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Preservation of fecal microbiome is associated with reduced severity of Graft-versus-Host Disease

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Abstract:
Following allogeneic hematopoietic cell transplantation (allo-HCT), the gastrointestinal (GI) tract is frequently affected by acute graft-versus-host disease (aGVHD), the pathophysiology of which is associated with a dysbiotic microbiome. Since microbial composition varies along the length of the GI tract, we hypothesized that microbiome features correlate with the pattern of organ involvement after allo-HCT. We evaluated 266 allo-HCT recipients from whom 1,303 stool samples were profiled by 16S ribosomal gene sequencing. Patients were classified according to which organs were affected by aGVHD. In the 20 days prior to disease onset, GVHD patients had lower abundances of members of the class Clostridia, lower counts of butyrate producers, and lower ratios of strict-to-facultative anaerobic bacteria (S/F anaerobe ratio) compared to allograft recipients who were free of GVHD. GI-GVHD patients showed significant reduction in microbial diversity pre-onset. Patients with lower GI-aGVHD had lower S/F anaerobe ratios compared to those with isolated upper GI-aGVHD. In the 20 days after disease onset, dysbiosis was observed only in GVHD patients with GI involvement, particularly those with lower-tract disease. Importantly, Clostridial and butyrate-producer abundance, as well as S/F anaerobe ratio were predictors of longer overall survival; higher abundance of butyrate producers, and higher S/F anaerobe ratio were associated with decreased risk of GVHD-related death. These findings suggest that the intestinal microbiome can serve as a biomarker for outcomes of allo-HCT patients with GVHD.

Conflict of interest: COI declared - see note

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Preservation of fecal microbiome is associated with reduced severity of Graft-versus-Host Disease

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Key points:

- Patterns of microbial dysbiosis can be detected in fecal samples of GI-aGVHD patients peri-aGVHD onset.

- Markers of microbial health pre-GVHD onset are associated with longer survival and lower risk of GVHD-related mortality after allo-HCT.

Abstract

Following allogeneic hematopoietic cell transplantation (allo-HCT), the gastrointestinal (GI) tract is frequently affected by acute graft-versus-host disease (aGVHD), the pathophysiology of which is associated with a dysbiotic microbiome. Since microbial composition varies along the length of the GI tract, we hypothesized that microbiome features correlate with the pattern of organ involvement after allo-HCT. We evaluated 266 allo-HCT recipients from whom 1,303 stool samples were profiled by 16S ribosomal gene sequencing. Patients were classified according to which organs were affected by aGVHD. In the 20 days prior to disease onset, GVHD patients had lower abundances of members of the class Clostridia, lower counts of butyrate producers, and lower ratios of strict-to-facultative anaerobic bacteria (S/F anaerobe ratio) compared to allograft recipients who were free of GVHD. GI-GVHD patients showed significant reduction in microbial diversity pre-onset. Patients with lower GI-aGVHD had lower S/F anaerobe ratios compared to those with isolated upper GI-aGVHD. In the 20 days after disease onset, dysbiosis was observed only in GVHD patients with GI involvement, particularly those with lower-tract disease. Importantly, Clostridial and butyrate-producer abundance, as well as S/F anaerobe ratio were predictors of longer overall survival; higher abundance of butyrate producers, and higher S/F anaerobe ratio were associated with decreased risk of GVHD-related death. These findings suggest that the intestinal microbiome can serve as a biomarker for outcomes of allo-HCT patients with GVHD.

INTRODUCTION

Graft-versus-host disease (GVHD) is a common complication after allogeneic cell transplantation (allo-HCT) that is initiated by donor immune cell activation against host tissue leading to organ damage. Acute GVHD (aGVHD) targets regions of the gastrointestinal (GI) tract, skin, and liver and may manifest in single or multiple organs. Involvement of the lower gastrointestinal (LGI) tract is associated with a lower likelihood of GVHD treatment response and a higher risk of mortality[1-4]. In contrast, GVHD that is isolated to the upper GI (UGI) tract or skin is commonly responsive to treatment and has negligible prognostic relevance for mortality[5,6].

Although the general immunopathology of GVHD has been investigated at length, the determinants of specific organ involvement in patients are not known. In mice with GVHD and in other colitis models, dendritic cells imprint tissue-specific homing molecules on T cells that drive trafficking to the GI tract. Less is known about specific homing to the upper vs. lower intestine, although expression patterns of homing molecules such as $\alpha_4\beta_7$ integrin, GPR15, CCR9, and CXCR3 in various T cell subsets have been implicated[7-10]. The pathways governing gut T cell trafficking in human GVHD are less clear, as are the factors that might vary between patients to explain patterns of organ involvement.

The GI tract harbors a dynamic population of microbial organisms, the composition of which increases in density and diversity along the length of the tract[11]. GI microbial colonization is relevant in allo-HCT since features of the intestinal microbiome such as diversity and dominance of specific bacteria have been associated with transplant-related mortality (TRM) and GVHD outcomes[12-19]. Studies evaluating microbiome disruption and diversity in the peri-engraftment period have demonstrated an association between dysbiosis and survival outcomes[13-18], which might be explained by an outgrowth of harmful organisms, a loss of

beneficial or homeostatic commensals, or a combination of both. We have previously reported that higher abundance of the anaerobic intestinal commensal *Blautia* (member of Clostridia class) in the peri-engraftment period is associated with reduced GVHD-related mortality and prolonged overall survival (OS)[15]. Moreover, the loss of members of class Clostridia has also been observed in mouse models of GVHD[12]. Some anaerobic commensals, among them many Clostridia (class), are producers of short-chain fatty acids (SCFA), a class of metabolites that include butyrate, propionate and acetate, as a byproduct of carbohydrate fermentation[20]. Several groups have observed lower concentrations of SCFA, specially butyrate, in patients with GVHD at peri-engraftment and at disease onset[19,21]. In addition, we observed lower circulating amounts of butyrate, amongst other SCFA, three months post-transplant in patients who went on to develop chronic GVHD (cGVHD)[22]. Microbiota-derived butyrate can modulate GI epithelial cell damage and mitigate GI aGVHD in mice by restoring intestinal epithelial cell junctional integrity and decreasing apoptosis[23-25]. Nonetheless, further research on the role of butyrate is needed, as one study linked the presence of butyrogenic bacteria to GVHD severity[26] and butyrate may also be toxic to intestinal stem cells[27]. These findings raise the possibility that microbial-derived metabolites have direct effects on GVHD target tissues and may affect disease outcomes. Several studies have linked intestinal injury to changes in the commensal flora in the gut[28,29] characterized by an outgrowth of such facultative anaerobes as Enterococcaceae (family)[14,18,25] and *Gemella* (genus)[30,31] along with a decrease in strict anaerobes, such as *Faecalibacterium prausnitzii (species)*[28] . However, it is unknown whether this loss of anaerobiosis also happens in the context of GVHD. We hypothesized that unique patterns of microbial dysbiosis correlate with organ-specific intestinal involvement in GVHD and consequently influence GVHD outcomes after allo-HCT.

METHODS

Patients and Graft Characteristics

Patients included in the analysis were all consecutive adult recipients of unmodified allografts at Memorial Sloan Kettering Cancer Center between 01/2011 and 02/2017 for the treatment of hematologic malignancies who had stool samples collected in our institutional fecal microbiome biobank. Patients provided written consent to biospecimen collection, and the analysis was approved by the institutional review board.

Study Definitions

aGVHD was diagnosed with histological confirmation as clinically appropriate. The International Bone Marrow Transplant Registry classification was used to guide aGVHD grading, except grades A-D were labeled as grades I-IV. Grading was reviewed by a transplant clinician panel[32]. Patients were classified in four cohorts according to aGVHD organ involvement by day 100 post allo-HCT: A) no GVHD in any organ, B) non-GI: skin and/or liver involvement without any GI tract involvement, C) UGI: UGI tract involvement without LGI tract, with or without skin/liver involvement, and D) LGI: any LGI tract involvement with or without any other organ involvement, including UGI aGVHD (**Table 1**). Relapse was defined as recurrence or progression of hematologic malignancy post-HCT. Causes of death were described according to the Copelan algorithm[33], TRM was defined as death from any cause not preceded by relapse. GVHD-related mortality included patients without disease recurrence being treated for GVHD at the time of death including those who died of infection. Relapse, OS, TRM and GVHD-related mortality were calculated from the time of GVHD onset to death.

