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Gut Microbiota Composition Correlates with Disease Severity in Myelodysplastic Syndrome

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ABSTRACT

The myelodysplastic syndrome (MDS) is a heterogeneous group of clonal disorders of hematopoietic progenitor cells related to ineffective hematopoiesis and an increased risk of transformation to acute myelogenous leukemia. MDS is divided into categories, namely lineage dysplasia (MDS-SLD), MDS with ring sideroblasts (MDS-RS), MDS with multilineage dysplasia (MDS-MLD), MDS with excess blasts (MDS-EB). The International Prognostic Classification System (IPSS) ranks the patients as very low, low, intermediate, high, and very high based on disease evolution and survival rates. Evidence points to toll-like receptor (TLR) abnormal signaling as an underlying mechanism of this disease, providing a link between MDS and immune dysfunction. Microbial signals, such as lipopolysaccharides from gram-negative bacteria, can activate or suppress TLRs. Therefore, we hypothesized that MDS patients present gut microbiota alterations associated with disease subtypes and prognosis. To test this hypothesis, we sequenced the 16S rRNA gene from fecal samples of 30 MDS patients and 16 healthy elderly controls. We observed a negative correlation between *Prevotella* spp. and *Akkermansia* spp. in MDS patients compared with the control group. High-risk patients presented a significant increase in the genus *Prevotella* spp. compared to the other risk categories. There was a significant reduction in the abundance of the genus *Akkermansia* spp. in high-risk patients compared with low- and intermediate-risk. There was a significant decrease in the genus *Ruminococcus* spp. in MDS-EB patients compared with controls. Our findings show a new association between gut dysbiosis and higher-risk MDS, with a predominance of gram-negative bacteria.

Keywords: Gut microbiota composition; Myelodysplastic Syndromes; 16S rRNA gene; Highrisk MDS; Elderly people

INTRODUCTION

The myelodysplastic syndrome (MDS) is a heterogeneous group of clonal disorders of hematopoietic progenitor cells characterized by cytopenia, dysplasia due to ineffective hematopoiesis, and associated increased risk of transformation to acute myeloid leukemia (AML) [1]. As most patients with MDS are elderly (median age range 65 to 70 years), its incidence and prevalence rise as the population ages [2].

Based on the risk of progression to AML, MDS is divided into high-and low-risk categories. Cytogenetic and molecular abnormalities, hemoglobin level, platelet and neutrophil counts, transfusion dependency, and percentage of blasts in the bone marrow are variables used to distinguish between high and low MDS risk [3,4]. Most importantly, the Revised International Prognostic Scoring System (R-IPSS), published by Greenberg et al in 2012 [4], is still the more robust way to predict the overall survival and risk of AML transformation.

The pathogenesis of MDS involves RNA splicing, epigenetic regulation, DNA repair system, mitotic and spindle genes, and chronic immune stimulation [5]. Chromosomal abnormalities are present in approximately 50% of de novo MDS, whereas somatic point mutations can be identified in up to 95% of cases [6]. Furthermore, an inflammatory microenvironment is present in MDS. This disease is characterized by immune dysfunction, stromal microenvironment, and cytokine imbalance [7.]

To our knowledge, no previous research assessed the role of the host gut microbiota in MDS. However, it is worth noting that earlier research has suggested that gut microbiota plays a role in hematopoiesis. Indeed, the gut microbiota has an impact on iron metabolism and hepcidin induction [8], platelet disorders [9], and post-transplant reactions [10]. Furthermore, besides hematopoiesis, gut microbiota regulates inflammation by influencing the differentiation of inflammatory cell types and cytokine production [11]. Hence, the knowledge about gut microbiota modifications in MDS patients will open new avenues for understanding the mechanisms underpinning this syndrome and developing novel therapeutic strategies, such as probiotics.

We hypothesized that MDS patients present gut microbiota alterations associated with disease subtypes. Therefore, our primary outcome was to identify and analyze the association between gut microbiota diversity in patients with MDS compared with the gut microbiota of the elderly without hematological disorders. Our secondary outcome investigated the association between gut microbiota profile in MDS patients and disease prognosis.

