. 2018). These bacteria comprise several intestinal residents that may become opportunistic pathogens (Kelly and LaMont 2008; Rajilic-Stojanovic and de Vos 2014), and indeed *Lactobacillus*, *Clostridium*, and *Citrobacter* have been detected in the gastric juice or gastric biopsies from patients taking acid suppressive drugs, and patients with intestinal metaplasia and gastric cancer (Sjostedt et al. 1985; Mowat et al. 2000; Dicksved et al. 2009; Aviles-Jimenez et al. 2014). In a recent study of nine gastritis and 11 gastric cancer patients from Taiwan, species of *Clostridium* and *Lactobacillus* were also found enriched in the gastric cancer microbiota (Hsieh et al. 2018).

Microbial dysbiosis was inversely correlated with the microbial diversity and was significantly higher in cancer than in gastritis, a finding that was validated in additional patient cohorts (Ferreira et al. 2018). Actually, microbial dysbiosis could distinguish gastric cancer and chronic gastritis patients in ROC analysis. Interestingly, microbial dysbiosis could discriminate gastric cancer better than individual genera, suggesting that alterations to the microbial community as a whole rather than particular bacteria contribute to the development of gastric cancer.

The role of the microbiota in the promotion of neoplasia is supported by data obtained in the insulin-gastrin (INS-GAS) transgenic mouse model. In comparison with germ-free INS-GAS mice, those harbouring a complex microbiota had higher levels of gastric inflammation, epithelial damage, oxyntic gland atrophy, hyperplasia, metaplasia, and dysplasia. When infected with *H. pylori*, INS-GAS mice that harboured a complex microbiota had more severe gastric lesions and an earlier development of gastrointestinal intraepithelial neoplasia (GIN) in comparison to *H. pylori*-infected germ-free INS-GAS mice (Lofgren et al. 2011). Furthermore, progression towards GIN occurred to a similar extent in *H. pylori*-infected INS-GAS mice with a complex microbiota and in *H. pylori*-infected INS-GAS mice colonized with a restricted microbiota consisting of only three species of commensal murine bacteria (*Clostridium* sp., *Lactobacillus*

murinus, and *Bacteroides* sp.) (Lertpiriyapong et al. 2014). These results suggest that colonization of the stomach with commensal bacteria from other locations of the GI tract may promote *H. pylori*-associated gastric cancer. Altogether, these studies highlight that there is a shift in the composition of the stomach microbiome from gastritis to gastric cancer, with a likely reduction of bacterial diversity, and with increased microbial dysbiosis in the cancerous stomach.

6 Revisiting Correa's Hypothesis of Gastric Carcinogenesis

In the multistep model of gastric carcinogenesis proposed by Pelayo Correa, persistent infection of the gastric mucosa with *H. pylori* initiates and perpetuates an inflammatory process that can progress to atrophic gastritis, intestinal metaplasia, dysplasia, and gastric cancer (Correa 1992). In this model, *H. pylori* infection plays an important role in the initial phases of the cascade. Indeed, *H. pylori* scarcely colonizes the severe atrophic stomach and may progressively disappear in gastric tissues at later steps of carcinogenesis (Correa 1992; Kuipers 1998). Analyses of the gastric microbiome have also described decreased relative abundance of *H. pylori* in gastric cancer (Dicksved et al. 2009; Eun et al. 2014; Ferreira et al. 2018; Hsieh et al. 2018), although this was not consistently observed or not reported (Yu et al. 2017; Coker et al. 2018).

