Microbiota dynamics in a randomized trial of gut decontamination during allogeneic hematopoietic cell transplantation

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Microbiota dynamics in a randomized trial of gut decontamination during allogeneic hematopoietic cell transplantation

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ABSTRACT

**Background.** Gut decontamination (GD) can decrease the incidence and severity of acute graft-versus-host-disease (aGVHD) in murine models of allogeneic hematopoietic cell transplantation (HCT). In this pilot study, we examined the impact of GD on the gut microbiome composition and incidence of aGVHD in HCT patients.

**Methods.** We randomized 20 pediatric patients undergoing allogeneic HCT to receive (GD) or not receive (no-GD) oral vancomycin-polymyxin B from day -5 through neutrophil engraftment. We evaluated shotgun metagenomic sequencing of serial stool samples to compare the composition and diversity of the gut microbiome between study arms. We assessed clinical outcomes in the 2 arms and performed strain-specific analyses of pathogens that caused bloodstream infections (BSI).

**Results.** The two arms did not differ in the predefined primary outcome of Shannon diversity of the gut microbiome at two weeks post-HCT (Genus, p=0.8; Species, p=0.44) or aGVHD incidence (p=0.58). Immune reconstitution of T-cell and B-cell subsets was similar between groups. Five patients in the no-GD arm had eight BSI episodes vs one episode in the GD arm (p=0.09). The BSI-causing pathogens were traceable to the gut in seven of eight BSI episodes in the no-GD arm, including *Staphylococcus* species.

**Conclusions.** While GD did not differentially impact Shannon diversity or clinical outcomes, our findings suggest that GD may protect against gut-derived BSI in HCT patients by decreasing the prevalence or abundance of gut pathogens.

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Despite advances in graft manipulation and acute graft-versus-host-disease (aGVHD)-prophylactic regimens, treatment-related complications remain major causes of morbidity and mortality in patients undergoing allogeneic hematopoietic cell transplantation (allo-HCT). In efforts to decrease the incidence and severity of aGVHD, gut decontamination (GD) with non-absorbable antibiotics in the peri-HCT time-period is administered in some clinical centers, largely based on limited preclinical studies. Specifically, mouse and human studies have demonstrated that non-absorbable antibiotics can debulk intestinal bacteria and that this can decrease aGVHD (1-5).

While the precise mechanism of how GD impacts aGVHD remains unknown, it is thought that decreasing microbial load or altering the microbiota composition can improve intestinal barrier integrity and decrease inflammation via interactions with the host immune system (reviewed in (6, 7)).

In addition to non-absorbable antibiotics, a distinct practice of using systemic antimicrobial prophylaxis has also been used with the intent of suppressing bacterial growth in the gut to alter clinical outcomes. For example, Beelen, et al., showed that patients randomized to receive ciprofloxacin plus metronidazole had a lower incidence of grade II-IV aGVHD compared to those randomized to receive ciprofloxacin alone (25% vs 50%, p<0.01) (8), suggesting a link between anaerobic bacteria and aGVHD. However, other studies have shown that the use of systemic antibiotics with activity against anaerobic bacteria is associated with an increased risk of aGVHD (9-11). If the effect of GD on the pathogenesis of aGVHD is related to alteration of the microbiota composition, then measuring the impact of GD on the microbiota may help to resolve these discordant findings.
Prior clinical trials for GD are confounded by the variations in the practice of GD and systemic prophylaxis across centers with no consensus regarding the choice of antibiotic regimen (reviewed in (7)). In addition, prior studies relied on culture-based approaches to measure microbiome composition. However, many organisms within the gut microbiota, such as strict anaerobes, are difficult to culture (7). Modern next generation sequencing (NGS)-based approaches enable more comprehensive profiling of the gut microbiome composition by overcoming the need to maintain viability of the organisms within the stool sample, and thus may help inform the specific impact of GD on the gut microbiota.

While aGVHD is the most prevalent and fatal treatment-related complication of HCT, bloodstream infections (BSI) are also an important cause of treatment-related morbidity and mortality in patients undergoing allo-HCT. The cumulative incidence of BSI in pediatric HCT patients is ~20% in the first 100 days (12) (ranging from 15% to 65% (12-16)), with 18% BSI-attributable mortality (range, 12% to 20%) (12), and an estimated healthcare cost of $40,000-$70,000 per BSI incident (13, 17). In adult HCT patients, a diverse gut microbiome is associated with a lower risk of chemotherapy-related BSI (16). Multiple studies have shown the microbiota composition may predict BSI in adult (16, 18) and pediatric HCT patients (19, 20), and pediatric patients undergoing chemotherapy for acute leukemia (21). GD has been explored in the granulocytopenic population as a strategy to reduce BSI (22), in patients in intensive care units (ICU) regardless of underlying diagnosis (23, 24), and to a limited extent in HCT patients, with no difference compared to historic controls (25). The ICU studies have reported conflicting results, with some studies showing a reduction in BSI (23), while others report no significant differences in Gram-negative bacteremia (24).

BSIs in HCT patients are the consequence of infectious pathogens entering the bloodstream through indwelling catheters, breakdown in the skin, and mucosal barrier injury (MBI) secondary
to conditioning chemotherapy and resulting neutropenia. Changes in the abundance of MBI-associated enteric strains can precede BSI episodes in patients undergoing HCT (16, 19, 26). In addition to MBI, different bacterial strains of the same species can respond differently to selective pressures (27), with certain strains being more fit than other closely related ones (28, 29). Being able to identify and subsequently analyze strain variation (30) could help define how BSIs occur in patients.