MATERIALS AND METHODS

Patients and Ethical Aspects

The institutional review board of the Federal University of Ceara/PROPESQ-UFC (number: 58761816.2.0000.5054) approved the study. All participants read and signed the written informed consent. Furthermore, the study complied with the ethical precepts of the research, based on Resolution 466/12 of Brazil's National Health Council and the Declaration of Helsinki.

This study included patients (9 men and 18 women) diagnosed with MDS aged 45 to 95 years who attended the Drug Research and Development Center (NPDM) of the Federal University of Ceara. MDS patients (n=27) were classified according to the 2016 WHO classification. Twenty-one patients were classified as early MDS (MDS-SLD, MDS-RS, MDS-MLD) and six as advanced MDS (MDS-EB1/EB2). Patients were also classified according to the R-IPSS score as low (very low + low, n=17), intermediate (n=5), and high risk (high + very high, n=5) (Table 1). The control group comprised 16 healthy elderly individuals. The exclusion criteria were patients with heart disease, pneumopathy, liver disease, history of alcoholism, psychiatric disorders, and those on chemotherapy or who had used antibiotics 30 days before the sample collection.

Before sample collection, the patients answered a questionnaire with their personal information, eating habits, medication use, intestinal symptoms, and pre-existing diseases. (See the questionnaire in Supplementary Methods.)

Sample collection and preparation

The stool samples were collected on the day of the patient's consultation. The samples were transported to the laboratory within an icebox (not exceeding four hours) and aliquoted in cryotubes. The samples were kept at −80 °C until the DNA extraction was performed.

DNA extraction

Firstly, the samples were vigorously homogenized using a MiniBeadBeater (BioSpec, Bartlesville, OK, USA) to effectively liberate bacterial DNA from complex fecal biomass (THOMAS, 2015). For this homogenization, we used samples around 0.3 g, 0.1 mm glass beads (BioSpec, Bartlesville, OK, USA) incubated at 95 °C in lysis buffer (QIAamp® Fast DNA Stool Mini Kit, QIAGEN Inc., Valencia, CA, USA).

After that, the DNA was extracted and purified from 0.2 g (total lysed humid weight) of stool using the QIAamp® Fast DNA Stool Mini Kit, according to the manufacturer's instructions.

We evaluated the quality of extracted DNA with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and confirmed by electrophoresis in 0.8% agarose gel with 1 × TAE buffer. In addition, the DNA was quantified by Qubit® 2.0 fluorometer using the dsDNA BR Assay kit (Invitrogen™, SP, Brazil). Extracted DNA was stored at −20 ºC until analysis.

Sequence processing and data analysis

We generated the amplicon library using primers (515F / 806R) targeting the V4 region of the 16S rDNA.

The conditions for PCR were as follows: 94 °C for 4 min to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 2 min, with a final extension of 10 min at 72 °C. In addition, a specific Illumina Nextera XT index pair (Illumina, San Diego, CA) was added to the purified PCR product for indexing. Thus, each 50 µL reaction contained: 23.5 µL of nuclease-free water (Certified Nuclease-free, Promega, Madison, WI, USA), 5.0 µL of 10× High Fidelity PCR Buffer (Invitrogen, Carlsbad, CA, USA), 4.8 µL of 25 mM MgSO4, 1.5 µL of dNTP (10 mM each), 5.0 µL of each Nextera XT index (Illumina, San Diego, CA, USA), 1.0 unit of Platinum Taq polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), and 5.0 µL of each purified PCR product. A final concentration of 10 pM purified amplicons together with 20% PhiX for loading in the MiSeq sequencer in accordance with the standard protocols by Illumina (San Diego, CA, USA).