The hypothesis of Correa contemplated that the loss of acid-secreting parietal cells in *H. pylori*-induced atrophic gastritis leads to higher gastric pH, and to proliferation in the stomach of bacteria that are capable of reducing nitrate to nitrite, to form N-nitroso compounds that are mutagenic (Correa et al. 1975; Correa 1992). Actually, significant intragastric bacterial overgrowth has been demonstrated in patients on long-term acid suppression by the use of proton pump inhibitors (PPIs) or histamine-2 receptor antagonists (Stockbruegger 1985; Sanduleanu et al. 2001). A recent investigation of 24 dyspeptic Italian patients, showed that although PPI treatment did not have a major influence in the gastric microbiota composition, an increase in the relative abundance of *Firmicutes*, namely *Streptococcus* was reported (Paroni Sterbini et al. 2016). In accordance with these findings, in a study analysing the metabolically active gastric microbial communities of 19 patients from the UK receiving PPI therapy and 20 individuals with normal stomach, relatively few alterations in the gastric microbiota were detected, but *Streptococcus* was significantly enriched in PPI-treated patients (Parsons et al. 2017). An enrichment in *Streptococcaceae* in the gut microbiota of PPI users has also been reported in two large studies (Imhann et al. 2016; Jackson et al. 2016). The enrichment of upper GI tract commensals observed in the stomach and in the gut, may be related with the disruption of the highly acidic barrier of the stomach induced by the acid suppressive therapy.

Likewise, the increase of the pH of the stomach due to decreased acid production as a result of parietal cell loss in *H. pylori*-associated atrophy, may generate a niche that becomes suitable to the establishment of a different microbiome (Plottel and Blaser 2011). One may speculate that this altered gastric microbiome, where *H. pylori* is less abundant or absent, and where commensal bacteria from other locations of the GI tract thrive, would act as continuous stimuli by maintaining the inflammatory process and/or inducing genotoxicity, thus promoting gastric carcinogenesis (Fig.1). This would in part explain the lack of success of *H. pylori* eradication in preventing progression of preneoplastic lesions and gastric cancer in patients with atrophy or intestinal metaplasia at baseline (Wong et al. 2004; Mera et al. 2018).

The microbiome of the cancerous stomach is functionally different from that of the stomach without cancer (Coker et al. 2018; Ferreira et al. 2018). Although only a very limited number of studies have addressed this aspect, predictive functional analyses have revealed that gastric cancer patients have an enrichment of several microbial pathways, including those related with membrane transport, carbohydrate digestion and absorption, carbohydrate metabolism, xenobiotics biodegradation and metabolism, and