We carried out the first prospective, randomized study of GD using oral, non-absorbable antibiotics (vancomycin and polymyxin B) to assess the impact of GD on the gut microbiome composition and diversity, and secondary outcomes including aGVHD and immune reconstitution. GD with vancomycin-polymyxin B in HCT patients was a long-standing institutional practice at Boston Children’s Hospital (BCH) due to their broad Gram-positive and Gram-negative coverage and lack of systemic absorption. However, the effect of vancomycin-polymyxin B on the gut microbial diversity during allo-HCT is not known. While historic studies suggest a trend that GD may decrease GVHD, studies in the last decade (9-11, 31) have suggested that decreased diversity of the gut microbiota leads to worse clinical outcomes. Given this discrepancy, we sought to compare the institutional practice of GD against the practice of no-GD to determine if there was a difference in Shannon diversity and clinical outcomes in a single center. In an exploratory analysis, we investigated whether GD is associated with a decreased incidence of BSI.
RESULTS

Patient characteristics are similar between the two study arms

Twenty pediatric patients undergoing allogeneic HCT were enrolled and randomized between March 2016 and June 2019 (Figure 1). Ten patients received GD per BCH standard practice with oral non-absorbable vancomycin-polymyxin B from day -5 relative to the transplant through neutrophil engraftment (dosing details in Supplemental Table 1; actual administration in Supplemental Figure 1), and 10 patients received no-GD (Figure 2). Baseline characteristics in the two arms were similar (Table 1). The median age at HCT for all patients was 15.2 years (range, 7.1-24.6). Most patients had underlying hematologic malignancies (n=15), received myeloablative conditioning regimens (n=17), and had bone marrow as the graft source (n=19). A median of seven stools (range, 3-18) were collected per patient starting pre-transplant through one-year post-transplant. 76% of the stool samples were collected within the first 30 days after HCT (Supplemental Figure 2).

Shannon diversity decreased similarly in patients with or without GD

To compare gut microbiome Shannon diversity between individuals on the two arms of the study, we first determined the taxonomic composition of the gut microbiota using the time-series stool collections. DNA was extracted from 147 patient stool samples and 142 (97%) were sequenced using whole-genome shotgun (WGS) short-read sequencing (Supplemental Figure 2; 5 of 147 (3%) had insufficient biomass to be sequenced). Libraries were sequenced to a median depth of 72.3x10^6 read pairs (range, 3.7x10^6-338x10^6) per stool sample. After pre-processing and quality control filtering of reads, a median of 16.9 x 10^6 (range, 4.3x10^4-81x10^6) high quality reads per sample were obtained (Supplemental Figure 3). In most cases where a large proportion of reads was removed during pre-processing (n=2 with less than 1 x 10^5 reads, n=14 of 142 (10%) with less than 1 x 10^6 reads), this was due to a very high proportion of human reads within the sample.
Taxonomic composition was determined using Kraken2 classification (32) against a database of all bacterial, fungal, and viral genomes contained in NCBI Genbank as of January 2020.

To determine if GD altered diversity of the gut microbiota, we focused on the primary endpoint of Shannon diversity at 2 weeks post-HCT. At this time point, stool specimens likely reflect changes induced by the conditioning regimen and gut decontamination in the GD arm, are collected prior to the development of inflammation and aGVHD (33), and usually precede the use of immunosuppressive medications for treatment of aGVHD. Shannon diversity, which is a measurement sensitive to the loss of rare taxa (34) and estimates microbial richness (e.g., the number of species) and evenness (e.g., the relative abundance of organisms within a sample), was calculated for each sample (Supplemental Figure 4). Consistent with previous studies (16, 31, 35), the median Shannon diversity of the gut microbiota at the species level prior to GD exposure was 3.6 (range, 2.1-4.5) for GD and 3.3 (range, 1.2-4.2) for no-GD, and decreased at 2-weeks post-transplant to 2.4 (range, 0.03-5.32) for GD and 3.1 (range, 2.1-3.7) for no-GD (Figure 3A, Supplemental Table 3, Supplemental Figure 5A for genus). Shannon diversity in the two arms was similar at baseline prior to GD exposure (Figure 3B for species (p=0.35), Supplemental Figure 5B for genus (p=0.32), Supplemental Table 4). At two-weeks post-transplant, there were no apparent differences in Shannon diversity between the GD and no-GD arms at the species (p=0.44, Figure 3B) or genus level (p=0.80, Supplemental Figure 5B), nor change in Shannon diversity from baseline (Supplemental Figure 6). Furthermore, in an exploratory analysis, there was similar Shannon (alpha) diversity between the two arms when extending the window beyond 30 days to include all samples in the study (Supplemental Figure 7). Beta diversity appeared similar between the two arms (Supplemental Figure 8), with the exception of a group of outliers in samples from three subjects with >45% relative abundance of Enterococcus faecium. As a control, stool samples from two healthy sibling donors were also collected to serve as a comparison to the HCT patients, which were similar to each other and the
HCT siblings at the time points collected (based on analysis of similarity (ANOSIM) in Supplemental Figure 8 legend). Based on these findings, there is no evidence of a significant difference in Shannon diversity in our intention-to-treat analysis of the gut microbiota between the two arms.

