Gastric Cancer Microbiome

Williams Fernandes Barra^a Dionison Pereira Sarquis^a André Salim Khayat^{a, b}
Bruna Cláudia Meireles Khayat^b Samia Demachki^c
Ana Karyssa Mendes Anaissi^{a, c} Geraldo Ishak^{a, d} Ney Pereira Carneiro Santos^a
Sidney Emanuel Batista dos Santos^a Rommel Rodriguez Burbano^{b, e}
Fabiano Cordeiro Moreira^a Paulo Pimentel de Assumpção^{a, d}

^aNúcleo de Pesquisas em Oncologia, Universidade Federal do Pará, Belém, Brazil; ^bInstituto de Ciências Biológicas, Universidade Federal do Pará, Belém, Brazil; ^cUnidade Laboratorial de Anatomia Patológica, Universidade Federal do Pará, Belém, Brazil; ^dServiço de Cirurgia Geral e do Aparelho Digestivo, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, Belém, Brazil; ^eHospital Ophir Loyola, Belém, Brazil

Keywords

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Abstract

Identifying a microbiome pattern in gastric cancer (GC) is hugely debatable due to the variation resulting from the diversity of the studied populations, clinical scenarios, and metagenomic approach. *H. pylori* remains the main microorganism impacting gastric carcinogenesis and seems necessary for the initial steps of the process. Nevertheless, an additional non-*H. pylori* microbiome pattern is also described, mainly at the final steps of the carcinogenesis. Unfortunately, most of the presented results are not reproducible, and there are no consensual candidates to share the *H. pylori* protagonists. Limitations to reach a consistent interpretation of metagenomic data include contamination along every step of the process, which might cause relevant misinterpretations. In addition, the functional consequences of an altered microbiome might be addressed. Aiming to minimize methodological bias and limitations due to small sample size and the lack of standardization of bioinformat-

ics assessment and interpretation, we carried out a comprehensive analysis of the publicly available metagenomic data from various conditions relevant to gastric carcinogenesis. Mainly, instead of just analyzing the results of each available publication, a new approach was launched, allowing the comprehensive analysis of the total sample amount, aiming to produce a reliable interpretation due to using a significant number of samples, from different origins, in a standard protocol. Among the main results, *Helicobacter* and *Prevotella* figured in the “top 6” genera of every group. *Helicobacter* was the first one in chronic gastritis (CG), gastric cancer (GC), and adjacent (ADJ) groups, while *Prevotella* was the leader among healthy control (HC) samples. Groups of bacteria are differently abundant in each clinical situation, and bacterial metabolic pathways also diverge along the carcinogenesis cascade. This information may support future microbiome interventions aiming to face the carcinogenesis process and/or reduce GC risk.

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Williams Fernandes Barra and Dionison Pereira Sarquis are co-first authors.

Introduction

Gastric cancer (GC) was responsible for >1,000,000 cases and about 783,000 deaths in 2018, representing the fifth most incident and the third leading cause of cancer death [1]. The microbiome, the group of microorganisms present in a defined environment [2], can distinguish different types of tumors, including GCs [3]. This review concerns the microbiome of gastric adenocarcinoma, which represents 95% of GC cases.

H. pylori is the infectious agent most related to cancer development [4], representing the cause of 89% of non-cardia GC [5]. However, the contribution of other bacteria to gastric carcinogenesis has been widely discussed [6, 7].

Nevertheless the significant improvement in stomach microbiome investigation technology and the discovery of plenty of microorganisms as part of healthy and unhealthy gastric microbiota, *H. pylori* remains the most critical stomach disease-related pathogen [8]. Evidence for non-*H. pylori* microorganisms' role in GC is almost consensual. However, there are no robust data to elect a unique candidate to share the holophotes with *H. pylori* in GC pathogenesis [9]. Many putative reasons limit the progress of knowledge in the cancer-related gastric microbiome, including the small number of studied cases, methodological variability, population diversity, and investigations performed in multiple clinical scenarios.

The main *H. pylori* effect seems to be related to the initial steps of carcinogenesis. Its dominance reduces along with the transition from benignant to malignant stages of the process, leading to an interpretation that *H. pylori* abundance usually represents nonmalignant lesions. In contrast, GC seldom shows high infection levels of this bacterium [7, 10]. The role of the microbiome in gastric carcinogenesis will be briefly discussed, focusing on possible clinical implications of the new data and the limitations for translation to daily practice.

The Investigation of the Gastric Microbiome

The possibility of uncovering the microbiota without needs to perform cultures greatly improved the role of microbiome discovery in diverse diseases, including cancer. Initially, gut microbiome was accessed by 16S rRNA (ribosomal RNA) sequencing, that is, searching for bacteria ribosomal sequences, in feces. Tissue microbiomes were then investigated directly by endoscopic or surgical biopsies, and robust metagenomic approaches widen the

discoveries to other microorganisms, in single experiments [6, 10].

