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HOST MICROBE INTERACTIONS

16S rRNA Gene Pyrosequencing Reveals Shift in Patient Faecal Microbiota During High-Dose Chemotherapy as Conditioning Regimen for Bone Marrow Transplantation

Emmanuel Montassier · Eric Batard · Sébastien Massart · Thomas Gastinne · Thomas Carton · Jocelyne Caillon · Sophie Le Fresne · Nathalie Caroff · Jean Benoit Hardouin · Philippe Moreau · Gilles Potel · Françoise Le Vacon · Marie France de La Cochetière

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Abstract Gastrointestinal disturbances are a side-effect frequently associated with haematological malignancies due to the intensive cytotoxic treatment given in connection with bone marrow transplantation (BMT). However, intestinal microbiota changes during chemotherapy remain poorly described, probably due to the use of culture-based and low-resolution molecular methods in previous studies. The objective of our study was to apply a next generation DNA sequencing technology to analyse chemotherapy-induced changes in faecal microbiota. We included eight patients with non-Hodgkin's lymphoma undergoing one course of BMT conditioning chemotherapy. We collected a prechemotherapy faecal sample, the day before chemotherapy was initiated, and a postchemotherapy sample, collected 1 week after the initiation of chemotherapy. Total DNA was extracted from faecal samples, denaturing high-performance liquid chromatography based on amplification of the V6 to V8 region of the *16S ribosomal RNA* (rRNA) gene, and 454-pyrosequencing of the *16 S rRNA* gene, using PCR primers targeting the V5 and V6 hypervariable *16S rRNA* gene regions were performed. Raw sequence data were screened, trimmed, and filtered using the QIIME pipeline. We observed a steep reduction in alpha diversity and significant differences in the composition of the intestinal microbiota in response to chemotherapy. Chemotherapy was associated with a drastic drop in *Faecalibacterium* and accompanied by an increase of *Escherichia.* The chemotherapy-induced shift in the intestinal microbiota could induce severe side effects in immunocompromised cancer patients. Our study is a first step in identifying patients at risk for gastrointestinal disturbances and to promote strategies to prevent this drastic shift in intestinal microbiota.

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E. Montassier (✉) · E. Batard · J. Caillon · N. Caroff · G. Potel · M. F. de La Cochetière
EA 3826 Thérapeutiques Cliniques et Expérimentales des Infections, Faculté de Médecine, Université de Nantes, 1 Rue G Veil, 44000 Nantes, France
e-mail: emmanuelmontassier@hotmail.com

S. Massart
DNA Vision S.A., 25 avenue Georges Lemaitre, Gosselies, 6041 Charleroi, Belgium

T. Gastinne · P. Moreau
Service d'Hématologie Clinique, Centre Hospitalier Universitaire de Nantes, 1 Place A Ricordeau, 44000 Nantes, France

T. Carton · S. Le Fresne · F. Le Vacon
Biofortis, Mérieux Nutrisciences, 3 Route de la Chatterie, 44800 Saint Herblain, France

J. B. Hardouin
EA 4275 Biostatistique, Recherche Clinique et Mesures Subjectives en Santé, Faculté de Médecine, Université de Nantes, 1 Rue G Veil, 44000 Nantes, France

M. F. de La Cochetière
INSERM, EA 3826 Thérapeutiques Cliniques et Expérimentales des Infections, Faculté de Médecine, Université de Nantes, 1 Rue G Veil, 44000 Nantes, France

Introduction

A wide diversity of microbial communities colonizes the human body as a result of millennia of coevolution, making humans 'superorganisms' [1, 2]. The largest and most

complex microbial ecosystem is hosted in the human gastrointestinal tract, with a density of microorganisms exceeding 10^{12} cells/mL of stool [3, 4]. The intestinal microbiota is composed of thousands of species-level phylotypes, dominated by two bacterial phyla: Firmicutes and Bacteroidetes [5, 6].

The human intestinal microbiota plays an essential role in host health by processing energy from food, protecting intestinal epithelial cells from injury, and promoting local and systemic immunity [7]. The human intestinal microbiota has been extensively studied in recent years using the culture-independent molecular method [8–16]. The next generation DNA sequencing technologies, including high-throughput 454 pyrosequencing, provide a large number of sequence reads in a single run, resulting in greater sampling depth and the detection of low-abundance taxa. The results of studies using high-throughput sequencing technologies have therefore revolutionized our understanding of intestinal microbiota under both healthy and disease conditions [13–21].