In paired-end mode, Illumina adapter sequences were trimmed from the already demultiplexed raw fastq files using Cutadapt v1.8. The quality of the reads was assessed using FastQC v.0.11.8 (Martin, 2013) and vsearch v2.10.4 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Subsequent analyses were performed within the R v3.5.3 environment (R Development Core Team, 2016), following DADA2 v1.11.1 (B. J. Callahan et al., 2016) package authors suggested pipeline and adjusting parameters to our data. The product was non-chimeric amplicon sequence variants (ASVs) (B. J. Callahan, Mcmurdie, & Holmes, 2017) table, which records the number of times each ASV (sequence differing by as little as one nucleotide) was observed in each sample. Taxonomy assignment of representative ASVs was performed with DADA2 against SILVA 132 reference database (B. Callahan, 2018). Finally, we performed downstream analyses using the phyloseq v1.26.1 package (Mcmurdie & Holmes, 2013) and graphical representations with ggplot2 v3.1(Wickham, 2009).

Analysis of α-diversity and species richness

Alpha-diversity estimators were calculated and tested for normality by the Shapiro-Wilk test. As the Shannon diversity and Simpson evenness were parametric, a one-way analysis of variance and Tukey's honestly significant difference (HSD) post hoc tests were used for multiple comparisons of means at a 95% confidence interval. For richness, Chao1 index, and Observed ASVs, we used the Kruskal-Wallis non-parametric test.

Statistical Analysis

Initially, the BIOENV tool (part of the vegan package for R) was used to select the subset of variables that best explain the variance of biological data, using biotic dissimilarity matrices and the data for each patient. Then, this subset of variables, defined for each sample, was used in the PERMANOVA test (adonis2 function of the vegan package), a non-

parametric method like the analysis of variance. PERMANOVA test uses permutation methods to test differences between groups to define their significance and correlation with biotic data [12]. Finally, P-value was set at P< 0.05.

RESULTS

Clinical characteristics, α-diversity, and the richness of gut microbiota in MDS patients

Table 1 presents the characteristics, WHO classification and R-IPSS score of the patients included in this study. Based on the analysis of α-diversity and richness of the samples, no differences were observed in the Shannon, Simpson, and Chao1 indices, comparing the median values obtained in the control and MDS groups. This indicates that the α-diversity and the richness of the samples are equivalents between the two groups studied (Figure 1).

Table 1: Clinical characteristics of MDS patients

Characteristics of MDS Patients	All Patients	Early MDS	Advanced MDS
MDS Patients number	27	21	6
Age, median (range)	74 (46 – 89)	73 (51-87)	77 (46-89)
Gender (number of patients)			
Female	18	15	3
Male	9	6	3
Hemoglobin g/dL, median (range)	8.7 (6.1 – 13.3)	8.6 (6.3 – 13.3)	7 (6.1 – 12.8)
ANC × 10 L−1, median (range)	1843 (117 – 5200)	1190 (711 - 2592)	2859 (117 – 5200)
Platelet (mm3) no. (%)			
<50000	6 (22%)	4 (19%)	2 (33%)
50000 – 100000	8 (30%)	7 (33%)	1 (17%)
>100000	13 (48%)	10 (48%)	3 (50%)
Karyotype, no. (%)			
Normal	10 (37%)	10 (48%)	0
Abnormal	17 (63%)	11 (52%)	6 (100%)
IPSS prognostic risk categories no. (%)			
Low			
Intermediated	17 (62%)	15 (71%)	2 (33%)
High	5 (19%)	5 (24%)	0
	5 (19%)	1 (5%)	4 (67%)
Transfusion dependence, no. (%)			
No dependence	23 (85%)	19 (90%)	4 (67%)
Dependence	4 (15%)	2 (10%)	2 (33%)

Early (MDS-SLD, MDS-RS, MDS-MLD); advanced (MDS-EB1/EB2) and R-IPSS score: low (very low + low); intermediate, and high risk (high + very high)

