Gut Microbiota Imbalance is Related to Sporadic Colorectal Neoplasms. A Pilot Study

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Abstract: (1) Background: Colorectal cancer (CRC) development is sustained by multiple factors including the gut microbiota, as suggested by a growing body of evidence. Most CRCs have a sporadic (non-hereditary) onset and develop from sporadic colorectal adenomas/polyp (SCA/P). In the present study, we investigated the characteristic of anaerobic microorganisms in stool samples obtained from 20 patients with SCA/P and 20 subjects without evidence of proliferative lesions at colonoscopy (Controls). (2) Material and Methods: We designed this clinical trial using adaptive randomization by minimization. Selective culture media and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry techniques were used to identify the components of microbiota. The data obtained revealed a different variability of gut microbiota in stool samples of controls and SCA/P subjects. (3) Results: The most interesting difference was observed for Bacteroides species, which represent the 50% of all bacterial species identified in the stool samples: two species, Bacteroides stercoris and Parabacteroides distasonis, were found only in the feces from control group, whereas Bacteroides fragilis and Prevotella melanigenica species were presents only in SCA/P patients. Among Gram+ bacteria also, specific species were found in the two groups of feces: Clostridium clostridioforme, Propionibacterium avidum and Pediococcus pentasaceus were identified only in controls, while Eubacterium limosum, Clostridium innocuum and Corynebacterium xerosis were identified in SCA/P stool samples only. (4) Conclusions: Our findings suggest that, compared to control stool samples, a different intestinal microbiota is present in SCA/P stool samples, that may create a micro-environment predisposing for the development of proliferative phenomena. As a consequence, gut microbiota manipulation could be a future target for personalized treatments.

Keywords: gut microbiota; dysbiosis; Bacteroides; probiotics; short-chain fatty acids (SCFAs), MALDI-TOF; intestinal polyps; colorectal cancer (CRC), personalized medicine; translational research
1. Introduction

Colorectal cancer (CRC) represents the third most common cause of cancer mortality worldwide. Experimental evidence supports the role of gut microbiota as an important contributor in the carcinogenic process [1]. The idea concerning the role of microbes in carcinogenesis is not new, but it received compelling evidence over the last two decades, mainly thanks to the studies on *Helicobacter pylori* [2]. Human colon contains the largest population of bacteria in the body (in excess of $10^{11}$ organisms per gram of wet weight) and the majority of these organisms are anaerobes. Among this population, 25% are *Bacteroides*, bile-resistant, gram negative bacilli [3]. Statistically, the relative abundance of five genera, *Bacteroides, Roseburia, Alistipes, Eubacterium* and *Parasutterella* has been identified in human gut microbiota [4]. A healthy balance between host and microorganism is kept for the optimal performance of metabolic and immune functions and to prevent disease development in intestinal and extra-intestinal regions, including the central nervous system [5]. Indeed, gut microbiota plays a pivotal immune function against pathogenic bacteria colonization and invasion and contributes to the maintenance of the intestinal epithelium integrity [6].

Microbiota variations could be principally due to host-related factors including body mass index (BMI), exercise frequency, ethnicity, cultural and dietary habits. As far as the last aspect, recent studies have shown that diet has an important role in shaping the structure of gut microbiota [7–9], which anaerobically ferments dietary components that are not completely digested and absorbed in the upper gastrointestinal tract [10]. Metabolites and antigens produced by gut microbiota may have significant roles in influencing the risk of developing colon cancer through their interactions with host metabolism and immunity [11,12].

It is widely accepted that there is no single causative agent identified in CRC [13]. However, there is evidence that a reduction of “protective” bacteria and an increase in some bacteria such as *Fusobacterium* sp. or *Bacteroides/Prevotella*, as well as age-related changes in microbiota have an impact on adenoma and cancer development [15]. Wang et al. [4] reported structural fecal bacterial segregations between CRC patients and healthy volunteers. Shen et al. demonstrated a higher abundance of Proteobacteria and a lower abundance of Bacteroidetes in CRC cases compared to healthy controls [14].

Most colorectal carcinomas develop from pre-cancerous lesions, namely sporadic colorectal adenoma/polyp (SCA/P), considered to have malignant potential [15]. At our knowledge, this is the first study comparing the gut microbiota anaerobic components in stool specimens from patients with SCA/P and subjects without precancerous lesions (controls).