Currently, the gastric microbiome can be accessed by endoscopic or surgical biopsies of the stomach mucosa. The main sequence strategies are whole genome sequencing, which includes every microorganism, or 16S rRNA sequencing that targets bacteria. The former is more expensive and requires more profound bioinformatics expertise for interpretation, while the latter is cheaper and easier to analyze, but restricted to bacteria and Archaea [11]. Gastric juice and feces are other potential sources of gastric microbiome samples, nevertheless rarely applied [12].

Most of the available gastric microbiome data come from 16S rRNA sequencing, though whole genome sequencing is becoming more usual. Theoretically, both strategies should reach similar results regarding the bacteria component of microbiota, which is the most studied microorganism in cancer microbiome research. Nevertheless, many methodological tricks cause few discrepancies when results are compared. These discrepancies contribute to hamper the understanding of GC microbiome interactions.

It should be noticed that both strategies provide the identification of microorganism DNA (from now on, will refer to bacteria, instead of microorganisms, since the majority of investigations focus exclusively on bacteria). However, the presence of bacterial DNA does not, necessarily, imply in host functional consequences, or either to the microenvironment.

Recently, new strategies to address the functional consequences of the cancer microbiomes are being applied, including the investigations of transcriptomes, proteomes, and metabolomes. These approaches aim to discriminate the microbiome effects and estimate interactions among the microorganisms and between these microorganisms and the host cells [13]. Few experiments are addressing GC microbiome functional interactions, and progress in this field may shed light on the actual mechanisms related to the microbiome carcinogenesis process.

Independently of the eligible strategy, a significant challenge needs to be faced, that is, the contamination misinterpretation. Every step of the process is susceptible to contamination, from sample collection to lab handling, and it is almost impossible to stay free of contaminants. Even sterile reagents are not free of contaminants since there is no need for viable microorganisms. However, just a few fragments of inert DNA can be sequenced and included in the analyses. The practice of sequencing “blank”

samples in each experiment runs minimizes misinterpretations and should be stimulated, together with using “bioinformatics” filters of contaminants, available in public data banks [3].

Healthy versus Unhealthy Gastric Microbiome

The concept of eubiosis means the supposed normal or equilibrated microbiome. The completely normal gastric mucosa is not well knowledged and accessible in clinical practice to allow a considerable number of analyses, harboring different populations, ages, and alimentary patterns, among other possible biases, to define the real normal gastric microbiome.

Most studies compare cancer versus noncancer samples to discriminate between “normal” and cancer microbiomes [6, 10, 13–26]. Regarding GC, some papers also include an intermediate stage represented by premalignant lesions, such as atrophy, metaplasia, and dysplasia, since for intestinal-type GC, these are recognized as steps of the carcinogenic process, according to Correa’s hypothesis [27].

H. pylori is the dominant bacteria in normal gastric mucosa and also in nonatrophic gastritis [28]. The microbiome diversity seems to be lower when *H. pylori* predominates and increases in parallel to *H. pylori* decrease, such as in the case of premalignant lesions and, possibly, in GC [19, 21].

The mechanisms related to *H. pylori* decrease along the carcinogenic process are still under debate [7]. The modification of the dominant gastric microbiome in GC could be a consequence of the newly established microenvironment, characterized by an increased pH, allowing the spread of opportunistic bacteria [6].

Others affirm that these new dominant bacteria are the real causal agents of GC, and the importance of *H. pylori* seems to be limited to a premalignant stage, providing the basis for subsequent modifications in the microenvironment, mainly the reduction of gastric acidity, secondary to *H. pylori*-related gastric corpus atrophy [21]. These microenvironment alterations are the supposed causes for both *H. pylori* reduction and the new carcinogenic-prone microbiome subsequent dominance [10, 15, 16].

Regarding diffuse-type GC, the lack of knowledge is broader since the carcinogenesis process is less understood. The absence of intermediate states between benign and GC patterns represents an additional barrier for elucidating the transition from normal to the cancer-related microbiome. Currently, diffuse-type GC-specific

microbiota cannot be described. There are few reports addressing microbiome differences among Lauren types. A need for augmentation of the number of analyzed cases, including full clinical information, may shed light on putative differences among histological, localization, and staging features [29].

The Importance of Stomach Acidity

The high stomach acidity represents a possible barrier for many microorganisms [30]. The stomach, and as a consequence, the usual gastric microbiome, is characterized by an acidic microenvironment, though selecting microorganisms able to adapt to such conditions. Inversely, in GA patients, the stomach pH is higher, so an important modification of the microenvironment is predicted, including the pattern of the resident microorganism.

As discussed, it is not clear if the microbiome modification is just a consequence of the microenvironment changes, without any role in the carcinogenesis process, or if the new microbiota present causal effects, favoring GC. Besides these unsolved questions, the current medical practice includes inhibition of acid secretion, thus implying in reducing stomach acidity, a condition closer to the GC microenvironment and probably to GC microbiota [31].