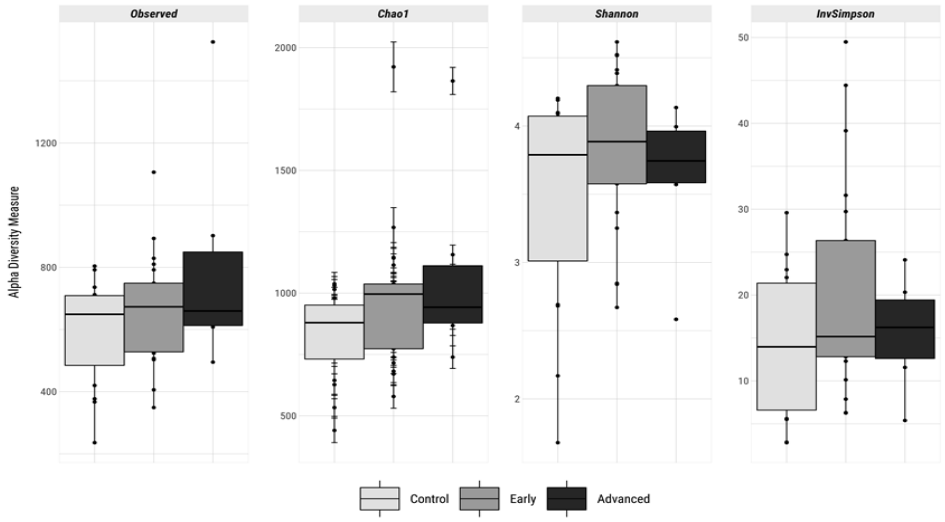

Figure 1. α-diversity and the richness of the samples between control and MDS groups

Based on the Spearman correlation test, there was a negative correlation (-0.42) between *Prevotella* spp. and *Akkermansia* spp. in MDS patients compared with the control group (Figure 2).