**No difference in cumulative exposure to antibiotics between GD and no-GD arms**

We sought to interrogate why gut microbiome Shannon diversity did not differ between the two arms. While adherence to vancomycin-polymyxin B varied (Supplemental Figure 1), we found no correlation between the proportion of GD doses received and Shannon diversity within the GD arm (Supplemental Figure 9). Unfortunately, the small sample size precludes a robust analysis comparing the microbial communities between patients with good adherence (>70% of planned doses, n = 4) versus those who had poor adherence (<30% doses, n=2).

As GD might impact pathogen colonization in the gut microbiota and possibly subsequent bloodstream translocation of these pathogens, we postulated that GD might be associated with decreased fevers and consequently decreased overall antibiotic exposure. In an exploratory analysis of the difference in systemic antibiotics between the two arms, we analyzed the clinical records based on individual antibiotics, class of antibiotics, and clinical indication including broad-spectrum antibiotics with anaerobic coverage (e.g., piperacillin-tazobactam, meropenem). We found that the duration of prophylactic and therapeutic antibiotic exposure (within 30 days post-HCT) was similar between the two treatment arms (Supplemental Table 5), including no difference in exposure to broad-spectrum antibiotics with anaerobic coverage (median 13 days (GD) versus 17 days (no-GD), \(p=0.68\)). Thus, it is possible that the impact of GD on the microbiota was small compared to the impact of systemic broad-spectrum antibiotics.

**Comparison of secondary clinical outcomes**
The pre-specified secondary outcomes of this study were stool frequency in the first 7 days, incidence of aGVHD in the first 100 days, relapse-free survival, overall survival (OS), and immune cell reconstitution. There was no apparent difference in the incidence of diarrhea in the first seven days post-transplant between the two treatment arms (Table 2, p=1.0). The overall incidence of grade II-IV aGVHD was 20%, with one patient in the GD arm and three patients in the no-GD arm (p=0.58, Table 2). The median day of onset of aGVHD was 38 days post-HCT (range 24-63). Of the 15 patients with a malignancy, three patients in the GD arm (n=7) and two patients in the no-GD arm (n=8) had malignant relapse within two years post-HCT; the 1-year relapse-free survival was 73±11.4% (n=15). The one-year OS was 100% (N=20, Supplemental Figure 10). No known harm from the GD treatment was seen. In summary, we found no differences in the rates of diarrhea, aGVHD, graft failure, relapse and relapse-free survival, or OS at 1 year in GD-treated individuals vs. no-GD in our study.

**Engraftment and immune reconstitution**

The median time to neutrophil engraftment was 26 days (interquartile range (IQR), 23.5-29.2) in the GD arm and 24 days (IQR, 19.2-28.0) in the no-GD arm (p=0.47). One patient in the no-GD arm had primary graft failure and underwent a second transplant on day +79. One patient in the GD arm had secondary graft failure and came off study at day +54. In a pre-specified secondary analysis, we examined reconstitution of peripheral blood lymphocyte subsets in the two arms, excluding the two patients with graft failure. The following analyses of T-cell reconstitution revealed similar outcomes in both groups: median CD4+ T-cell count at three months post-HCT (126.5 in GD arm vs 182.1 cells/µL in no-GD arm; p=0.24; Figure 4A); median CD4+ T-cell count at six months post-HCT (median, 206.5 in GD arm vs 248.2 cells/µL in no-GD arm; p=0.70; Figure 4A); ratio of regulatory T-cells to conventional T-cells (Treg:Tcon) in the 12 months post-HCT (Figure 4B); naïve T-cell fraction within CD4+ Tcon (Figure 4D). Taken together, these findings
suggest that gut decontamination does not adversely affect thymic function. Recovery of CD8+ T-cells and natural killer cells was similar between the study arms (Figures 4C and 4F, respectively). CD19+ B-cell concentration at 12 months was a median of 903.5 cells/µL (IQR, 814.0-984.8) in the GD arm compared to a median of 223.9 cells/µL (IQR, 190.1-333.3) in the no-GD arm (uncorrected p=0.016; not significant after a stringent Bonferroni-adjusted alpha level of 0.0045 given 11 biomarkers were tested) (Figure 4E). Of note, none of the patients in the no-GD arm received rituximab as part of their conditioning regimens or as part of post-transplant therapy. While it is difficult to draw conclusions from this small study, future, larger studies may further illuminate if and how immune reconstitution is influenced by gut decontamination and the microbiota.

**Incidence of bloodstream infection (BSI)**

In an exploratory analysis, we noted a trend of fewer BSIs in subjects enrolled in the GD compared to the no-GD arm. During the 100-day study period, a total of nine BSI episodes occurred in six patients; eight in the no-GD arm and one in the GD arm (p=0.09, Table 2 and Supplementary Table 2). Five of the six patients had a BSI within the first 31 days. In a post-hoc exploratory comparison, the cumulative incidence of BSI was higher in the no-GD arm compared to GD arm (Figure 5, p=0.0483, Gray’s test). Seven of the nine (78% of total) BSI episodes were from the no-GD arm and occurred before the day of neutrophil engraftment.