Gastrointestinal disturbances are common in cancer patients, particularly in patients with haematological malignancies, owing to the intensive cytotoxic treatment given in connection with bone marrow transplantation (BMT) [9–11, 22, 23]. Clinically, patients experience mouth ulceration, abdominal pain, nausea, vomiting, abdominal bloating and diarrhoea. Delayed treatment and reduced dosages of chemotherapeutics are often necessary as a result of these side effects, which ultimately contribute to suboptimal treatment of the cancer [22, 23]. These severe side effects are a result of chemotherapy-induced diffuse intestinal injury characterized by inflammation and apoptosis (i.e. mucositis) [24]. However, the changes in intestinal microbiota during chemotherapy and their potential impact on the onset of mucositis remain poorly described. Chemotherapy has been shown to increase Gram-negative and beta-glucuronidase-producing bacteria, especially *Escherichia coli*, in experimental studies [8, 9, 25]. Moreover, four cycles of chemotherapy decreased anaerobic bacteria in pediatric patients [10]. However, these studies used culture-based and low-resolution molecular methods, focused on the most dominant microbial community members, providing an incomplete understanding of chemotherapy-induced alterations of intestinal microbiota. A previous study introduced next generation sequencing technologies to analyse changes in human faecal microbiota during chemotherapy, with or without antibiotics [11]. They applied *16S ribosomal RNA* (rRNA) gene pyrosequencing in two patients with acute leukaemia, who were receiving two different chemotherapy regimens. Nevertheless, the results of this study are limited by high individual variation and the use of concomitant antibiotherapy.

Here, we describe the chemotherapy-induced changes of faecal microbiota using *16S rRNA* gene pyrosequencing in a cohort of patients undergoing BMT conditioning chemotherapy.

Materials and Methods

Study Location and Population

We enrolled eight consecutive patients (5 men and 3 women) with non-Hodgkin's lymphoma undergoing BEAM BMT conditioning chemotherapy in the oncology–haematology department of Nantes University Hospital, France. The mean age (±SD) was 50.5 (±10.8) years. All the patients had a previous history of chemotherapy. Other characteristics of the included patients are reported in Table 1. We excluded patients treated with antibiotics and probiotics and patients who received nasal tube feeding or parenteral nutrition during the study period, as these factors are well described as impacting the intestinal microbiota [19, 25–27]. However, several patients received antibiotics before the onset of the study anfaecal sample collection, as shown in Table 1. These patients received oracillin (penicillin V) and cotrimoxazole as antibiotic prophylaxis. Prophylactic oracillin administration prevents streptococcal bloodstream invasion and cotrimoxazole is the agent of choice for preventing *Pneumocystis* infection in immunocompromised hosts [28]. Note that fluoroquinolone prophylaxis was not used in our patients.

BEAM BMT conditioning chemotherapy is a standard 5-day protocol that includes high-dose carmustine (bischloroethylnitrosourea), etoposide, aracytin and melphalan. The mechanism of action of these four anti-neoplastic agents is summarized in Table 2.

Written informed consent was obtained from all the patients participating in the study. The clinical protocol and informed consent documents were approved by the Nantes University Hospital Ethics Committee (identification of the protocol: BRD/10/04-Q).

Faecal Samples

Two faecal samples were collected from each patient: a prechemotherapy sample (S1), collected the day before chemotherapy was administered, and a postchemotherapy sample (S2), collected 1 week after the chemotherapy treatment. After homogenization with a sterile spatula, approximately 1 g of stool was transferred to a sterile tube and immediately stored at −80 °C for subsequent molecular analysis.

DNA Extraction and Purification

The genomic DNA extraction procedure was based on the QIAamp DNA Stool Minikit (Qiagen) with slide modifications, as previously described [29–31]. Briefly, we first added a lysis step for Gram-positive bacteria: 200 mg of faecal sample were homogenized in 180 µL of lysozyme buffer and incubated for 30 min at 37 °C. Mechanical lysis then took place by adding 1.220 mL of ASL buffer and 300 mg of glass

Table 1 Characteristics of the eight patients included in the study

Name of the patients	Sex	Age	Body mass index	Antibiotic prophylaxis received before the onset of the study	Other condition	Previous history of chemotherapy	Gastrointestinal disturbance during hospitalization	Other clinical events
P1	Female	65	32.8	Oracillin, cotrimoxazole	None	Yes	Nausea, vomiting, oral mucositis and neutropenic enterocolitis	Folicullitis
P2	Female	48	21.1	Oracillin	None	Yes	Diarrhoea, nausea, vomiting and oral mucositis	None
P3	Male	45	25.8	Oracillin, cotrimoxazole	None	Yes	Diarrhoea, nausea, vomiting and oral mucositis	Colonization with *P. Aeruginosa*, perianal cellulitis
P4	Male	40	22.8	Oracillin	Proximal deep-vein thrombosis	Yes	Nausea, oral mucositis	*E. coli* acute pyelonephritis with bacteremia
P5	Female	56	25.8	Cotrimoxazole	None	Yes	Diarrhoea	*Enterococcus* acute pyelonephritis
P6	Male	36	26.9	None	None	Yes	Diarrhoea	None
P7	Male	65	24.8	Oracillin, cotrimoxazole	Hepatitis B	Yes	Oral mucositis	*E. coli* bacteremia
P8	Male	49	24.1	Oracillin	None	Yes	Diarrhoea, oral mucositis and oesophagitis	Pneumonia, acute pulmonary oedema, acute atrial fibrillation

