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A Multi-OMIC approach to locally advanced gastric cancer: the MIMETIC study

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Abstract

Stato dell'arte: il carcinoma dello stomaco rappresenta una delle principali cause di morte per cancro. Nonostante il progresso nelle tecniche chirurgiche e l'utilizzo di trattamenti multimodali integrati, come la chemioterapia e la radioterapia perioperatorie in pazienti idonei, i tassi di recidiva e conseguentemente di morte riguardano ancora circa un terzo dei pazienti. Numerosi parametri biologici e clinici sono stati testati per guidare la scelta terapeutica nella malattia localmente avanzata resecabile, con intento curativo, ma permangono aspetti controversi in pratica clinica. Per questo motivo lo studio di nuovi promettenti parametri, tra cui il microbiota, valutati in contemporanea nello stesso paziente, rappresenta una possibile chiave di volta per selezionare sottogruppi specifici di pazienti e migliorare la loro durata di vita.

Metodi: Nel presente progetto, abbiamo ideato e condotto uno studio osservazionale prospettico che ha riguardato pazienti affetti da carcinoma dello stomaco localizzato o localmente avanzato, resecabile d'emblée o dopo chemioterapia neoadiuvante. Di questi pazienti abbiamo raccolto dati clinici, nutrizionali, anatomo-patologici e radiologici ed abbiamo raccolto e stoccato campioni biologici (sangue, feci, saliva e tessuto) per condurre analisi traslazioni riguardanti la composizione del microbiota e la determinazione di markers di attivazione dei neutrofili (NETs).

Risultati: I dati da noi raccolti suggeriscono che un progetto multi-omico che permetta la creazione di una piattaforma di integrazione di dati clinici e traslazionali sia fattibile. Abbiamo osservato una diversità in termini di composizione di microbiota tra pazienti affetti da tumore e soggetti sani e abbiamo posto le basi per lo studio dei NETs come potenziale parametro di risposta ai trattamenti o di ripresa di malattia.

Conclusioni: l'applicazione di un approccio multi-omico per la caratterizzazione di pazienti con carcinoma dello stomaco in fase precoce risulta fattibile e rappresenta un importante ambito di futuro sviluppo, con l'obiettivo di migliorare la prognosi. Lo studio del microbiota e dei NETs in questo contesto clinico rappresentano promettenti nuove linee di ricerca. Tuttavia, ulteriori sforzi sono necessari al fine di poter meglio definire la ripercussione clinico-pratica di tale modalità e il suo reale impatto sulla durata di vita dei pazienti con carcinoma gastrico in stadio precoce.

Introduction

Gastric cancer (GC) represents the fourth cause of cancer deaths worldwide[1]. The overall 5-year survival rate of GC is poor, since more than 60% of the patients are diagnosed with an advanced stage of disease, manageable only with palliative treatments. In early-stage and locally advanced setting, radical surgery represents the only chance for cure[2]. However, patients with gastric and esophago-gastric junction (EGJ) adenocarcinoma treated with curative intent have a poor prognosis, with a marked decrease in survival moving from localized to locally advanced stages[3]. In this context, several strategies could be added to tumor resection, to increase survival rates. Peri-operative chemotherapy significantly improved prognosis of patients with locally advanced (LA) resectable gastric and EGJ adenocarcinoma (generically defined gastric cancer [GC] through the manuscript) compared to surgery alone and it is considered the standard of care in Western countries[4]. Beside these improvements, a relevant proportion of patients still experiences disease recurrence, mainly leading to metastatic spread and early death. Histologic features and nodal involvement are the main aspects that guide the choice of systemic treatment in the curative setting, whereas molecular biomarkers do not still have sufficient clinical reliability[5]. Currently available molecular parameters that provide prognostic information include human epidermal growth factor receptor 2 *(HER2)* and Microsatellite Instability (MSI). However, even in these specific subgroups, many patients experience shorter survival rates, potentially related to more complex biological pathway which limit durable responses. Therefore, there is a strong clinical unmet need to identify novel predictive and prognostic biomarkers and to investigate multi-omic strategies which could stratify patients and improve survival.

Background and rationale

Management of localized and locally advanced gastric cancer

Patients with GC are often pauci- or asymptomatic in early stages. Symptoms are not specific and usually do not lead to urgent evaluation. Therefore, less than half of patients diagnosed with GC is eligible for curative treatment due to late presentation and/or severe comorbidities. Initial diagnostic workup includes physical examination, endoscopy and contrast enhanced computed tomography (CT) scan of the chest, abdomen, and pelvis. Fluoro-deoxy-glucose (FDG)- Positron Emission Tomography (PET)-CT is not routinely recommended, while laparoscopy with peritoneal washing for tumor cytology is recommended for patients with resectable GC, potentially eligible for perioperative chemotherapy. According to clinical staging, endoscopic resection could be proposed for very early gastric cancer (EGC) stage IA (T1a) if clearly confined to the mucosa and well-differentiated G1-2 histology. From stage IB, radical gastrectomy is the gold standard procedure. For tumors with an expansive growth pattern (including intestinal histotype) the recommended proximal margin of resection is 3 cm, that should be increased to 5 cm for those with an infiltrative growth pattern (including poorly cohesive/diffuse histotypes). Subtotal gastrectomy, for distal tumors, could be proposed if a satisfactory proximal resection margin can be obtained. Nodal dissection is crucial in GC surgery and its extent has been widely debated. The current AJCC/UICC TNM (8th edition) classification recommends a minimum of 15 lymph nodes analyzed to have a reliable staging. According to Eastern randomized trials, D2 resection (which consists in removing lymph nodes along the proper or common hepatic artery, splenic artery, or coeliac axis) in addition to D1 resection is associated with superior outcomes compared to a less extensive one[6]. However, despite an optimal surgical approach, most GC relapse. Therefore, multimodal therapies are the standard for stage IB disease and above. Perioperative (neo- adjuvant + adjuvant) therapy represents the standard treatment for LAGC based on the results of the MAGIC and FFCD trials [7,8]. More recently, the taxane-containing FLOT [docetaxel, oxaliplatin, leucovorin and 5-fluorouracil (5-FU) regimen] showed superiority over ECF in terms of histologic response, relapse-free survival (RFS), and overall survival (OS)[4]. This treatment regimen has increased survival rates up to 15% after 5 years of follow-up,

becoming the new standard of care in Western countries. The greatest benefit from perioperative chemotherapy seems to be derived from the pre-operative part [neoadjuvant chemotherapy (NAC)] since a relevant quote of patients (even in clinical trials) is not able to complete all planned cycles of post-operative chemotherapy, mainly because of higher rate of side effects and worse clinical conditions after surgery. The role of adjuvant chemotherapy after GC surgery has always been controversial. A doublet chemotherapy with fluoropyrimidine plus oxaliplatin for a duration of six months is the most widely used regimen[9]. The benefit in OS from the addition of chemotherapy has been proven in different trials and an individual-patient data meta-analysis confirmed an absolute benefit of 6%[10]. After adequate R0 surgery, postoperative chemo-radiation (CRT) is not recommended, following negative results from CRITICS and ARTIST trials[11, 12]. The addition of radiotherapy to chemotherapy in case of R1 should be discussed in a multidisciplinary setting since a balance between systemic risk and local relapse should be well considered. According to the results of a Dutch trial, CRT in R1 unfit patients was associated with a limited improvement in survival compared with no further treatment[13]. On the other side, adjuvant CRT can be considered in patients who did not receive preoperative chemotherapy and an appropriate D2 lymphadenectomy[9].

Histology and molecular profiling of gastric cancer

Around 90% of GC are adenocarcinomas (ACs). The most used histopathological classification schemes for gastric cancer are the World Health Organization (WHO), the Japanese Gastric Cancer Association classifications, and those proposed by Nakamura and colleagues and Lauren[6, 14-16]. All these classifications are similar: the WHO classification recognizes five main histological subtypes: tubular, papillary, poorly cohesive (including signet ring cell and other subtypes), mucinous and mixed ACs, while Lauren encompasses three main subtypes: intestinal, diffuse and mixed. In addition to histologic features, key relevance has been recently given to the identification of molecular profiles of GC which are crucial for a better understanding of tumor subtypes and the identification of clinically relevant biomarkers. The Cancer Genome Atlas (TCGA) research network identified four molecularly distinct GC: EBV positive,

microsatellite instability-high (MSI-H), genomically stable (GS) and tumors with chromosomal instability (CIN)[17]. Each subtype is enriched for selected molecular abnormalities, with some overlap. The CIN subtype is enriched for copy number changes in key receptor tyrosine kinase oncogenes such as HER2, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 2 (FGFR2) and MET. However, these determinations still do not have a defined role to guide treatment decision in locally advanced (LA) setting. Based on positive phase III trial data, HER2 status and programmed death-ligand 1 (PD-L1) combined positive score (CPS) should be evaluated in patients with metastatic GC to tailor first-line treatment in combination with chemotherapy. Emerging data from clinical trials suggest that immunotherapies such as programmed cell death protein 1 (PD-1) inhibitors are efficient in GC. Evaluation of PD-L1 expression in patients with GC using CPS has been proposed, where a cut-off of 1 would indicate positive PD-L1 expression; the prevalence of PD-L1 CPS 1 tumors is between 50% and 60%[18]. A CPS cut-off of 5 represents a validated threshold for OS benefit of nivolumab plus standard platinum- and fluoropyrimidine-based first-line ChT[19]. Different antibodies for staining of PD-L1 in GC are used. In a recent study, PD-L1 22C3 and 28-8 pharmDx assays, both tested on the same platform (hardware), were highly comparable at CPS cut-offs of 1, 10 and 50, providing evidence for the potential interchangeability of the two PD-L1 assays in GC. However, these results were not confirmed in another study, which suggested that scoring PD-L1 CPS with the 28-8 assay may result in higher PD-L1 scores and a higher proportion of PD-L1 positivity compared to the 22C3 and other assays. Until stronger evidence of inter-assay concordance is determinated, caution should be taken when treating the assays as equivalent[20]. Microsatellite instability high (MSI-H)/mismatch repair deficiency (dMMR) are associated with better prognosis in localized stages of GC[21]. There is an ongoing debate on whether MSI/MMR should be used in order to tailor peri operative ChT. As MSI-H/dMMR is associated with a high response rate and improved benefit from immunotherapy compared with ChT in stage IV GC[22], MSI/MMR status should be assessed in patients with LA and unresectable or metastatic GC to tailor treatment accordingly[23].

Role of diet and lifestyle in gastric cancer

Gastric carcinogenesis arises because of a complex interaction between host and environmental factors. It is well established that dietary, lifestyle and metabolic factors are in cause in GC development. Smoking has been implicated as a risk factor for non-cardia cancer. Furthermore, host genetic polymorphisms have an impact on host responses to gastric inflammation and acid secretion, thereby interacting with H. pylori infection and other environmental factors in gastric carcinogenesis. Although dietary, lifestyle and metabolic risk factors have been identified, and addressing these lifestyle and metabolic risk factors may contribute to health, the actual impact in modulating cancer response and outcomes is still debated. Results from epidemiological studies reported that dietary factors may play an important role in GC etiology[24]. While the role of grilled/barbecued meat and fish, processed meat and fruit remains controversial, there is convincing evidence for other food groups. The recent 2018 WCRF/AICR expert report concluded that high intake of alcoholic drinks and salt-preserved foods are strongly associated with an increased GC risk. To examine the relationship between dietary consumption and GC risk, food frequency questionnaires are usually used. Although these tools are subject to measurement errors[25], they are practical tool in the epidemiological research. They enable the assessment of long-term dietary intake in a relatively simple, cost-effective and time-efficient manner. Malnutrition is an independent predictor of increased morbidity and mortality[26]. Additionally, weight loss and sarcopenia lead to higher chemotherapy-induced toxicity[27]. Moreover, neoadjuvant chemotherapy (NAC) and chemoradiation therapy, which often worsen a patient's nutritional status, have become a standard treatment. In some cases, anti-cancer treatments may induce weight gain; on the other hand, overweight and obesity represent a risk factor for metabolic syndrome, and they may foster disease recurrence. Therefore, it is challenging to estimate how anti-cancer treatments affect nutritional status and vice versa. These serious changes in nutritional status are also associated with an important deterioration in quality of life[28] and can affect the ability to resist infection and recover from surgery. Screening for nutritional risk as early as possible allows the identification of patients at risk of becoming malnourished. Recent literature suggests that screening should be done at diagnosis or at hospital admission and then after repeated during

treatment course, if needed[29]. When nutritional risk is present, screening should be followed by comprehensive nutritional assessment to better determine the course of nutritional intervention. However, there is no consensus on the best method to perform this assessment, but SGA (Subjective Global Assessment) and PG-SGA (Patient Generated-Subjective Global Assessment) have been validated for nutritional assessment in adult oncology patients[30].

The SARC-F has been developed as a possible rapid diagnostic test for sarcopenia. In cancer patients at risk for malnutrition, sarcopenia and cachexia, muscle mass should be assessed. Methods available are dual X-ray absorptiometry (DEXA), CT-scans at the level of the 3rd vertebra or bioimpedance analysis (BIA). A nutritional intervention is most effective at a pre-cachexia, compared to a late stage of cachexia.[31]. Sarcopenia is a highly prevalent disease and might promote several adverse health-related outcomes. Previous studies suggested that cancer patients with pre-therapeutic sarcopenia had higher risk of postoperative complications, chemotherapy-induced toxicity, and poorer survival than those without sarcopenia[32]. Therefore, further knowledge is strongly warranted to realize the actual impact of dietary- and lifestyle-factors in GC risk and to examine if nutritional disorders are able to negatively impact prognosis and outcomes of anticancer treatments.

Preclinical and clinical evidence of microbiota in gastric cancer

The human microbiota describes the microbial taxa associated with humans and consists of as much as 10–100 trillion microbial cells harbored by each person in the different parts of the body. Bacteria comprise most of the biomass and diversity in the human gut, but viruses, archaea and eukaryotes are also present[33]. The composition of the human microbiota varies depending on different anatomical sites, age, environmental factors such as diet, antibiotic use, and diseases[34] (figure 1).