Based on the hypothesis that gastric microbiota related to lower acidity, as is the case of GC, are only a consequence of a new microenvironment, with no deleterious effect on the stomach mucosa, prescribing inhibitors of gastric secretion, despite causing a significant change in the microenvironment, should not affect GC risk [32]. Inversely, if lower acidity-related stomach microbiota play a role in GC, the prolonged use of gastric acid secretion inhibitors might favor GC carcinogenesis and should be avoided [33]. Much published data are defending both hypotheses, and in-depth investigations addressing this issue are necessary to elucidate if the prolonged medical elevation of gastric pH could trigger microbiota modification and, if this new microbiota could, eventually, increase GC risk.

H. pylori and Gastric Microbiome

H. pylori is considered a type 1 carcinogen by the World Health Organization (WHO), irrespective of Lauren’s type classification [34]. Eradication of *H. pylori* re-

Table 1. Studies on the microbiome and GC with 16S rRNA sequencing, published between 2018 and 2020 [6, 21, 22, 24–26, 66]

Paper	N	Country	Samples	Phyla	Genera	Main results
Wang et al. [66]	132	China	Gastric mucosal biopsy	Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria	<i>Acinetobacter</i> , <i>Aquicola</i> , <i>Haemophilus</i> , <i>Halomonas</i> , <i>Helicobacter</i> , <i>Lactobacillus</i> , <i>Prevotella</i> , <i>Shewanella</i> , <i>Sphingomonas</i> , <i>Streptococcus</i> , and <i>Vaiilonella</i>	IN and GC had lower microbial richness and diversity <i>H. pylori</i> sequences were detected in all mucosa samples. Intestinal type of GC and IN had a reduced abundance of <i>H. pylori</i>
Chen et al. [24]	62	China	Gastric mucosal biopsy	Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, Fusobacteria	<i>Streptococcus</i> , <i>Peptostreptococcus</i> , <i>Prevotella</i> , <i>Prevotella_7</i> , <i>Acinetobacter</i> , <i>Bacillus</i> , <i>Selenomonas</i> , <i>Lachnoanaerobaculum</i> , and <i>Sphingomonas</i> and the species <i>Acinetobacter baumannii</i> , <i>P. aeruginosa</i> , <i>Prevotella oris</i> , and <i>Prevotella denticola</i>	<i>H. pylori</i> sequencing-positive group showed greater bacterial diversity GC had increased community richness and diversity Metabolic enzymes related to denitrification were enriched in the gastric microbiota of the cancer group
Liu et al. [26]	276	China	Gastric mucosal biopsy		<i>Streptococcus</i> , <i>Selenomonas</i> , <i>Fusobacterium</i> , <i>Propionibacterium</i> , and <i>Corynebacterium</i> were enriched in the tumoral microhabitat	<i>H. pylori</i> was significantly decreased in tumoral sites
Gunathilake et al. [25]	556	Korea	Gastric mucosal biopsy		<i>H. pylori</i> , <i>Propionibacterium acnes</i> (<i>P. acnes</i>), and <i>Prevotella copri</i> (<i>P. copri</i>)	GC had higher abundance of <i>H. pylori</i> , <i>P. acnes</i> , and <i>P. copri</i> . <i>L. lactis</i> was higher in the controls
Chen et al. [24]	62	China	Gastric mucosal biopsy	Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, Fusobacteria	<i>Streptococcus</i> , <i>Peptostreptococcus</i> , <i>Prevotella</i> , <i>Prevotella_7</i> , <i>Acinetobacter</i> , <i>Bacillus</i> , <i>Selenomonas</i> , <i>Lachnoanaerobaculum</i> , and <i>Sphingomonas</i> and the species <i>Acinetobacter baumannii</i> , <i>P. aeruginosa</i> , <i>Prevotella oris</i> , and <i>Prevotella denticola</i>	GC had a higher number of OTUs and increased community richness and diversity
Hsieh et al. [22]	27	Taiwan	Gastric mucosal biopsy		<i>Clostridium</i> , <i>Fusobacterium</i> , and <i>Lactobacillus</i>	<i>H. pylori</i> were significantly lower in the GC
Ferreira et al. [6]	291	Portugal, China, and Mexico	Gastric mucosal biopsy	Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria	<i>Phyllobacterium</i> , <i>Achromobacter</i> , <i>Citrobacter</i> , <i>Lactobacillus</i> , <i>Clostridium</i> , <i>Rhodococcus</i>	GC microbiota had a higher microbial dysbiosis index than that of patients with CG
Coker et al. [21]	207	China, Mexico	Gastric mucosal biopsy	Fusobacteria	<i>Parvimonas micra</i> , <i>Dialister pneumosintes</i> , <i>Slackia exigua</i> , <i>Peptostreptococcus stomatis</i> , <i>Prevotella intermedia</i> , <i>Fusobacterium nucleatum</i> , <i>Prevotella oris</i> , and <i>Catonella morbi</i>	GC had increased oral bacteria. IM and GC had significantly reduced microbial richness. More interactions were seen among gastric microbes in <i>H. pylori</i> -negative
CG, chronic gastritis; IM, intestinal metaplasia; IN, intramucosal neoplasia; GC, gastric cancer; OTUs, operational taxonomic units.						