Analysis of Fecal Samples

Stool samples from an institutional fecal microbiome biobank were analyzed. Samples collected after a fecal microbiota transplant were removed from the analysis[34]. DNA was purified using bead-beating in phenol-chloroform as previously described[35]. Amplification of Genomic 16S ribosomal RNA V4/V5 regions were PCR-amplified and sequenced on the Illumina

platform. Sequences were mapped to amplicon sequence variants (ASVs) using DADA2[36] and assigned taxonomical identity according to the NCBI 16S database. Microbial α-diversity was computed using the reciprocal Simpson index[37]. Exposure to a list of gut-microbiome-perturbing antibiotics[34] was assessed according to the interval of sample collection (7 days prior to the first sample collected to 1 day prior to the last sample collected) **(Supplemental Figure S1**) and classified according to published data on bacterial inhibition[16,38-46] **(Supplemental File ST1)**. Organisms were classified as strict vs. facultative anaerobes by collapsing AGORA (assembly of gut organisms through reconstruction and analysis) annotations[47] into categories according to their predicted oxygen metabolism: the terms “anaerobes” and “strict anaerobes” were categorized as **strict anaerobes,** and the terms “aerotolerants, “obligate aerobes”, “facultative anaerobes”, “microaerophile/anaerobes”, “aerobes”, “nanaerobes”, “facultative anaerobes” were grouped as **facultative anaerobes**. The microbiome was classified at the genus level when all species within it belonged to the same category, whereas species level distinctions were applied otherwise. The curated list of predicted butyrate producers was adapted from Haak *et al*.[48]. Predicted metabolic functions were computationally derived from 16S rRNA sequences using PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states)[49]. Metagenomic shotgun sequencing was conducted as previously described[50]. In summary, samples were extracted, sheared to 650bp, prepared using the Illumina TruSeq DNA library kit and sequenced using the Illumina HiSeq system with coverage of 10-20x10^6 reads per sample and read length of 100 bp paired-end. Human sequence contaminants were removed using BMTagger and HUMAnN 3.0 and MetaCyc[51,52] were used for annotation of microbial metabolic pathways and relative expression considered at counts per million (cpm).

Statistical Analysis

A Wilcoxon rank-sum test was used to compare microbial associations across groups. A false discovery rate (FDR) correction was applied to these tests when comparing taxa at genus

level, along with PICRUSt and shotgun associations, across GVHD-defined groups. The associations were further summarized by calculating the fold-change in microbiota features across groups. For the primary analysis of transplant outcomes, associations between microbial markers and OS, GVHD-related mortality, TRM and relapse were estimated by first categorizing GVHD patients above and below the median value for each marker within the pre-onset window. As a secondary analysis, the markers were analyzed continuously and log-transformed, as appropriate. For OS, associations were estimated using Cox proportional hazards regression. The incidence of GVHD-related mortality and TRM were estimated using cumulative incidence functions, treating relapse and death unrelated to GVHD as competing events for GVHD-related mortality and relapse for TRM. A Fine and Gray model was used to further adjust for gender, age, and conditioning regimen. All statistical analyses were performed using R (version 3.6.2).

Data Sharing Statement

Sequencing data have been deposited in NCBI databases; accession identifiers are tabulated in **Supplemental File ST2.**

RESULTS

Patient Demographics and aGVHD

A total of 135 patients who developed grade I-IV aGVHD by day 100 after HCT had stool samples collected near the time of onset. GVHD occurred at a median onset of 32 days (range 13-86). The proportion of grade II-IV and III-IV aGVHD was 47% and 13%, respectively. The GI tract was the organ most commonly involved (106/135), followed by skin (67/135), and liver (2/135). Patients predominantly had grade II aGVHD. Notably, grade IV aGVHD occurred only in patients with LGI involvement. We hypothesized that the reason the LGI tract may be spared in some patients is due to protective features of the colonic microbiota. To explore this, we

grouped these patients according to the GVHD target organ affected (**Table 1**), including non-GI (n = 29), UGI (n = 53), and LGI (n = 53). As we have previously reported that allo-HCT patients have microbiome compositions that are distinct from those of healthy volunteers before[18], during[18], and for at least one year following transplantation[22] , we included as controls a set of 1066 fecal samples from 131 recipients of unmodified allografts who did not develop GVHD. The non-GI group had predominantly skin involvement whereas the UGI group had isolated UGI or UGI combined with skin involvement. LGI cases had predominantly LGI combined with UGI involvement with or without skin. **Table 2** summarizes the clinical variables of the groups. The no-GVHD group received more chemotherapy-based conditioning and fewer cord blood grafts, whereas the UGI group received predominantly unrelated donor and cord blood grafts. Non-GI and LGI cases were more likely to have a reduced intensity conditioning and an HLA-matched unrelated donor or cord blood graft.

Genomic 16S sequences were generated from 1303 stool specimens from 266 patients (average, 4.89 samples per patient). Stool specimens were grouped into pre-onset (day -20 to day -1 relative to onset of GVHD) and post-onset (day 0 to day 20 relative to GVHD onset) bins for the analyses; this resulted in an overall sampling range of day -3 to day 102 relative to HCT. For patients without GVHD, samples were included when collected in the same overall sampling window relative to HCT (**Supplemental Figure S1A-C**). When patients had samples collected on multiple days within a sampling window, the average of each microbiome feature across all samples were taken. In the event that more than one sample was collected on the same day from the same patient, one sample from that day was randomly selected for the analysis and others omitted.

Dysbiosis in GVHD

To map patterns of microbial injury to specific organ involvement in aGVHD we first characterized microbial differences between cohorts both pre- and post-onset. Analysis of

microbial composition at genus level in samples collected pre-onset revealed distinct bacterial compositions between those with and without GVHD. Microbial disruption in samples from patients with GVHD consisted mainly of a lower abundance of anaerobes of the class Clostridia. The genus *Blautia* was 3.4-fold and 3.6-fold lower in the UGI and LGI group respectively vs. the no-GHVD group ($P \leq 0.001$ for both), while the genus *Anaerostipes* was 37.2-fold, 4.6-fold and 26.4-fold lower in the non-GI, UGI and LGI group respectively vs. the no-GHVD group ($P \leq 0.001$ for all) (**Figure1A-C; Supplemental File, ST3**). Compared to the no-GVHD group, GVHD patients also had lower abundances of other Clostridia including the genera *Eubacterium, Coprococcus* and *Ruminococcus*. In contrast, among these pre-onset samples there were no significant differences in genus-level abundances between patients with GVHD (**Supplemental Figure 2A-C**).

Microbial disparities continued for GI-GVHD patients 20 days post-onset compared to their no-GVHD counterparts (**Figure 2A-B; Supplemental File, ST4**). Although non-GI patients had a similar flora composition compared to the no-GVHD group at post-onset (**Supplemental Figure 2D**), UGI patients showed a respective 3-log fold and 14.2-fold reduction of *Coprococcus* and *Parabacteroides* compared to no-GVHD controls ($P \leq 0.001$) (**Figure 2A**). The LGI cohort continued to show features of microbiome disruption post-onset when compared to the no-GVHD group, including decreased abundance of various Clostridia genera, such as *Blautia* and *Erysipelatoclostridium* (5.5-fold and 9.2-fold, respectively; $P \leq 0.001$ for both) (**Figure 2B**). Although non-GI and UGI groups had similar microbial composition (**Supplemental Figure 2E**) non-GI patients had higher relative abundances of *Erysipelatoclostridium*, *Blautia*, and *Anaerostipes* compared to the LGI group (11.3-fold, 8.2-fold and 3.5-fold; $P < 0.05$ for all) (**Figure 2C**). Similarly, the UGI cohort microbiome was distinct in comparison to the LGI group with increased relative abundance of *Blautia* and *Erysipelatoclostridium* (5.2-fold and 6.6-fold; $P < 0.01$ for both) *(***Figure 2D**). Taken together,

these observations indicate distinct changes in the microbiota correlate with organ involvement both pre- and post-GVHD onset.

Summary Markers of Microbiome Composition and aGVHD Onset

Although specific attributes of microbial composition vary widely between patients after transplantation several studies have described similar microbial shifts in GHVD, such as a loss members of the class Clostridia and reduction of diversity[12,14,17,19,34]. We sought to determine whether summary indices of microbial composition could serve as indicators of dysbiosis peri-GVHD onset. Prior to aGVHD onset, intestinal α-diversity was significantly lower in patients with GI tract involvement ($P \leq 0.001$) compared to the no-GVHD cohort, while the non-GI group had a similar microbiota diversity to the no-GVHD group ($P = 0.241$) (**Figure 3A**). Post-onset, LGI patients exhibited significantly lower diversity when compared to no-GVHD and non-GI patients ($P < 0.001$ and $P = 0.023$, respectively).

Since butyrate has been implicated in the pathophysiology of GVHD we next analyzed the aggregated abundance of predicted butyrate producers (**Supplemental File, ST5)** in fecal samples of patients with or without GVHD. No-GVHD patients had a higher abundance of predicted butyrate producers when compared to all GVHD groups at pre-onset, including the non-GI ($P = 0.016$), UGI and LGI ($P < 0.001$) **(Figure 3B).** Furthermore, after onset, only patients with LGI GVHD maintained lower abundance of butyrate producers when compared to the other cohorts including no-GVHD ($P < 0.001$), non-GI ($P = 0.002$) and UGI ($P = 0.029$).