2. Material and Methods

2.1. Study Design

The study is aimed to evaluate possible differences in gut microbiota composition comparing patients with pre-cancerous lesions end/or sporadic colorectal adenomas in respect of healthy subjects. A clinical trial using adaptive randomization by minimization was been conducted. The protocol study was approved by the Ethics Committee of the University of Bari, Protocol Record Policlinico Hospital, Bari, ClinicalTrials.gov Identifier: NCT03417258.

2.2. Patient Enrollment

We performed an analysis of anaerobic gut microbiota in subjects that performed colonoscopy at the Gastroenterology Unit, Policlinico University Hospital, Bari, and showed pre-cancerous lesions end/or sporadic colorectal adenomas (Group SCA/P, 20 subjects). In addition, the same analysis was performed in 20 subjects, without proliferative lesions, matched for age and sex (controls). All the participants underwent colonoscopy within the previous 1–3 months before the evaluation of their faecal specimens. Written informed consent was obtained from all study subjects.

The inclusion criteria were: subjects of both sexes aged between 50 and 75 years, undergoing colonoscopy. The exclusion criteria were: age < 50 and >75 years, previous diagnosis of colon cancer...
or inflammatory bowel diseases (IBD), hereditary intestinal tumours, ongoing infections, intake of drugs that could alter the intestinal microbiota during the last 4 weeks, creatinine clearance below 60 mL/min, liver failure.

2.3. Microbiological Study

Fecal samples were collected from each subject and immediately analyzed for the presence of the anaerobic bacteria. The stool samples were inoculated on both Schaedler blood agar and on Bile Esulin Agar (BEA) as selective isolation medium for anaerobic bacteria \textit{Bacteroides} spp. After incubation at 37 °C for 72 h in an anaerobic jar, strict anaerobes were chosen. Thereafter, after grown on both culture plates, identification of the different colonies was performed by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), an innovative technology for the rapid and accurate identification of bacterial and fungal isolates in clinical settings [16]. MALDI-TOF technology has been developed to profile, within minutes instead of the usually required 36–48 h of the traditional approaches, based on the pure culture of the microorganism, bacterial proteins from whole cell extracts and to obtain a bacterial fingerprint able to discriminate microorganisms from different genera and species [17]. The technology is automated, high throughput, and applicable to a broad range of common as well as rare bacteria and fungi.

2.4. Statistical Analysis

Where indicated, individual comparisons were performed using Student’s t-test. The results obtained are expressed as mean ± S.D. Statistical comparison among groups was determined using analysis of variance. Statistical significance was ascribed to the data when \( p < 0.05 \).

3. Results

In Table 1, demographical and anthropometric parameters of the patients involved in the study, matched for sex, age and BMI, are reported. No statistical difference was detected between the two reported groups of subjects.

| Pts. Data | SCA/P   | Control | \( p \) Value |
|-----------|---------|---------|--------------|
| Age       | 63.4 ± 8| 64.5 ± 4| 0.56         |
| Sex       | M:14, F:6| M:11, F:9| 0.61         |
| BMI       | 29.8 ± 5.4| 30.3 ± 7.2| 0.76         |
| Weight (Kgs)| 80.5 ± 12.3| 77.3 ± 13.8| 0.45         |

Table 2 describes the different anaerobic species identified in the stool samples of the 40 subjects participating to the study. Our results show that a total of 9 Gram− and 9 Gram+ species of anaerobic bacteria were present in the feces of these subjects. It is worth noting that all species of Gram− anaerobic bacteria are part of the \textit{Bacteroides} family which represents the 50% (9/18) of all bacterial species identified.
Table 2. Fecal anaerobic bacteria species isolated from the subjects involved in the study.

| Gram+ Bacteria                | Gram- Bacteria |
|-------------------------------|----------------|
| Bifidobacterium spp (Bif.)    | Bacteroides ovatus (B.o.) |
| Clostridium clostridiforme (C.c.) | Bacteroides stercoris (B.s.) |
| Clostridium innocuum (C.i.)   | Bacteroides thetaotaomicron (B.t.) |
| Clostridium ramosum (C.r.)    | Bacteroides uniformis (B.u.) |
| Clostridium sardelli (C.s.)   | Bacteroides vulgatus (B.v.) |
| Propionibacterium avidum (Pr.a.) | Prevotella melaninigenica (Pre.m.) |
| Eubacterium limosum (Eu.l.)   | Parabacteroides distasonis (Pa.d.) |
| Pediococcus pentosaceus (Pe.p.) | Parabacteroides merdae (Pa.m.) |