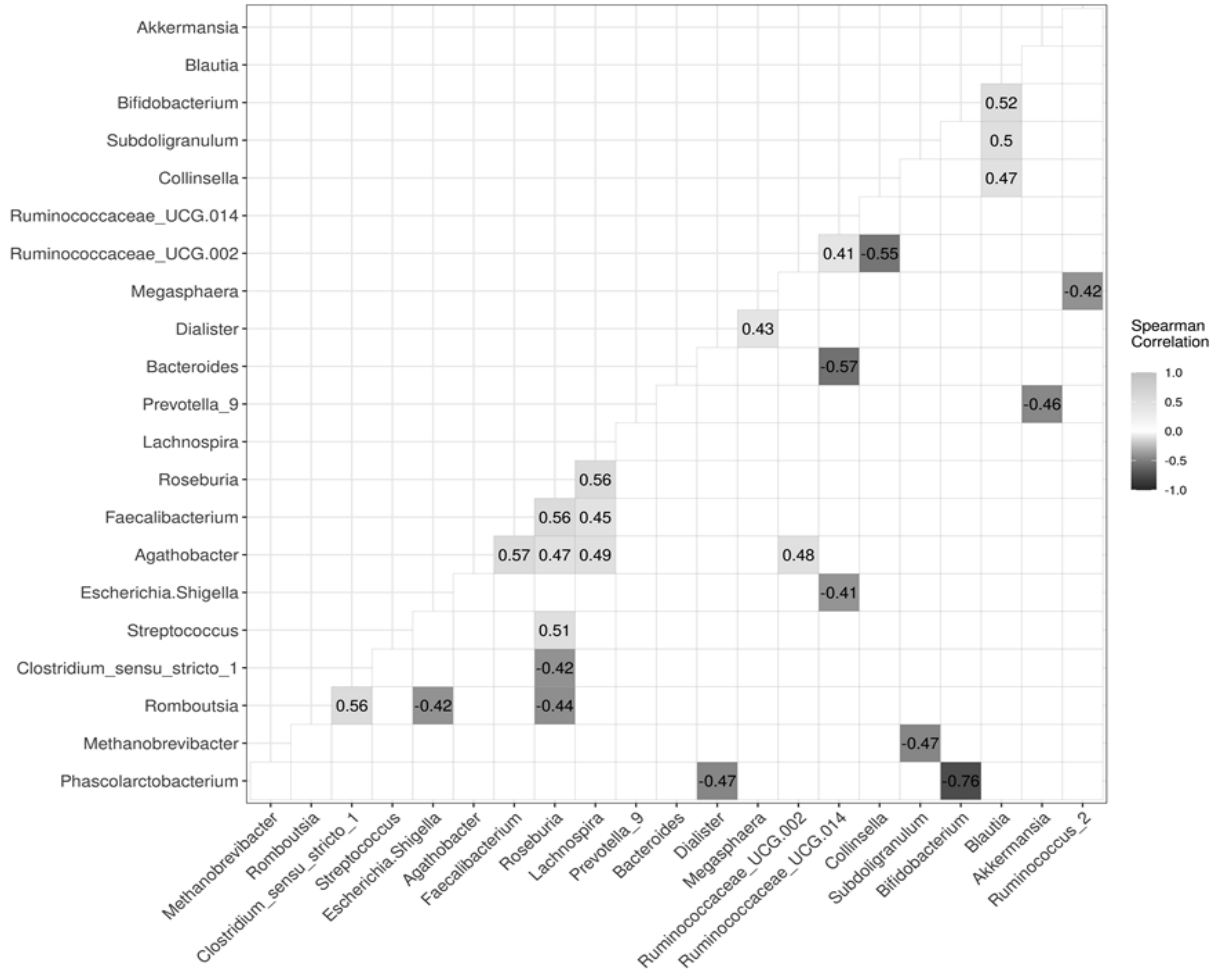

Figure 2. Correlations between the composition of the fecal microbiota of control and MDS groups based on Spearman correlation test

High-risk patients based on R-IPSS have a unique profile: increase of *Prevotella* and decrease in *Akkermansia*

High-risk patients presented a significant increase in the genus *Prevotella* spp. in relation to the other risk categories (P= 0.011) (Figure. 1A). On the other hand, there was a significant reduction in the abundance of the genus *Akkermansia* in the group of high-risk patients in comparison with low- and intermediate-risk (P = 0.005) (Figure 3C).

MDS subtypes according to WHO 2016

Regarding the genus distribution, there was a significant decrease in the genus *Ruminococcus* in MDS-EB1/EB2 patients compared with controls (P= 0.004) (Figure 3B).