beads. The mixture was shaken vigorously using BioSpec Products Inc. Minibeadbeater-16. The homogenized sample was heated at 95 °C for 5 min and centrifuged for 1 min at 13,000 rpm. The supernatant was transferred into a 2-mL tube, and an Inhibitex tablet was added. After dissolution, the sample was incubated for 1 min at room temperature and centrifuged for 6 min at 13,200 rpm. The supernatant was transferred into a 1.5-mL tube and centrifuged for 3 min at 13,200 rpm. Then, 200 μL of supernatant was mixed with 15 μL of proteinase K and 200 μL of AL buffer and incubated at 70 °C for 10 min to remove protein and polysaccharides. Two hundred μL of ethanol were added, and the solution was mixed by vortexing. The complete lysate was applied to the column and centrifuged for 2 min at 13,200 rpm. After two washing processes, one with 500 μL of AW1 and the other with 500 μL of AW2 buffer, DNA was eluted by adding 200 μL of buffer AE. DNA quality was assessed by gel electrophoresis and spectrophotometry measuring OD ratio 260/280. The extracted DNA aliquots were stored at −20 °C.

Denaturing High-Performance Liquid Chromatography (dHPLC)

The V6 to V8 region of the *16S rRNA* gene was amplified using universal primers, U968-GC (5′ CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5′ GCG TGT GTA CAA GAC CC), in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). A polymerase chain reaction (PCR) was performed using HotGoldStar Taq polymerase (Eurogentec). PCR mixtures of 25 μL contained 1X PCR Buffer, 2.5 mM of $MgCl_2$, 0.2 mM each of dNTP, 0.4 μM of primers U968-GC and L1401, 2.5 U of HotGoldStar Taq polymerase, and approximately 2 ng of DNA. PCR was performed in a T3 thermocycler (Biometra) using the following parameters: one cycle at 95 °C for 7 min, followed by 30 cycles of 93 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, with an additional extension at 72 °C for 30 min. Then the PCR products were checked on an agarose gel, purified and reconditioned before injection into the dHPLC system. The purified PCR products containing bacterial *16S rRNA* gene segments were separated on a DNASep® HT cartridge (Transgenomic) as described previously [12, 30, 31]. Optimal separation with dHPLC was completed at an oven temperature of 62.2 °C and flow rate of 0.9 mL/min. The gradient was formed with WAVE® optimized buffer A, consisting of 0.1 M triethylammonium acetate (TEAA) and WAVE® optimized buffer B, consisting of 0.1 M TEAA in 25 % acetonitrile, according to the manufacturer's instructions. Separated bacterial *16S rRNA* gene amplicons were detected and visualized with an HSX-3500 fluorescence detector using a WAVE®-optimized HS staining solution I. The 16S ribosomal amplicons of the dominant microbiota were then converted into a profile with peaks, each peak accounted for one amplicon (SI Fig. 1).

High-Throughput 454 DNA Pyrosequencing

PCR Amplification of V5V6 Region of Bacterial 16S rRNA Genes

For each sample, we amplified *16S rRNA* genes using a primer set corresponding to primers 784F (AGGATTAGATACCC

Table 2 Mechanism of action and antimicrobial activity of the four anti-neoplastic agents used in our study

	Etoposide (inhibitor of cell division)	Melphalan (alkylating agent)	Cytarabine (anti-metabolite)	Carmustine (alkylating agent)
Mechanism of action [49]	- Inhibits DNA topoisomerase II, thereby inhibiting DNA re-ligation. This causes critical errors in DNA synthesis at the premitotic stage of cell division and can lead to apoptosis of the cancer cell - Cell cycle dependent and phase specific, affecting mainly the S and G2 phases of cell division	- Attachment of alkyl groups to DNA bases forming monoadducts, preventing DNA synthesis and RNA transcription from the affected DNA - DNA damage via the formation of cross-links which prevents DNA from being separated for synthesis or transcription - Induction of mispairing of the nucleotides leading to mutations	- Acts through direct DNA damage and incorporation into DNA - Exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and under certain conditions blocking the progression of cells from the G1 phase to the S-phase - Acts through the inhibition of DNA polymerase	- Causes cross-links in DNA and RNA, leading to the inhibition of DNA synthesis, RNA production and RNA translation (protein synthesis) - Binds to and modifies glutathione reductase
MIC Gram-positive bacteria [42, 43]	10–100 μg/mL	0	0	0
MIC Gram-negative bacteria [42, 43]	0	0	0	0
MIC anaerobic bacteria [42, 43]	10-100 μg/mL	0	0	0
MIC yeast [42, 43]	0	0	0	100–1000 μg/mL