A common feature of the normal human intestinal microbiome is a predominance of obligate anaerobic bacteria[53,54] whereas expansion of facultative anaerobes has been linked to GI inflammatory conditions[55]. We and others have observed expansion of facultative anaerobes, for example those from genus *Enterococcus*, as a significant risk factor for the development of aGVHD and GVHD-related mortality[14,25,56]. Thus, we sought to determine the ratio of strict-to-facultative (S/F) anaerobes among GVHD groups, defined by annotating the taxa in this dataset

against a reference predicted metagenomic functional classification[47]**.** All GVHD groups at pre-onset had significantly lower S/F anaerobe ratio when compared to the no-GVHD group; non-GI (P = 0.006), UGI (P = 0.001) and LGI ($P \leq 0.001$) cohorts (**Figure 3C**), which was driven both by a low relative abundance of strict anaerobes and high relative abundance of facultative anaerobes (**Supplemental Figure S3A-C)**. Notably, LGI patients also had a lower S/F anaerobe ratio (P = 0.044) (**Figure 3C**) and less strict anaerobes (P = 0.039) (**Supplemental Figure S3B**) prior to onset when compared to UGI patients, indicating increased severity of dysbiosis in LGI patients pre-onset when compared to their UGI counterparts. At post-onset UGI and LGI patients maintained a decreased S/F anaerobe ratio compared to no-GVHD controls (P = 0.013 and $P < 0.001$, respectively) while LGI patients also had a lower S/F anaerobe ratio compared to their non-GI (P = 0.004) counterparts.

We also investigated potential cofounders to microbial composition within our data such as antibiotic exposure, infection, and nutritional health. Given the heterogeneity in the different antibiotics to which the patients were exposed, we classified the antibiotics as having medium-to-high vs. low microbiome-disrupting potential close to sample collection (Methods) (**Supplemental File, ST1)**. Nearly all patients received some type of microbiome-disrupting antibiotic during the sampling windows **(Supplemental Figure 4)**. The proportion of patients exposed to antibiotics with medium-to-high-microbiome perturbation potential was lower in the no-GVHD cohort compared those with GHVD pre-onset, while post-onset the proportion of non-GI GVHD patients exposed to these antibiotics was lower compared to those with lower-GI GVHD (**Supplemental File, ST6)**. Exposure to this group of antibiotics was also associated with reduced α-diversity, relative abundance of butyrate producers and S/F anaerobe ratio **(Supplemental Figure 5A-C),** which was independent of the occurrence of blood stream infection (BSI) prior to GVHD onset. **(Supplemental Figure 5D-G, ST7).** Finally, we also explored other potential confounding clinical variables, including nutritional status, modus of nutrition, viral enteritis and *C. difficile* infection (**Supplemental File, ST8-11)**. While all groups

displayed similar patterns of weight loss and associated decrease in BMI after HCT, patients with GI-GVHD had a greater decline in nutritional status as assessed by nutritional risk index (NRI)[57-59] after GVHD onset (**Supplemental Figure 6A-F**). This index correlated only weakly with microbial features in samples **(**Spearman *R* = 0.3) (**Supplemental Figure 6G-I**). Exposure to total parenteral nutrition (TPN) was associated with lower diversity and lower S/F anaerobe ratio within the no-GVHD group and lower diversity and relative abundance of butyrate producers in the upper GI GVHD group prior to GVHD onset **(Supplemental Figure 6J-L)**. Notably, in a subset analysis that excluded any patient who received TPN, those with GVHD had lower diversity, lower relative abundance of butyrate producers, and lower S/F anaerobe ratios when compared to no-GVHD controls **(Supplemental Figure 6M-O, ST11)**. We observed no significant association between *C. difficile* infection prior to GVHD onset with microbiota features in GVHD patients **(Supplemental Figure 5H-J, ST9)**.

Together, our data suggest that summary indices of pre- and post-onset microbial injury, such as bacterial diversity, abundance of butyrate producers and S/F anaerobe ratio, are sensitive markers of organ-specific GVHD.

Metagenomic Pathways Analysis

We next explored functional features of the fecal microbiome by extrapolating metabolic pathway abundances from 16S-based taxonomic profiles using the PICRUSt algorithm. We recovered six main metabolic pathways that were classified according to the MetaCyc database[52]: carbohydrate degradation, nucleotide and amino acid biosynthesis, antibiotic resistance, vitamin K_2 biosynthesis, and fermentation including SCFA metabolism (**Figure 4-5**). Patients with GI-GVHD had increased abundance of pathways associated with vitamin K_2 biosynthesis, nucleotide biosynthesis and antibiotic resistance and reduced abundance of pathways related to amino acid biosynthesis when compared to no-GVHD patients at pre-onset ($P < 0.05$ for all) **(Figure 4A-B; Supplemental File, ST12)**. Interestingly, although UGI GVHD

patients showed increased presence of pathways broadly associated with fermentation prior to onset ($P < 0.05$), LGI-GVHD patients had lower abundance of butyrate-specific pathways when compared to no-GVHD controls **(Figure 4B, bold green)**, which is consistent with our findings of composition at the genus-level and aggregated abundance of butyrate producers.

PICRUSt analysis of samples at post-onset suggested that microbial metabolic dysfunction was most apparent in LGI patients (**Figure 5A-B; Supplemental File, ST13)**. LGI-GVHD patients continued to show increased abundance of metabolic pathways linked to antibiotic resistance ($P = 0.02$) and vitamin K_2 ($P = 0.005$) and lower abundance of amino acid biosynthesis pathways ($P < 0.001$) when compared to no-GVHD patients (**Figure 5A-B)**. Moreover, we also found reduced abundance of butyrate-specific pathways in LGI-GVHD patients at post-onset when compared to no-GVHD controls (**Figure 5B, bold green)**.

To confirm these PICRUSt observations, we next analyzed the metabolic genome profiles of samples from a subset of 131 patients belonging to no-GVHD (n = 68), non-GI (n = 8), UGI (n = 23), LGI (n = 32) cohorts that had undergone whole metagenomic shotgun sequencing (**Supplemental Figure S1, S7, S8**). Prior to onset, the microbiome of LGI-GVHD patients had higher overall abundance of pathways associated with carbohydrate degradation and lower abundance of those associated with amino acid biosynthesis (**Supplemental Figure S7A-C; Supplemental File, ST14**) which partially corroborated our PICRUSt data. Post-GVHD onset, the LGI group had increased abundance of broad gene pathways associated with carbohydrate degradation and decreased abundance of genes associated with amino acid biosynthesis and various fermentation pathways linked to butyrate production (**Supplemental Figure S8A,B; Supplemental File, ST15**). As such, metagenomics confirmed a pattern of compromised butyrate production and abundance differences in various other metabolism-related pathways in the intestinal microbiota, particularly in LGI patients post onset.

Microbial Biomarkers in Survival Outcomes

Lastly, we investigated the association of microbial features measured in the 20 days prior to GVHD with clinical outcomes after GVHD onset. We analyzed the association between Clostridia, predicted butyrate producers and S/F anaerobe ratio prior to GVHD onset with OS, GVHD-related mortality, TRM and relapse. Lower mortality risk in the first 2-years of GVHD onset was associated with high S/F anaerobe ratio (HR, 0.46; 95%CI, 0.24-0.86; *P* = 0.016), and high relative abundance of predicted butyrate producers (HR, 0.45; 95%CI, 0.24-0.84; *P* = 0.012) (**Figure 6A; Supplemental File, ST16).** While not significant as a binary variable (*P* = 0.106), the higher relative abundance of Clostridia was associated with a reduced risk of death when analyzed as a continuous predictor (HR, 0.75; 95%CI, 0.57-0.98; *P* = 0.037). Higher abundance of predicted butyrate producers and S/F anaerobe ratio were both associated with lower risks of GVHD-related mortality (HR, 0.34; 95%CI, 0.13-0.91; *P* = 0.032 and HR, 0.34; 95%CI, 0.12-0.95; *P* = 0.04; respectively) (**Figure 6B)**. Additionally, 2-year TRM was significantly lower in patients with high abundance of butyrate producers (*P* = 0.013) and S/F anaerobe ratio (*P* = 0.035) (**Supplemental Figure S9A)**. Moreover, older age, female sex and non-ablative conditioning (vs. reduced intensity) were also associated with reduced survival in multivariate comparisons **(Supplemental File, ST17)** while no associations were observed between these summary microbial markers and incidence of relapse (**Supplemental Figure S9B**). In summary, these data suggest that global biomarkers of microbial health prior to onset may be useful tools in predicting outcomes in patients with GVHD.

DISCUSSION

The intestinal microbiome is a crucial component of gut health and its relationship with the host includes exchange of factors and nutrients that promote gut homeostasis and modulate host immunity. Various studies in mice and humans have demonstrated that the intestinal microbiome composition is particularly relevant for allo-HCT outcomes, including GVHD[12-19]. This study focused on the fecal microbiome of adult allo-HCT recipients with or without aGVHD,

according to organ involvement. LGI GHVD patients had worse microbial injury than patients from other groups. Low abundance of Clostridia members in fecal samples of allo-HCT patients has been described previously in the peri-engraftment period[12,19]. Here we extend this finding to the 20 days prior to onset of GVHD, specifically for Clostridia members such as *Ruminococcus*, *Anaerostipes*, *Eubacterium,* and *Blautia.* In post-onset samples, we only observed this pattern of dysbiosis in those with GI-GVHD. Increased abundance of *Blautia* has been associated with improved OS and reduced GVHD after HCT[15], and was reduced significantly in GI-GVHD patients. Furthermore a study evaluating the immunological consequences of mono-colonization of germ-free mice with *Ruminococcus gnavus*, a species suggested to belong to the *Blautia* genus[60], reported increased colon frequencies of regulatory T cells and innate lymphoid cells[61]. These cells are of particular interest in GVHD as regulatory cells may reduce GVHD in patients and preclinical models [62,63], and innate lymphoid cells can promote intestinal regeneration through secretion of interleukin-22 and ameliorate aGVHD[64,65].