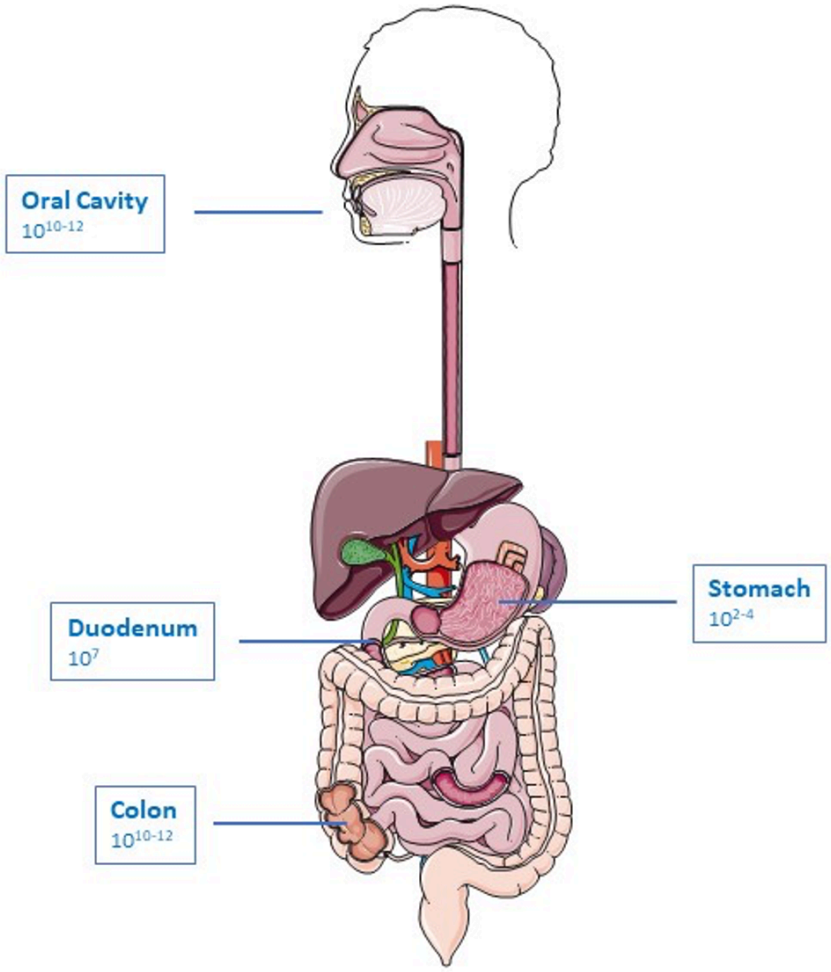


Figure 1. The human gut microbiota.

Exploring microbial communities in the human gut requires taxonomic classification and gene functional profiling. Metagenomics is the study of microbial communities in their original living places and refers to sequencing the entire genomes of all microbes present in a sample. When addressing a microbial ecosystem, the presence and abundance of specific bacterial strains are usually weighed by alpha diversity and beta diversity. Alpha diversity is a measure of microbes' variation within samples: more in deep, it defines how many taxa are present in the samples at qualitative (number of taxa, as richness) and quantitative (different taxa abundances, as evenness) level. For instance, the Shannon diversity index combines richness and diversity, by measuring both the number of species and the inequality between species abundances. The beta diversity shows the difference between microbial communities between samples and can be quantified by Bray–Curtis dissimilarity, Jaccard distance and UniFrac methods[35].

Because of the low pH (median 1.4) the stomach has a lower microbial load (10^2-10^4 colony-forming units [CFU]) compared to the small intestine and the colon (10^{10}-10^{12} CFU)[36]. The composition of the commensal microbiota is influenced by numerous factors including age, sex, geographical area, diet, the use of antibiotics, proton pump inhibitors (PPI), H2-antagonists and concomitant diseases[37]. Any condition that results in an increase in gastric pH greater than 4 (e.g. long-term use of PPI, H2 blockers, or chronic gastritis) may favor bacterial overgrowth[38], even though with traditional study techniques, such as isolation and culture, only a limited number of bacteria can be identified. Zilberstein and colleagues conducted one of the first studies assessing the composition of gastric microbiome by culture analysis in 20 healthy individuals[39]. The most frequently identified bacteria were *Veillonella* spp, *Lactobacillus* spp, and *Clostridium* spp. Unfortunately, up to 80% are not cultivable; therefore, the use of genomic techniques (polymerase chain reaction [PCR] or next generation sequencing [NGS]) can provide a better portrait of the gastric microbiome[40]. Bik and colleagues in 2006 investigated bacterial diversity by the use of a small subunit 16S ribosomal deoxyribonucleic acid (rDNA) clone library approach and analyzed near 2000 sequences generated by broad-range bacterial PCR from 23 gastric endoscopic biopsy samples[41]. The five most dominant phyla under normal conditions were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria. Li and colleagues evaluated the gastric microbiota of 10 healthy subjects, by cloning and sequencing 16S ribosomal ribonucleic acid (rRNA). The most abundant genera were *Streptococcus*, *Prevotella*, *Neisseriae*, *Haemophilus* and *Porphyromonas*[42]. It has been recently shown that gastric juice displays a different microbial community compared with the gastric mucosa. The abundance of *Helicobacter pylori* and Proteobacteria was higher in mucosa specimen, while Actinobacteria, Bacteroidetes, and Firmicutes were more represented in gastric juice[37]**.** The relationship between *Helicobacter pylori* infection and other gastric microbiota is still controversial. Osaki and colleagues conducted an *in vivo* study to investigate the effect of *Helicobacter pylori* on the rest of the gastric microbiota[43]. The authors divided infected Mongolian gerbils into different groups according to *Helicobacter pylori* positivity one year after infection, together with a negative control. In the *Helicobacter pylori*-negative group, reduced numbers of *Bifidobacterium* species, *Clostridium coccoides* and *Clostriduim leptum* subgroups, and increased abundance of

Atopobium cluster were observed. In a population of 12 patients, positive *Helicobacter pylori* status was correlated with augmented relative richness of bacteria from the Proteobacteria, Spirochetes and Acidobacteria, and with decreased abundance of Actinobacteria, Bacteroidetes and Firmicutes[44]. These results were in contrast with Bik and colleagues' study that reported no difference in the gastric microbiota according to *Helicobacter pylori* status[41]. It should be taken into account that the observed differences between populations are likely influenced by multiple factors, encompassing diet and life-styles rather than the time since *Helicobacter pylori* infection, the consequent chronic inflammation and atrophic gastritis, which in turn favor colonization by opportunistic bacteria. Overall, these findings led the authors to conclude that disease and ethnicity might have a greater impact than *Helicobacter pylori* on the composition of gastric microbiota.

Novel evidence suggests a potential mechanisms of GC carcinogenesis also beyond *Helicobacter pylori* [45]. For example, insulin-gastrin (INS-GAS) transgenic mice represent a unique model for studying the pathogenesis of GC, since the overexpression of circulating gastrin levels is associated with development of atrophic gastritis (AG) and gastric intramucosal neoplasia (GIN). INS-GAS mice infected with *Helicobacter pylori* spontaneously developed AG and GIN in 80% of the cases within 6-7 months post-infection[46]. Interestingly, Lofgren and colleagues observed that in *Helicobacter pylori*-infected INS-GAS mice, less severe gastritis and late onset of GIN were observed, compared to *Helicobacter pylori*–infected INS-GAS mice with composite gastric microbiota[47]. A subsequent study by Lertpiriyapong and colleagues evaluated the risk of developing gastric lesions after *Helicobacter pylori* infection in INS-GAS mice with different microbiota compositions: germ-free, restricted microbiota (*Lactobacillus*, *Clostridium* and *Bacteroides*) or complex[48]. Remarkably, restricted and complex microbiota were associated with significant expression of inflammatory and cancer-related genes, including *TNF-a*, Prostaglandin E Receptor 4 (*Ptger4)* and *Tgf-β*, and determined an increased risk of gastric pathology compared with germ free INS-GAS mice. Furthermore, Lee and colleagues observed that anti-inflammatory and antibiotic treatment prevent the progression from severe dysplasia to GC in *Helicobacter pylori* infected INS-GAS

mice. Taken together, these data suggest a complicit role between *Helicobacter pylori* and other taxa in promoting GC.

Several studies aimed to investigate the composition of gastric microbiota in patients with different pathologic conditions at different stages of the gastric carcinogenic route. Recently, Ferreira and colleagues evaluated gastric microbiota in 54 patients affected by GC and 81 ones with chronic gastritis by 16S rRNA gene profiling, using NGS[49]. Gastric cancer microbiota exhibited lower microbial diversity, by reduced abundance of *Helicobacter* and *Neisseria* and by the enrichment of intestinal bacteria (*Achromobacter*, *Citrobacter*, *Phyllobacterium*, *Clostridium*, *Rhodococcus* and *Lactobacillus*) compared with chronic gastritis. Similarly, Coker and colleagues conducted a 16S rRNA gene analysis of samples from a Chinese cohort of 81 patients with different gastric diseases (superficial gastritis, atrophic gastritis, intestinal metaplasia and GC)[50]. The authors observed an enrichment and network centralities in *Peptostreptococcus stomatis*, *Streptococcus Anginosus*, *Parvimonas micra*, *Slackia exigua* and *Dialister pneumosintes* taxa in microbiota of patients with GC with respect to samples from patients with superficial gastritis. Another similar study showed an increased bacterial load in patients in *Helicobacter pylori* positive patients compared with negative cases as well as in GC patients compared with chronic gastritis. Five genera, with potential pro-tumorigenic activity, were enriched in GC: *Lactobacillus*, *Escherichia–Shigella*, *Nitrospirae*, *Burkholderia fungorum*, and *Lachnospiraceae*[51].

To elucidate the dysbiotic change during gastric carcinogenesis, Park and colleagues used 16S rRNA gene profiling to analyze the gastric juice of 88 patients with gastritis, gastric adenoma and early/advanced GC[52]. A progressive decrease in the alpha diversity and significant difference in microbial composition was observed in the different steps from gastritis to GC. More precisely, a significant reduction in the abundance of *Akkermansia* and *Lachnospiraceae NK4A136* was reported in the GC cases alongside an enrichment with *Lactobacillus* and *Veillonella*. Similarly, Liu and colleagues performed a large analysis on 1270 tissue samples, pooled by 10 public datasets, based on 16S rRNA sequencing of tumor biopsies with the goal of mapping the gastric microbiota[53]. Besides reporting a reduced diversity in GC samples compared to the other pre-cancerous conditions, the authors identified four GC-associated bacteria, such

as *Fusobacterium*, *Peptostreptococcus*, *Streptococcus*, and *Veillonella*. To date, the role of specific bacteria other than *Helicobacter pylori* in GC development has not yet been established. Nevertheless, different studies of GC samples showed a significant higher abundance of *Lactobacillus* species, in some cases originating from the oral cavity, such as *Lactococcus* and *Lactobacillus* genera[54]. While a causal pathogenic mechanism could not be proved, the Authors proposed that the production of lactic acid might represent an energy substrate that favors cancer cell proliferation and tumor progression. Findings from other studies also showed that GC microbiome was characterized by the presence of *Nitrospirae* species, a group of N-Nitrosamine compound generating bacteria*,* which are able to promote the conversion of nitrite to nitrosamine, hence enhancing the genotoxic risk[49, 51].

Emerging data suggest that *Fusobacterium nucleatum*, an anaerobic Gram-negative bacterium, known as a commensal of the oral cavity, is correlated with different diseases after a downstream migration into the gastrointestinal tract[55]. Chen and colleagues indeed reported that *Fusobacterium nucleatum* was more abundant in GC tissues than in normal mucosa and was more like to be observed in elderly than younger patients ($P = 0.041$), apart from being associated with tumor lymphocyte infiltration[56]. Contrasting results were reported about the correlation of *Fusobacterium nucleatum* with survival outcomes in GC[57]. While its carcinogenic role has yet to be elucidated, it has been reported that *Propionibacterium acnes* could modulate the immune response by the production of short fatty acids in patients with lymphocytic gastritis[58] Recently, Park and colleagues demonstrated that *Rhizobiales* were enriched in patients with intestinal metaplasia respect of chronic gastritis[52]. In addition, the authors found an up-regulation of T4SS genes in intestinal metaplasia cases. As previously described, T4SS is involved in the intracellular transport of cytotoxin-associated gene A (CagA), one of the main *Helicobacter pylori* virulence factors thus potentially increasing the risk of GC development[59]. While there is an increasing number of studies supporting the oncogenic role of various bacteria species, less is known about the protective role exerted by specific bacteria. A recent study reported that *Sphingobium yanoikuyae,* which can degrade carcinogenic compounds, thus reducing the risk of GC initiation, was found to be less abundant in the GC-associated microbiota[60], thus opening novel scenarios that deserve further investigation.

Preclinical evidence of NET-osis in cancer

Neuthophil Extracellular Traps (NETs) are unique web-like structure, originated from neutrophils which act as the first defense of the organism against external stress, playing a role in removing foreign pathogens zhong. The progression of NET formation was first described in 2004[61]; neutrophils are activated by external factors such as lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) and then release intracellular DNA, histones, and granule proteins such as myeloperoxidase (MPO) and neutrophil elastase (NE)[62]. Although firstly described as an antimicrobial response to infection, NETosis is also involved in non-infectious diseases, including cancer, thrombosis and autoimmunity[63]. The interaction between tumor cells and NETs includes different pathways. Several receptors and signal pathways associated with growth, and metastasis could be activated by NETs, to shape the characteristics of the tumor. NETs also seem to enhance the malignancy of cancer[64], by acting on High mobility group box 1 (HMGB1), a protein widely distributed in the body that has been discovered to have a pro-inflammatory function, thus activating the nuclear factor- kappa B (NF-kB) signaling pathway upon binding to the receptor for advanced glycation end products (RAGE) on the tumor cell surface and promoting tumor secretion of interleukin-8 (IL-8)[65]. In contrast, IL-8 recruits neutrophils and promotes the production of NETs, thereby creating positive feedback [66]. Furthermore, the binding of NETs to tumor cells can also induce tumor cells to acquire resistance to death as well as enhanced invasiveness by activating the TLR4/9-COX2 pathway. According to Albrengues et al. NETs can also "wake up" dormant tumor cells through metalloproteinase (MMP) and NE, facilitating metastasis and recurrence[67]. Xiao et al. reported that cathepsin C (CTSC), the protease produced by tumor cells, can activate proteinase 3 (PR3) on the neutrophil membrane, to promote interleukin-1b (IL-1b) and activate NF-kB, which can upregulate interleukin-6 (IL-6) and CCL3, recruit neutrophils, and promote the production of reactive oxygen species (ROS) in neutrophils to induce NET formation[68]. The extracellular vesicles (EVs) derived from the tumor are deemed to be associated with the growth of cancer and modulate the TME and immune function[69].

Aim of the present project

Our study aims to create a unique platform to integrate clinical, biologic, and radiologic data regarding patients with resectable GC. This innovative approach looks at either implementing the data source in resectable GC and mapping the complex interaction among the aforementioned features (nutrition-microbiome-genomics), in order to sharpen the actual precision medicine toward a patient-centric model. Moreover, we plan to address the potential role of NETs in GC development and, eventually, as predictive biomarkers for response to treatments.

Part of the current project was developed and carried out with research funds provided by Istituto Europeo di Oncologia, IEO, Milan.

Methods

Clinical setting

Study work-plan

The MIMETIC trial is an observational, prospective study in patients with resectable GC (including Siewert I) which are candidate to receive either peri-operative treatments or upfront radical surgery followed by adjuvant treatments, if recommended. Potentially eligible patients are diagnosed with localized or LA GC (from stage IB to III according to TNM VIII edition) that will receive pre-operative treatment for a maximum of 2 months with FLOT or weakened regimens (i.e. FOLFOX, CAPOX), according to local practice, followed by surgery (if restaging demonstrates a resectable disease) and, lastly, post-operative chemotherapy (with FLOT regimen or weakened regimens [i.e. FOLFOX, CAPOX, Fluoropirimidine monotherapy] for two months or observation, according to clinical indication.