MIC minimum inhibitory concentrations

TGGTA) and 1061R (CRRCACGAGCTGACGAC), targeting the V5 and V6 hypervariable *16S rRNA* gene region (~280 nt region of the *16S rRNA* gene), as previously described [32]. The forward primer contained the sequence of the Titanium A adaptor (5′-CCATCTCATCCCTGCGTGTC TCCGACTCAG-3′) and a barcode sequence. The reverse primer contained the sequence of the Titanium B adaptor (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′). For each sample, a PCR mix of 100 μL was prepared containing a PCR buffer, 2 U of KAPA HiFi Hotstart polymerase blend, and dNTPs (Kapabiosystems), 300-nM primers (Eurogentec), and 60-ng DNA. Thermal cycling consisted of an initial denaturing step at 95 °C for 5 min, 25 cycles of denaturing at 98 °C for 20 s, annealing at 56 °C for 40 s, extension at 72 °C for 20 s, and a final extension step at 72 °C for 5 min. The amplicons were visualized using 1 % agarose gels and GelGreen nucleic acid gel stain (Biotium) in a 1XTEA buffer. The amplicons were purified using the Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions.

Amplicon Quantitation, Pooling, and Pyrosequencing

Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen) following the manufacturer's instructions. Assays were carried out using 10 μL of the cleaned PCR product in a total reaction volume of 200 μL in black, 96-well microtiter plates. Fluorescence was measured on a Perkin-Elmer Victor Plate reader using the 485/530-nm excitation/emission filter paired with a measurement time of 0.1 s. Following quantitation, the cleaned amplicons were combined in equimolar ratios in a single tube. The final pool of DNA was precipitated on ice for 45 min following the addition of 5-M NaCl (0.2 M final concentration) and two volumes of ice-cold 100 % ethanol. The precipitated DNA was centrifuged at 7,800×*g* for 40 min at 4 °C, and the resulting pellet was washed with an equal volume of ice-cold 70 % ethanol and centrifuged again at 7,800×*g* for 20 min at 4 °C. The supernatant was removed, and the pellet was air-dried for 10 min at room temperature and then resuspended in 100-μL nuclease-free water (Ambion). The final concentration of the pooled DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher). Pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche) using titanium chemistry.

Sequence Analysis

The *16S rRNA* raw sequence data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) v. 1.6.0 pipeline (http://qiime.sourceforge.net) to obtain the taxonomic composition and diversity of the samples [33]. The sequences

were demultiplexed and quality-filtered using default QIIME parameters, including chimera identification and removal using chimeraslayer. After sample assignation of the reads, primer and tag sequences were removed before operational taxonomic units (OTUs) clustering. OTU clustering was done at a 97 % similarity threshold. Taxonomic identities were assigned using an RDP classifier (v. 10.28) included in the QIIME v 1.6.0 with a confidence threshold of 0.8 [34]. A variable number of sequences were obtained per sample. Therefore, the sequence data were rarefied at 8,000 sequences per sample to account for this variation for Chao and Shannon index calculations (i.e. alpha diversity) and the principle coordinates analyses (PCoA) using the phylogeny-based unweighted Unifrac distance metric (i.e. beta diversity) [35].

Statistics

Identification of OTUs that were significantly different in abundance before and after chemotherapy was carried out in QIIME using a paired *t*-test with a Bonferroni correction. To determine whether any groups of samples contained significantly different bacterial communities, the analysis of similarities (ANOSIM) in R (version 2.15.3) using a Vegan package was conducted. Using the unweighted UniFrac distance matrix, distances were grouped as 'within group' or 'between group'. Significance levels were calculated by comparing the *R* statistic against the distribution generated from 10,000 permutations of the randomized dataset.

The data were entered into a custom database (Excel, Microsoft Corp) and analysed using Matlab R2007b (Mathworks) and R (version 2.15.3). Quantitative data were reported as mean±SD or median [first and third quartile]. Categorical data were reported as percentage and 95 % confidence interval. Intrapatient differences before and after chemotherapy at a phylum and genus level were compared using multiple Wilcoxon rank tests for paired data. A p value less than 0.05 was considered to be statistically significant. Differences between prechemotherapy and postchemotherapy bacterial profiles following dHPLC were analysed by principal component analysis and hierarchic cluster analysis. Group clustering was performed on the pairwise distance matrix.

Results

Changes in the Faecal Microbiota during Chemotherapy Characterized With dHPLC

The cluster analysis of bacterial profiles indicated one cluster containing only S2 samples and a second cluster that contained all S1 samples and three of the eightS2 samples (Fig. 1a). We also performed a principal component (PC) analysis (PCA) of the dHPLC fingerprints. PC1 and PC4 accounted for 46.1 % and 8 % of total variance, respectively. The score plot of PC1 and PC4 showed that S1 samples were grouped together and separate from the S2 samples (Fig. 1b). Both cluster analysis and PCA of the dominant bacteria dHPLC fingerprints identified two groups of samples that were highly dependent on whether they were collected before or after the chemotherapy, demonstrating a shift in patient faecal microbiota populations during a 5-day high-dose chemotherapy protocol.