The heterogeneity of microbial compositions across populations makes identifying associations between specific microbes and HCT outcomes complex. We therefore investigated whether broad markers of intestinal dysbiosis could be used as surrogates to characterize organ involvement in GVHD. We first evaluated microbiome diversity, which was decreased in patients with GI-GVHD pre-onset, and continued post-onset for the LGI cohort. These findings corroborate studies by our group and others demonstrating that lower diversity pre-transplant and peri-engraftment is associated with GVHD and survival outcomes[13-15]. Moreover, we found an association between exposure to antibiotics with high potential to disrupt the flora at pre-onset and dysbiosis in the gut which is in agreement with other studies[14,16]. The observational design of this study prevents definitively dissociating potential effects of antibiotics themselves on the microbiome from potential effects of the underlying condition the antibiotics were used to treat. Antibiotic-associated dysbiosis metrics were observed comparably in the overall cohort

and in subsets excluding those with bloodstream infections, pointing to a prominent role for antibiotics in mediating microbiome injury in this population.

A diverse microbial community is important for the production of microbial metabolites with beneficial effects on the host. Metabolites such as SCFAs, bile acids, vitamins and tryptophan-derived molecules are important for intestinal gut health and immune regulation[64,66-68]. The SCFA butyrate has an important role in maintaining the epithelial barrier[69] and regulating local intestinal immunity by inducing differentiation of colonic T-regs[70]. Importantly, in preclinical models butyrate administration could mitigate acute and chronic GVHD[23,22]. We found that the relative abundance of predicted butyrate producers was lower prior to onset in GI-GVHD patients when compared to no-GVHD controls. Intestinal outgrowth of facultative anaerobes is also a significant risk factor for the development of aGVHD and worse survival after allo-HCT[14,25,56]. All GVHD groups had a significantly lower S/F anaerobe ratio pre-onset when compared to no-GVHD cases, which persisted post-onset in GI-GVHD patients. Our findings extend previous reports that intestinal domination by facultative anaerobes is associated with worse aGVHD[14,25,56].

In order to uncover microbial metabolites with potential to modulate organ involvement in GVHD we further investigated metabolic pathway abundances in the fecal samples via PICRUSt and shotgun metagenomics. Patients without GVHD showed increased predicted abundance of pathways associated with carbohydrate degradation, amino acid biosynthesis, and fermentation, including butyrate-specific pathways, whereas antibiotic resistance and nucleotide biosynthesis pathways were increased in GI-GVHD patients both pre and post-onset. Notably, we observed a reduction in pathways responsible for fermentation to butyrate in patients with GI-GVHD, particularly the LGI, which is consistent with our findings on reduced predicted butyrate producers within these cohorts. These findings are in agreement with preclinical studies, which have suggested a protective effect of butyrate in GVHD[23]. However this protective effect may depend on the degree of intestinal damage and butyrate concentration available[26,27].

Finally, we also demonstrate the clinical importance of these markers of microbial health after allo-HCT, where higher relative abundance of class Clostridia, predicted butyrate producers and S/F anaerobe ratio were associated with significant improvement in transplant outcomes including overall survival, GVHD-related mortality and TRM. In conclusion, we found significant associations between microbial diversity, the abundance of butyrate producing taxa and the S/F anaerobe ratio with GI-GVHD, particularly LGI-GVHD. Our findings have practical implications for the development of microbiome-derived biomarkers and prophylactic and therapeutic interventions in aGVHD.

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Author Contributions: D.M.P., M.B.S., J.U.P. and M.R.M. vdB. designed the study, analyzed the data, and wrote the manuscript. M.B.S., A.D., S.D., A.G. and T.F., performed statistical analysis. G.M. J.S., and G.K.A. collected the data. G.A., E.F., L.A.A., and R.J.W. performed research. R.S., S.D., E.F., L.A.A., R.J.W., H.A, O.M., M.A.P., and Y.T. wrote the manuscript.

Conflict of Interest:

D.M.P. has served as advisory board member for Evive Biotechnology (Shanghai) Ltd (formerly Generon [Shanghai] Corporation Ltd), Kadmon Corporation, CareDx, and Ceramedix. J.U.P. reports research funding, intellectual property fees, and travel reimbursement from Seres Therapeutics, and consulting fees from DaVolterra, CSL Behring, and from MaaT Pharma. He serves on an Advisory board of and holds equity in Postbiotics Plus Research. He has filed intellectual property applications related to the microbiome (reference numbers #62/843,849, #62/977,908, and #15/756,845). M.R.M.vdB has received research support and stock options from Seres Therapeutics and stock options from Notch Therapeutics and Pluto Therapeutics; he has received royalties from Wolters Kluwer; has consulted, received honorarium from or participated in advisory boards for Seres Therapeutics, WindMIL Therapeutics, Rheos Medicines, Merck & Co, Inc., Magenta Therapeutics, Frazier Healthcare Partners, Nektar Therapeutics, Notch Therapeutics, Forty Seven Inc., Ceramedix, Lygenesis, Pluto Therapeutics, GlaskoSmithKline, Da Volterra, Vor Biopharma, Novartis (Spouse), Synthekine (Spouse), and Beigene (Spouse); he has IP Licensing with Seres Therapeutics and Juno Therapeutics; and holds a fiduciary role on the Foundation Board of DKMS (a nonprofit organization). M.B.D.S has no conflict of interest. R.S. received consultancy fees from Medexus and MyBiotics. Memorial Sloan Kettering Cancer Center (MSK) has financial interests relative to Seres Therapeutics.

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Table 1. GVHD clinical groups by aGVHD organ involvement

GVHD group	Organ involvement
No GVHD (n = 131)	None
Non-GI (n = 29)	Skin: 27 (93%)
	Skin/liver: 2 (7%)
UGI (n = 53)	UGI: 34 (64%)
	UGI/skin: 19 (36%)
LGI (n = 53)	LGI: 7 (13%)
	LGI/UGI: 26 (49%)
	LGI/skin: 1 (2%)
	LGI/liver: 1 (2%)
	LGI/UGI/skin: 17 (32%)
	LGI/UGI/skin/liver: 1 (2%)

Abbreviations: GVHD, graft-versus-host disease; GI, gastrointestinal; LGI, lower gastrointestinal; UGI, upper gastrointestinal

Table 2. Patient demographics (n = 266)

	No-GVHD (n = 131)	Non-GI (n = 29)	UGI (n = 53)	LGI (n = 53)	P-value
Median age (range)	57 (24-77)	53 (23-72)	55 (23-74)	53 (21-78)	0.378
Male sex, n (%)	98 (75%)	17 (59%)	29 (52%)	27 (51%)	0.004
Diagnosis, n (%)					
Acute leukemia/ MDS	54 (41%)	20 (69%)	34 (64%)	25 (47.2%)	0.004
Lymphoma/CLL/LGL/T-PLL	74 (57%)	8 (28%)	17 (32%)	23 (43.4%)	
CML/ MM	3 (2%)	1 (3%)	2 (4%)	5 (9.4%)	
Conditioning Regimens					
Myeloablative & reduced intensity, n (%)					0.003
TBI-based*	40 (30.5%)	12 (41%)	29 (54.7%)	26 (49%)	
Chemotherapy-based**	40 (30.5%)	12 (41%)	29 (54.7%)	26 (49%)	
Nonmyeloablative n (%)	57 (43.5%)	13 (45%)	20 (37.7%)	23 (43%)	
Cy/Flu/TBI 200 cGy	34 (26%)	4 (14%)	4 (7.6%)	4 (8%)	
Donor, n (%)					
MRD	52 (40%)	2 (7%)	11 (21%)	5 (9.4%)	<0.001
MUD/MMUD	43/3 (35%)	11/- (38%)	11/1 (22.5%)	18/5 (43.3%)	
Haploidentical	7 (5%)	4 (14%)	3 (5.5%)	2 (4%)	
Cord blood	26 (20%)	12 (41%)	27 (51%)	23 (43.3%)	
Donor-recipient HLA-match,n n n (%)					
8/8	97 (74%)	13 (45%)	23 (43%)	23 (43.5%)	<0.001
7/8	3 (2%)	2 (7%)	1 (2%)	5 (9.5%)	
<7/8	31 (24%)	14 (48%)	29 (55%)	25 (47%)	
Stem cell source, n (%)					
BM	12 (9%)	3 (10.3%)	6 (11%)	3 (6%)	<0.001
PBSC	93 (71%)	14 (48.3%)	20 (38%)	27 (51%)	
Cord blood$^{\alpha}$	26 (20%)	12 (41.4%)	27 (51%)	23 (43%)	
GVHD prophylaxis					
CNI/MTX/ +/- siro +/- other$^{\beta}$	96 (73%)	13 (45%)	22 (39%)	24 (45%)	<0.001
CSA/MMF	26 (20%	12 (41%)	29 (52%)	23 (43.5%)	
PTCy based	9 (7%)	4 (14%)	5 (9%)	6 (11.5%)	
GVHD severity					
Grade I		11 (38%)	-	-	<0.001
Grade II	N/A	7 (24%)	50 (94%)	32 (60%)	
Grade III		11 (38%)	3 (6%)	10 (19%)	
Grade IV		-	-	11 (21%)	

P-values compare the distribution of covariates (rows) across all cohorts with the exception of GVHD severity, which only includes those with GVHD and were obtained using a Fisher's exact test for categorical variables and Kruskal–Wallis for age as a continuous variable.