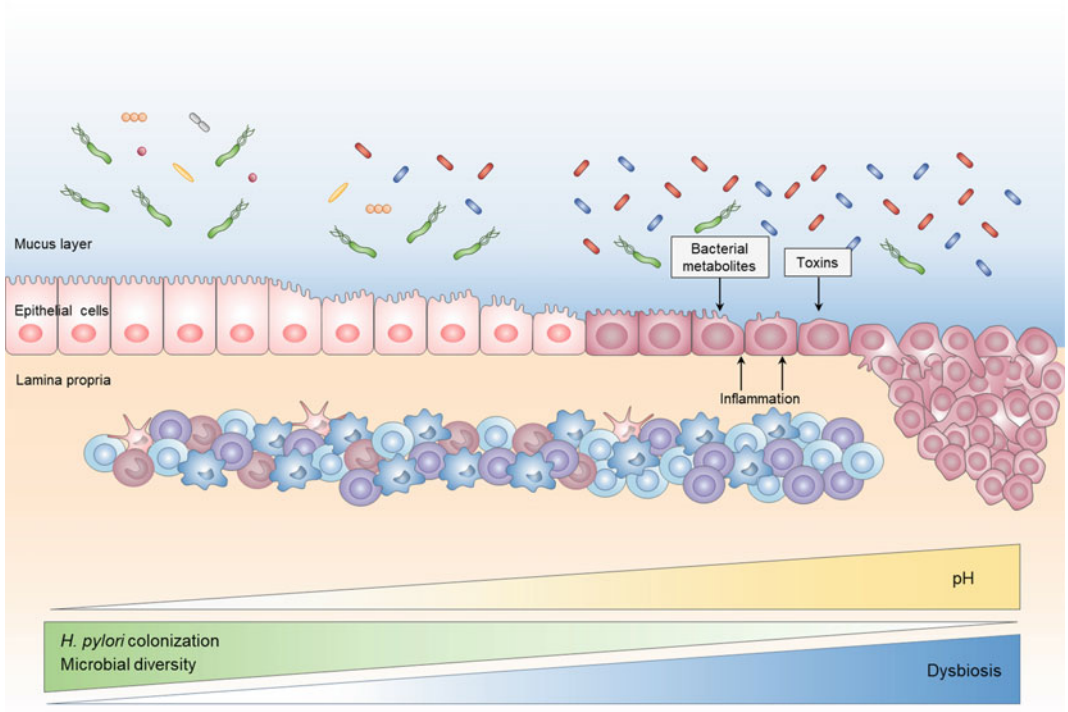

Fig. 1 Model for microbial dysbiosis in gastric cancer development. *H. pylori* infection triggers and perpetuates an inflammatory response in the gastric mucosa that, in some of the infected individuals, leads to loss of acid-secreting parietal cells with increase of the gastric pH. In this altered environment, *H. pylori* colonization decreases, and bacteria from other locations of the GI tract establish in the gastric niche, resulting in dysbiosis. This dysbiotic microbiome, characterized by reduced microbial diversity, may promote the development of gastric cancer by sustaining inflammation and/or inducing genotoxicity. Bacteria: green, *H. pylori;* orange, pink and grey, resident mucosa-associated microbiota; blue and red, dysbiotic microbiota; Inflammatory cells: dark blue, macrophages; pink, dendritic cells; dark pink, monocytes; light blue, CD4 T-lymphocytes; violet, CD8 T-lymphocytes

lipid metabolism (Tseng et al. 2016; Castano-Rodriguez et al. 2017; Coker et al. 2018; Ferreira et al. 2018). Findings are, however, relatively divergent between studies and results should therefore be interpreted with caution.

To revisit Correa's hypothesis that nitrate-reducing bacteria contribute to malignant transformation of the atrophic stomach by increasing the concentrations of nitrite and of N-nitroso compounds, we have assessed the functional features of the microbiome involved in these reactions (Ferreira et al. 2018). By fully reconstituting the metagenomes, based on the profiles obtained from the 16S rRNA gene sequences, we showed that in comparison with chronic gastritis, the gastric cancer microbiome had an increased representation of nitrate reductase and of nitrite reductase functions, the enzymes that respectively reduce nitrate to nitrite and nitrite to nitric oxide. The four genera *Citrobacter*, *Achromobacter*, *Clostridium* and *Phyllobacterium* were identified as the major contributors to these functions (Ferreira et al. 2018). Interestingly, and in agreement with our observations, are those of a follow-up study conducted in Taiwan to evaluate the effects of subtotal gastrectomy as a treatment for early-stage gastric cancer. The alteration of the gastric environment by the surgery led to significant changes in the gastric microbial community, and

nitrate reductase, nitrite reductase, and other functions related to nitrosation were enriched in the gastric microbiome before, but not after subtotal gastrectomy (Tseng et al. 2016). These data suggest that the gastric cancer microbiome has the potential to produce carcinogenic N-nitroso compounds. Additional features linked to the dysbiotic microbiome may be involved in the promotion of a carcinogenic environment in the stomach. Microbial metabolites and toxins, as well as inflammation by-products generated by the dysbiotic microbiome, may directly induce host cell damage or interfere with host signalling pathways that influence cell turnover and survival, thus increasing the risk for gastric malignant transformation (Fig.1).

7 Conclusions

Despite the recent advances in the investigation of the human gastric microbiome, research in this area remains limited. Although a number of papers about the microbiome of the stomach in the context of gastric carcinogenesis have been published, caution should be taken with the interpretation of the results of very distinct technical approaches. Additionally, differences in the geographic origin, genetic background, and environmental exposures of the populations should be taken into consideration.

While it is clear that the microbial community present in gastric cancer is distinct from that present in chronic gastritis, research conducted on the microbiome of the histological stages that precede gastric cancer is still lacking. Studies in large and clinically well-defined patient populations will be key to determine the role of microbial dysbiosis in progression to cancer. The shift from descriptive to functionally based studies that investigate the effects of specific taxa and/or bacterial derived-metabolites in the gastric mucosa, will allow gaining insights into the mechanisms that lead to dysbiosis-associated genotoxicity and inflammation. Uncovering these mechanisms will create the grounds for translating microbiome research into prevention, diagnosis, and treatment improvements to control and decrease gastric cancer burden.

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