All patients enrolled are required to perform the following procedures at baseline and during pre-specified timepoints:

- food frequency questionnaire. Past dietary consumption is measured at baseline using the validated and self-administered food frequency questionnaire (FFQ) developed for the European Prospective Investigation into Cancer and Nutrition Italian section (EPIC) study. It records daily intake of foods and nutrients over the previous year.

- clinical assessment. General Physical Examination, Vital Signs, Physical Measurements (temperature, blood pressure, pulse), Demographics and Medical History, Performance Status (according to Karnofsky or ECOG Scale). The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Version 3.0 (EORTC QLQ-C30) is administered at each time point.

- nutritional status. Anthropometric measure (body weight, height and BMI), Nutrition Risk Screening 2002 (NRS-2002), Patient Generated Subjective Global Assessment (PG-SGA), which is a patient-reported instrument for assessment of nutrition status, Prognostic nutritional index calculated as follows: 10 x serum albumin (g/dL) + 0.005 x total lymphocyte count in the peripheral blood repeated 24-hr recalls, SARC-F simple questionnaire.

- blood samples. complete blood hematology, chemistry, and markers (hemoglobin, platelet count, RBC, WBC including differential, creatinine, alkaline phosphatase, ALT, AST, GGT, LDH, total bilirubin, total proteins, HDL cholesterol, albumin, Na+, K+, Ca++, Mg++, aPTT, INR, C-reactive protein, CEA, CA 19.9). Hepatitis B surface antigen (HBVs Ag), and hepatitis C antibody (HCV Ab) or hepatitis C RNA (HCV RNA) are assessed only at baseline. Plasma samples are collected at baseline and stored at the IEO Biobank.

- endoscopy and biopsy. Upper digestive endoscopy with a core biopsy or excisional biopsy are performed to obtain tissue for diagnosis, histological classification, molecular biomarkers (e.g. HER2 status). Six more samples are taken from tumor tissue and surrounding normal mucosa (1 to 3 cm far from tumor site) and are shipped to IEO Campus for microbiota analysis. Endoscopy will be performed again one year after surgery.

- imaging assessment: Staging of tumor - to detect local/distant metastases - is performed with CT thorax and abdomen-pelvis ± FDG-PET, if clinically indicated.

- salivary swab and fecal samples. Kit for at-home fecal and salivary samples self-collection are provided during the visit during the follow up.

- upper digestive ultrasound ± fine needle aspiration of suspected lymph nodes, only if clinically indicated from MB for an accurate assessment of T and N stage at baseline.

- laparoscopy ± washing, only if clinically indicated from MB to rule out occult metastatic disease involving peritoneum/diaphragm.

- surgical procedures. Surgery is performed according to local guidelines and after multidisciplinary consensus. Surgical biopsy is reviewed by an experienced pathologist, and histology should be reported according to the World Health Organization (WHO) criteria. Six more samples are taken from tumor tissue and surrounding normal mucosa and shipped to IEO Campus for microbiota analysis.

Study design is visually represented in Figure 2.

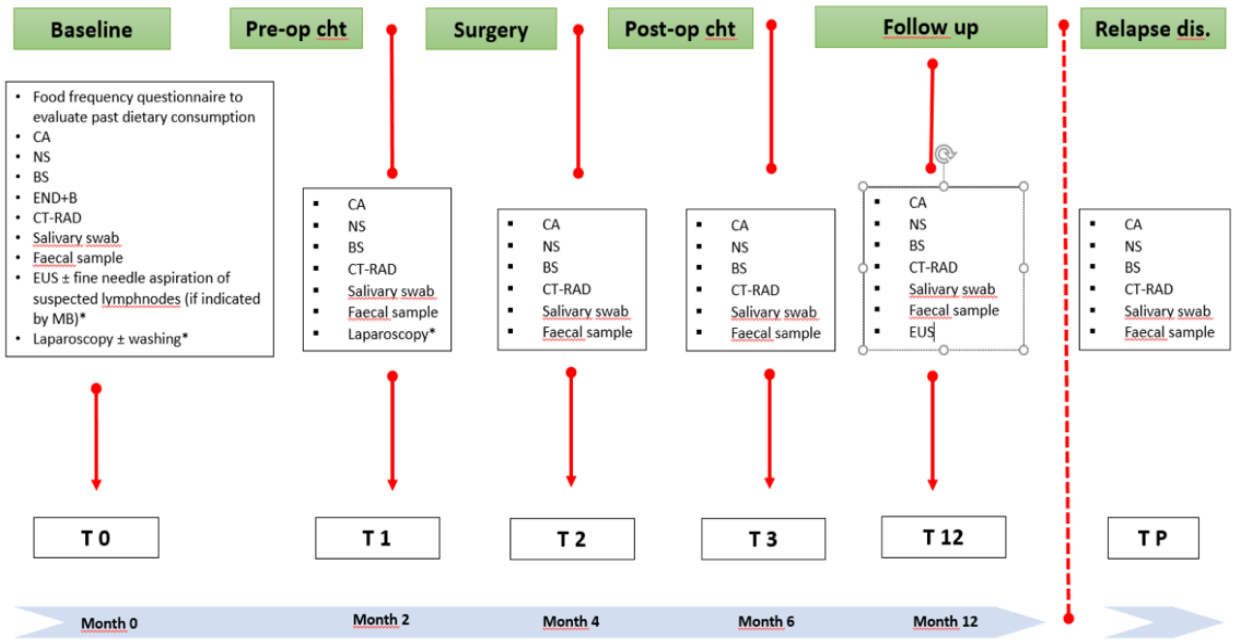


Figure 2. MIMETIC trial design with timepoints and relative collection of samples.

Pathology and molecular analysis

Esophago-gastric junction and gastric adenocarcinoma was classified into intestinal, diffuse, or indeterminate type according to Lauren classification or into tubular, papillary, mucinous and poorly cohesive (including signet ring cell carcinoma), according to WHO 2010 classification.

Molecular analyses were performed at the Division of Pathology of IEO and included MMR status defined on immunohistochemistry (IHC), throughout the use of antibodies against MLH1, MSH2, MSH6, and PMS2 and by PCR analysis ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A, and SULF2 ("IdyllaTM MSI Mutation Assay"). Human epidermal growth factor receptor status was determined by IHC, and samples with HER-2 status IHC 2 + underwent HER-2 FISH analysis for amplification, according to clinical practice. The analysis was carried out on endoscopic (if available) and surgical samples for the neoadjuvant subset of patients, and only on surgical sample for the primary resected subgroup. Programmed death ligand 1 determination was performed by IHC with the 22C3 pharmDX (Dako 22C3) kit. Results were reported as CPS, which represents the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. Final CPS score were presented according to clinical threshold (<1, 1-5, 5-10, >10).

Statistical plan

The MIMETIC study is an exploratory trial, therefore we do not propose *a priori* assumptions and hypotheses on which to base the calculation of sample size. Most of the signal-searching analyses described here will be done primarily to generate new hypotheses. However, since immunologic dysregulation in response to the resident microbiome may lead to tumor growth, we based our hypothesis on the idea that microbiota diversity may be associated with pathological response. Thus, if we assume that high diversity is associated with higher response to treatment, a sample size of 70 achieves 80% power to detect a difference of 20% between the percentage of responses in the two groups, defined by microbiota diversity. The percentage in the group with high diversity was assumed to be 15% under the null hypothesis and 45% under the alternative. The proportion in group with low diversity was assumed to be 15%. The test statistic used is the two-sided Z test with pooled variance. The significance level of the test was targeted at 5%. Descriptive statistical analyses were performed for association with clinical outcomes (significance at p-value<0.05). Survival outcomes including relapse-free survival (RFS) and overall survival (OS) were calculated using Kaplan–Meier method and log-rank tests. Statistical analyses were performed using SPSS software (version 28.0.1.0).

Pre-clinical setting

Microbiota analysis

Sample processing

Fecal samples were processed for 16S sequencing to compare the microbiota structure and composition at baseline and during therapy and between groups of interest.

Briefly, DNA was extracted from feces of gastric patients and healthy donors using the DNeasy PowerSoil Pro kit (Qiagen), after which the V3-V4 region of 16S was amplified. Libraries were prepared following the 16S sequencing library preparation protocol (Illumina) and sequenced by a 2x250 bp paired end chemistry on a MiSeq platform.

16S sequence pre-processing

For each 16S sequencing run, data were filtered and denoised using the DADA2 plug-in[70] in qiime2 (qiime2-2018.11)[71] where parameters were set to trim sequences at the 5' region by the length of the primer, to generate a counts table of amplicon sequence variants (ASVs) for that dataset. Phylogenetic tree reconstruction for downstream diversity analyses was done with the q2-fragment-insertion plug-in[72], using SILVA 128 database[73] as reference sequence. For taxonomy assignment the q2-feature-classifier plug-in was used. Briefly, full-length reference sequences from the SILVA 132 database were downloaded from the SILVA resources page for qiime (https://www.arb-silva.de/download/archive/qiime), after which the sequences were used to train a Na.ve-Bayes classifier using the fit-classifier-na.ve-bayes function[74]. The trained classifier was run on the representative sequences output of DADA2 using the classify-sklearn function to generate taxonomic assignments for each ASV.

16S microbiota analysis

All downstream analyses were performed in R, after exporting the taxonomy table, ASV counts table, phylogenetic tree, and metadata in R and converting into a phyloseq object[75]. Alpha and beta diversity analyses were performed using the vegan package, using counts rarefied by the lowest sequence depth within a given dataset. For computing higher level taxa ratios, raw counts were first aggregated to that level before rarefaction. Group differences based on alpha-diversity and counts ratio were computed by Wilcoxon rank sum test; beta-diversity differences were computed from the distance matrices by PERMANOVA, checking for balance in dispersion by PERMDISP. All reported significant p-values by PERMANOVA were checked to have non-significant dispersion.

NETs determination

The amount of Citrullinated Histone H3 in patients' sera was measured by using the Citrullinated Histone H3 (Clone 11D3) Sandwich ELISA Kit (Cayman, 501620), following the manufacturer's instruction. Briefly, 1:2 diluted sera were incubated with the plate-coated monoclonal antibody specific for citrullinated histone H3[76]. After washing, a second monoclonal antibody (HRP conjugate) was added to

the wells. After performing another wash and adding the HRP substrate TMB followed by the Stop Solution, the plate was read at a wavelength of 450nm.

Results

Clinical and molecular characteristics

Patients' characteristics

In this preliminary report, we included the first 37 patients. Median age of the cohort was 68 years old (IQR 61-73), with 23 (62.2%) males. At the time of diagnosis, seven patients were current smokers, 15 were previous smokers (11 patients stopped less than six months before the diagnosis, four more than six months) and 15 patients never smoked. Four patients had a positive history for HCV infection treated with specific therapy. No patients presented with HBV or HIV infection. In our cohort, 28 patients (75.6%) were taking PPI at the time of enrollment, mainly due to not specific symptoms appeared before the diagnosis of GC. First histology was tubular in 16 cases, poorly cohesive in 11, mixed in 5, papillary in one. In 4 cases the initial subtype was not specified. All patients underwent CT-scan and gastroscopy, while video-laparoscopy was performed in 15 patients (40.6%). In seven cases, FDG-PET/CT was performed to rule out distant metastasis (18.9%). According to clinical stage, 2 patients were diagnosed with stage I disease, 11 with stage II (4 stage IIA and 7 stage IIB) and 24 patients with stage III.

All patients underwent surgical resection. Complete R0 resection was performed in 34 patients (92%), two cases were R1 and one R2. At pathologic staging, 3 patients achieved a pathologic complete response (pCR), 3 cases were pathologic stage I, 17 ones were stage II and 11 were stage III. Additionally, 3 cases were classified IV A for omental/peritoneal diffusion. Cytology on peritoneal lavage was performed in 23 cases with only one microscopic tumor cells. Post-surgical complications occurred in 9 cases (24.3%), 3 in the primary surgery group and 6 in the neoadjuvant group, mainly due to anastomotic leakage. One patient died within 30 days from surgery, for complication related to anastomotic leakage.

Post-operative chemotherapy was delivered in 20 patients (54.5%), 6 patients received exclusively adjuvant chemotherapy (16.2%) and 9 (24.3%) did not receive any systemic treatments neither before nor after surgery, principally because of inadequate clinical conditions. The median follow-up was 20 months. At the data cut off (May 31st 2023), 7 patients had died and 30 were alive.

Molecular features

Molecular profiling was performed on endoscopic samples: 6 cases were defined as mismatch repair deficient (16.2%), and the results were confirmed by PCR as microsatellite instable. Eight tumors out of 37 (21.6%) were classified as HER2 positive. No cases presented both MSI-H and HER2 positive signature. Programmed death ligand 1 results were available for 15 patients (40.5%): 2 cases were negative; 3 cases were CPS positive (1-10) and 10 were CPS positive > 10. The same determinations were performed also on surgical specimens. Six cases were classified as mismatch repair deficient/microsatellite instable, completely consistent between pre- and post- chemotherapy. Seven tumors were defined as HER2 positive (18.9%) with one discordant case. On surgical samples, PD-L1 status was assessed in 28 cases (75.5%): four tumors were CPS negative (14.2%) and 24 were CPS positive (2 CPS 1-4, 7 CPS 5-10 and 15 CPS > 10).

Neoadjuvant cohort

Twenty-two patients (59.5%) received NAC before surgery. The most used regimen was FLOT in 20 cases, while two patients were treated with platinum based- doublet (XELOX). Three patients (13.6%) received anti-HER2 agents together with chemotherapy as part of a clinical trial. The overwhelming majority of patients (95.5%) completed all planned cycle of treatment, only one cases early discontinued the treatment due to toxicity. Eleven patients (50%) experienced at least one G3 toxicity, principally represented by leukopenia, neutropenia, nausea/vomiting, and diarrhea; a dose reduction due to side effects was required in 10 cases (45.5%). After neoadjuvant chemotherapy, one patient achieved a clinical CR (4.5%), 12 a partial response (PR) meaning a reduction in primary tumor dimensions or nodal involvement (54.5%) and 8 (36.4%) a radiologic stability. One patient had a progression of the disease during pre-operative chemotherapy. All patients underwent surgery. A R0 resection was achieved in 20 patients (91.0%), one case was R1 and one R2 (plus CTM positive in the peritoneal lavage). Fifteen patients (68.2%) obtained a downstaging from pre-operative stage with 3 pCR (13.6%), while 7 did not experience any downstaging. Tumor regression grade (TRG) 1 according to Becker was observed in 8 patients, TRG 2 in 6 case and TRG3 in 8. In this cohort, 8 patients did not receive any post-operative

treatment (36.4%), mainly for surgical complications. FLOT was the most used regimen in 11 patients out 14 (78.6%), and 3 patients discontinued the treatment for side effects. Overall, 9 patients out 22 (40.9%) received all pre-planned cycles without any dose reductions.