Abbreviations: BM, bone marrow; CNI, calcineurin inhibitor; CML, chronic myeloid leukemia; cyclosporine-A; GI, gastrointestinal; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; LGI, lower gastrointestinal; MUD, matched-unrelated donor; MTX, methotrexate; MMUD, mismatched-unrelated donor; MM, multiple myeloma; MMF, mycophenolate mofetil; MA, myeloablative; MDS, myelodysplastic syndrome; NMA, non-myeloablative; n, number; PBSC, peripheral blood stem cell;

CSA, PTCy, post-trans plant cyclophosphamide; RI, reduced intensity; Siro, sirolimus; TBI, total body irradiation; UGI, upper gastrointestinal;

*Includes Cyclophosphamide/ TBI 1375 cGy, Fludarabine/ TBI 1375 cGy, Cyclophosphamide/ Thiotepa/ TBI 1375 cGy, Fludarabine/ Cyclophosphamide/ TBI 1320-1375 cGy, Cyclophosphamide/ Fludarabine/ Thiotepa/ TBI 400 cGy.

**Includes Melphalan/ Fludarabine, Fludarabine/ Busulfan, Melphalan/ Thiotepa/ Fludarabine, Busulfan/ Melphalan, Busulfan/ Fludarabine/ Cyclophosphamide, Clofarabine/ Thiotepa /Melphalan, Busulfan/ Cyclophosphamide.

[α]Includes 33 patients who received cord blood graft combined with a haploidentical *ex vivo* CD34+ selected T-cell depleted graft as a myeloid bridge as part of protocol (NCT01682226).

[β]Other includes Bortezomib, Maraviroc and Mycophenolate mofetil. Five patients did not receive methotrexate but Tacrolimus/Sirolimus/MMF (n = 1), Tacrolimus/MMF (n = 2), and Tacrolimus/Sirolimus (n = 2).

Figure Legends

Figure 1. Allo-HCT patients with GVHD developed microbial disruption pre-onset GVHD. (A-F) Volcano plot [-$\log_{10}$(FDR p-value) versus $\log_2$(fold change)] representation of microbial dysbiosis of pre-onset GVHD (day -20 to -1 relative to onset) and no-GVHD (day -3 to +102 relative to allo-HCT) fecal samples. Plot includes taxa with mean relative abundance within samples > 0.1% and highlights the 20 genera with strongest FDR corrected p-value significance (≤ 0.05). Highlighted taxa abbreviations: p-phylum, f-family, o-order, g-genus, Proteo-Proteobacteria. (A-C) No-GVHD patients had higher abundance of commensals belonging to the class Clostridia when compared to non-GI, UGI and LGI cohort.

Figure 2. Allo-HCT patients with GI-GVHD developed microbial disruption post-onset GVHD. (A-D) Volcano plot [-log10(FDR p-value) versus log2(fold change)] representation of microbial dysbiosis of post-onset GVHD (day 0 to +20 relative to onset) and no GVHD (day -3 to +102 relative to allo-HCT) fecal samples. Plot includes taxa with mean abundance within samples > 0.1% and highlights the 20 genera with strongest FDR corrected p-value significance (≤ 0.05). Highlighted taxa abbreviations: p-phylum, f-family, o-order, g-genus, Proteo-Proteobacteria. (A,B) No-GVHD compared to UGI and LGI patients had increased abundance of class Clostridia commensals as *Coprococcus* and *Blautia* respectively, post-GVHD onset. (C,D) Non-GI and UGI patients also had increased relative abundance of *Blautia* and *Erysipelatoclostridium* when compared to LGI patients.

Figure 3. GVHD patients have reduced diversity, abundance of butyrate producers and loss of anaerobiosis pre- and post-GVHD onset. (A) Pre- and post-onset aGVHD microbial analysis show reduced diversity in GI-GVHD patients. (B) GI-GVHD patients also had lower

686 relative abundance of predicted butyrate-producing strains when compared to the other cohorts,
687 while this was also found for LGI patients when compared to non-GI and UGI patients post-
688 onset. (C) GVHD patients also had lower strict-to-facultative (S/F) anaerobe ratio in comparison
689 to No-GVHD patients, while the LGI, in specific, showed lower ratio when compared to UGI and
690 non-GI patients, respectively pre- and post-onset.
691
692 **Figure 4. GVHD patients present distinct pre-onset predicted PICRUSt pathways.** (A)
693 Radar chart representation of PICRUSt predicted functional pathway relative abundance from
694 patients without GVHD and GVHD patients at pre-onset. Relevant pathways belonged to six
695 main metabolic categories: carbohydrate degradation, nucleotide biosynthesis, amino acid
696 biosynthesis, antibiotic resistance, vitamin K_2 biosynthesis, and fermentation. Axis represents
697 fold change relative to no-GVHD patients.(B) Heatmap with hierarchical clustering of statistically
698 significant (FDR p-value ≤ 0.05) unique PICRUSt pathways. Figure displays pathways found in
699 a minimum of 5% samples within all groups. UGI and LGI GVHD patients show increased
700 pathways associated with general antibiotic resistance, vitamin K_2 metabolism and nucleotide
701 biosynthesis, with reduced representation related to amino acid biosynthesis compared to no-
702 GVHD patients. There was reduced presence of butyrate-producing specific pathways in LGI
703 patients (Bold). *FDR p-value ≤ 0.05; **FDR p-value ≤ 0.01; ***FDR p-value ≤ 0.001
704
705 **Figure 5. GVHD patients present distinct post-onset predicted PICRUSt pathways.** (A)
706 Radar chart representation of PICRUSt predicted functional pathways belonging to six main
707 metabolic pathways: carbohydrate degradation, nucleotide biosynthesis, amino acid
708 biosynthesis, antibiotic resistance, vitamin K_2 biosynthesis, and fermentation for GVHD patients
709 post-GVHD onset relative to no-GVHD controls. Axis represents fold change relative to no-
710 GVHD patients. (B) Heatmap with hierarchical clustering of statistically significant (FDR p-value
711 ≤ 0.05) unique PICRUSt pathways. Figure displays pathways found in a minimum of 5%

712 samples within all groups. LGI GVHD patients show increased abundance of pathways
713 associated with general antibiotic resistance and vitamin K_2 metabolism with lower abundance
714 of pathways linked to amino acid biosynthesis relative to no-GVHD patients. Lower abundance
715 of pathways specific to butyrate production was also found in LGI patients compared to no-
716 GVHD controls (Bold). *FDR
717 p-value ≤ 0.05; **FDR p-value ≤ 0.01; ***FDR p-value ≤ 0.001
718
719 **Figure 6. Pre-onset GVHD clostridia abundance, butyrate producers, and S/F anaerobe**
720 **ratio predicts survival in allo-HCT patients.** (A) Overall Survival (OS) and (B) GVHD-related
721 mortality. Patients were stratified according to the median relative abundances of class
722 Clostridia, butyrate producers and S/F anaerobe ratio prior to GVHD onset. Figure shows
723 univariable (UVA) and multivariable (MVA) tests of associations for microbial determinants as
724 binary or continuous variables. The proportional hazards multivariable regression models (OS)
725 and the Fine and Gray multivariable regression models (GVHD-related mortality) were adjusted
726 for gender, age and conditioning regimen. Data corroborates the association of higher relative
727 abundance of butyrate-producing bacteria and S/F ratio to increased OS and reduced GVHD-
728 related mortality.

Figures

Figure 1.

Pre-onset

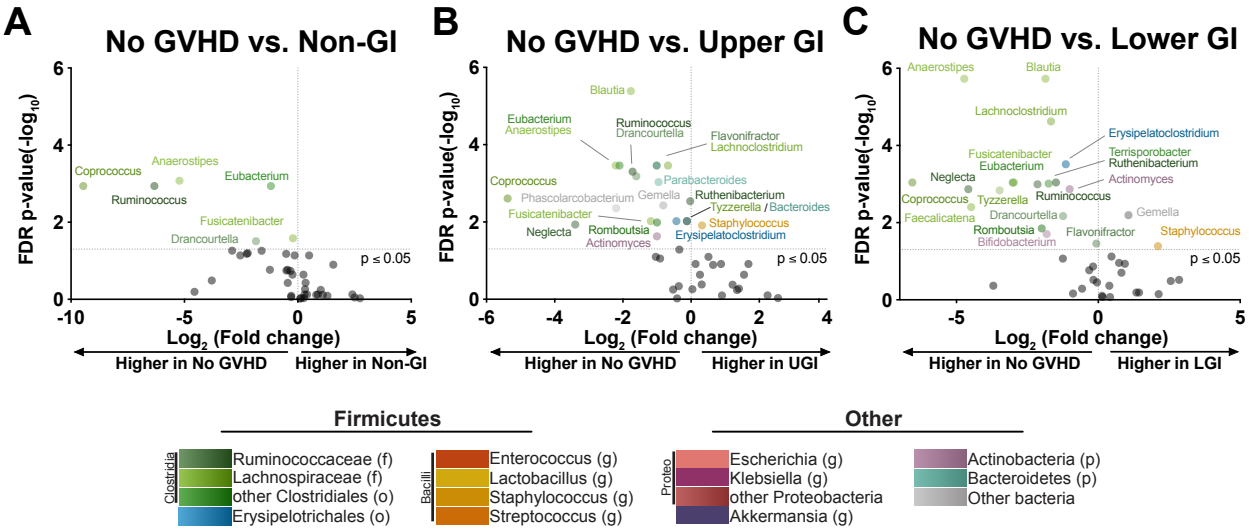


Figure 2.