Fig. 1. Main changes in gastric microbiota during carcinogenesis adapted according to the Correa cascade. At the bottom, the Correa cascade. Above, the changes in the microenvironment and microbiome generally mentioned, possibly related to the stages of the cascade.

duced rates of metachronous GC after endoscopic resection of early GC or high-grade adenoma [35] and the risk of GC in persons with *H. pylori* infection who had first-degree relatives with a history of GC [36].

Several studies show a progressive decline in the prevalence of infection with *H. pylori* in different parts of the world, partly consistent with a reduction in GC [37]. However, *H. pylori* infection seems necessary but not sufficient for GC development, since only a small fraction of those infected end up developing GC [38]. Since *H. pylori* does not cause GC in all infected patients, the participation of other infectious agents and genetic and environmental factors, such as eating habits, must be relevant in gastric carcinogenesis.

Evaluation of gastric microbiome, in patients with gastritis, showed that the *H. pylori*-positive gastritis group had significantly lower bacterial richness, as compared to *H. pylori*-negative gastritis (HpN) or controls. The HpN group showed the enrichment of Firmicutes, Fusobacteria, Bacteroidetes, and Actinobacteria phyla. Based on abundance, *Streptococcus* sp. and *Haemophilus parainfluenzae* were significantly associated with the HpN (odds ratio 18.9 and 12.3, respectively) [39].

Several authors have studied the change in the microbiota according to gastric carcinogenesis, mainly for Lauren's intestinal type [6, 10, 13–26]. The results are diverse, and some are conflicting mainly due to nonstandardization of techniques, limited case series, and data treatment (shown in Table 1). Generally, in parallel to advances in gastric carcinogenesis from normal epithelium to GC, there is a reduction in the abundance of *H. pylori*, an increase in the frequency of oral and intestinal microbiota, and an increase in microbial diversity (shown in Fig. 1).

Exploring Combined Information from Available Gastric Microbiome Data

Considering that the reduced number of cases and different methodological strategies can hamper the interpretation of gastric microbiome data, we launched a tentative effort to overpass these barriers by joining available raw data from selected publications. Instead of looking at each publication's results, we reanalyzed the whole group, that is, we included every case from every selected publication and performed a new independent analysis, addressing the whole group.

Besides evaluating tumor and nontumor samples, the pooled analysis allowed a more robust exploration of premalignant conditions, due to looking at a higher number of cases under a unique methodological approach. Aiming to address the most frequently described patterns of gastric microbiomes in benign, premalignant, and GC, we performed an analytical review of the 16S rRNA sequencing available dataset.

We identified a total of 8 eligible datasets from the literature and the NCBI BioProject repository, sequenced by the Illumina platform (shown in Table 2). We obtained 1,825 samples, distributed as follows: 119 healthy controls (HC); 20 functional dyspepsia (FD); 350 chronic gastritis (CG); 84 intestinal metaplasia (IM); 75 intraepithelial neoplasia (IN); 538 adjacent (ADJ); and 639 Gastric Cancer (GC). For analysis purposes, data from the project PRJNA428883 originally presented as normal (>5 cm apart from the tumor) were grouped within the peritumor (2–5 cm adjacent to the tumor tissue) and called ADJ since these are not normal samples, as previously discussed [40, 41]. In addition, we carry out a data analysis

Table 2. Database with available data for microbiome analysis by 16S rRNA

BioProject	Author*/institution (year)	Country	SRA experiments	Groups	16S rRNA region	Platform
PRJEB21497	Castañero-Rodriguez et al. [18]	Singapore and Malaysia	32	20 FD 12 GC	V4	Illumina MiSeq 2 × 250 bp
PRJEB26931	Wang (2020)	China	310	56 HC 41 CG 54 IM 75 IN 84 GC	V4	Illumina MiSeqPE300
PRJNA310127	Yu et al. [20]	China and Mexico	291	157 CG 134 GC	V3–V4	Illumina MiSeq
PRJNA313391	Das (2017)	India	120	30 CG 30 IM 60 GC	V3–V4	Illumina HiSeq2500
PRJNA532731	Chen et al. [24]	China	123	62 CG 61 GC	V4–V5	Illumina HiSeq2500
PRJNA545207	Hospital of Nanchang University (2019)	China	63	63 HC	V3–V4	Illumina MiSeq
PRJNA508819	Zhejiang University (2018)	China	180	60 CG 61 ADJ 59 GC	V3–V4	Illumina MiSeq
PRJNA428883	Liu et al. [26]	China	706	477 ADJ** 229 GC	V3–V4	Illumina MiSeq
Total			1,825	20 FD, 119 HC, 350 CG, 84 IM, 538 ADJ, 75 IN, 639 GC		

HC, healthy control; FD, functional dyspepsia; CG, chronic gastritis; IM, intestinal metaplasia; IN, intramucosal neoplasia; ADJ, adjacent; GC, gastric cancer. * Related publication. ** Grouping of “normal” and “peritumoral” samples from this project as both are from cancer patients.

after grouping HC + FD (as controls) and CG + IM + IN (as preneoplastic), keeping the ADJ and GC groups.