In the NAC cohort, median RFS was 19.5 months (95% CI 15.7-23.4) and median OS was 24.6 months (95% CI 22.0-27.1). Those resulted statistically significantly longer for patients with tumor downstaging compared to patients without downstaging [RFS: NR vs 8 months (95% CI: 6.17–9.28), log-rank p value = 0.03; OS: NR, log-rank p value = 0.005] (Fig. 3A,B).

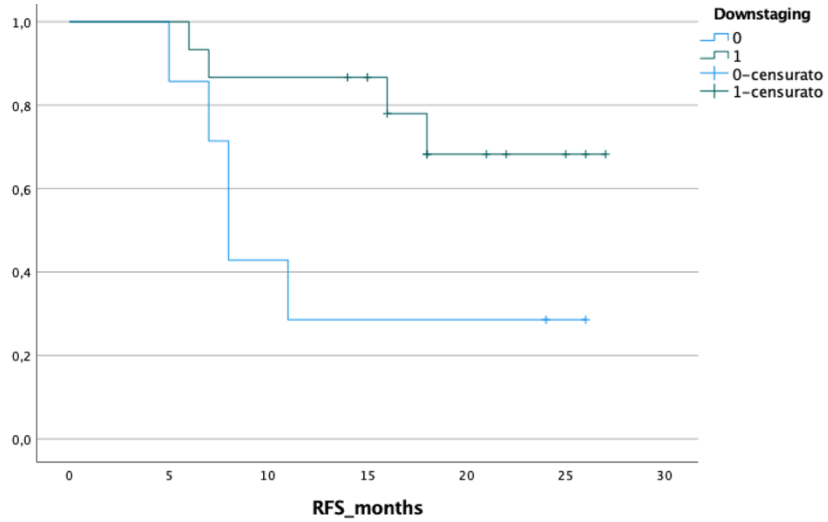


Figure 3A. Recurrence-free survival (RFS) for patients with downstaging vs no downstaging. 0 = no downstaging; 1 = yes downstaging

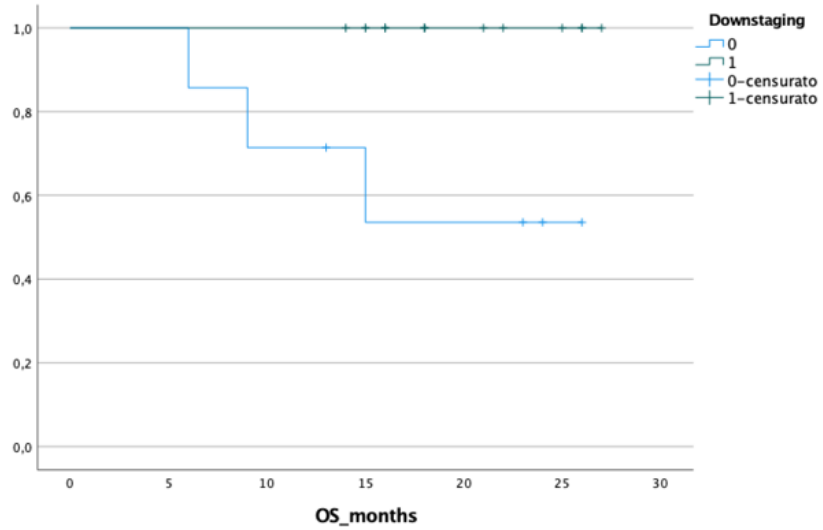


Figure 3B. Overall survival (OS) for patients with downstaging vs no downstaging. 0 = no downstaging; 1 = yes downstaging

According to pCR status, RFS and OS were better for patients achieving pCR compared to patients without pCR [RFS: log- rank p value = 0.23; OS: log rank p value = 0.46] (Fig. 4A,B)

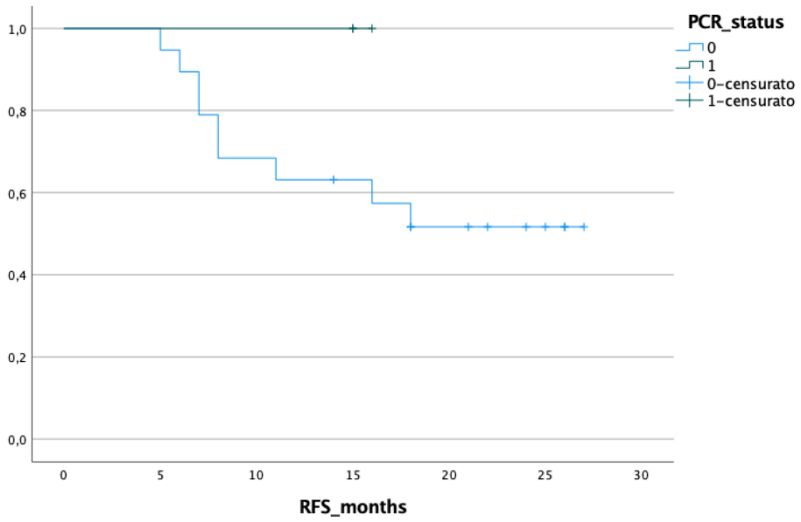


Figure 4A. Recurrence-free survival (RFS) for patients achieving pathologic complete response (pCR) vs no pCR. 0 = no pCR; 1 = yes pCR.

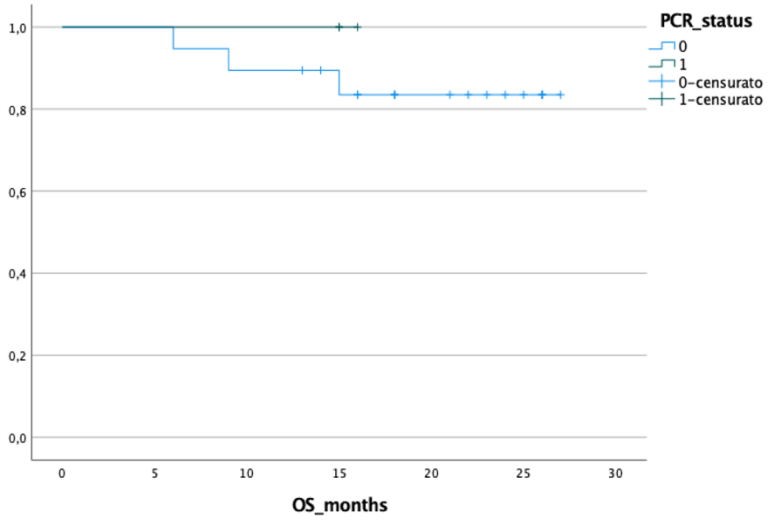


Figure 4B. Overall survival (OS) for patients achieving pathologic complete response (pCR) vs no pCR. 0 = no pCR; 1 = yes pCR.

Survival analysis

Considering the entire population, mean EFS was 20.3 months (95% CI 17.3-23.4) and mean OS was 23.3 months (95% CI 20.7-25.8). Fig.5 and fig. 6.

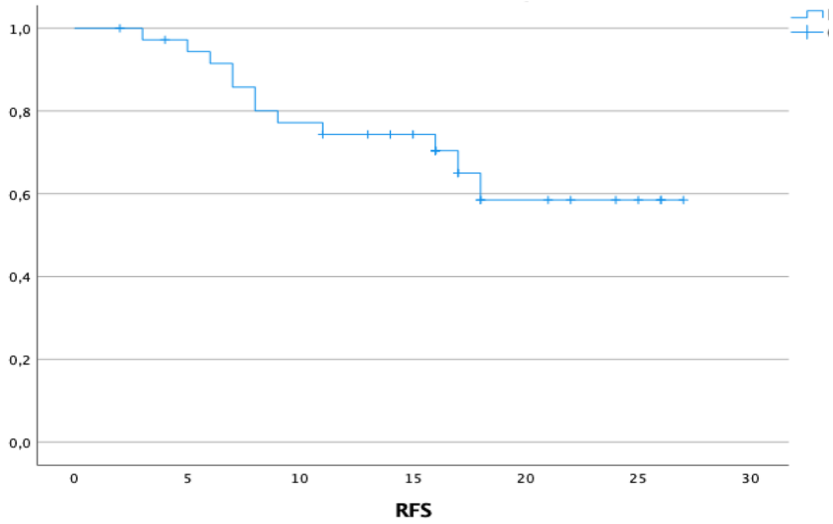


Figure 5. Recurrence-free survival (RFS) the entire population.

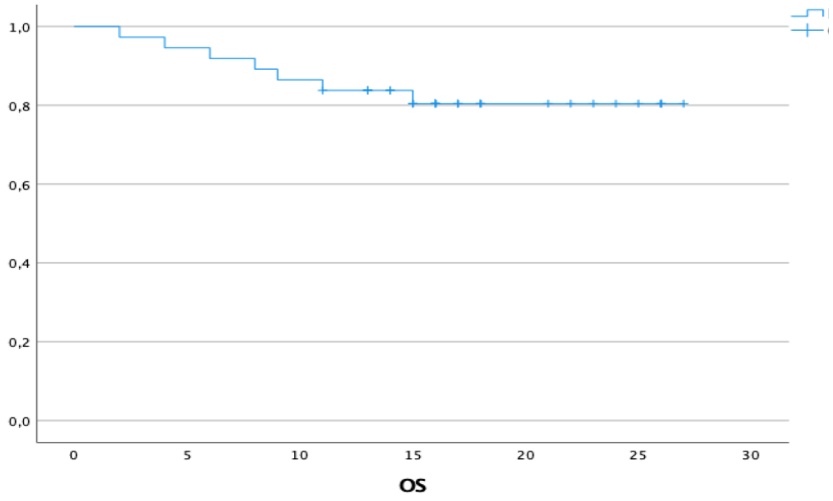


Figure 6. Overall survival (OS) for the entire population.

According to molecular profiling, MSI-H subgroup experienced shorter RFS and OS compared to MSS [RFS: 8 months (95% CI 1.9-14.0) vs NR, log-rank p value = 0.027; OS: 15 months (95% CI 3.2-26.7) vs NR, log-rank p value = 0.043]. Fig 7A,B

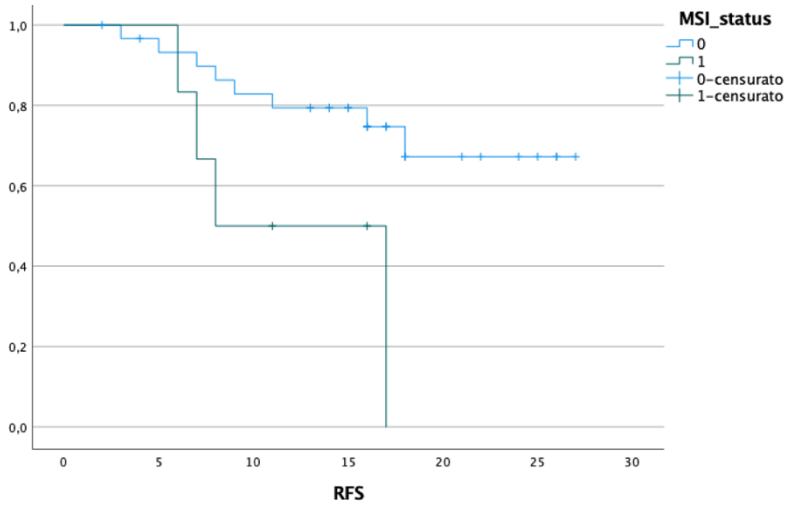


Figure 7A. Recurrence-free survival (RFS) for the entire population according to MSI status. 0 = MSS; 1 =MSI

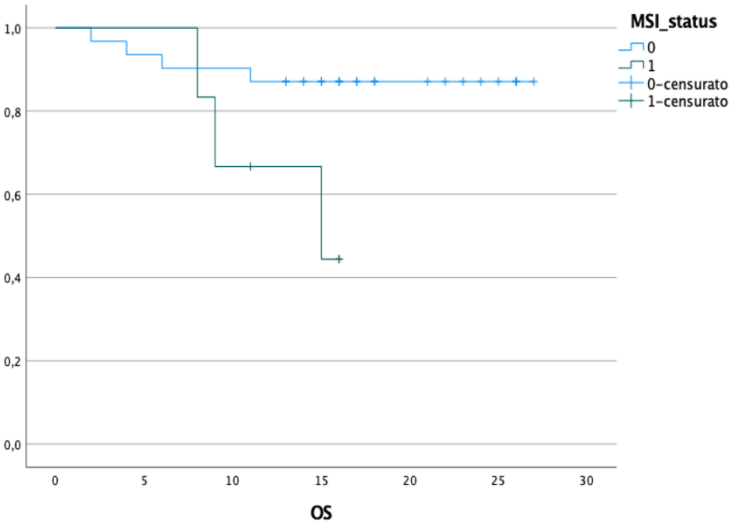


Figure 7B. Overall survival (OS) for the entire population according to MSI status. 0 = MSS; 1 =MSI

Moreover, no statistical differences were observed in RFS and OS according to HER2 status [RFS: log-rank p value = 0.27; OS: log-rank p value = 0.58]. Fig. 8A,B

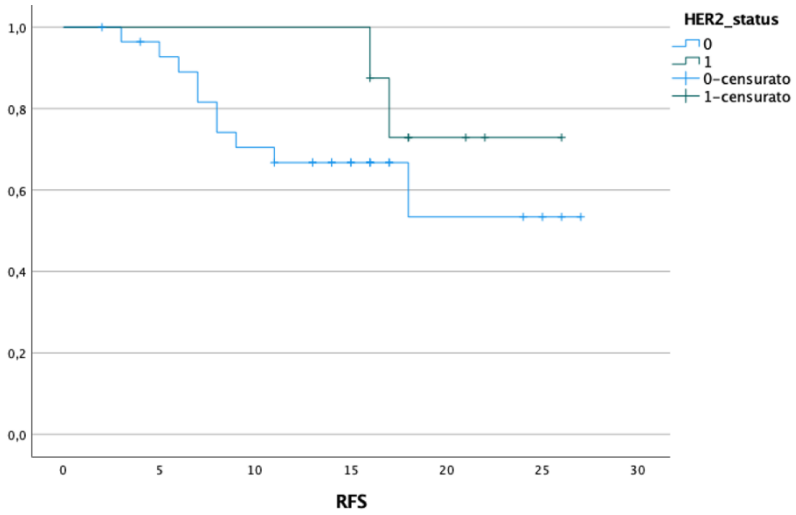


Figure 8A. Recurrence-free survival (RFS) for the entire population according to HER2 status. 0 = HER2 negative; 1 = HER2 positive

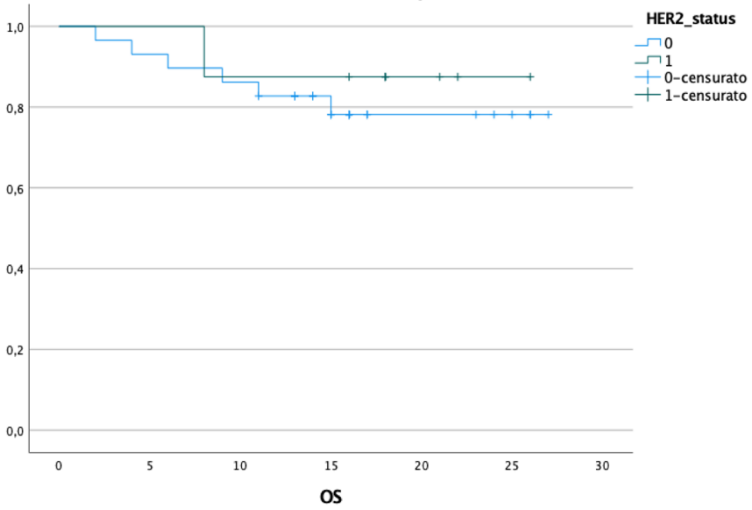


Figure 8B. Overall survival (OS) for the entire population according to HER2 status. 0 = HER2 negative; 1 = HER2 positive

Nutritional assessment

Thirty-three out of 37 patients (89.2%) underwent nutritional assessment. Median body weight was 72.1 kg (47.9 - 98.1, IQR 65.5 – 81.7) and the median Body Mass Index (BMI) was 27.3 (24.5 – 28.4). The MUST score (Malnutrition Universal Screening Tool) was 0 for 20 patients (60.6%), 1 for 7 patients (21.2%) and 2 for 6 ones (18.1%). All patients were < 4 according to the SARC-F score. In the overall population, the median percentage of daily calories intake was 69% (IQR 60% - 80%) with a median recall of daily calories of 1472 Kcal (IQR 1020-1658 Kcal) and a median ratio of Kcal/Kg of body weight of 20 (IQR 17-24). The median percentage of protein intake was 58% (IQR 46 – 66). The median recall of daily protein assumption was 60 g (IQR 44 – 75), with a ratio of protein/Kg of weight of 0.9 g (IQR 0.7 – 1.0). The median serum albumin level was 4.0 g/dl (3.4 – 5.0, IQR 3.8 – 4.2) with 3 missing.