Figure 1 reports the different gut microbiota species (Gram− and Gram+) recognized in the fecal samples of the two groups of subjects. In controls and SCA/P patients the total number of bacterial species identified was similar (7 Gram− and 6 Gram+). However, among Gram- bacteria, all anaerobic bacteria belonging to the Bacteroides genus, two species, Bacteroides stercoris and Parabacteroides distasonis, were found only in the feces from control group, whereas Bacteroides fragilis and Prevotella melaninigenica species were present only in SCA/P patients. Moreover, it appears evident, among the 5 bacterial species present in both groups of subjects, that the detection of Bacteroides ovatus (B.o.), Bacteroides uniformis (B.u.) and Bacteroides vulgatus (B.v.) was tripled, doubled and slightly higher, respectively, in control group as compared to SCA/P patients, while the presence of Parabacteroides merdae (Pa.m.) was double in the SCA/P group as compared to control subjects. Similar presence was evidenced for Bacteroides thetaotaomicron (B.t.) in both groups of fecal samples.

As far as the Gram+ bacteria identified in the two group of subjects (Figure 1), similarly to what observed in Gram- bacteria, specific bacterial species were found in the two groups: Clostridium clostridioforme (C.c.), Propionibacterium avidum (Pr.a.) and Pediococcus pentosaceus (Pe.p) were identified in control group only, while Eubacterium limosum (Eu.l.), Clostridium innocuum (C.i.) and Corynebacterium xerosus (Cor.x.) were identified in SCA/P feces only.

Among the bacteria present in both groups of feces, Bifidobacterium spp (Bif.) resulted tripled in control group compared to SCA/P group, while similar was the presence of Clostridium ramosum (C.r.) and Clostridium sardelli (C.s.) in both group of fecal samples.
The RPDPPH test is a cost-effective, rapid, and simple acellular method thus offering the same advantages of the OP assays. In the future, the combined application of all these tests to wider sample sets will permit to better understand redox equilibria among PM native species that might occur during sample storage, extraction phase, and/or application of oxidative potential assays. Furthermore, it is worth considering that reducing species could reasonably react with oxidizing ones, also when PM gets in contact with biological systems, thus exerting a possible opposition to oxidative stress. The availability of a suitable assay for routinely estimate the amount of reducing species in PM samples could constitute a new and potentially useful tool for acquiring new insight in the field of its redox behavior and health effect.

Supplementary Materials:
The following are available online at www.mdpi.com/xxx/s1, Figure S1: Meteorological data collected from 29th to 17th March-April 2019 at Cassana (FE), Italy: wind speed (a), rainfall (b), temperature (c), wind direction (d) and pressure (e), Figure S2: Oxidative potential obtained through 2′,7′-dichlorofluorescin (OPDCFH), ascorbic acid (OP AA) and dithiothreitol (OP DTT) assays on PM 2.5 filters collected at Cassana (FE), Italy, from 29th March to 17th March-April 2019. Values below limits of detection (LODs) are not shown.

4. Discussion

Microbiota manipulations by administration of probiotic have been demonstrated to be effective in a number of clinical conditions (e.g., respiratory, oral, urogenital, and intestinal) to ameliorate the history of either infective or non-infective diseases, probably modulating the local and systemic immune functions [18–23]. Herein, for the first time, we report a description of the anaerobic population of gut microbiota in stool samples from controls subjects and patients with sporadic colorectal adenoma/polyp (SCA/P). Our attention to the anaerobic species has been driven by numerous data present in literature reporting a strong correlation between stool anaerobic bacteria and CRC. Zhu et al., reviewing the relationship between microbiota and the development of CRC, reported highest levels of carcinogen production in association with gut anaerobic bacteria [23]. Kwong and coll reported that genera Bacteroides, the 25% of anaerobic bacteria [3], are strongly associated with CRC, stating also that CRC development is associated with Bacteroides fragilis [24]. Farther, epidemiological studies reported that incidence and mortality of CRC higher in African American (AA) than in Caucasian American (CA) associated to the more abundant presence of Bacteroidetes in AAs than in CAs patients [25].
Our data revealed that a different gut microbiota pattern, particularly evident for the *Bacteroides* genus, was found in the two group of subjects studied. However, our data suggest other possible important implications. First of all, the two groups of stools are characterized by different species of bacteria, *Bacteroides stercoris* and *Parabacteroides distasonis* for control group and *Bacteroides fragilis* and *Prevotella melaninigenica* for SCA/P group. Data from literature assign specific roles to these bacteria in relation to CRC. *Parabacteroides distasonis* (Pa.d.) has been reported abundant in mice’s stools and inversely associated with intestinal tumor burden and IL-1β concentrations [26]. In their report, the Authors attributed to Pa.d. anti-inflammatory and anti-cancer properties that are likely mediated by the suppression of TLR4 and Akt signaling, as well as promotion of apoptosis [27].