Raw fastq files of each dataset were downloaded and extracted with NCBI SRA Toolkit [42] and were processed using QIIME2-2019.2 pipeline. The analyses were divided into different categories based on the denoising methods and the bacterial databases used for taxonomic assignment. Denoising was performed by using DADA2 (Divisive Amplicon Denoising Algorithm 2) [43]. Quality filtered sequences were clustered against the Greengenes v13_8 database using q2-vsearch-cluster-features-closed-reference [44] into operational taxonomic units (OTUs) performed at 97% identity. Singleton OTUs were removed from the OTU table.

There was no treatment over rRNA 16S hypervariable regions since all included sequenced V3, V4, or V5 re-

gions, and according to Martinez-Porchas et al. [45], the sensitivity and specificity among those regions are similar. All statistical analysis and graphics were performed using R 3.6.1 version. The sequencing process privileges the most prevalent bacteria, since small differences in abundance converge to highly significant differences in read counts, due to exponential amplification.

Instead of solely considering the read counts, or the relative abundance, we moved to adopt the ranking position, aiming to minimize these biased differences. In addition, considering that abundant bacteria in few samples might also bias the ranking average, this was mitigated by including the bacterial frequency among the samples. Accordingly, a score [$s = a \times (1.1 - f)$] was created, where s is the score value; a is the mean of the taxon ranking position in each sample in a given condition; and f is the tax-

Fig. 2. Venn diagram showing genera in each group. The diagram was created from the 15 genera with the best score shown in Table 3, described as present or absent. When analyzing the 7 available conditions (HC, FD, CG, IM, IN, GC, and ADJ), 3 genera were present in all conditions (*Helicobacter*, *Prevotella*, and *Sphingomonas*). Gathering in 4 groups (control, preneo, ADJ, and GC), 6 genera were found in common (*Prevotella*, *Helicobacter*, *Pseudomonas*, *Halomonas*, *Shewanella*, and *Sphingomonas*). HC, healthy control; FD, functional dyspepsia; CG, chronic gastritis; IM, intestinal metaplasia; IN, intraepithelial neoplasia; GC, gastric cancer; ADJ, adjacent (control [HC + FD], preneo [CG + IM + IN], GC, and ADJ).

Table 3. Bacterial list, according to the proposed pipeline, including both abundance and ranking position scores ($n = 1,825$)

Order	HC	FD	CG	IM	IN	GC	ADJ
1	<i>Prevotella</i>	<i>Methylobacterium</i>	<i>Helicobacter</i>	<i>Lactobacillus</i>	<i>Halomonas</i>	<i>Helicobacter</i>	<i>Helicobacter</i>
2	<i>Agrobacterium</i>	<i>Sphingomonas</i>	<i>Prevotella</i>	<i>Sphingomonas</i>	<i>Shewanella</i>	<i>Prevotella</i>	<i>Prevotella</i>
3	<i>Sphingobium</i>	<i>Streptococcus</i>	<i>Lactobacillus</i>	<i>Halomonas</i>	<i>Prevotella</i>	<i>Streptococcus</i>	<i>Sphingomonas</i>
4	<i>Helicobacter</i>	<i>Helicobacter</i>	<i>Streptococcus</i>	<i>Helicobacter</i>	<i>Neisseria</i>	<i>Halomonas</i>	<i>Halomonas</i>
5	<i>Porphyromonas</i>	<i>Prevotella</i>	<i>Pseudomonas</i>	<i>Prevotella</i>	<i>Lactobacillus</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
6	<i>Pseudomonas</i>	<i>Acinetobacter</i>	<i>Sphingomonas</i>	<i>Bradyrhizobium</i>	<i>Helicobacter</i>	<i>Sphingomonas</i>	<i>Bacteroides</i>
7	<i>Halomonas</i>	<i>Enhydrobacter</i>	<i>Halomonas</i>	<i>Neisseria</i>	<i>Rothia</i>	<i>Lactobacillus</i>	<i>Streptococcus</i>
8	<i>Neisseria</i>	<i>Sediminibacterium</i>	<i>Neisseria</i>	<i>Vibrio</i>	<i>Haemophilus</i>	<i>Shewanella</i>	<i>Acinetobacter</i>
9	<i>Zoogloea</i>	<i>Fusobacterium</i>	<i>Porphyromonas</i>	<i>Pseudomonas</i>	<i>Bacillus</i>	<i>Acinetobacter</i>	<i>Shewanella</i>
10	<i>Shewanella</i>	<i>Corynebacterium</i>	<i>Bacillus</i>	<i>Porphyromonas</i>	<i>Actinobacillus</i>	<i>Corynebacterium</i>	<i>Faecalibacterium</i>
11	<i>Rhodobacter</i>	<i>Bacteroides</i>	<i>Acinetobacter</i>	<i>Shewanella</i>	<i>Actinomyces</i>	<i>Bacillus</i>	<i>Bifidobacterium</i>
12	<i>Vogesella</i>	<i>Lactobacillus</i>	<i>Haemophilus</i>	<i>Haemophilus</i>	<i>Sphingomonas</i>	<i>Neisseria</i>	<i>Chryseobacterium</i>
13	<i>Sphingomonas</i>	<i>Veillonella</i>	<i>Bradyrhizobium</i>	<i>Actinobacillus</i>	<i>Aggregatibacter</i>	<i>Leptotrichia</i>	<i>Bacillus</i>
14	<i>Erythromicrobium</i>	<i>Brevibacterium</i>	[<i>Prevotella</i>]	<i>Aggregatibacter</i>	<i>Granulicatella</i>	<i>Veillonella</i>	<i>Oscillospira</i>
15	<i>HB2-32-21</i>	<i>Chryseobacterium</i>	<i>Corynebacterium</i>	<i>Rothia</i>	<i>Porphyromonas</i>	<i>Bacteroides</i>	<i>Veillonella</i>