**Species-level evidence that the BSI-causing bacterium is present in the gut microbiome**

Given the interesting trend of increased BSI incidence in the no-GD vs. GD arm, we hypothesized that GD may decrease the burden of pathogens in the gut microbiota that can translocate across the mucosal barrier and subsequently cause a BSI. The gut microbiota can be a reservoir of BSI-causing pathogens in this patient population (16, 19, 26, 36, 37); thus, we asked whether patients
in the no-GD arm had BSI-causing pathogens within their gut microbiota before or during the time of infection.

In seven of nine of the BSI episodes, we were able to identify the BSI species in the gut (relative abundance >0.1%) within four days of the BSI (Figure 6 and Supplemental Figure 11, patients C03, C04 (two of three BSIs), C10, C11, C20, C22 (one of two BSIs)). We observed typically enteric bacteria causing a BSI in three patients (*Klebsiella oxytoca* in patient C04, *Escherichia coli* in C10, and *Enterococcus faecium* in C22). In addition, we observed increasing relative abundance of *Leclercia adecarboxylata* in the gut prior to the BSI in patient C03 (Supplemental Figure 11A). Interestingly, three independent BSI episodes were caused by organisms in the genus *Staphylococcus*, which is typically categorized as non-enteric, non-mucosal barrier injury (non-MBI) related (38). In each of these cases, *Staphylococcus* was found in the intestinal microbiota within four days of the BSI episode (Figure 6A and C, patient C04 (two independent BSIs 89 days apart, at 35% and 0.1% relative abundance) and C20 at 61% relative abundance). By contrast, we detected *Staphylococcus aureus* in <0.005% of the total reads in stool samples from three independent healthy controls (Supplemental Figure 4).

Two of the nine BSI episodes (a *Bacillus* bacterium and *Rothia dentocariosa*) are less likely to be derived from the lower gut (Supplemental Figure 11B and Figure 6D). While there was a rise in the relative abundance of the genus *Bacillus* in the stool sample (Supplemental Figure 11B, patient C11), the BSI isolate from C11 was subsequently sequenced and found to be *Lysinibacillus fusiformis* (also called *Bacillus fusiformis*, Supplementary Table 2), which was not detected in any of the stool samples of this patient. This suggests that either the BSI did not originate from the distal gut or was low enough in abundance to preclude taxonomic identification at the species level. The typically oral microbe *Rothia dentocariosa* was detectable at a low abundance in the fecal sample early in the first transplant of patient C22, and was undetectable.
after day +15, suggesting this BSI either originated from a location other than the distal gut, or was in low abundance in the lower gastrointestinal tract at the time of the BSI (Figure 6D).

Strain-level evidence that multiple BSI isolates are identical or nearly identical to those found in the gut microbiota around the time of the BSI

In the previous analysis, we found temporal concordance between species in the gut microbiota and the subsequent BSI, which suggested possible bacterial translocation across a damaged intestinal epithelium. If the BSI and gut strain were identical, this would support the gut being a likely source of infection. Thus, we looked for the presence of concordant strains of the BSI-causing pathogens within the gut. To carry out this strain analysis, we used inStrain (30), a tool that can account for multiple strain populations in metagenomic sequencing data. Specifically, the population average nucleotide identity (popANI) metric only calls single nucleotide polymorphisms (SNPs) in positions where two samples do not share any alleles (30). For example, two organisms of the same bacterial species will share >95% ANI (five mismatches for every hundred bases compared) (30); two microbes are considered nearly identical if they have >99.9999% ANI (one mismatch for every one million bases compared).

For this analysis, we generated whole genome sequencing data from 11 different BSI isolates from nine BSI episodes (Supplementary Tables 2 and 6); all strains analyzed are from no-GD patients except for one strain from patient C11 from the GD-arm. A median of 32.4x10⁶ (range, 26.4 to 46.6x10⁶) reads were generated for each BSI isolate and 15.6x10⁶ (range, 7.5 to 23.5x10⁶) reads remained after pre-processing (estimated median 545-fold genome coverage). Draft genomes from BSI organisms were assembled and used as references for the inStrain comparison (assembly statistics in Supplementary Table 7). Sequencing reads from all stool and BSI isolates were mapped against a patient’s BSI draft genome, and samples with at least 20,000 mapping reads were retained. SNPs were called with inStrain profile, and pairs of samples were
compared with inStrain compare. Pairs of samples that had at least 50% of the genome covered at a depth of at least five reads were considered for further analysis.

Going through the most notable cases individually (Table 3), no-GD patient C04 had two independent Staphylococcus aureus BSIs on day +5 and day +94 (Figure 6A). The S. aureus in the stool sample from day +12 was identical to the BSI on day +5 (100% popANI, zero SNPs detected in 2.8 Mb of sequence compared (the size of the S. aureus genome is 2.8 Mb); Table 3, Supplemental Figure 12A). The S. aureus BSI on day +94 was nearly identical to the stool sample on day +12 (99.9999% popANI; 2 SNPs between samples). Patient C04 also had detectable Klebsiella oxytoca in the stool (0.3% relative abundance on day +12; 5% on day +18) and had a BSI with the same strain of K. oxytoca (100% popANI with 6 Mb compared (the K. oxytoca genome is 6.02 Mb)) on day +18 (Figure 6A). However, as Klebsiella was in lower abundance and not sequenced to high enough depth in samples prior to day +18, we were unable to determine whether the strain was present prior to BSI. Interestingly, while the clinical microbiology lab identified that the two Klebsiella strains from the blood culture had different sensitivity to ceftriaxone (Supplementary Table 6), the two isolates were identical by inStrain (100% popANI, zero SNPs) at the genomic level.