CRC development has been associated with increased presence of *Bacteroides fragilis* (B.f.) in gut microbiota [24]. This Gram-negative, facultative anaerobe specie constitutes an appreciable proportion of the human gastrointestinal tract microbiota and, as typical of most gram-negative bacilli, it secretes an unusually complex mixture of neurotoxins, including the extremely pro-inflammatory lipopolysaccharide [11]. In this regard, it is important to underline the presence of the Gram+ *Pediococcus pentasaceus* (Pe.p.) in fecal samples of control subjects since this specie is able to eliminate the lipopolysaccharide (LPS) components, which can cause intestinal inflammation [28], that is a biological condition known to predispose to CRC development [29,30]. Furthermore, *Pediococcus pentasaceus*, found only in controls, showed antiproliferative activity on colon cancer cells directly adhering to those and triggering the bioproduction of short-chain fatty acids (SCFAs), mainly butyric and propionic acids, suggesting for the use of this bacteria specie as an alternative bio-prophylactic and biotherapeutic strategy for colon cancer [31]. Finally, we detected *B. fragilis* only in subjects with endoscopic pre-cancerous lesions (SCA/P).

What metabolic situation can derive from a shift of gut microbiota or, in general, by the induction of dysbiosis? From a biochemical point of view, dysbiosis has some common characteristics: the number of bacteria that produce SCFAs is reduced. This condition is negative because the SCFA strengthen the intestinal barrier and the immune system in the defense against pathogens. At the same time, levels of harmful microorganisms rise; these include bacteria, mostly Gram-negative bacteria, that lysing lipopolysaccharide (LPS) in the gut lumen facilitate its entry into bloodstream and may lead to a deleterious pro-inflammatory systemic immune response [32].

It is also known that dysbiosis leads to an increase of bacterial populations that stimulate tumorigenesis and contribute to epithelial carcinogenesis and tumor progression by altering metabolic properties (such as bile acid and butyric acid) and inflammatory process. In many human intestinal diseases, such as Crohn’s disease and ulcerative colitis, significant changes in gut microbiota have been reported [33–35]. In these pathological conditions, as well in CRC patients [4], again, one of the major biological effects observed is a reduction/substitution of butyrate-producing bacteria. Butyrate, one of the SCFAs, is a major metabolite in colonic lumen arising from bacterial fermentation of dietary fiber and has been shown to be a critical mediator of the colonic inflammatory response. Butyrate has been implicated in the protection against colitis and CRC by reducing oxidative damage to DNA, inducing apoptosis in DNA-damaged cells, inhibiting tumor cell growth, and decreasing the activity of co-carcinogenic enzymes [36,37]. Butyrate possesses both preventive and therapeutic potential to counteract inflammation-mediated ulcerative colitis (UC) and colorectal cancer. Emerging data suggest that certain groups of bacteria might promote whereas others might protect against colon cancer. Indeed, *Fusobacterium nucleatum* seems to play a central role in the tumor microenvironment as its abundance correlates with cancer progression as well as the dysbiotic tumor microbiota composition including *Bacteroides* spp, the enterotoxigenic *Bacteroides fragilis* in particular, and *Prevotella* species, may also promote colorectal cancer by stimulating exaggerated immune responses via Th17 cells [38]. Andersen and coworkers reported an increase of acetic and butyric acids in colon contents of pigs, when a commercially available probiotic strain *Pediococcus pentosaceus* was administrated [39], a Gram+ coccus again present only in fecal samples of control subjects.
Concerning the greater presence of *Bacteroides vulgatus* and *Bacteroides uniformis* in control stool samples compared to SCA/P subjects, it is worth mentioning the findings by Wang, which studied the discrepancies of gut microbiota between healthy subjects and CRC patients, reporting a structural segregation between the two populations [4]. In his report, Wang identified forty-eight operational taxonomic units (OTUs) by redundancy analysis as key variables significantly associated with the structural difference. One OTU closely related to *Bacteroides fragilis* (98.1% similarity) was enriched in the gut microbiota of CRC patients, whereas two OTUs related to *Bacteroides vulgatus* (100% and 98.7% similarity, respectively) and one to *Bacteroides uniformis* (97.5% similarity) was enriched in that of healthy volunteers. Revisiting our data, it is clear that *Bacteroides fragilis* is present only in the fecal samples of the SCA/P subjects, while *Bacteroides vulgatus* and *Bacteroides uniformis* are predominant in the feces of control subjects confirming, in part, the data coming from Wang’s report [4].