A score [$s = a \times (1.1 - f)$] was created, where s is the score value; a is the mean of the taxon ranking position in each sample in a given condition; and f is the taxon frequency in each condition. HC, healthy control; FD, functional dyspepsia; CG, chronic gastritis; IM, intestinal metaplasia; IN, intramucosal neoplasia; ADJ, adjacent; GC, gastric cancer.

on frequency in each condition. By using this score, these discrepancies were minimized.

Analysis of statistically significant differences in the relative abundance of taxa was performed using linear discriminant analysis effect size [46]. Metagenome functional prediction was performed using PICRUST. The predicted functions were categorized with the Kyoto Encyclopedia of Genes and Genome. The most frequent genera in each type of gastric samples, after applying the proposed strategy of performing a whole analysis using

the totality of samples, and the proposed pipeline are shown in Table 3.

Mainly, *Helicobacter* and *Prevotella* figure in the “top 6” of every group. *Helicobacter* is the first in the CG, GC, and ADJ groups, while *Prevotella* is the leader of HC samples. There were some genera presented among the top 15 of >1 group.

In an attempt to evaluate the genera in each situation, the Venn diagram was produced (shown in Fig. 2a, b). Although *Sphingomonas* appears in the top 15 of every

Fig. 3. Genera diversity by the Shannon index. A bimodal behavior was observed in the assessment of alpha diversity. There is initially a reduction in diversity among the samples from HC to CG, followed by an increase in diversity along the carcinogenesis from CG to IN and one final drop in diversity in GC. HC, healthy control; FC, functional dyspepsia; CG chronic gastritis; IM, intestinal metaplasia; IN, intraepithelial neoplasia; GC, gastric cancer; ADJ, adjacent. Kruskal-Wallis test; ns, nonsignificant; * p -adj < 0.05; ** p -adj < 0.01; *** p -adj < 0.001.

group, there are many reports considering this genus as contaminants [47].

Nevertheless applying a strategy to minimize biases, it is necessary to take care while looking at metagenomic results. The majority of publications score the most important bacteria according to their abundance. These procedures may result in errors due to the amplification of some bacteria during the sequencing process, which substantially favors that bacteria, corrupting the final result. As an example, *Ochrobactrum* appears as the top one

in CG and IM and the second one in GC, if abundance is taken as the unique criteria. By adding the frequency score, this bias disappears since while being present in high abundance in a few samples, it is absent in the vast majority of the remaining samples.

In addition, it is critically necessary to stay alert regarding eventual lab-related discrepancies. There are some bacteria present in the majority of samples handled in a specific lab, which might be caused by contamination or even a unique feature of such a population that should

Fig. 4. Differently abundant genera between the conditions of gastric carcinogenesis (control, Preneo, and GC) and adjacent tissue.

not be generalized. For example, the samples from FD included in this analysis came from a unique lab (PRJEB21497) and presented *Ochrobactrum* in every sample that should be the situation contamination discussed above.

A bimodal behavior was observed in the assessment of alpha diversity by the Shannon index. There is initially a

reduction in diversity among the samples from HC to CG, followed by an increase in diversity along the carcinogenesis from CG to IN and one final drop in diversity in GC (shown in Fig. 3). This pattern might represent intestinal-type carcinogenesis, and the final drop could be influenced by diffuse-type GA inclusion in this group. It should be highlighted that although many reports refer to

adjacent to tumor samples as normal controls, this group, as previously reported by our team [40, 41], is much closer to GC than to normal samples [48, 49].

There is a significant similarity between GC and ADJ microbiota (adjusted p value <0.01 ; hypergeometric test). Accordingly, 2 hypotheses can raise from this observation: (i) since adjacent to tumor samples and GC samples are paired collected from GC patients, they may have similar microbiota, that is, it is a representation of the patient's stomach microbiome; (ii) there is a "field cancerization effect," a condition related to a carcinogenic insult reaching a wider area in the stomach including both the cancer tissue and surrounding noncancer mucosa. Regarding this second hypothesis, the microbiota could be a causal condition, favoring the carcinogenic process, or a consequence of the cancer establishment.

While looking at bacterial markers for each group, it was possible to demonstrate the peculiar distribution of those bacteria in each sample type group. Accordingly, the bacterial profile might identify specific conditions such as control (HC + FD), preneoplastic (CG + IM + IN), GC, and ADJ (Fig. 4). Although looking promising for helping the understanding of eventual bacterial-related steps along the carcinogenesis cascade, the presented results seem not to be currently strong enough to support this correlation for clinical translation.