Post-onset

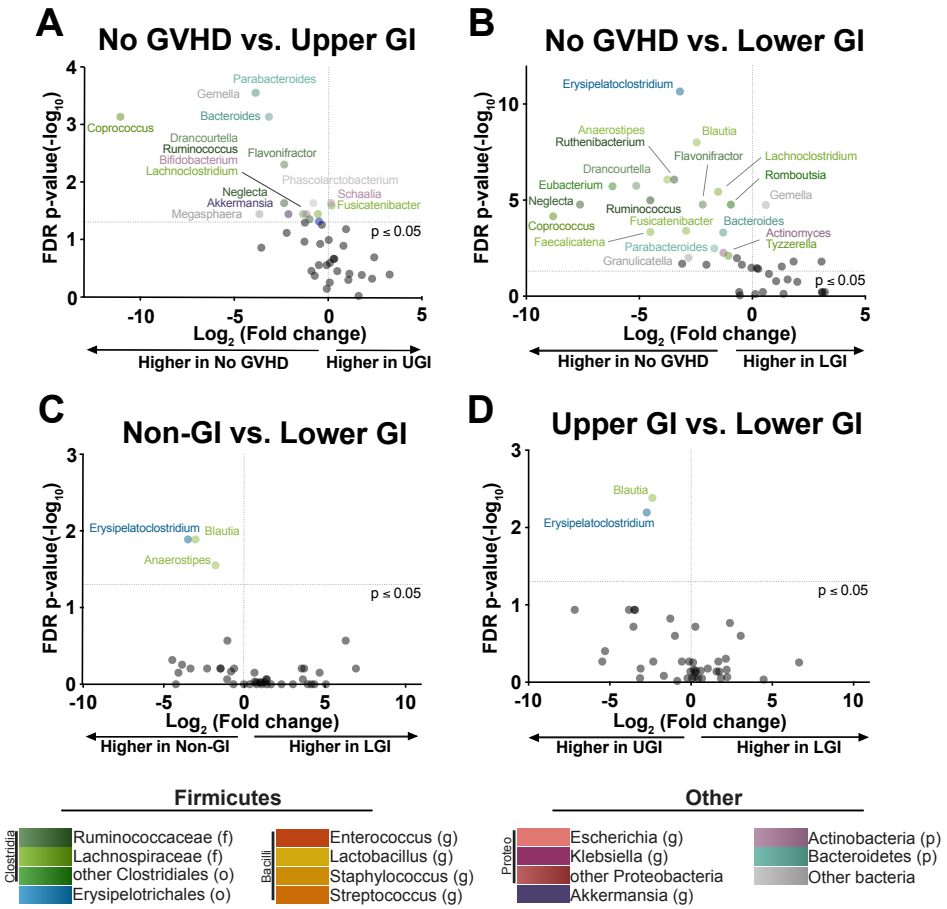


Figure 3.

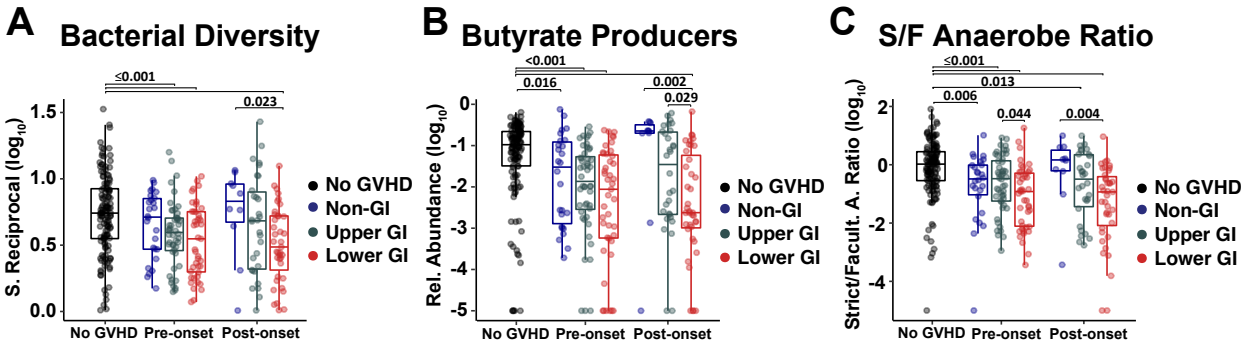

A
Bacterial Diversity
S. Reciprocal (log10)
≤0.001
0.023
No GVHD
Pre-onset
Post-onset
No GVHD
Non-GI
Upper GI
Lower GI
B
Butyrate Producers
Rel. Abundance (log10)
<0.001
0.016
0.002
0.029
C
S/F Anaerobe Ratio
Strict/Facult. A. Ratio (log10)
≤0.001
0.013
0.006
0.044
0.004

Figure 4.