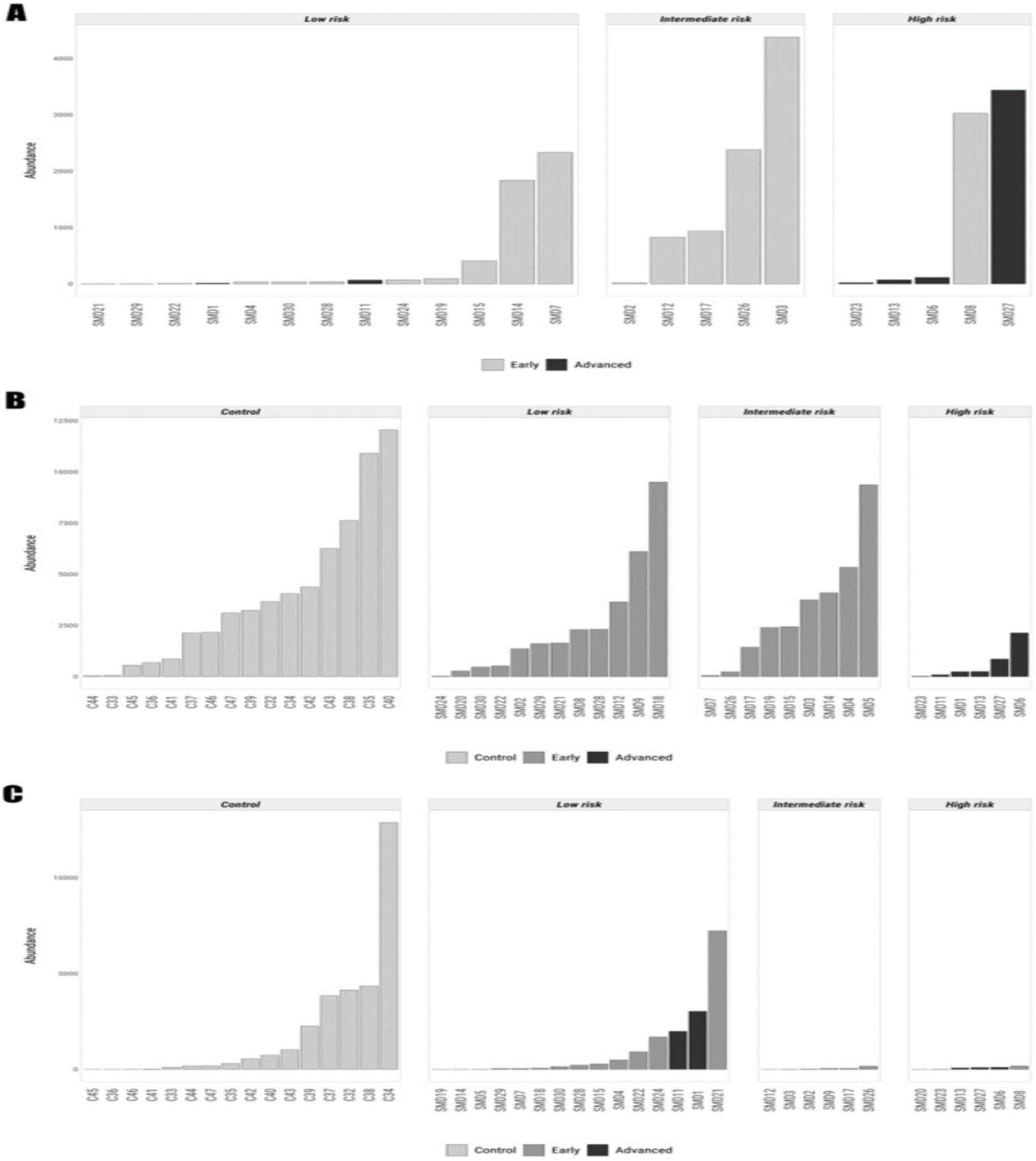

Figure 3. Distribution of the genus *Prevotella* (A), family Ruminococcaceae (B), and genus *Akkermansi*a (C) according to the revised International Prognostic Scoring System (R-IPSS) prognostic score in MDS patients and control samples. Patients are identified according to their MDS category

DISCUSSION

Our findings show gut microbiota alterations in MDS patients. Furthermore, high-risk patients presented the main alterations, providing novel mechanisms underpinning this syndrome's severity.

A wide range of inflammatory disorders present with gut microbiota composition changes, as observed in human and animal studies[13]. Furthermore, gut microbiota influences hematopoiesis[14], as supposed by Huijuan and collaborators, 2022[15], who concluded that the gut microbiota alterations in patients with MDS, which may be involved in the immunopathogenesis of the disease, probably cause an immune imbalance in MDS.

No significant difference was observed between the composition of the intestinal microbiota and the sex of the patients. Considering that most patients analyzed were female, despite the prevalence of MDS being described as higher in men[16]. It is also important to highlight that the composition of the intestinal microbiota of transfusion-dependent patients was compared with that of the non-transfusion-dependent group, to analyze whether there was any interference from this type of treatment. However, no significant difference was observed between these two groups of patients.