Microbiome Metabolism and Carcinogenesis

Stomach microbiome dysbiosis might lead to bacterial networks with specific functional interactions, including metabolic effects capable of provoking local production of carcinogens [18]. The higher prevalence of non-*H. pylori* GC-related microbiota able to produce carcinogenic N-nitroso compounds, secondary amines from food, strengthens the hypothesis of a role for these non-*H. pylori* microorganisms in GC [14, 24, 50].

Aiming to investigate the prevalent metabolic patterns along the GC carcinogenesis, 3 steps of this process were evaluated: nonmalignant samples (control); premalignant; and GC groups, and additional analysis of ADJ samples was also included. Nevertheless, relevant to the understanding of microbiome metabolism's role along the carcinogenesis process, these pathways are all predicted according to the identified bacteria, that is, if a DNA of a specific bacterium is identified, an inferred pathway is proposed, without any functional validation. Predominant metabolic pathways could be associated with each group (shown in Fig. 5).

Fig. 5. Most predominant bacterial metabolic pathways in each group.

The analysis of the main bacterial metabolic patterns according to each sample origin group can distinguish these groups and might be related to specific contributions to the carcinogenesis pathways (shown in Fig. 5). Notably, the involvement of amino acid metabolisms or degradation seems to be associated with preneoplastic lesions, and such metabolites have been associated with gastric carcinogenesis [51].

Interestingly, in the cancer group, associations among genes related to sugar metabolism, possibly linked to energy production, such as sucrose and galactose metabolizers, and glycolysis, which is known to be related to tumor promotion/progression, were observed [52]. The association with genes linked to metabolic enhancement, and increased cellular activity, can also be observed. The predominance of signal transduction genes, phosphotransferases, RNA transport, and ribosomal biogenesis can influence the tumor environment, or even the host cell itself [53].

Usually, the microbiota investigation depends on the microorganism's DNA, obtained from tumor samples. Nevertheless, though being informative about the presence of a given microorganism, this cannot be interpreted as representative of a functional interaction between the microorganism and the host. Regarding GC, *H. pylori* is the most abundant microorganism in nonmalignant states that precedes GC and trends to scarce in final steps of carcinogenesis. One of the hypotheses to this phenomenon is a possible modification in *H. pylori* shape and functional profile, since as stomach pH increases, due to gastric atrophy, the microenvironment turns to be hostile, provoking a change in *H. pylori* shape to a coccoid aspect, as well as modifying the functional status, including a reduction of urease production [54]. Consequently, clinical tests fail to identify *H. pylori*, since they are based on pathologic identification of bacteria morphology and/or urease test.

Although resulting negative in clinical tests, *H. pylori* DNA is still present in molecular investigations since even the coccoid shapes retain spiral *H. pylori* DNA. At this point, the presence of *H. pylori* DNA probably has a different meaning of the same DNA identified in the premalignant state, representing, at this time, the shape and functional profile of the latter. Considering this so representative example, it is reasonable to consider that this interpretation could also happen in plenty of other microorganisms that even having their DNA linked to a given metabolic pathway might have different functional roles.

The target to be identified is not the microorganism per se, but the active functional pathway that might influence the carcinogenesis process, which may be shared by

different microorganisms. On the other side, the functional consequences of a given microorganism hazards or benefits cannot be assigned by host DNA analyses, but by modification of host's gene transcription.

Gut Microbiota Role

The gut microbiota impact many cancer types and also may influence GC carcinogenesis, as well as GC treatment responses and prognosis. The importance of gut microbiota to cancer-immune interactions is gaining evidence, paralleling the increased introduction of immunotherapy in clinical practice.

Gut microbiota differ between neoplasms of the gastrointestinal tract. Stools of patients with GC had elevated Enterobacteriaceae levels, and those with rectal neoplasms had lower levels of Bifidobacteriaceae. A lower abundance of Lactobacillaceae was seen in patients with colon neoplasms. The abundance of Lactobacillaceae was higher in stools of gastrointestinal tract patients sampled after cancer treatment than samples collected before any treatment [55].

Distal gastrectomy affected postoperative gut microbiota composition, resulting in higher abundances of *Escherichia/Shigella*, *Veillonella*, and *Clostridium XVIII* and lower abundances of *Bacteroides* [56]. Alterations on fecal microbiota, especially the dominant phyla of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, may be involved in *H. pylori*-related gastric lesion progression [57]. Others showed that a change to oral-like microbiome occurs after distal gastrectomy, with an increase in *Escherichia-Shigella*, *Enterococcus*, *Streptococcus*, and other typical oral cavity bacteria (*Veillonella*, *Oribacterium*, and *Mogibacterium*) abundance [58]. Changes in the composition of the intestinal microbiome that occur after bariatric surgery can have long-term metabolic consequences that need to be elucidated [59, 60].