We correlated survival outcomes to nutritional parameters. No statistical differences were observed for RFS and OS according to BMI, nor high vs low (with respect to median value) [RFS log rank p value = 0.60; OS log rank p value = 0.38] nor < 25 vs 25-30 vs > 30 [RFS log rank p value = 0.45; OS log rank p value = 0.82]. (Figure 9A,B). Similarly, RFS and OS did not differ if analyzed for MUST score [RFS: log rank p value = 0.79; OS log rank p value = 0.44]. Even clustering MUST score in 0 vs 1-2, no statistical differences were observed [RFS log rank p value = 0.65; OS log rank p value = 0.69] (Figure 10A,B).

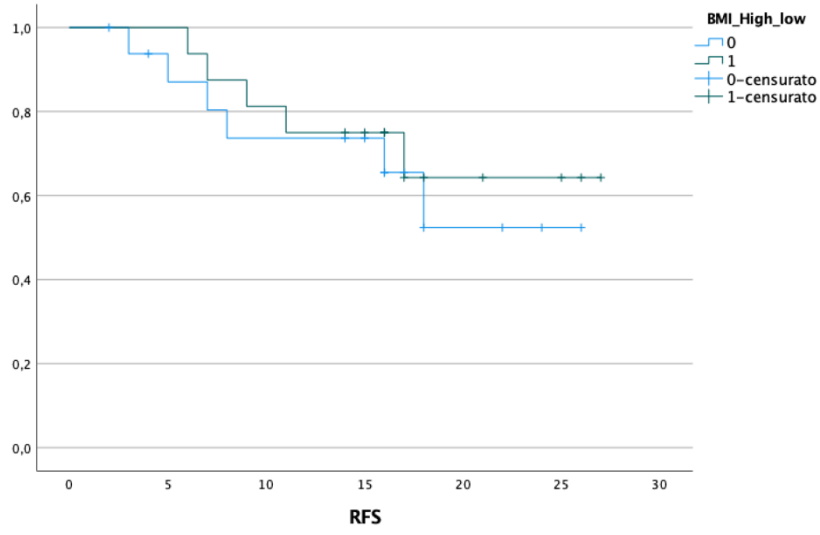


Figure 9A. Recurrence-free survival (RFS) according to BMI high or low. 0 = BMI lower median value; 1 = BMI above the median value.

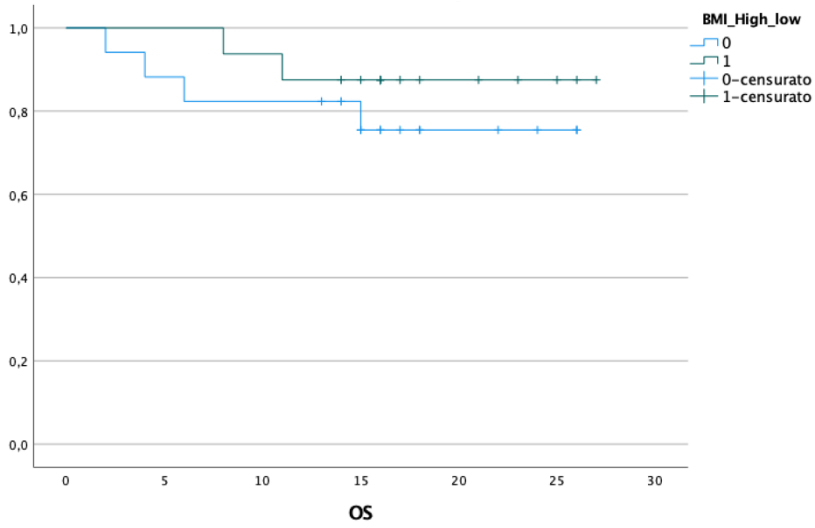


Figure 9B. Overall survival (OS) according to BMI high or low. 0 = BMI lower median value; 1 = BMI above the median value.

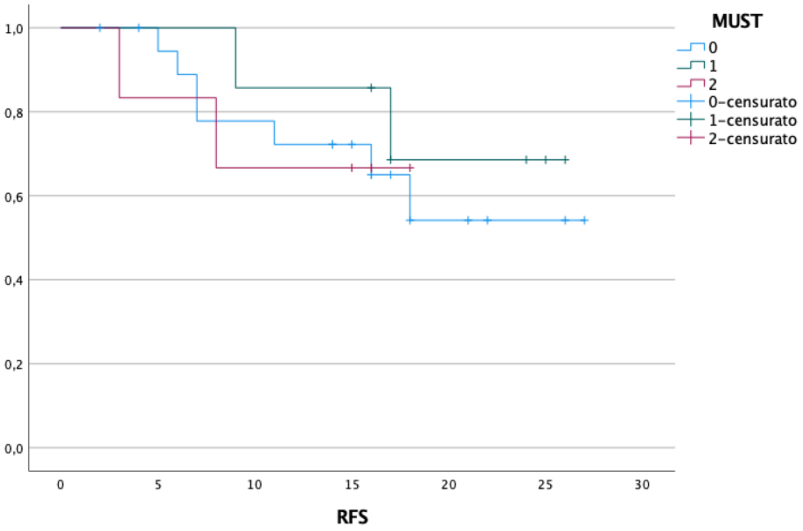


Figure 10A. Recurrence-free survival (RFS) according to MUST score. 0 = MUST score 0; 1 = MUST score 1; 2 = MUST score 2.

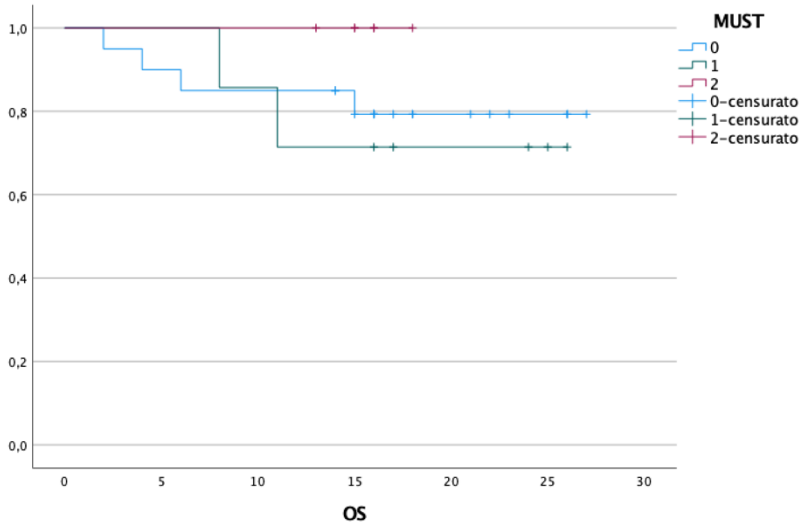


Figure 10B. Overall survival (OS) according to MUST score. 0 = MUST score 0; 1 = MUST score 1; 2 = MUST score 2.

Microbiota analysis

We compared fecal microbiota obtained from the first 35 GC patients with a cohort of healthy subjects (HS) collected at our Institution. We performed several diversity measures which include alpha-diversity (absolute diversity), beta-diversity (relative diversity) and differential abundance. We also compared patients within the two cohorts (neoadjuvant vs primary surgery) for the same measures.

Main taxa associated with healthy/gastric cancer at baseline and alpha-diversity metrics are reported in figure 11 and 12. When comparing GC patients and HS, we observed a significant difference in terms of Beta-diversity (Unweighted/Weighted UniFrac) and in the Firmicutes/Bacteroidetes (F/B) ratio, as shown in figures 13 a-c

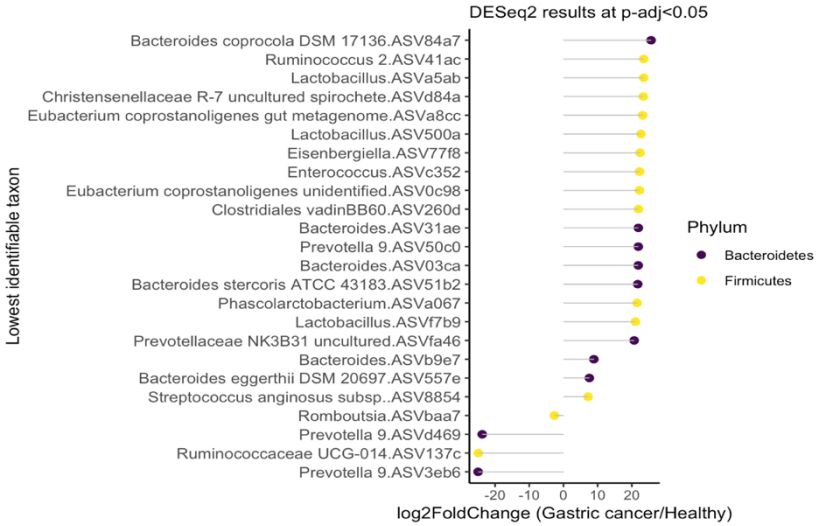


Figure 11. Taxa associated with HS and GC fecal samples at baseline.

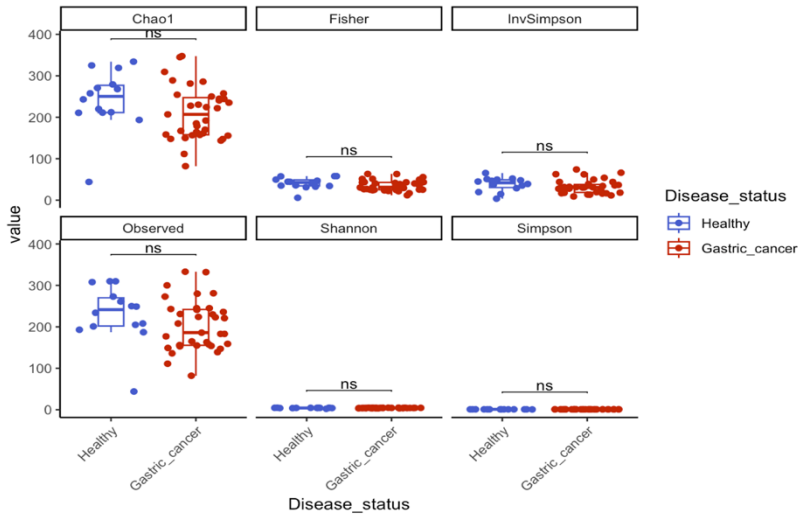


Figure 12. Alpha diversity of fecal samples at baseline.

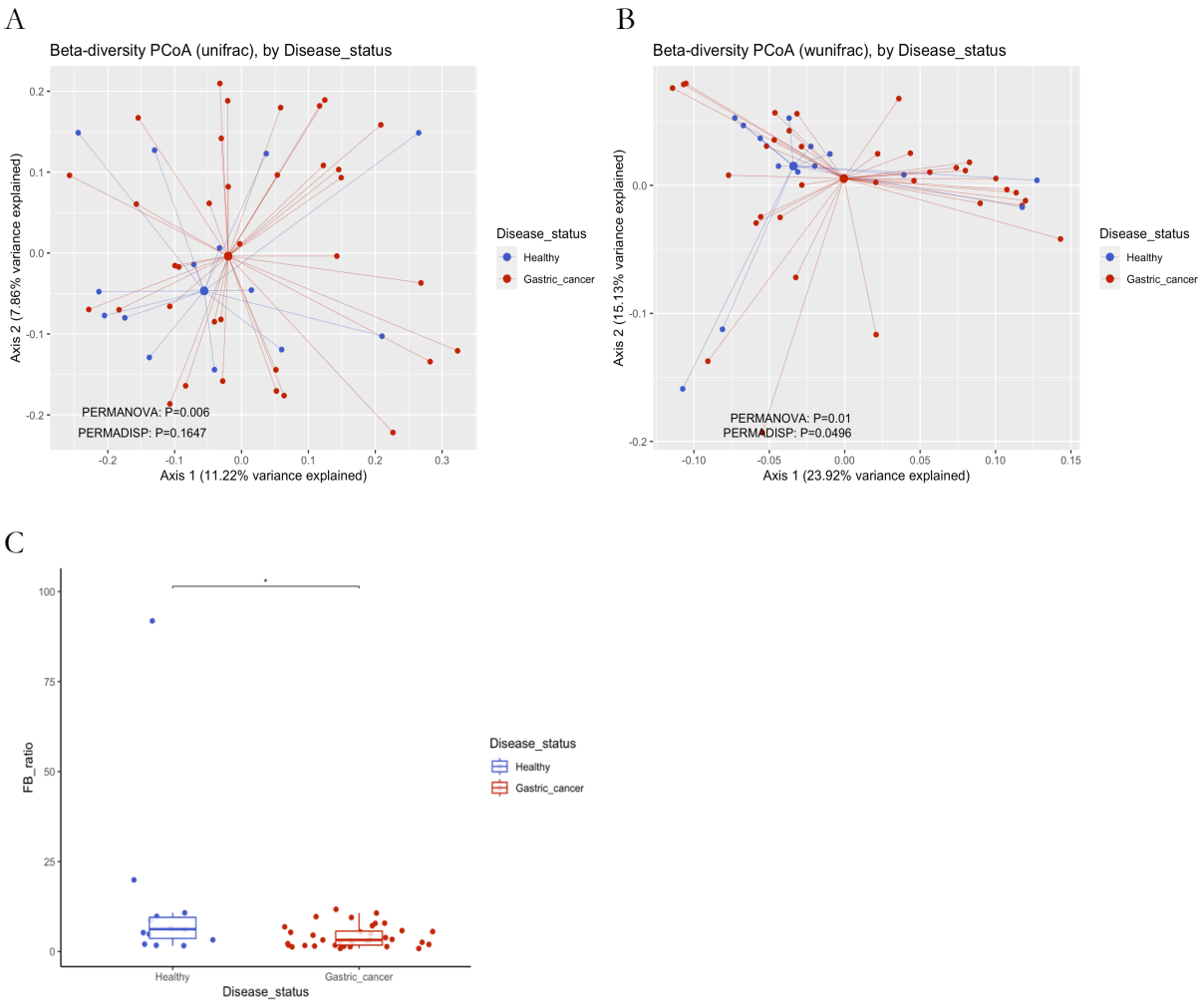


Figure 13 A-C. Beta-diversity metrics (unifrac, wunifrac) and Firmicutes/Bacteriodes (F/B) ratio for GC patients compared to HS at baseline.