Our results revealed that high-risk MDS patients significantly increased the genus *Prevotella*. Notably, among the vast composition of the human intestinal microbiota, the exacerbated abundance of the genus *Prevotella*, known as a biomarker of one of the three proposed human intestinal enterotypes[17], has been associated with the manifestation of inflammatory diseases, such as rheumatoid arthritis[18], and inflammatory bowel disease[19]. Our first defense against infection is initiated by innate immunity, and the recognition of pathogens occurs by pattern recognition receptors (PRRs) expressed in hematopoietic and no hematopoietic cells. Under normal conditions, hematopoietic cells (HSC) are quiescent. At the same time, in response to inflammation, they proliferate and signal through toll-like receptors (TLRs). The sustained exposure to TLR signals is associated with loss of normal HSC function, negatively influencing these cells towards apoptosis and myeloid differentiation. This mechanism has been an important phenomenon in bone marrow disorders such as myelofibrosis, acute myeloid leukemia, and MDS[20].

Prevotella genus, more precisely the *P. intestinalis* species, promotes inflammation and intestinal dysbiosis, an effect shared with other intestinal bacteria. These damaging effects of *Prevotella* on the host's physiology can be caused by several factors, including direct effects of this bacteria, or mediated by modification of intestinal microbiome composition. Thus, the abundance of this genus is interpreted as a marker of a new microbiome pattern, potentially inflammatory. Furthermore, the increase in the severity of the intestinal inflammatory process is associated with changes in the microbial ecosystem and the metabolites produced in this microenvironment [21.]

Previous studies have shown that inflamed tissues of mice colonized with *P. intestinalis* present increased levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α, in addition to higher levels of pro-inflammatory chemokines, with consequent neutrophil infiltration. It is also well demonstrated that colonization by *Prevotella* shapes the host's immunity, causing a reduction in IL-18 production. Furthermore, previous studies have shown that reducing IL-18 production by 1.3-1.5 times is sufficient to disturb the homeostasis of the intestinal microenvironment[22]. MDS has been constantly reported with increased IL-6 and TNF-α, which also may predispose to apoptosis, a critical phenomenon related to anemia in MDS. These cytokines create bone marrow with modifications called the inflammasome. The prototypical example of a hematological disease caused by bone marrow inflammation is aplastic anemia, similar to hypoplastic MDS [23].

Prevotella spp. also influences the production of short-chain fatty acids (SCFAs). SCFAs are beneficial for intestinal health since they are a source of energy for cells and contribute to maintaining intestinal barrier function, being indicated even as protectors against the development of colorectal cancer and in the control of local inflammatory processes[24]. The intestinal production of SCFAs is influenced by the composition of the diet and the microbiota [25]. Hence, there is an inverse relationship between the prevalence of *P. intestinalis* and the levels of SCFAs

produced in the intestine[20]. Furthermore, Iljazovic 2020 and collaborators[21] showed that the intestinal colonization of *Prevotella* sp. in mice causes a decrease in the relative abundance of the phyla Bacteroidetes and Firmicutes (Lachnospiraceae and Ruminococcaceae), which may explain the reduction in the production of SCFAs. The Ruminococcaceae is an important family of bacteria producing SCFAs[26].

Regarding MDS, we know that DNA methylation is prevalent in tumor suppressor genes, principally MDS-EB1/EB2. In this context, the use of Azacytidine and decitabine aims to allow the activation of tumor suppressor genes. It is known that SCAFs may modify epigenetics, can function as histone deacetylase (HDACs) inhibitors[27], which is related to the suppression of malignant transformation and the stimulation of apoptosis of precancerous cells. Therefore, positively influencing this hypomethylating agents commonly used to treat MDS-EB1/EB2.

The reduction in the genus *Ruminococcus,* observed here in advanced MDS patients, i.e., MDS-EB1/EB2 subtype, may increase the inflammatory processes constantly reported in bone marrow disorders. We do not know if this effect is related to reducing SCFAs, but this result creates a new idea that deserves further evaluation.