Patient C10 in the no-GD arm had an E. coli BSI on day +8 (Figure 6B). An identical (100% popANI, 5 Mb compared (the E. coli genome is 5.12 Mb)) strain was found in the six stool samples collected from days +1 to +32. However, a different E. coli strain was present in the stool at day -4 (99.7% popANI to BSI and other stool samples, with approximately 13,303 different SNPs, Table 3, Supplemental Figure 12B). Thus, at least two different strains of E. coli were observed in this patient at the different timepoints. It is possible that the BSI causing strain was present at day -4, but was simply below our limit of detection in this earlier sample. The two E. coli BSI samples on
the same day (+8) from the clinical microbiology laboratory (Supplemental Table 6) were nearly identical to each other, with one SNP detected (99.9999% popANI).

Patient C22 in the no-GD arm experienced two BSIs after a second transplant (Figure 6D: Rothia dentocariosa on day +6 and Enterococcus faecium on day +20, relative to second transplant). In the eighteen stool samples from this patient, R. dentocariosa achieved a maximum of 0.1% relative abundance without enough sequencing coverage to conduct an inStrain comparison. By contrast, E. faecium in patient C22 was at >92% relative abundance at the time of the E. faecium BSI (Figure 6D). In the samples from day +23 of the first transplant through the end of the study (14 samples total), E. faecium in the gut was nearly identical (>99.9999% popANI) to the BSI strain, suggesting that the same strain was present in this patient’s microbiota through the course of two transplants, and likely eventually caused BSI (Figure 6D, Table 3, Supplemental Figure 11C).

In patients C03 (no-GD arm) and C11 (GD arm), the organism (Leclercia adecarboxylata and Lysinibacillus fusiformis, in C03 and C11 respectively) found in the BSI was either undetectable or at low abundance and did not have sufficient sequencing depth and coverage in the stool samples to make a conclusion regarding strain specificity using the inStrain comparison (Table 3). No-GD patient C20 experienced a Staphylococcus epidermidis BSI on day +23, with a rise in relative abundance in the gut microbiota from 3.7% on day +15 to 61% on day +25 (Figure 6C), suggesting the BSI originated from the gut, however, we were unable to do strain-level analysis as the original BSI-causing isolate was archived, but upon sequencing was identified to be E. coli, a likely contaminant of the culture (Table 3).

Based on these findings, at least five of the nine BSIs are identical or nearly identical to a species found in the gut microbiota using the popANI genome comparison of inStrain. Two additional BSIs
(Leclercia adecarboxylata from patient C03, and Staphylococcus epidermidis from patient C20) may also have originated from the gut based on an increase in the relative abundance of the bacteria around the time of the BSI, although strain level confirmation of this prediction is lacking. This suggests that in up to seven of the nine BSIs, the infection-causing pathogen is present in the gut in patients from the no-GD arm; by contrast, none of the BSI-causing pathogens are present in the gut in patients from the GD arm (Figure 6, Supplemental Figures 11 and 12). Collectively, these data demonstrate that the gut microbiota is a reservoir for pathogens traditionally derived from the gut, and that microbes like Staphylococcus, that are not typical gut bacteria, may subsequently cause a gut-derived BSI in pediatric allo-HCT patients.
Due to the lack of evidence supporting a clear benefit, GD is not recommended as a standard practice for the prevention of aGVHD or bacteremia. An informal survey of transplant centers in the United States in 2017 indicated that approximately 40% of adult and pediatric centers routinely practiced GD (7). As part of this study, we surveyed 101 pediatric HCT centers in the U.S.A. and Canada regarding their GD practices in 2019. Of the 32 centers that responded, only a small proportion (3 of 32; 9.4%) of centers were using GD as part of the aGVHD prophylaxis regimen (Supplemental Document 1). The practice of GD in patients undergoing allo-HCT is based on murine data, as well as early single-arm and retrospective studies suggest that using GD to alter the intestinal microbiota may protect against aGVHD (2, 5, 8). In this study, the first prospective randomized trial of GD in pediatric allo-HCT recipients, we investigated how GD with oral vancomycin-polymyxin B alters the microbial composition on a species and strain level using NGS. We also characterized secondary clinical outcomes and identify BSI-causing pathogens traceable to the gut either temporally or by strain-specific comparative genomics.