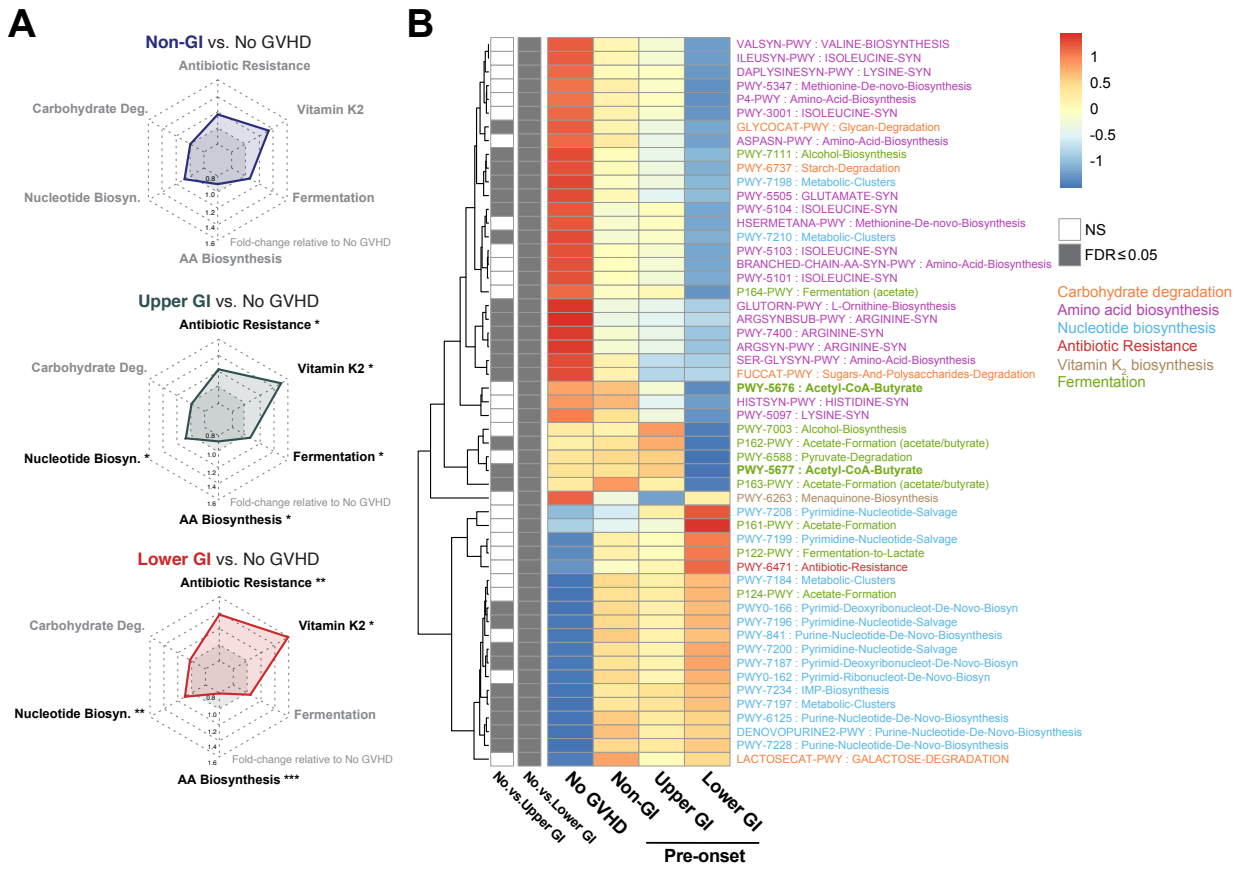

A
Non-GI vs. No GVHD
Antibiotic Resistance
Vitamin K2
Fermentation
AA Biosynthesis
Nucleotide Biosyn.
Carbohydrate Deg.
Fold-change relative to No GVHD
Upper GI vs. No GVHD
Antibiotic Resistance *
Vitamin K2 *
Fermentation *
AA Biosynthesis *
Nucleotide Biosyn. *
Carbohydrate Deg.
Fold-change relative to No GVHD
Lower GI vs. No GVHD
Antibiotic Resistance **
Vitamin K2 *
Fermentation
AA Biosynthesis ***
Nucleotide Biosyn. **
Carbohydrate Deg.
Fold-change relative to No GVHD
B
VALSYN-PWY : VALINE-BIOSYNTHESIS
ILEUSYN-PWY : ISOLEUCINE-SYN
DAPLYSINESYN-PWY : LYSINE-SYN
PWY-5347 : Methionine-De-novo-Biosynthesis
P4-PWY : Amino-Acid-Biosynthesis
PWY-3001 : ISOLEUCINE-SYN
GLYCOCAT-PWY : Glycan-Degradation
ASPASN-PWY : Amino-Acid-Biosynthesis
PWY-7111 : Alcohol-Biosynthesis
PWY-6737 : Starch-Degradation
PWY-7198 : Metabolic-Clusters
PWY-5505 : GLUTAMATE-SYN
PWY-5104 : ISOLEUCINE-SYN
HSERMETANA-PWY : Methionine-De-novo-Biosynthesis
PWY-7210 : Metabolic-Clusters
PWY-5103 : ISOLEUCINE-SYN
BRANCHED-CHAIN-AA-SYN-PWY : Amino-Acid-Biosynthesis
PWY-5101 : ISOLEUCINE-SYN
P164-PWY : Fermentation (acetate)
GLUTORN-PWY : L-Ornithine-Biosynthesis
ARGSYNBSUB-PWY : ARGININE-SYN
PWY-7400 : ARGININE-SYN
ARGSYN-PWY : ARGININE-SYN
SER-GLYSYN-PWY : Amino-Acid-Biosynthesis
FUCCAT-PWY : Sugars-And-Polysaccharides-Degradation
PWY-5676 : Acetyl-CoA-Butyrate
HISTSYN-PWY : HISTIDINE-SYN
PWY-5097 : LYSINE-SYN
PWY-7003 : Alcohol-Biosynthesis
P162-PWY : Acetate-Formation (acetate/butyrate)
PWY-6588 : Pyruvate-Degradation
PWY-5677 : Acetyl-CoA-Butyrate
P163-PWY : Acetate-Formation (acetate/butyrate)
PWY-6263 : Menaquinone-Biosynthesis
PWY-7208 : Pyrimidine-Nucleotide-Salvage
P161-PWY : Acetate-Formation
PWY-7199 : Pyrimidine-Nucleotide-Salvage
P122-PWY : Fermentation-to-Lactate
PWY-6471 : Antibiotic-Resistance
PWY-7184 : Metabolic-Clusters
P124-PWY : Acetate-Formation
PWY0-166 : Pyrimid-Deoxyribonucleot-De-Novo-Biosyn
PWY-7196 : Pyrimidine-Nucleotide-Salvage
PWY-841 : Purine-Nucleotide-De-Novo-Biosynthesis
PWY-7200 : Pyrimidine-Nucleotide-Salvage
PWY-7187 : Pyrimid-Deoxyribonucleot-De-Novo-Biosyn
PWY0-162 : Pyrimid-Ribonucleot-De-Novo-Biosyn
PWY-7234 : IMP-Biosynthesis
PWY-7197 : Metabolic-Clusters
PWY-6125 : Purine-Nucleotide-De-Novo-Biosynthesis
DENOVOPURINE2-PWY : Purine-Nucleotide-De-Novo-Biosynthesis
PWY-7228 : Purine-Nucleotide-De-Novo-Biosynthesis
LACTOSECAT-PWY : GALACTOSE-DEGRADATION
1
0.5
0
-0.5
-1
NS
FDR≤0.05
Carbohydrate degradation
Amino acid biosynthesis
Nucleotide biosynthesis
Antibiotic Resistance
Vitamin K2 biosynthesis
Fermentation
No.vs.Upper GI
No.vs.Lower GI
No GVHD
Non-GI
Upper GI
Lower GI
Pre-onset

Figure 5.

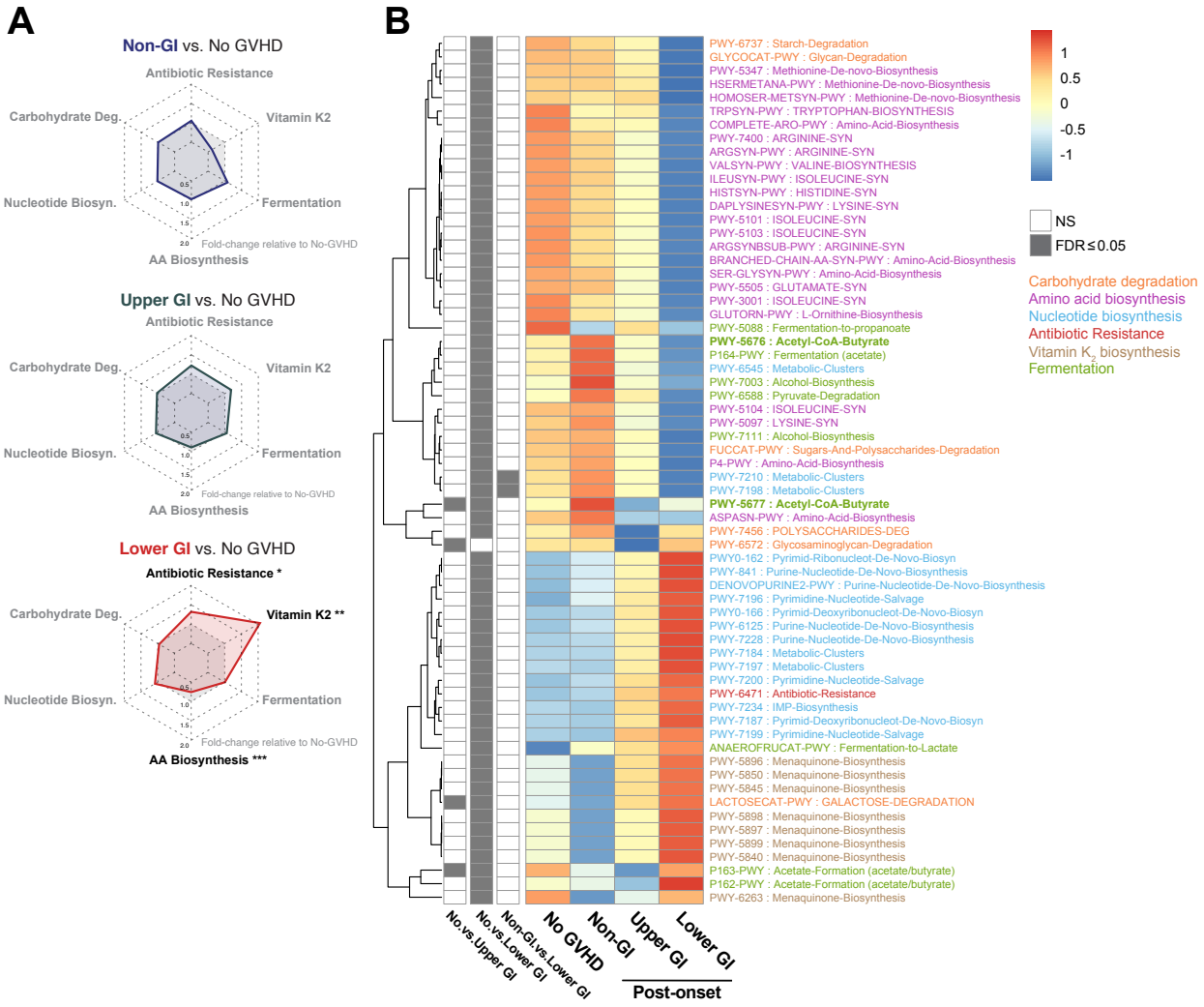
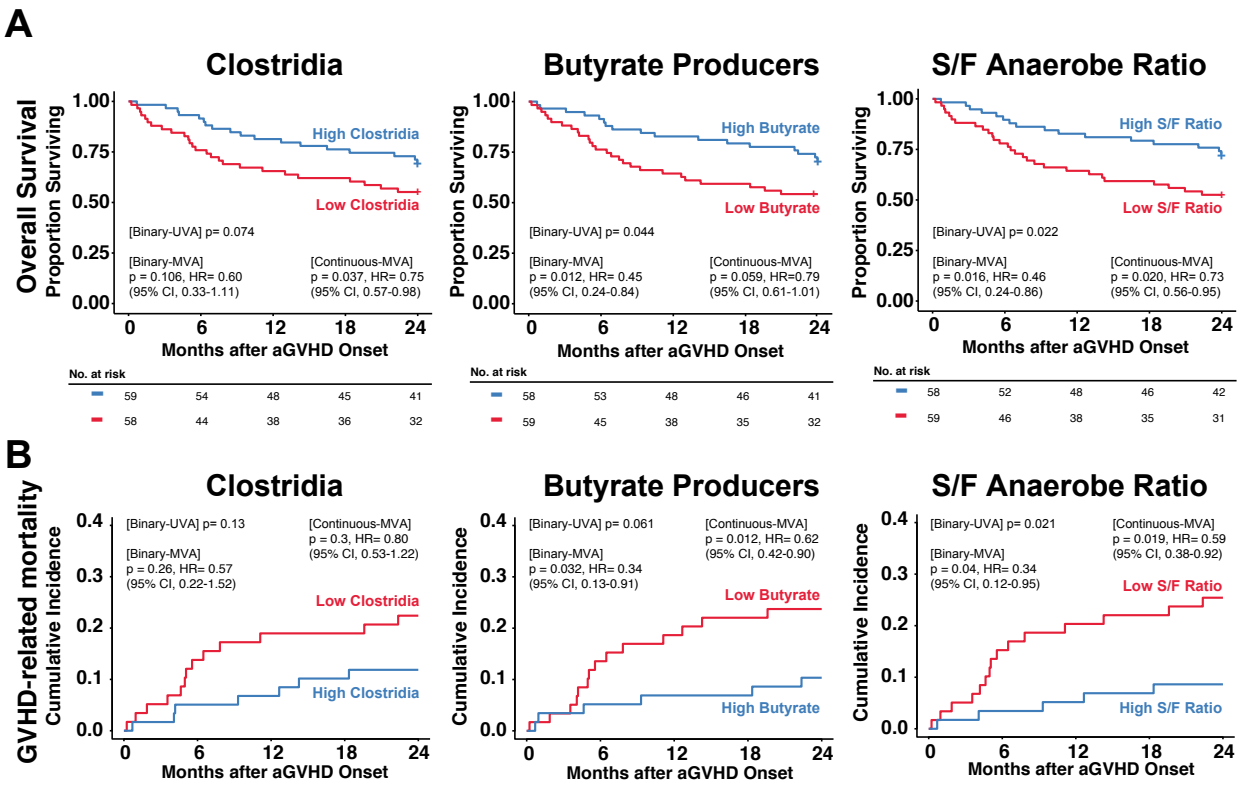