Similarly, a significant difference was maintained after therapy at T1 in Beta-diversity, all metrics. However, no difference was observed between HS and GC at T1 in terms of F/B ratio. (Figure 14, A-D and 15).

A B

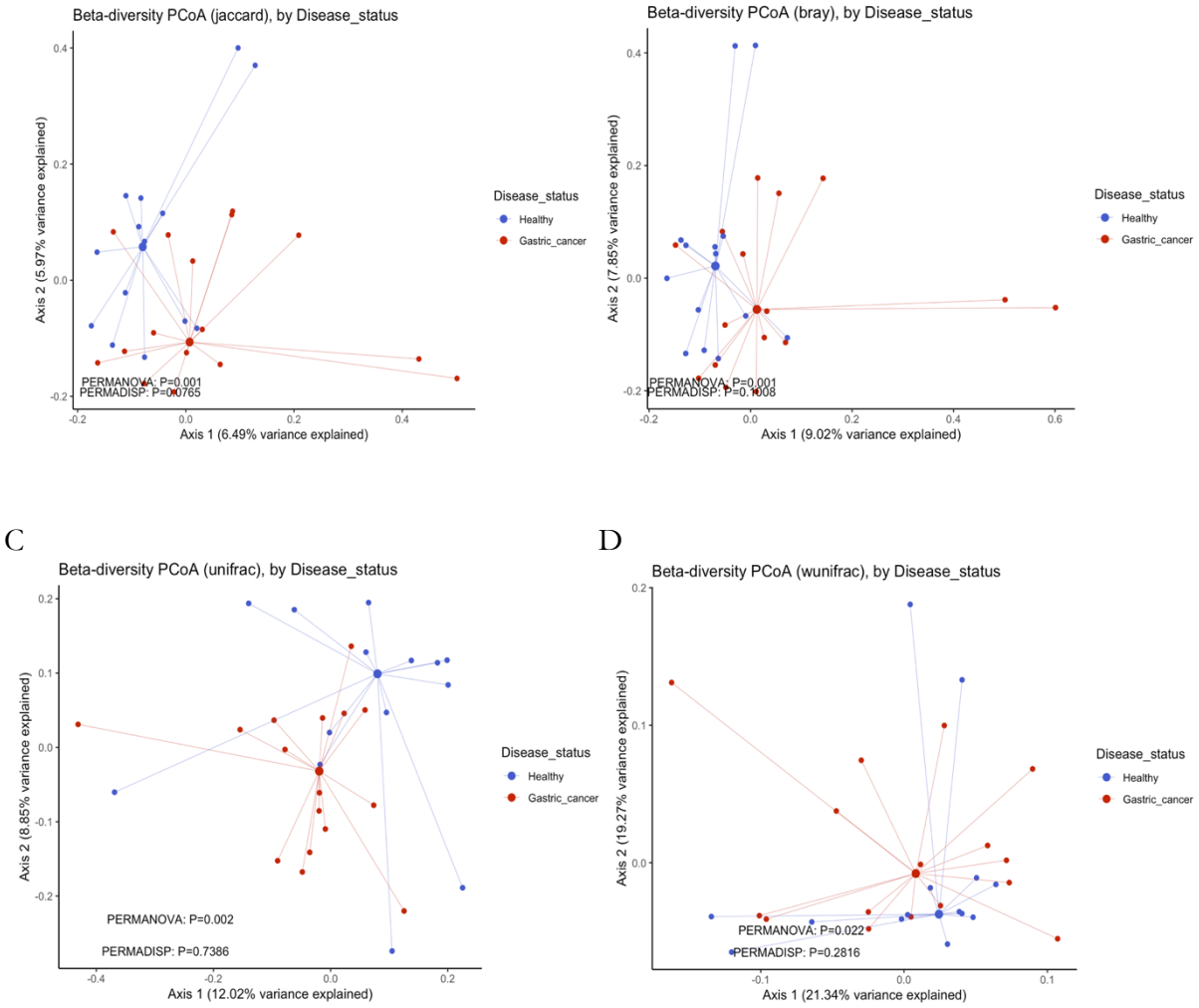


Figure 14 A-D. Beta diversity metrics for GC patients compared to HS at T1. A: Jaccard; B: Bray; C: unifrac; D: wunifrac.

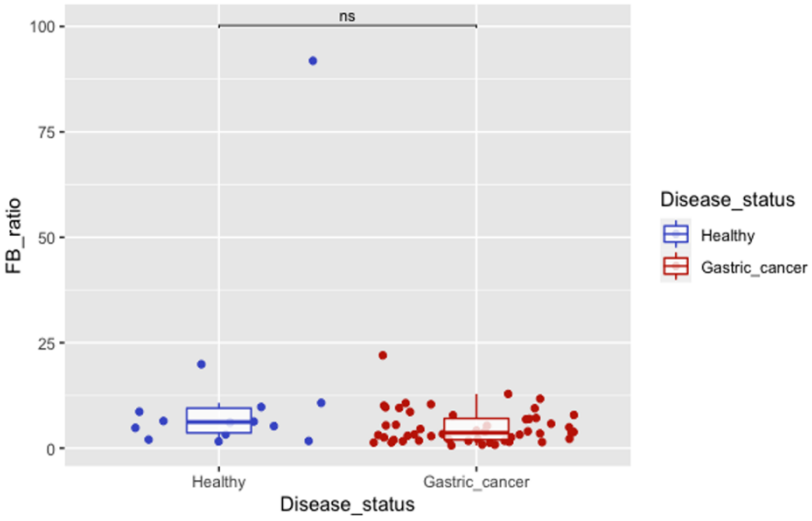
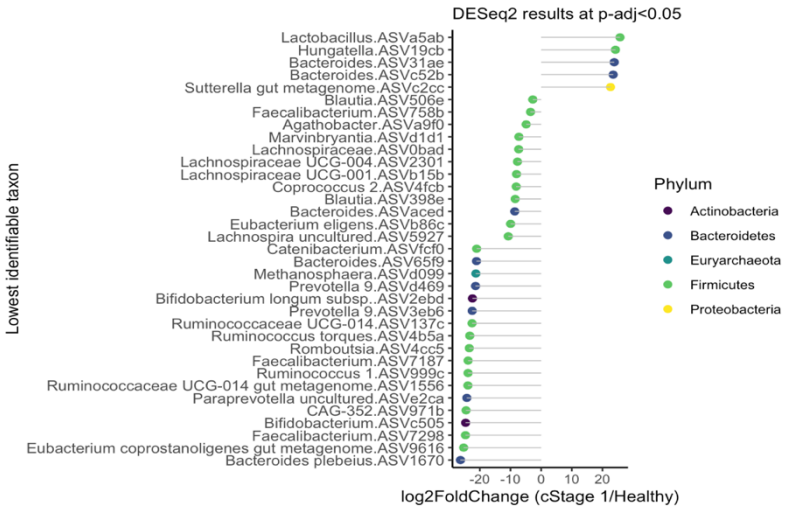


Figure 15. Firmicutes/Bacteriodes (F/B) ratio for GC patients compared to HS at T1. No significant differences were observed.

We assessed microbiota composition according to clinical stage. Differential abundance at baseline for HS/stage I and HS/stage II-III are reported in figure 16A,B. We observed a significant difference at baseline for both stage I and stage II-III vs Healthy (beta-diversity- all metrics, Figure 17 A-H). On the contrary, the F/B ratio was significantly different at baseline only for clinical stage II-III compared to HS (Figure 18A,B).

A



B

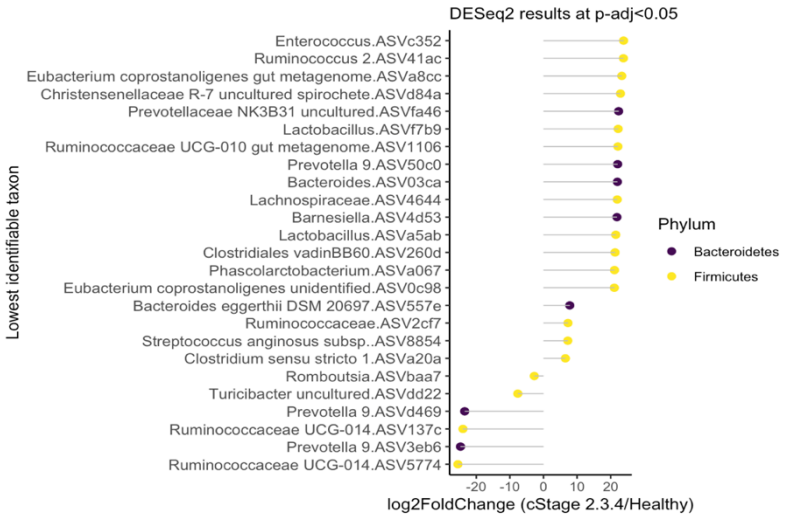
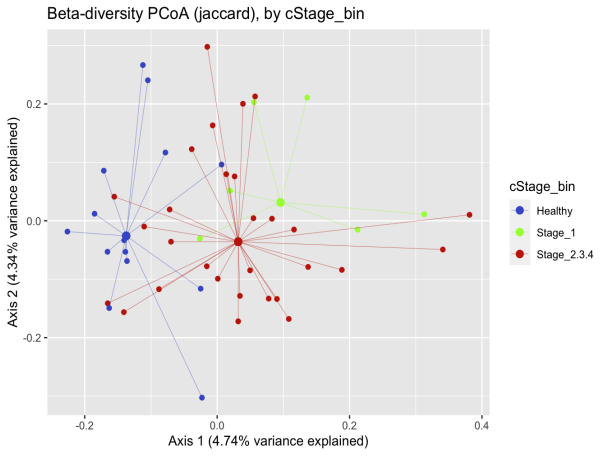
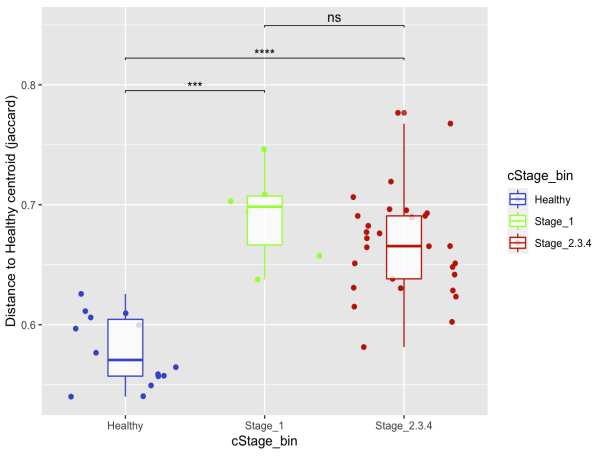


Figure 16 A,B. Taxa associated with GC patients compared to HS according to different baseline clinical stages. A: taxa for HS/GC at stage I; B: taxa for HS/GC at stage II-III

A

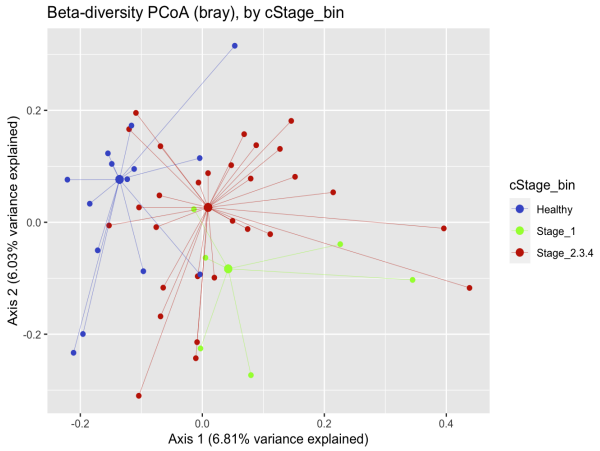


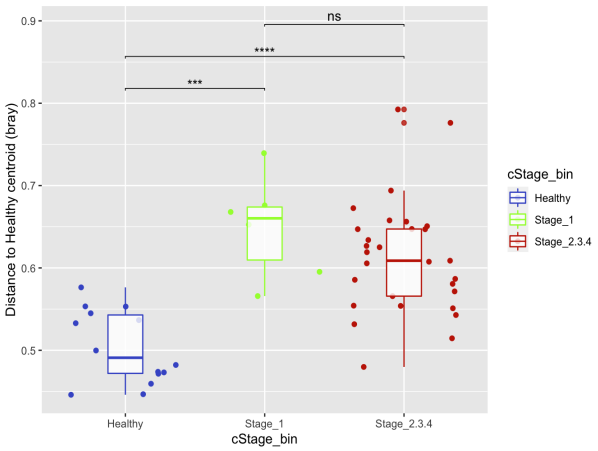
B



C

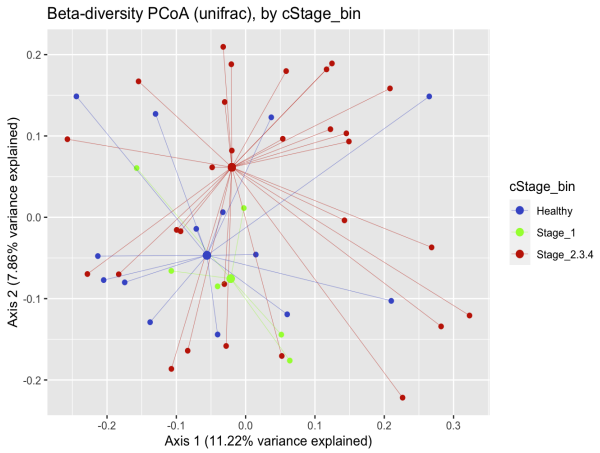
D

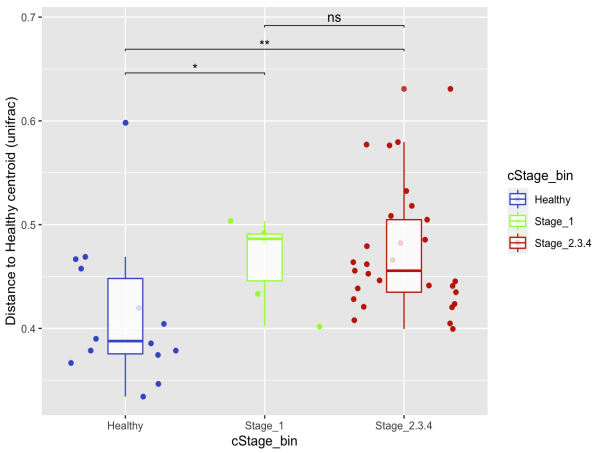

Beta-diversity PCoA (bray), by cStage_bin
Axis 2 (6.03% variance explained)
Axis 1 (6.81% variance explained)
cStage_bin
Healthy
Stage_1
Stage_2.3.4