We show that there is no appreciable difference in gut microbiome Shannon diversity during the peri-transplant period between GD and no-GD arms. While subjects in the GD arm had variable administration of vancomycin-polymyxin B, the changes in diversity at 2 weeks did not correspond to the amount of GD the individual subject received. Furthermore, GD does not appear to lead to a decreased use of systemic antibiotics, as the time of exposure to prophylactic and therapeutic antibiotics was similar between the two arms. Thus, the equivalent exposure to systemic antibiotics in the two arms may have masked the impact of GD on Shannon diversity within the gut microbiome. Alternatively, while Shannon diversity is sensitive to loss of rare taxa (34), a different analysis with a future larger study, such as a mixed effect linear model, may be able to evaluate if there is a differential abundance of species between the GD and no-GD arms.
Despite promising evidence from earlier studies indicating that non-absorbable antibiotics were associated with a lower incidence of GVHD (1, 5), recent data suggests a more complex picture. For example, one retrospective report suggests that choice of antibiotics is critical, as cefuroxime, tobramycin, and nystatin in the GD arm was associated with an increased risk of developing aGVHD compared to non-GD (39). Several studies have expanded this concept showing that systemic broad-spectrum antibiotics and loss of microbial diversity (specifically commensal organisms) are associated with gastrointestinal (GI)-GVHD and GVHD-related mortality (10, 31). While our study is not powered to assess a difference in aGVHD incidence, we found that three patients had grade III-IV aGVHD in the no-GD arm vs one patient in the GD with grade II aGVHD. However, this trend could be attributed to the higher number of matched unrelated donors in the no-GD arm compared to the GD arm (n=5 vs n=2, respectively). Collectively these data leave an open question as to whether GD decreases the risk of grade III-IV aGVHD.

Based on prior studies suggesting interactions between the gut microbiota and development of circulating immune cells, we examined immune reconstitution of T- and B-cell subsets over the first year post-HCT. Although the reconstitution of T-cell and B-cell subsets was similar between the study arms after a Bonferroni correction, there is an interesting trend in the absolute CD19+ B-cell count at 12 months between the arms (Figure 4E). This trend cannot be explained by in vivo depletion as none of the patients in the no-GD arm received rituximab post-HCT. Several reports demonstrate extensive crosstalk between the microbiota and B-cell diversity (40-42), and that early B-lineage development in mice is influenced by the gut microbiome (43). While difficult to draw inferences with the small numbers here, an analysis of immune reconstitution and potential implications for infection risk should be fully characterized in a larger study.
While the impact of GD on infection-related outcomes was not part of our pre-specified primary or secondary analyses, we postulated that GD with oral vancomycin-polymyxin B may have an impact on decreasing the rate of BSIs that originated from the gut compared to no-GD (Supplemental Figure 14, and Supplemental Discussion). In an exploratory analysis, we found that all BSIs where the pathogen was found concomitantly in the gut were observed in the no-GD arm (BSIs did not appear dependent upon systemic corticosteroid therapy as 3 of 9 BSI episodes had concurrent corticosteroid therapy at the time of BSI). Interestingly, historical publications reporting on GD demonstrated that many of the Gram-negative bacteria isolated from the BSIs were still sensitive to the drugs used for decontamination (22), and a meta-analysis of ICU patients showed no increase in antimicrobial resistance with selective decontamination (44). The primary mechanism of GD reducing bacteremia in critical care patients is thought to be through limiting the growth of select bacteria, including bacteria such as Enterococcus and from the phylum proteobacteria (45), which includes the genera Escherichia and Stenotrophomonas. No prospective study in pediatrics has been conducted to date with a standard therapy without GD arm that is appropriately powered to address if GD decreases the risk of BSI. Furthermore, there have been very limited studies to compare systemic antibiotic prophylaxis to non-absorbable gut decontamination (including (46), and a meta-analysis (47)). The largest pediatric study to demonstrate a reduction in bacteremia was conducted in children undergoing initial therapy for acute leukemia and used levofloxacin for systemic prophylaxis, rather than gut decontamination; a second arm of the study did not reach the level of statistical significance for children undergoing HCT (48). Given the above findings, there is insufficient data supporting a clear benefit of GD in humans in HCT at this time. However, a larger trial may inform the question of whether oral vancomycin-polymyxin B decreases the burden of pathogens in the gut microbiota that can then translocate across the mucosal barrier and subsequently cause a BSI.
HCT patients often have injured mucosae secondary to conditioning chemotherapy and neutropenia, increasing their risk of mucosal barrier injury-laboratory confirmed bloodstream infection (MBI-LCBI). Due to their immunocompromised status and central venous catheters, HCT patients are also at risk for non-MBI-LCBI infections, including central line-associated BSI (CLABSIs). This is an important distinction as MBI-LCBI’s are not prevented by improved central venous catheter care when compared to CLABSI (12, 49-51). Given that there is approximately 18% mortality attributable to BSI in this patient population (12), being able to identify and develop methods to decrease this rate could have a profound impact on patient survival during HCT. We demonstrate here that the bacterial species responsible for the BSI are found in the gut in seven of nine BSI episodes. While many of those identified in the gut microbiome are Gram-negative enteric organisms, we also found evidence of classically non-MBI-LCBI organisms such as Staphylococcus species. These data, along with previous work (19, 26, 36, 37, 52), strongly suggest that the gut can serve as a reservoir for pathogens in HCT recipients. Overall, there may be skin- and nares-resident organisms such as S. aureus and S. epidermidis that can colonize and grow to high relative abundance in the gut microbiomes of severely immunocompromised hosts. Furthermore, while our study focused on young patients, it is possible that reduction of gut-derived BSI may be more relevant for improving HCT outcomes in older patients.