A
Non-GI vs. No GVHD
Antibiotic Resistance
Vitamin K2
Fermentation
AA Biosynthesis
Nucleotide Biosyn.
Carbohydrate Deg.
Fold-change relative to No-GVHD
Upper GI vs. No GVHD
Lower GI vs. No GVHD
Antibiotic Resistance *
Vitamin K2 **
AA Biosynthesis ***
B
PWY-6737 : Starch-Degradation
GLYCOCAT-PWY : Glycan-Degradation
PWY-5347 : Methionine-De-novo-Biosynthesis
HSERMETANA-PWY : Methionine-De-novo-Biosynthesis
HOMOSER-METSYN-PWY : Methionine-De-novo-Biosynthesis
TRPSYN-PWY : TRYPTOPHAN-BIOSYNTHESIS
COMPLETE-ARO-PWY : Amino-Acid-Biosynthesis
PWY-7400 : ARGININE-SYN
ARGSYN-PWY : ARGININE-SYN
VALSYN-PWY : VALINE-BIOSYNTHESIS
ILEUSYN-PWY : ISOLEUCINE-SYN
HISTSYN-PWY : HISTIDINE-SYN
DAPLYSINESYN-PWY : LYSINE-SYN
PWY-5101 : ISOLEUCINE-SYN
PWY-5103 : ISOLEUCINE-SYN
ARGSYNBSUB-PWY : ARGININE-SYN
BRANCHED-CHAIN-AA-SYN-PWY : Amino-Acid-Biosynthesis
SER-GLYSYN-PWY : Amino-Acid-Biosynthesis
PWY-5505 : GLUTAMATE-SYN
PWY-3001 : ISOLEUCINE-SYN
GLUTORN-PWY : L-Ornithine-Biosynthesis
PWY-5088 : Fermentation-to-propanoate
PWY-5676 : Acetyl-CoA-Butyrate
P164-PWY : Fermentation (acetate)
PWY-6545 : Metabolic-Clusters
PWY-7003 : Alcohol-Biosynthesis
PWY-6588 : Pyruvate-Degradation
PWY-5104 : ISOLEUCINE-SYN
PWY-5097 : LYSINE-SYN
PWY-7111 : Alcohol-Biosynthesis
FUCCAT-PWY : Sugars-And-Polysaccharides-Degradation
P4-PWY : Amino-Acid-Biosynthesis
PWY-7210 : Metabolic-Clusters
PWY-7198 : Metabolic-Clusters
PWY-5677 : Acetyl-CoA-Butyrate
ASPASN-PWY : Amino-Acid-Biosynthesis
PWY-7456 : POLYSACCHARIDES-DEG
PWY-6572 : Glycosaminoglycan-Degradation
PWY0-162 : Pyrimid-Ribonucleot-De-Novo-Biosyn
PWY-841 : Purine-Nucleotide-De-Novo-Biosynthesis
DENOVOPURINE2-PWY : Purine-Nucleotide-De-Novo-Biosynthesis
PWY-7196 : Pyrimidine-Nucleotide-Salvage
PWY0-166 : Pyrimid-Deoxyribonucleot-De-Novo-Biosyn
PWY-6125 : Purine-Nucleotide-De-Novo-Biosynthesis
PWY-7228 : Purine-Nucleotide-De-Novo-Biosynthesis
PWY-7184 : Metabolic-Clusters
PWY-7197 : Metabolic-Clusters
PWY-7200 : Pyrimidine-Nucleotide-Salvage
PWY-6471 : Antibiotic-Resistance
PWY-7234 : IMP-Biosynthesis
PWY-7187 : Pyrimid-Deoxyribonucleot-De-Novo-Biosyn
PWY-7199 : Pyrimidine-Nucleotide-Salvage
ANAEROFRUCAT-PWY : Fermentation-to-Lactate
PWY-5896 : Menaquinone-Biosynthesis
PWY-5850 : Menaquinone-Biosynthesis
PWY-5845 : Menaquinone-Biosynthesis
LACTOSECAT-PWY : GALACTOSE-DEGRADATION
PWY-5898 : Menaquinone-Biosynthesis
PWY-5897 : Menaquinone-Biosynthesis
PWY-5899 : Menaquinone-Biosynthesis
PWY-5840 : Menaquinone-Biosynthesis
P163-PWY : Acetate-Formation (acetate/butyrate)
P162-PWY : Acetate-Formation (acetate/butyrate)
PWY-6263 : Menaquinone-Biosynthesis
1
0.5
0
-0.5
-1
NS
FDR≤0.05
Carbohydrate degradation
Amino acid biosynthesis
Nucleotide biosynthesis
Antibiotic Resistance
Vitamin K2 biosynthesis
Fermentation
No.vs.Upper GI
No.vs.Lower GI
Non-GI.vs.Lower GI
No GVHD
Non-GI
Upper GI
Lower GI
Post-onset

Figure 6.


A
Clostridia
Overall Survival
Proportion Surviving
1.00
0.75
0.50
0.25
0.00
High Clostridia
Low Clostridia
[Binary-UVA] p= 0.074
[Binary-MVA]
p = 0.106, HR= 0.60
(95% CI, 0.33-1.11)
[Continuous-MVA]
p = 0.037, HR= 0.75
(95% CI, 0.57-0.98)
0 6 12 18 24
Months after aGVHD Onset
No. at risk
59 54 48 45 41
58 44 38 36 32
Butyrate Producers
Proportion Surviving
High Butyrate
Low Butyrate
[Binary-UVA] p= 0.044
[Binary-MVA]
p = 0.012, HR= 0.45
(95% CI, 0.24-0.84)
[Continuous-MVA]
p = 0.059, HR=0.79
(95% CI, 0.61-1.01)
Months after aGVHD Onset
No. at risk
58 53 48 46 41
59 45 38 35 32
S/F Anaerobe Ratio
Proportion Surviving
High S/F Ratio
Low S/F Ratio
[Binary-UVA] p= 0.022
[Binary-MVA]
p = 0.016, HR= 0.46
(95% CI, 0.24-0.86)
[Continuous-MVA]
p = 0.020, HR= 0.73
(95% CI, 0.56-0.95)
Months after aGVHD Onset
No. at risk
58 52 48 46 42
59 46 38 35 31
B
Clostridia
GVHD-related mortality
Cumulative Incidence
0.4
0.3
0.2
0.1
0.0
[Binary-UVA] p= 0.13
[Binary-MVA]
p = 0.26, HR= 0.57
(95% CI, 0.22-1.52)
[Continuous-MVA]
p = 0.3, HR= 0.80
(95% CI, 0.53-1.22)
Low Clostridia
High Clostridia
0 6 12 18 24
Months after aGVHD Onset
Butyrate Producers
Cumulative Incidence
[Binary-UVA] p= 0.061
[Binary-MVA]
p = 0.032, HR= 0.34
(95% CI, 0.13-0.91)
[Continuous-MVA]
p = 0.012, HR= 0.62
(95% CI, 0.42-0.90)
Low Butyrate
High Butyrate
Months after aGVHD Onset
S/F Anaerobe Ratio
Cumulative Incidence
[Binary-UVA] p= 0.021
[Binary-MVA]
p = 0.04, HR= 0.34
(95% CI, 0.12-0.95)
[Continuous-MVA]
p = 0.019, HR= 0.59
(95% CI, 0.38-0.92)
Low S/F Ratio
High S/F Ratio
Months after aGVHD Onset