Several authors suggest that an altered composition in the gut microbiota, including the loss of microbiota diversity (LOMD), determines a predisposing microenvironment for dysbiosis. LOMD appears as a common feature of most dysbioses and it is a constant finding of intestinal dysbiosis [40]. In digestive diseases such as Crohn’s disease [41,42], irritable bowel syndrome, with [43] or without diarrhea [44] and colorectal cancer [45], LOMD is constantly observed. The duodenum-associated microbiota of celiac disease patients is also less diversified as previously reported also by our group [46,47]. According to this hypothesis, the LOMD may lead to an unstable microbial ecosystem favoring the emergence of dysbiotic microbiota and giving rise to immunological or metabolic diseases.

5. Conclusions

In conclusion, MALDI-TOF MS is a tool for rapid, accurate, and cost-effective identification of cultured bacteria and fungi in clinical microbiology. We agree with literature data suggesting that there is no single causative bacterial organism responsible in CRC. However, we believe that there is sufficient evidence suggesting that the reduction of protective bacteria or, vice versa, an increase of noxious strains, induced by diet in association with lifestyle and age, could influence the development of proliferative lesions. Apart from SCFAs produced by gut bacteria, the control of pathogenic species in the intestine could be determined by natural dietary compounds (i.e., polyphenols, essential oils, etc.) and a lot of immune regulators and mediators, partly tested in daily practice [48–50].

Future studies will enable to better understand both pro-carcinogenic and anti-carcinogenic mechanisms and will shed lights on the rational manipulation of the human microbiota and on the use of prebiotics, probiotics, or dietary modifications to improve health conditions.

The role of the diet in influencing the composition of the human microbiota is certainly not to be neglected. It is worldwide accepted that diet can influence the composition of the human microbiota, and that the supplementation with specific dietary resistant starch (RS) may lead to changes in fecal microbiota profiles associated with improved bowel health [51].

We know for sure that bacterial species might be greatly influenced by the presence of different nutrients, fibers etc. in diet and this aspect need to be resolved together with the concept of high variability and poor number of subjects included in the study.

Having this in mind, the manipulation of oncogenic, high risk gut microbiota may became an effective tool for cancer prevention, as well as treatment possibly based on fecal transplantation and lifestyle modifications.

However, this study was aimed to establish how different is the microbiota in subjects with or without intestinal polyps, regardless of dietary habits. So, for this study we just asked to the participants if they have had always the same dietary habits.

An evaluation of dietary habits would have made sense if we wanted to establish the type of foods that promote the development of certain bacterial species. But this aspect was beyond the scope of our study. In addition, although BMI was comparable between the two groups, no information is provided concerning the dietary habits in the weeks before assessment of intestinal microbiota.
**Ethical Statement:** The protocol study was approved by the Ethics Committee of the University of Bari, Protocol Record Policlinic Hospital, Bari, ClinicalTrials.gov Identifier: NCT03417258. Written informed consent was obtained from all study subjects.

**Author Contributions:** Conceptualization, L.P., M.B. and L.S.; Methodology, L.P., M.B. and L.S.; validation, L.P., M.B. and A.M.; investigation, L.P., M.B., A.M. and L.S.; resources, A.D.L.; data curation, M.B., M.T.V., and L.S.; writing—original draft preparation, L.P., M.B. and A.M.; writing—review and editing, L.P., M.B. and L.S.; visualization, M.B. and M.T.; supervision, A.D.L.; project administration, L.P., M.B. and L.S.; funding acquisition, M.B. and A.D.

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