Given this well-known set of changes promoted by the predominance of *Prevotella* in the intestinal microenvironment, these changes can affect the intestinal barrier homeostasis [21]. Indeed, changes in the intestinal barrier contribute to bacterial translocation and metabolites into the individual's bloodstream. Based on our findings, we can speculate that changes in the intestinal barrier caused by *Prevotella* in MDS patients may be related to alterations in IL-18 since this cytokine has a protective role in preventing dysbiosis [28], promoting the integrity and regeneration of the epithelial barrier[29]. To date, there is no study evaluating intestinal barrier permeability in MDS patients. Despite this, MDS patients present a higher expression of the CD40 receptor on monocytes, contributing to bone marrow failure through CD40–CD40L interactions with T helper cells[30]. Since lipopolysaccharides (LPS) are regulators of CD40 expression in macrophage[31], we can infer that the gut dysbiosis detected here in MDS patients is causing bacterial translocation and influencing the peripheral immune response of these patients by LPS release to the plasma. Therefore, intestinal permeability, IL-18, and LPS plasma levels in MDS patients must be evaluated in future studies since this mechanism may contribute to the inflammatory alterations observed in these patients.

Notably, TLR-4, which mediates the recognition of LPS, is the best-described receptor in MDS. The constant activation of TLR-4 is associated with DNA damage induced by reactive oxygen species (ROS), generating the accumulation of genotoxic components[32] and, consequently, chromosomal abnormality[33]. Indeed, LPS activation of TLR-4 receptors may justify some inflammatory alterations observed in MDS, such as the NLRP3-mediated inflammasome activation that triggers the permanent release of pro-inflammatory cytokines such as IL-1β[34].

Thus, we can assume that an imbalance in the composition of the microbiota and the maintenance of the intestinal barrier may result in changes in the hematopoiesis process, which would be even more severe for patients with MDS, which already presents alterations in hematopoiesis. Furthermore, a recent study shows that microbial content, originating from translocation events, circulating in the blood in patients with myeloid malignancy such as MDS, interferes with disease subtypes [35].

Still concerning the intestinal barrier, interestingly, our study found a negative correlation between the genera *Prevotella* and *Akkermansia* in SMD MDS patients. The *A. muciniphila* controls the mucus barrier in the intestinal epithelium that functions as a protective physical barrier by acting against toxic metabolites in the intestine and controlling translocation events. Therefore, this species acts by degrading mucin when it is in excess and stimulating its production by goblet cells of the intestinal epithelium, when it is scarce[36]. Of utmost importance, the use of *A. muciniphila* as microbial therapy for altering immunotherapy response was reported by Routy et al[37]. They demonstrated reversion of rapid tumor growth and antibiotic-induced dysbiosis using anti-programmed death 1 immunotherapy plus *A. muciniphila*. Although still

rare, this type of therapy is growing, and its evidence increases daily [36].

The present study has some limitations since we did not perform experiments to detect plasma LPS, IL-18 alterations, and SCFA levels, which would help us explain the mechanisms suggested here.

In conclusion, the present study's findings show a new association between dysbiosis (with the predominance of gram-negative bacteria) and higher-risk MDS. This dysbiosis can contribute to a systemic inflammatory profile in these patients, hindering clinical management and a favorable outcome for the patient's survival. Thus, we can propose that intestinal dysbiosis, observed in higher-risk MDS patients, can contribute to LPS translocation to the bloodstream leading to TLR-4 and NLRP3 activation. This activation results in chronic inflammatory alterations observed in MDS patients, promoting DNA damage and chromosomal abnormalities. This study opens new avenues for studying the influence of gut microbiota composition in response to hypomethylating agents in MDS.

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CONFLICT OF INTEREST

The authors declare no conflict of interests. The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

The institutional review board of the Federal University of Ceara/PROPESQ-UFC (number: 58761816.2.0000.5054) approved the study. All participants read and signed the written informed consent. Furthermore, the study complied with the ethical precepts of the research, based on Resolution 466/12 of Brazil's National Health Council and the Declaration of Helsinki.

Consent to participate

Written informed consent was obtained from each participant and is available under request directly to giovanna@ufc.br.

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Availability of data and material

16S data is available under request directly to Prof. Giovanna Riello (giovanna@ufc.br).

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