The use of probiotics had significantly reduced the inflammation indexes (leukocyte), while it markedly enhanced the immunity (lymphocyte) and nutrition (albumin and total protein) indexes. Patients with GC had a stomach microbial diversity increase via enhancing the number of pathogens such as *Streptococcus*, *Peptostreptococcus*, and *Prevotella* and reducing the abundance of *Bifidobacterium*. Again, distal gastrectomy changed intestinal microbial diversity, and the uses of probiotic combinations reduced the ratio of Firmicutes/Bacteroidetes at the phylum level. At the genus level, the probiotic combinations significantly enhanced the probiotic bacteria *Bac-*

teroides, *Faecalibacterium*, and *Akkermansia* and lowered the richness of *Streptococcus* [61]. During *H. pylori* eradication, probiotic supplementation may reduce the antibiotic-induced alteration and imbalance of the gut microbiota composition [62].

Gut microbiome composition can interfere in primary resistance to immune checkpoint inhibitors (ICIs) targeting programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1), recently introduced to GC therapeutic arsenal. Antibiotics inhibited the clinical benefit of ICIs in patients with advanced cancer. In vivo, germ-free or antibiotic-treated mice had better antitumor effects of PD-1 block after fecal microbiota transplantation (FMT) from cancer patients who responded to ICIs, while FMT from unresponsive patients failed. In addition, patient stool sample metagenomics analyses at diagnosis revealed correlations between clinical responses to ICIs and the relative abundance of *Akkermansia muciniphila*. Oral supplementation with *A. muciniphila* after FMT with nonresponder feces restored the efficacy of PD-1 blockade [63]. Interestingly, patients with non-small cell lung cancer who had higher antibiotic-immunotherapy exposure ratio defined as “days of antibiotics/days of ICIs” had worse progression-free survival and overall survival than the others [64]. The impact of interventions in GC patients’ gut microbiota might integrate therapeutic strategies in the newer future.

Conclusion

The discovery of gastric microbiota profiles in each clinical condition, from normal stomach to premalignant and GC onset, may impact the understanding of the GC carcinogenesis process, moving the management of these conditions to an innovative approach. There is a need for standardization of microbiome exploration, from sample collection to bioinformatics pipelines, aiming to allow robust interpretation of the role of stomach microbiota, as well as the consequences of medical interferences on the individual microbiome pattern. Currently, besides the importance of *H. pylori* in GC carcinogenesis, additional microorganisms seem to play a role in both GC and premalignant lesions. By performing a comprehensive analysis of publicly available metagenomic data, from various clinical steps of gastric carcinogenesis, it was possible to infer that the gastric microbiota change along the carcinogenesis process, allowing the identification of different specific clinical situations according to their most abundant groups of bacteria. In addition, metabolic pathways,

resulting from bacterial metabolism, are differently active along the carcinogenesis process. These global analyses of evaluable data, besides bringing more robust information about the GC microbiota, reinforce an urgent need to standardize techniques for the production, analysis, and publication of metagenomics data, in an attempt to apply this knowledge in clinical practice.

Perspectives

The deep access to microbiome data, while bringing plenty of new information about the healthy and unhealthy stomach, created a big expectation regarding the possibility of interfering in such microbiomes with therapeutic goals. Attempts to change high-risk microbiota, both by diet modification, antibiotics, probiotics, and even microbiota transplantation, are being considered as possible treatment strategies. Unfortunately, regarding GA, except for *H. pylori* infection prevention or eradication, the absence of clear targets to avoid or treat hampers the clinical interventions targeted to gastric microbiota, although these constitute valuable perspectives to improve GA control.

Currently, in addition to *H. pylori* infection knowledge, EBV whenever present among gastric microbiota implies in important clinical consequences, since EBV-related GC is currently recognized as a molecular GC type, according to the TGCA classification. Besides molecular and histological peculiarities, EBV-type GC seems to be implicated in different therapeutical approaches, such as immunotherapy. The on-track discoveries might find out other microorganisms’ implications in GC process.

Among the most promising on-track innovations in the microbiota field studies is the possibility of performing a liquid microbial biopsy. Recently, the capacity of identifying bacterial circulating DNA, coincident with that bacteria identified in matched tumor samples, opened new horizons for the clinical application of microbiota data in daily practice [65]. Our team is working on clinical tests for both screening asymptomatic individuals from a high-risk GA region and treatment response evaluation, since we found circulating bacteria DNA similar to that found in primary tumors from different sites, including GA. Currently, standardization of methodology, from sample collection to final interpretations, and looking at functional interactions beyond the exclusive recognition of bacteria DNA might shed light on the role of gastric microbiota in the management of GA, from prevention to treatment strategies.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

P.P.A. and W.F.B. conceived the manuscript. A.S.K., B.C.M.K., S.D., A.K.M.A., G.I., N.P.C.S., S.E.B.S., and R.R.B. did the literature review. D.P.S. and F.S.M. performed the bioinformatics analysis. W.F.B., P.P.A., A.S.K., D.P.S., and F.S.M. wrote the manuscript. W.F.A., A.S.K., B.C.M.K., S.D., and A.K.M.A. produced the final format of the manuscript. All authors have reviewed and approved the manuscript in its final state.

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