While the findings presented here suggest that GD is generally safe and has a relatively limited impact on the gut microbiome, there are several limitations to our study. First, this is a single center study with a small sample size. While originally powered to detect a difference of gut microbiome Shannon diversity of 4.0 in the no-GD arm and 2.8 in the GD arm based on changes in adult HCT patients (16), the magnitude of the change was less than anticipated. Some of this disparity is likely due to confounding variables like systemic antibiotics creating an even larger heterogeneity of the microbiota data than originally anticipated. Poor adherence to the oral
vancomycin-polymyxin B regimen, likely due to palatability, also may have lessened the effect between the arms. We also do not consider the microbiota from other sites in the body (e.g., skin) and thus cannot definitively exclude the skin or other sites as the source of the BSI.

Despite these limitations, the prospective design of this study, the standardized collection of samples, the application of NGS technologies and the use of inStrain to compare BSI genomes and fecal metagenome assemblies (MAGs) enable us to investigate the impact of GD vs. no-GD at a detailed level. In particular, whole genome sequencing of stool and bacterial isolates, along with use of the inStrain algorithm, allows for the confident and accurate comparison of bacterial isolates and metagenomes to identify identical or nearly identical microbial strains. In addition, by using a genome-wide comparison, we provide the framework to subsequently interrogate strain specificity which can have important impacts on antibiotic resistance and pathogenicity of particular organisms (e.g., exploiting metabolic pathways or secreted proteins of specific strains).

A larger study could test the model that GD decreases the number of, or eliminates entirely, bacteria that can potentially be pathogenic (Supplemental Figure 14) and translocate across the mucosal barrier. In addition, bacteria that caused the infections may be dependent on other bacteria that are susceptible to vancomycin-polymyxin B. Microbes in the gut live in communities, and when keystone species are lost, it may lead to fundamental changes in the community through a ‘cascading’ effect. While efforts have been made to use the microbiota composition to predict the risk of a BSI (18, 20, 21), it remains an open question whether targeting of microbes can be achieved to decrease the risk for BSI in the allo-HCT population (53). In conclusion, in this phase 2 RCT of 20 patients, we noted that all BSIs traced to the gut were found in the no-GD arm. Furthermore, Staphylococcus was found in the gut suggesting that expanding the number of organisms defined as MBI-LCBI for allo-HCT patients is an important step for detecting and subsequently designing ways to mitigate the risk of BSI during HCT. Finally, these data suggest
that oral vancomycin-polymyxin B GD may protect against post-HCT BSI by decreasing the prevalence or abundance of pathogens that can translocate across the mucosal barrier and subsequently cause gut-derived BSIs, a finding that will need to be verified in a larger trial.
METHODS

Cohort selection and study design
We performed a randomized phase 2 trial (+/- gut decontamination, ClinicalTrials.gov Identifier: NCT02641236, CONSORT diagram in Figure 1) examining the impact of gut decontamination (GD) with oral vancomycin-polymyxin B on intestinal microbiota composition of allogeneic HCT patients compared to no-GD. Eligibility included any recipient, ages ≥ 4 years to 30 years (Adult: 18-30 years, pediatric: 4-17 years) and toilet-trained, of 9/10 or 10/10 matched bone marrow allogeneic-HCT, or 4/6, 5/6 and 6/6 matched cord blood allogeneic HCT. Stool from two healthy sibling donors were collected as a comparison group (ages ≥ 4 years and toilet-trained). Detailed eligibility criteria are available in Supplementary document 2. Enrolled patients were randomized (1-to-1) to either “GD” or “no-GD.” The primary endpoint was microbial (Shannon) diversity at two weeks post-HCT. The trial was powered to detect a difference in Shannon diversity index of 1.2 (4.0 for no-GD vs 2.8 for GD) with a one-sided t-test and alpha=0.05, and assuming a standard deviation of 0.9 based on (16). An intention-to-treat comparison of GD vs no-GD was performed using the Wilcoxon rank-sum test. Participants assigned to the GD arm received non-absorbable, oral vancomycin-polymyxin B capsules according to body surface area (BSA); 375mg/m² BSA of vancomycin and 187mg/m² of polymyxin B (see Supplemental Table 1 for details). Random allocation of anonymous identifier, enrollment, and assignment completed under the supervision of the principal investigator (J.S.W.) of the clinical trial. Oral gut decontamination began on day -5 relative to the hematopoietic cell infusion date (day 0) and continued through neutrophil engraftment, defined as an absolute neutrophil count ≥ 500 cells/mm³ for three consecutive days. Patients in no-GD received the institutional standard practice including all other supportive care as did patients in the GD arm. Antifungal and antiviral prophylaxis was used at the discretion of the treating physician generally starting at day -9 (e.g., fluconazole) and day -5 (e.g., acyclovir) respectively (administration data in Supplemental Figure 13). Use of any agent (e.g.
sulfamethoxazole/trimethoprim, pentamidine) for prophylaxis of *Pneumocystis jirovecii* pneumonia was permitted. Secondary endpoints include the frequency of diarrhea (>3 stools per day) in the first seven days post-HCT, incidence of grade ≥ 2 aGVHD during the first 100 days post-transplant, survival, malignant disease relapse at one year after study entry, progression free survival (defined as time from randomization to the earlier of progression of malignant disease or death due to any cause at one year after study entry), and immune reconstitution. Exploratory outcomes include bacteremia during the first 100 days post-transplant. Clinical records including details of the transplant type, conditioning regimen and prophylaxis medications for aGVHD, antibiotic administration, microbiological information (including BSI and antibiotic resistance data), clinical symptoms (including aGVHD and diarrhea), and outcomes (relapse, death, and aGVHD) were obtained from the patient chart.