Changes in Faecal Microbiota during Chemotherapy Characterized with *16S rRNA* Gene Pyrosequencing

Faecal Sample Collection and Bacterial Sequences, Alpha and Beta Diversity

Of the faecal samples collected, a total of 200,110 high-quality *16S rRNA*-encoding sequences were identified. The mean number of sequences obtained per sample was 12,506.9± 2,307.2 (range 7,956–18,672). Using the QIIME pipeline, we identified 1,649 OTUs. Rarefaction measurements, reported in Fig. 2, showed a significant reduction in OTUs, Chao and Shannon indices ($p<0.001$) during chemotherapy. OTUs that were significantly different in abundance before and after chemotherapy were reported in SI Table 1. Following the Bonferroni correction for multiple comparisons, a total of 52 bacterial taxa varied significantly between the prechemotherapy and postchemotherapy samples.

PCoA plots based on the unweighted UniFrac distance matrix, indicated separation of samples between prechemotherapy and postchemotherapy faecal samples (ANOSIM, $R=0.97$, $p<0.001$) (Fig. 3).

Thus, we demonstrated a shift in patient faecal microbiota during a 5-day high-dose chemotherapy protocol.

Changes in Faecal Microbiota at the Phylum Level

Seven phyla were identified, as summarized in SI Fig. 2. Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria represented more than 98 % of all *16S rRNA* gene sequences. The analyses of the cumulative percentage of phyla sequences are reported in Fig. 4. The vast majority of sequences detected in the S1 samples were members of the phyla Firmicutes (73.9 % ±4.4), whereas the majority of sequences detected in the S2 samples were members of the phyla Bacteroidetes (51 % ±7.3). Firmicutes and Actinobacteria decreased, whereas Bacteroidetes and Proteobacteria increased during chemotherapy ($p=0.008$). Moreover, the difference in the Firmicutes to Bacteroidetes ratio (F/B ratio) before and after chemotherapy (6.6±6.3 vs 0.7±0.6, respectively, $p=0.008$) suggests a drastic shift in bacterial colonization of faecal microbiota after chemotherapy.

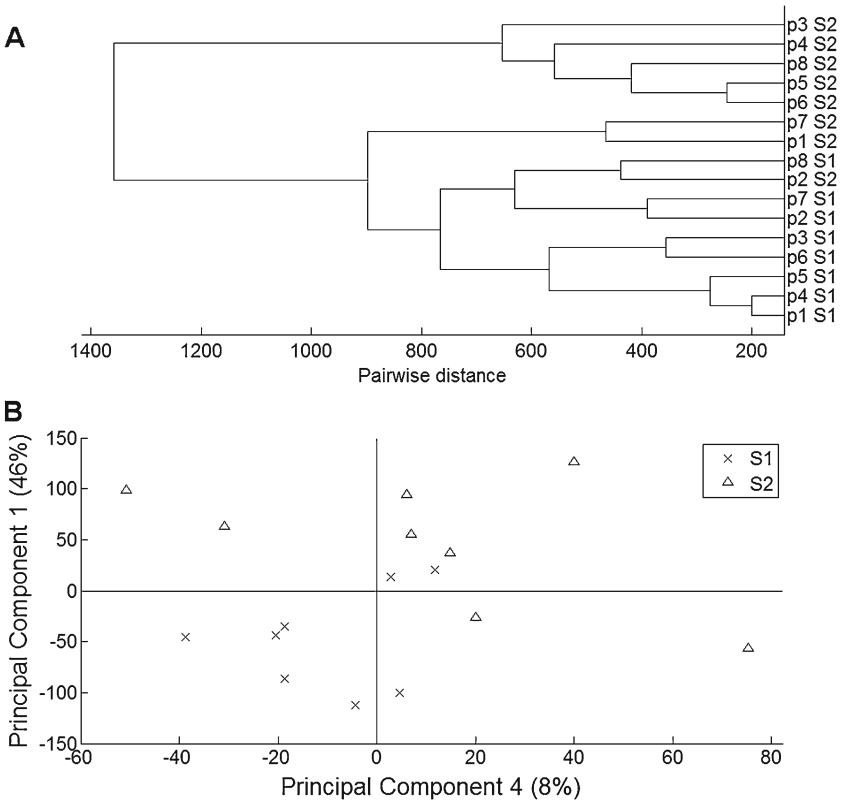

Fig. 1 Denaturing high-performance liquid chromatography (dHPLC) fingerprint analysis of the V6 to V8 region of the *16S rRNA* gene **a** Cluster analysis of dHPLC fingerprints in faecal samples from 8 patients (p1 to p8). One cluster contained only S2 samples whereas the second cluster contained all S1 samples and three of the eight S2 samples **b** Score plot of the principal component (PC) analysis of dHPLC fingerprints. PC1 and PC4 separated S1 from S2 samples