Distance to Healthy centroid (bray)
ns
****
***
Healthy
Stage_1
Stage_2.3.4
cStage_bin

E

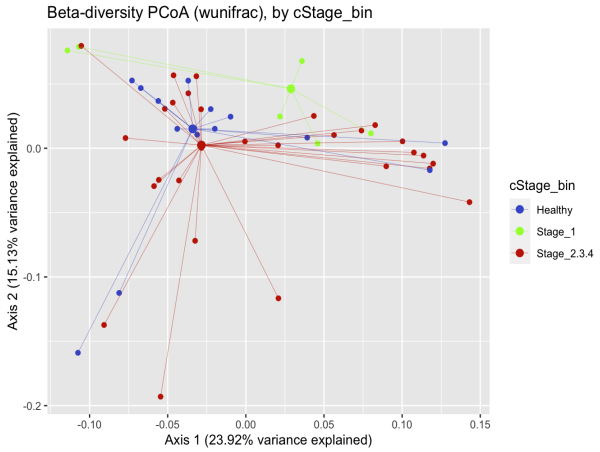
F


Beta-diversity PCoA (unifrac), by cStage_bin
Axis 2 (7.86% variance explained)
Axis 1 (11.22% variance explained)
cStage_bin
Healthy
Stage_1
Stage_2.3.4


Distance to Healthy centroid (unifrac)
ns
**
*
Healthy
Stage_1
Stage_2.3.4
cStage_bin

G

H


Beta-diversity PCoA (wunifrac), by cStage_bin
Axis 2 (15.13% variance explained)
Axis 1 (23.92% variance explained)
cStage_bin
Healthy
Stage_1
Stage_2.3.4

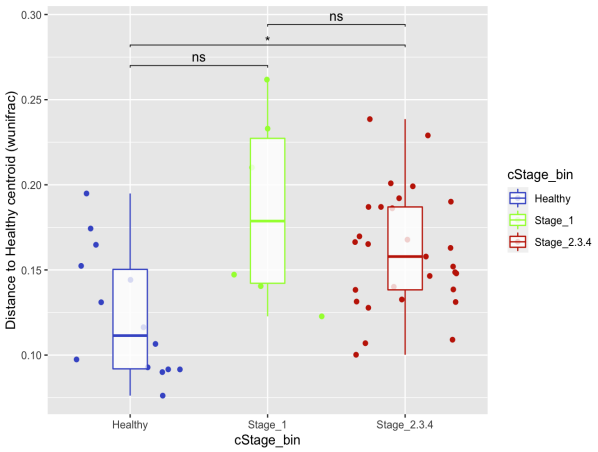

Distance to Healthy centroid (wunifrac)
ns
*
ns
Healthy
Stage_1
Stage_2.3.4
cStage_bin

Figure 17A-H. Beta-diversity metrics for GC and HS at baseline and at T1, according to clinical stages. A: jaccard; B: distance to healthy centroid by jaccard; C: bray; D: distance to healthy centroid by bray; E: unifrac; F: distance to healthy centroid by unifrac; G: wunifrac; H: distance to healthy centroid by wunifrac.

A B

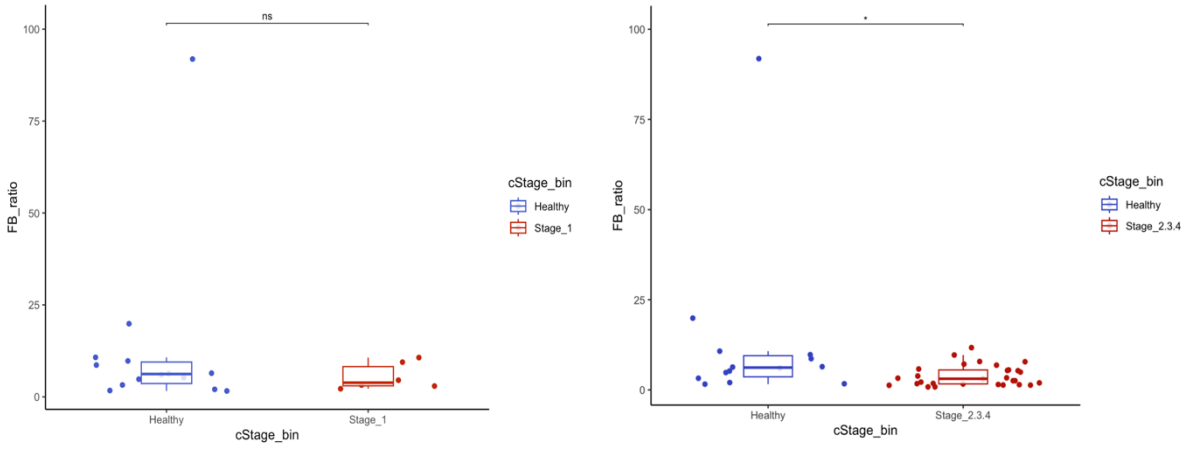


Figure 18A,B. Firmicutes/Bacteriodes (F/B) ratio comparing HS to GC according to clinical stages. A: F/B ratio HS vs GC stage I; B: F/B ratio HS vs GC stage II-III.

Focusing only on GC patients, the overall population included 15 patients in the primary surgery cohort and 20 in the NAC cohort. Among the top taxa observed in the samples, the most dominant are Ruminococcus and Parabacteroides merdae based on median relative abundance across samples (figure 9M). In both cohorts, S. Anginonus was one of the dominant taxa (Figure 19), even though it ranked #15 in terms of median relative abundance (0.0028261361067149 vs 0.0184137489325363 for Parabacteroides merdae).

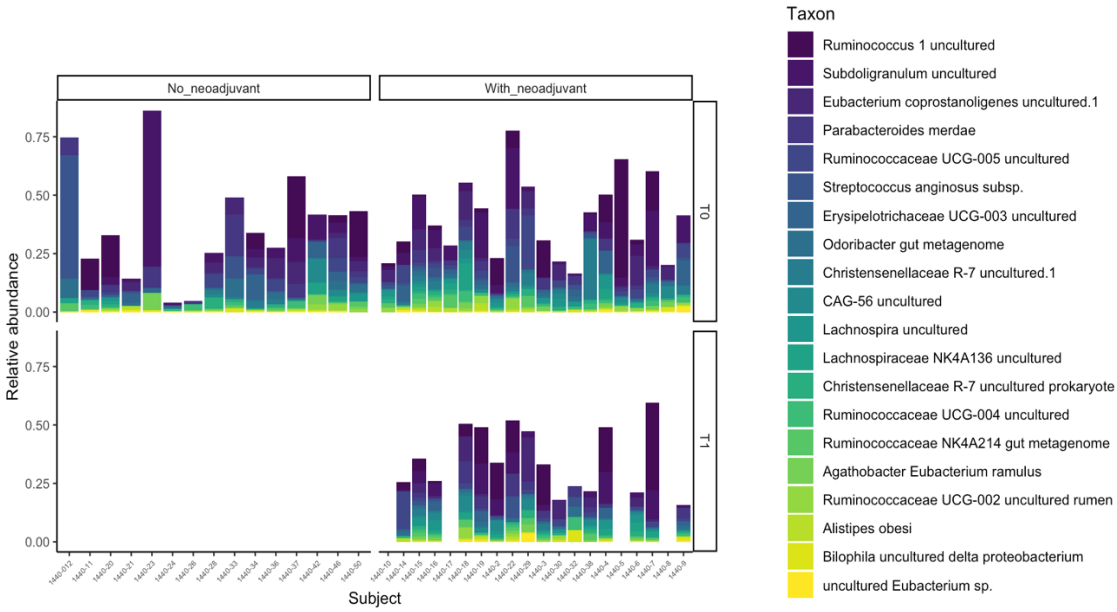


Figure 19. Taxa barplots of GC patients. Left column indicated patients treated with primary surgery, right column patients treated with NAC.

We evaluated also differences in microbiota between patients receiving NAC and patient treated with primary surgery (PS). Baseline differential abundance for NAC/PS are shown in figure 20.

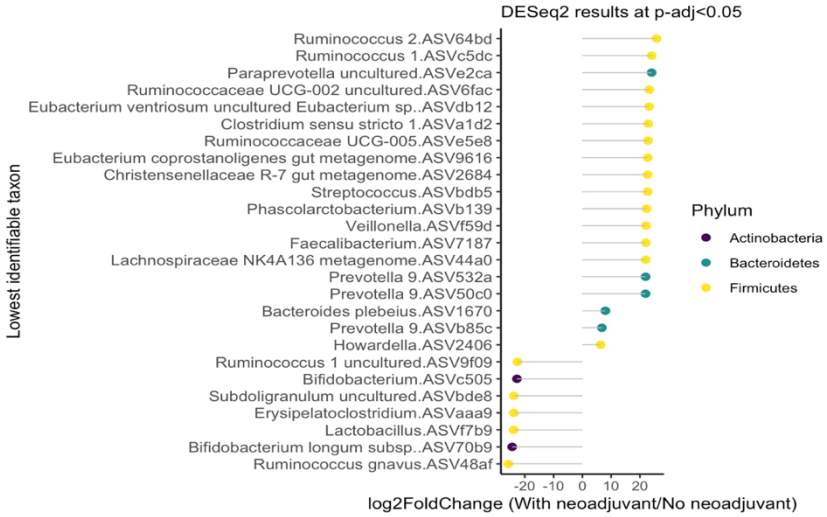


Figure 20. Taxa associated with fecal samples of NAC/PS

In this context, we observed a significant difference in some alpha-diversity metrics and in unweighted and weighted UniFrac (figure 21 and 22A,B).

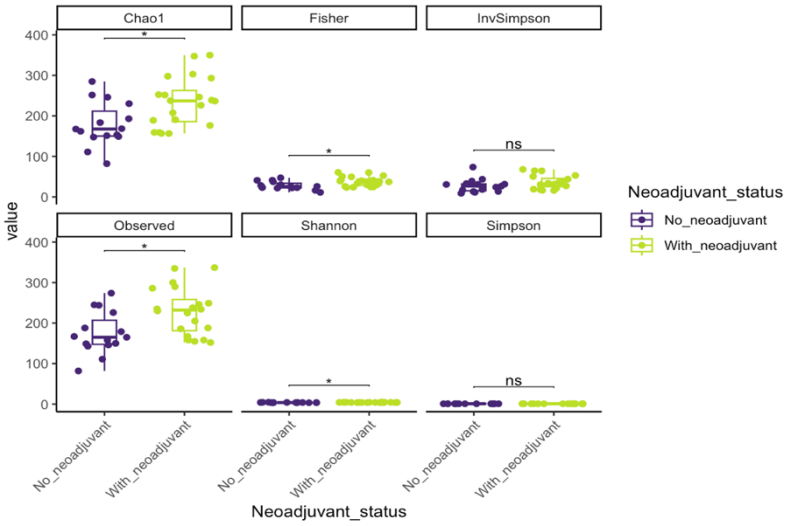


Figure 21. Alpha diversity metrics differences for patients who received NAC or primary surgery.

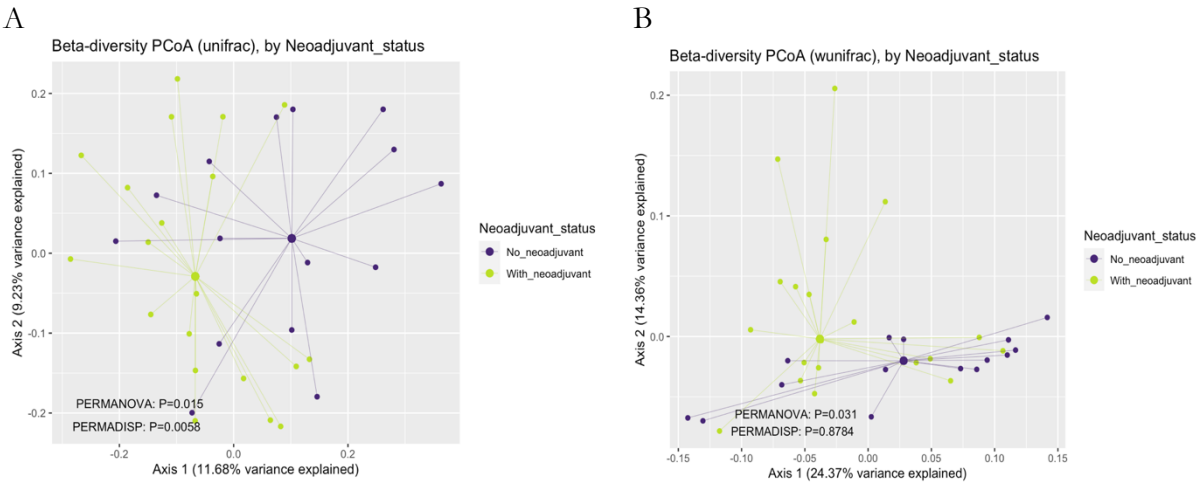


Figure 22A,B. Beta diversity metrics for patients who received NAC or primary surgery. A: unifrac; B: wunifrac.

Lastly, we tested the potential impact of a PPI use on fecal microbiota (figure 23). A significant difference in term Beta-diversity (Weighted UniFrac) and F/B ratio was seen between PPI-users vs PPI-non-users at T1 (figure 24A,B).

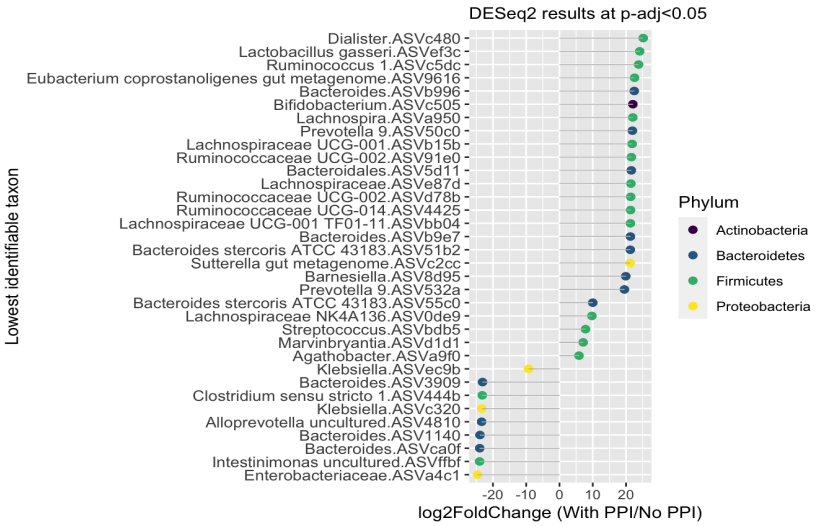


Figure 23. Taxa observed in GC patients according to PPI use (yes/no).