Changes in Faecal Microbiota at the Genus Level

Sequence analyses from the S1 and S2 samples suggest the prescence of 230 different genera. The most frequent genera found in each patient are shown in SI Fig. 3. Figure 5 illustrates the relative abundance of genera that significantly changed in response to chemotherapy. We revealed a drastic increase in the proportion of *Bacteroides* (30.3 %±5.8 vs

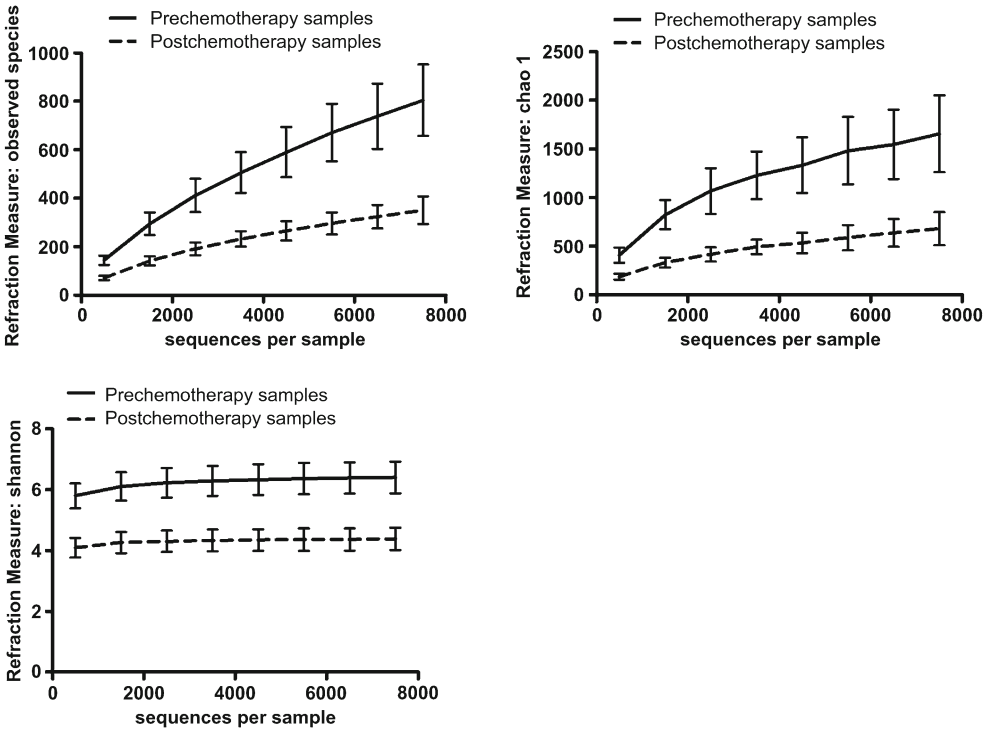

Fig. 2 Rarefaction analysis of *16S rRNA* gene sequences obtained from faecal samples of the patients. *Lines* represent the average of each group (prechemotherapy and postchemotherapy samples), while the *error bars* represent the standard deviations

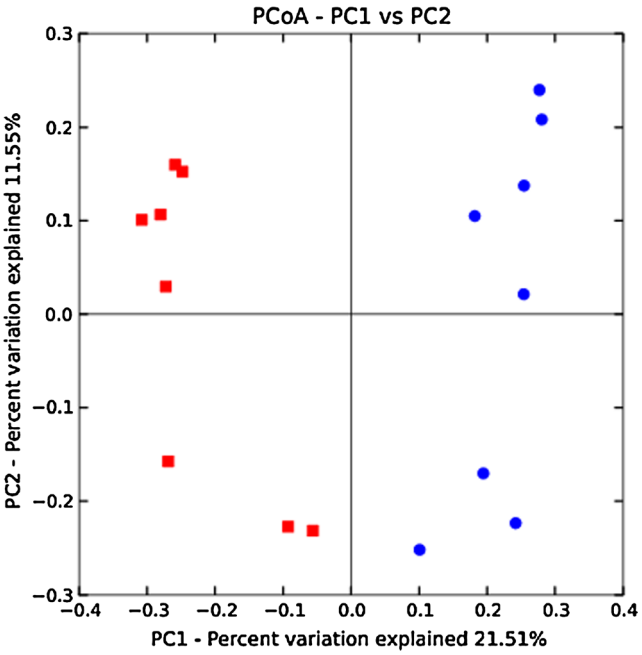

Fig. 3 Principal coordinate analysis (PCoA) of unweighted UniFrac distances between S1 (*red*) and S2 (*blue*) faecal samples. The percentage of variation explained by each PCoA is indicated on the axes

13.3 %±3.7 of the sequences, respectively, p=0.008) and an increase in the proportion of *Escherichia* (6.9 %±0.4 vs 0.6 % ±0.4, respectively, p=0.008) after chemotherapy, relative to before. We found a steep decrease in the proportion of *Blautia* (0.0 %±0.0 vs 5.9 %±0.7, respectively, p=0.008), *Faecalibacterium* (8.0 %±3.0 vs 17.2 %±3.1, respectively, p=0.04) and *Roseburia* (0.0 %±0.0 vs 6.9 %±1.6, respectively, p=0.008) after chemotherapy, relative to before. We also highlighted a pronounced decrease in the proportion of *Bifidobacterium* after chemotherapy, relative to before (0.1 %±0.0 vs 1.8 %±0.9, p=0.04).

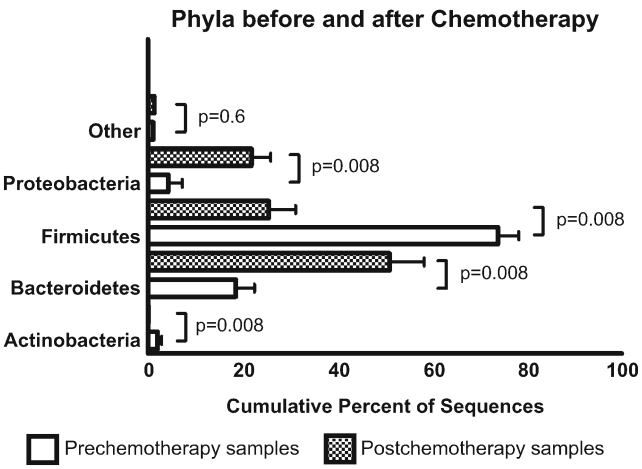

Fig. 4 Cumulative percentage of the phyla sequences detected during the prechemotherapy period and postchemotherapy samples compared using multiple Wilcoxon rank tests for paired data

Moreover, as a result of the drastic shift in the F/B ratio during chemotherapy, Gram-negative bacteria were more abundant after chemotherapy (64.2 %±3.4) than before (23.2 %±4.3). Thus, during chemotherapy, we observed a shift in the Gram-positive to Gram-negative bacteria ratio (p<0.001).

Furthermore, less abundant bacterial genera sequences (<0.01 %) appeared after chemotherapy. These genera were *Abiotrophia*, *Anaerococcus*, *Anaerofustis*, *Arcanobacterium*, *Corynebacterium*, *Finegoldia*, *Megasphaera*, *Methanosphaera*, *Mobiluncus*, *Paenibacillus*, *Parascardovia*, *Peptostreptococcus*, *Pyramidobacter*, *Rothia*, *Staphylococcus* and *Varibaculum*.

Discussion

By studying a homogeneous cohort of patients, we described a shift in faecal microbiota populations in response to intensive chemotherapy, using *16S rRNA* gene pyrosequencing. Firstly, we observed a steep reduction in alpha diversity during chemotherapy. Moreover, we observed significant differences in the composition of intestinal microbiota during chemotherapy, as shown by PCoA plots based on the unweighted UniFrac distance matrix.

We also found a drastic decrease in Firmicutes bacteria, which has also been found in the intestinal microbiota of mice following broad-spectrum antibacterial therapy [36]. This phylum was found more abundantly in obese patients and may increase the host's ability to harvest energy from the diet [14]. Thus, a chemotherapy-associated decrease in the abundance of Firmicutes has strong implications for cancer patients regarding the availability of energy. Moreover, we observed a steep reduction in the abundance of *Faecalibacterium*, a member of the Firmicutes. A previous study in patients with Crohn's disease found that a reduction of a major member of Firmicutes, *Faecalibacterium prausnitzii*, is associated with a higher risk of postoperative recurrence of ileal Crohn's disease. Further, *F. prausnitzii* exhibited anti-inflammatory effects both in vitro and in vivo in mice [37]. This implies that the subjects in the current study may have fewer intestinal microbiota with anti-inflammatory properties as a result of chemotherapy. These findings suggest that chemotherapy-associated alterations in intestinal microbiota may be involved in the pathophysiology of mucositis, indicating an inability to maintain intestinal microbiota with a protective role against inflammation [23].

Moreover, the pronounced increase of *Bacteroides* during chemotherapy was similar to the effect of broad-spectrum antibacterial therapy on murine intestinal microbiota, and contributed to the inversion of the F/B ratio, that has also been reported in Crohn's Disease [36, 37]. Furthermore, the decrease in Firmicutes was associated with a drastic increase in