A B

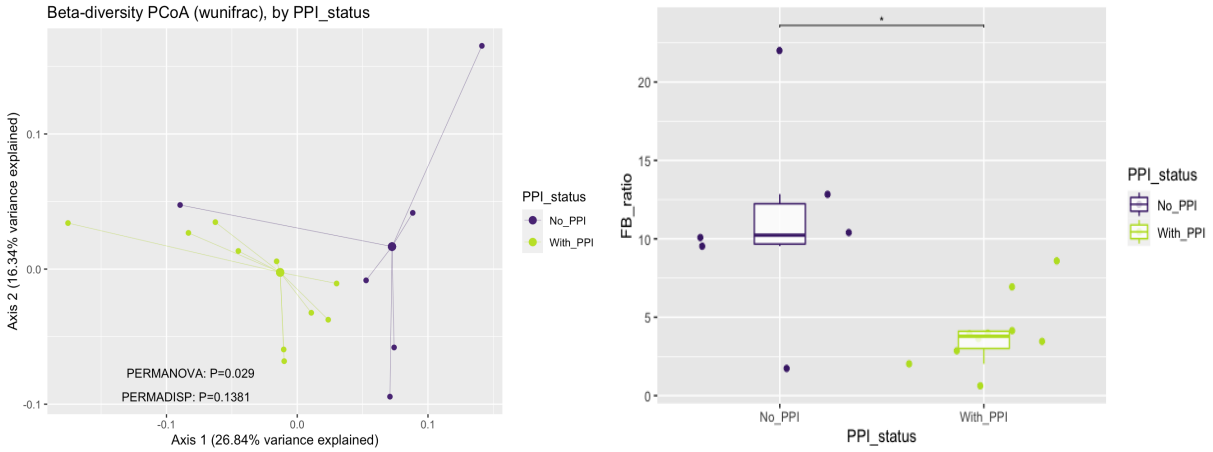


Figure 24A,B. Beta diversity and F/B ratio for GC patienbts according to PPI use (yes/no). A: wunifrac; B: F/B ratio was significant lower in PPI users compared to no-PPI subgroup.

NETs analysis

Using the sample cohort of enrolled patients, we performed an exploratory analysis trying to assess NETs level on serum. The determination of NETs was done in a limited subgroup of patients at different timepoints. Serum quantification of NETs was obtained at baseline (T0), at the end of pre-operative chemotherapy (T1), after surgery (T2) and at progression (PD). Median concentration at baseline was 4.73 ng/ml. Results from the first 15 patients showed that cit-H3 levels change across the different timepoints, following the course of the disease. In seven cases with no evidence of disease, NETs levels were lower in subsequent timepoints compared to baseline. In two patients who experienced tumor relapse, cit-H3 levels at progression were higher than previous timepoints, suggesting a potential relationship with tumor burden. Conversely, in one patient (1440-39) NETs levels were lower at the time of relapse compared to a previous timepoint (figure 25).

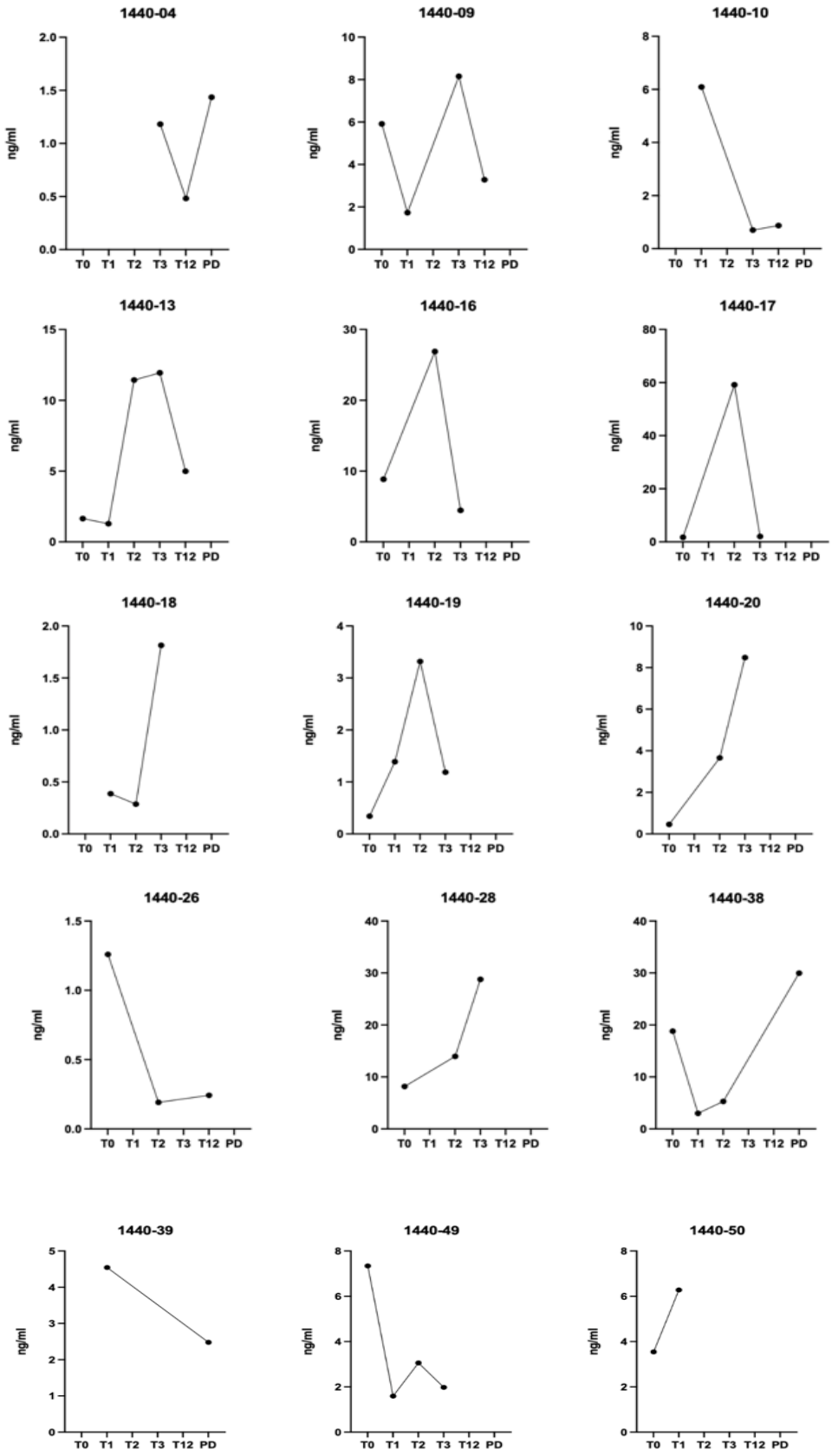


Figure 25. Citrullinated Histone H3 (cit-H3) quantified by ELISA at different timepoint in 15 patients enrolled in the MIMETIC trial.

Discussion

In the MIMETIC study, we prospectively established a multi-omic platform for LAGC patients and investigated the interaction between molecular, nutritional and microbiome characteristics and clinical outcomes. In this preliminary report, we observed a relative abundance of S. Anginosus in GC fecal samples compared to healthy subject and higher rates of MSI-H and HER2 tumors compared to historic controls, which deserve further investigation. In addition, the analysis of serum cit-H3 levels seems to be correlated with tumor burden and may represent a potential tool to be addressed. Larger sample size and longer follow up are needed to draw more solid conclusions.

Gastric cancer remains a lethal disease, with percentages of survival at 5 years for localized disease that do not reach 30%. Progresses in molecular biology of GC have been done through the last decades and currently molecular biomarkers such as MSI-H, PD-L1 and HER2 are crucial for metastatic disease. However, no conclusive data are still mature to be used in clinical practice to guide the treatment selection in the locally advanced setting. Our 16% rate of MSI-H resectable LAGC is remarkable, compared to current knowledge, which describe a prevalence for MSI-H subgroup in GC patients undergoing upfront surgical treatment of less than 10%[77]. Similarly, HER2 positivity rate was around 20%, which is in line with percentage expected for metastatic disease, but higher than previous report in the same setting[78]. These results should be re-affirmed in the complete population of the trial. According to molecular features, MSI-H is an established positive prognostic factor in GC, and in this specific subgroup, the addition of peri-operative or adjuvant chemotherapy to surgery did not result in a benefit in terms of OS, according to a post-hoc analysis from randomized controlled studies[23]. The detrimental impact of chemotherapy on the immune system, which is very active in MSI-H phenotypes and could inhibit residual micro-metastasis, is one rationale that has been suggested. In our cohort, MSI-H patients treated with NAC experienced the worst clinical outcomes, even though the number of cases is very low, and therefore data should be intended as hypothesis generating. In the NAC cohort of our study, two third of the population experienced a pathologic downstaging, with a 13% of pCR. Pathologic CR after NAC is a reliable prognostic surrogate for survival in several cancer, including GC[79]. However, while we did

not display any statistical differences in survival according to pCR status (mostly attributable to the paucity of patients), a significant survival benefit in OS and EFS has been observed in patients achieving downstaging after chemotherapy, compared to those without downstaging. A longer follow up will be crucial for the identification of subgroups of patients who could benefit the most from peri-operative management.

It is well established that malnutrition increases patients' morbidity and mortality in many different cancers[80]. Numerous best practice guidelines, propose routine screening for individuals with gastrointestinal cancer using validated techniques to efficiently diagnose and treat malnutrition. In our study, patients have been evaluated at baseline by the MUST score and treated accordingly, with around 20% of the total having a score of 2, which indicates a high risk of malnutrition. According to the different definitions of malnutrition and different assessments, the prevalence in GC patients ranges from 20 to 80% and it represent a detrimental factor for survival[81]. In our dataset, a higher risk of malnutrition at baseline did not correlate with significant worst clinical outcomes and confirm the strong added value of an early nutritionist referral, before starting any anti-cancer treatment.

One of the cornerstones of our study is the analysis of microbiota. We analyzed fecal samples from GC and HS at baseline and after therapy and we observed a significant difference in terms of composition, with an enrichment in S. Anginosus in GC. This result is consistent with previous literature evidence[50, 82] S. Anginosus is a Gram positive anaerobe firstly discovered in 1998 in GC tissue[83] and its abundance is typically observed in GC tissue, and is not affected by the location of the tumor and the microecological environment. Studies on the microorganisms associated with GC have also been conducted on the oral cavity and intestine, and Streptococcus has been identified in all three sites, suggesting a potential significant role in of GC. Furthermore, a recent work tested the determination of S. Anginosus (together with S. Constellatum) in stool samples as prevention tool[82]. These results are in line with our preliminary report, that should be confirmed. We tried to describe conceivable difference in microbiota according to clinical stage. We observed a difference in the Firmicutes/Bacterioides (F/B) ratio, which is significantly lower at baseline of clinical stage II-III patients as compared to HS. Instead, F/B ratio of clinical stage

patients does not change when compared to HS. The F/B ratio is known to play a potential effect on gut homeostasis preservation and its imbalance is observed in several diseases, as obesity and inflammatory bowel disease (IBD) and cancers[84]. Similarly, a lower F/B ratio was observed in patients with breast cancer, when compared to HS[85]. With the caution related to low numbers, our results are consistent and deserve to be pursued, to assess the implication of F/B ratio modification in gastric cancer development and prognosis. We also evaluated the impact of PPI use on fecal microbiota, and we detected an enrichment in *Lactobacillus Gasseri*, and *Ruminococcus*, among other taxa. The use of PPI has been associated with a higher risk factor of GC development due to hypergastrinaemia, which may induce hyperplasia, even though studies assessing this association suffer from methodologic bias. Currently, results are still not univocal, since a population-based cohort study showed a HR 1.45 (95% CI 1.06 to 1.98) compared to H2Ras[86], while a recent metanalysis found no association between PPIs and GC after adequate control for confounding factors[87]. In our dataset *Lactobacillus Gasseri* was one of the most abundant taxa in PPI users and similar results have been presented by Hovo and colleagues which show an increase in *Lactobacillus* including *L. gasseri*, *L. fermentum*, the *L. reuteri* subgroup, and the *L. ruminis* subgroup after 4 and 8 weeks of treatment[88]. Additionally, we did not observe any significant differences in term of alpha diversity, in line with previous reports[86]. Differences in microbiota composition according to PPI use should be further explored, together with the relationship with response to treatments.

Lastly, we performed NETs determination on serum of GC patients. We observed a trend to decreased level of NET-citH3 after chemotherapy compared to baseline. Conversely, increased level of NETs have been identified in samples obtained at the time of radiologic tumor relapse or progression. Several studies confirmed the relationship between and NETs and cancer through several ways, including HMGB1 production, activation of TLR9-dependent pathways and "waking up" dormant tumor cells through metalloproteinase (MMP)[67, 89]. Analogously, tumor cells can enhance the formation of NETs, secreting some cytokines and EVs[90]. Beside these aspects, NETs have been recently identified as important players of the tumor microenvironment which could influence recurrence and response to therapy. In our study, in the majority of patients experiencing relapse, NETs detected on blood were higher compared to

previous timepoints. This is in line with previous reports suggesting a strong pre-clinical rational because NETs can be present not only early in the pre-metastatic microenvironment but also in the peripheral blood[91] (REF). Increased formation of NETs is detected also after surgery since tissue damage activates the immune and coagulation systems for the healing process[92]. Consistently, in our experience, NETs were higher at post-surgery compared to the end of neoadjuvant chemotherapy in around half of the patients, even in the absence of any disease recurrence.

Our study presents several limitations: firstly, the low number of patients included in this initial report and the relatively short follow up. Secondly, the lack of salivary samples, which prevents from a complete map and comparison of oral, gastric, and intestinal microbiota. Lastly, the absence, at this time, of peripheral blood neutrophil-to-lymphocyte ratio (NLR), which can be a useful biomarker to be taken into account when analyzing NETs results. Higher number of patients, longer follow up and supplemental data are required for more solid statements. Baseline clinical stages and drugs interaction should be also assessed for limiting patients' heterogeneity.

Conclusion

In conclusion, our study provides initial results on the feasibility of a multi-omic approach in LAGC. Microbiota analysis on fecal samples from patients diagnosed with GC presents a relative abundance in *S. Arginosus*, compared to healthy subjects, consistent with literature reports. These results deserve to be further analyzed to determinate potential modifications in microbiome composition and their relationships with treatments. Moreover, the higher percentage of patients with MSI-H observed (around 16%) represents a crucial point that requires additional research, for the identification of different subgroups of patients, leading to personalized therapeutic approaches. In addition, our study highlighted that NETs determination is feasible in LAGC and our data shows that their levels may correlate with response and disease recurrance. Based on these preliminary results, there is a rational for a prospective longitudinal assessment of NETs.

Therefore, the MIMETIC study paves the way to the creation of a multi-omic platform which allows to gather data from several fields in a unique tool. This strategy could help clinicians to tailor optimal multimodal strategies for LAGC patients, with the goal of improving outcomes. The study is currently ongoing enrolling patients to obtain more robust evidence to move forward this approach into the clinical practice.

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