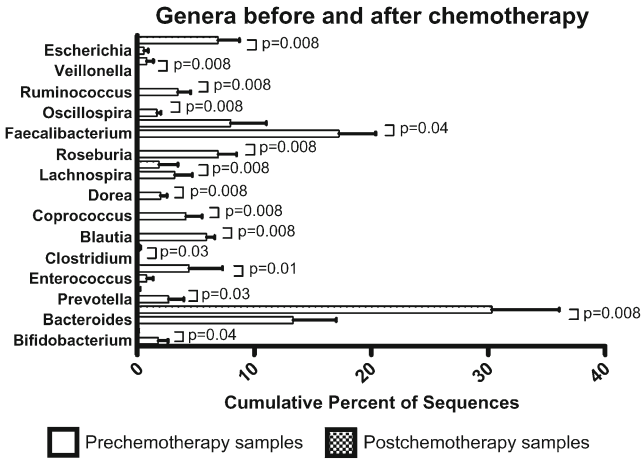

Fig. 5 Cumulative percentage of genera sequences detected in the prechemotherapy and postchemotherapy samples, compared using multiple Wilcoxon rank tests for paired data

Proteobacteria, as a result of increased *Escherichia*. Importantly, recent data have reported that *Escherichia coli* is the most frequently isolated pathogen in bacteremic cancer patients [38]. These results are consistent with a chemotherapy-induced increase in beta-glucuronidase-producing bacteria, especially *E. coli*, previously shown in murine intestinal microbiota [8, 25]. Thus, these chemotherapy-associated microbiota alterations may allow the establishment of potentially pathogenic organisms, through the pro-inflammatory properties of lipopolysaccharides for example, increasing the potential for acquired resistance to antibacterial agents. Importantly, these findings have not been previously reported in humans [25, 36].

Futhermore, we reported a decrease in *Bifidobacterium* in the postchemotherapy samples of our patients. A decreased relative abundance of *Bifidobacterium* has already been reported in inflammatory bowel disease patients [39]. Moreover, another study found that intestinal inflammation improved in mice that consumed a specific fermented milk product containing *Bifidobacterium lactis*, suggesting the eventual use of strategies for altering intestinal microbiota in favour of bifidobacteria to prevent potential adverse effects [40]. Thus, probiotics may be used to modulate the intestinal microbiota, inducing colonization resistance against pathogens and influencing host immune responses [41]. The results of our study support the fact that *Bifidobacterium* as a probiotic agent could be of interest for cancer patients receiving high doses of chemotherapy, to prevent the drastic shift in intestinal microbiota.

Moreover, we described the mode of action of each of the four antineoplastic agents used in our study and their impact on in vitro pure cultures of bacteria. As summarized in Table 2, based on in vitro data, the direct influence of antineoplastic agents on micro-organisms appears to be low. Only one drug, Etoposide, showed appreciable antibacterial activity at achievable plasma levels, specifically on Gram-positive bacteria [42, 43]. Its known mechanism of action is an inhibition of DNA topoisomerase II. Thus, it could very well be that the changes in faecal microbiota during chemotherapy, as reported in our study, were partly due to the topoisomerase inhibitor. However, a stronger antibacterial effect due to regional concentration of drugs, such as the supposed one in the gastrointestinal tract, cannot be excluded so far. Little is known regarding gastrointestinal accumulation of antineoplastic agents after intravenous administration.

Our study has several limitations. Firstly, we did not collect faecal samples from healthy volunteers or from hospitalized patients without chemotherapy. Moreover, we cannot evaluate the impact of the antibiotic prophylaxis given prior the study period as we did not collect faecal samples before hospitalization. Secondly, we used faecal samples to extrapolate the changes in intestinal microbiota similar to many of the studies investigating human intestinal microbiota [10–16]. Although several studies have reported that faecal microbiota differs from the adherent microbiota, collection of biopsy samples is difficult in immunocompromised patients, giving rise to technical difficulties and ethical questions [11, 44]. Thirdly, there is currently an on-going debate on the variability that can occur during a pyrosequencing assay [45]. Ideally, biological replicates of a single sample should be processed in parallel during the whole assay. Nevertheless, the cost of this option can be prohibitive, and alternatives have been proposed like performing PCR in replicates before pooling the PCR amplicons [46]. Even if it is becoming more popular, it is not yet set as a standard [47, 48]. The absence of replicates might therefore correspond to a limitation in our study. Finally, we did not complete the experiments, in particular regarding the anti-inflammatory properties of *F. prausnitzii*, a result obtained previously in a mouse model, which may provide some insight into the direct effects of chemotherapy on the immunomodulatory response of micro-organisms from the intestinal microbiota [38].

Conclusion

Following BMT conditioning chemotherapy in cancer patients, we observed (i) alterations in the diversity of faecal microbiota, (ii) a reduction in the abundance of organisms with anti-inflammatory properties, and (iii) dysbiosis characterized by a significant establishment of *Escherichia*. These chemotherapy-induced changes in the faecal microbiota may have strong implications for immunocompromised cancer patients. Future work will be directed at correlating clinical consequences with microbiota alterations in a larger cohort of patients, specifically gastrointestinal disturbances and bacteremia.

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Accession Number The V5–V6 16S rDNA bacterial sequences analysed in this paper have been deposited in the GenBank Short Read Archive (Accession number: SRA116522).

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