Stools samples were collected and stored immediately at 4°C and frozen at -80°C in cryovials within 24 hours of collection. Stool samples were collected as follows: weekly (+/- 3 days) prior to neutrophil engraftment; monthly (+/- 2 weeks) after neutrophil engraftment until six months; at six months and one year (+/- 1 month); within 48 hours of aGVHD diagnosis or positive blood culture.

**Flow cytometry and immune profiling**

Phenotypic analyses of lymphocytes subsets were performed at: pre-transplant, 1, 2, 3, 6, 9, and 12 months after transplant. Briefly, 50µL of EDTA whole blood was subjected to red blood cell lysis using 1x BD-PharmLyse (BD Biosciences) and subsequently incubated with fluorochrome-conjugated monoclonal antibodies with individual subsets enumerated in a FACSCanto-II flow cytometer and analyzed using BD-FACSDiva (both from BD Biosciences) and FlowJo software (TreeStar) as described previously (54). CD4Treg were defined as CD3+CD4+CD25^med-high^CD127^low^; CD4Tcon as CD3+CD4+CD25^neg-low^CD127^med-high^; B cells as CD19+, cytotoxic T cells as CD8+; and natural killer (NK) cells as CD56+CD3-. Within CD4Treg and CD4Tcon,
subsets were defined as follows: naïve T cells (CD45RO-CD62L+), CM (CD45RO+CD62L+), and EM (CD45RO+CD62L-).

**BSI antibiotic susceptibility testing**

Antibiotic susceptibility testing on isolates from bloodstream infections was performed by the Clinical Microbiology Laboratory at Boston Children’s Hospital (BCH) / Dana-Farber Cancer Institute (DFCI), except for colistin (polymyxin E), which was performed at Stanford Health Care Clinical Microbiology Laboratory using the disk elution test as previously described (55). Minimal inhibitory concentrations (MIC) for Enterobacteriales were interpreted using breakpoints according to Clinical and Laboratory Standards Institute (CLSI) (56) and colistin (polymyxin E) using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (57).

**BSI isolate cultures**

BSI isolates from HCT patients were obtained from the Clinical Microbiology Laboratory at DFCI. Isolates were grown on agar slants, transferred to Luria-Bertani (LB) broth, and grown to saturation at 37°C. Bacteria were pelleted by hard spin (10,000g) followed by removal of the supernatant and frozen at -80°C until DNA extraction.

**DNA extraction and whole genome shotgun (WGS) metagenomic sequencing of stool samples and BSI isolates**

Genomic DNA was extracted from stool samples and BSI cultures using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Cat. #19593) per the manufacturer’s instructions with the following modifications: in suspension buffer, samples were heated to 95°C and subjected to seven rounds of bead-beating for 30 seconds, alternating with cooling on ice for 30 seconds prior to addition of proteinase K and lysis buffer. DNA concentration was measured using Qubit Fluorometric Quantitation (DS DNA High-Sensitivity Kit, Cat. #Q32851, Thermo Fisher Scientific). DNA
sequencing libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina) and
Nextera FLEX (Illumina) for samples unable to be prepared with Nextera XT due to low biomass.
DNA library fragment length distributions were quantified via Bioanalyzer 2100 instrument (Agilent
Technologies) using the High Sensitivity DNA kit (Cat.#5067-4626, Agilent Technologies) per the
manufacturer’s protocol.

Libraries were pooled with unique dual sample indices to avoid barcode swapping (58) and
sequenced on Illumina HiSeq4000 or NovaSeq P150 platforms with a read length of 150bp.
Sequencing performed by Novogene (Sacramento, CA).

For microbiota analysis, a healthy donor stool was used as a positive control for batch-to-batch
variation, a known microbiota community (ZymoBIOMICS microbial community standard,
Cat#D6300) to determine microbial extraction bias, and a negative control carried through the
entire DNA extraction, library preparation, and sequencing.

**Computational methods**

**Preprocessing**

Fecal and BSI short-read WGS metagenomic sequencing reads were preprocessed to remove
sequencing adapters, PCR artifacts and duplicate reads, and any reads mapping to the human
genome, using established workflows available at:
Briefly, sequenced reads were deduplicated using SuperDeduper (60) and trimmed using
TrimGalore v0.6.5 (61) with a minimum quality score of 30 for trimming and minimum read length
of 60. All reads that aligned to the human genome (hg19) were removed using BWA v0.7.17 (62)
with final results of pre-processing read-counts shown in Supplemental Figure 3. Sequences then
underwent quality control using FastQC v0.11.9 (63). Bioinformatics workflows were implemented using Snakemake (64).

Classification with Kraken2 and diversity calculations

Short-read data was taxonomically classified using Kraken2 (32) against a database of all bacterial, fungal, and viral genomes in the NCBI GenBank database assembled to complete genome, chromosome, or scaffold quality as of January 2020. Species abundance was estimated using the Bracken (65) database, built using a read length of 150 and \textit{k-mer} length of 35. Workflows are available at: https://github.com/bhattlab/kraken2_classification (66). Diversity of the microbes was calculated using Vegan v2.5-7 (67).

Assembly and binning

Short-read sequences from stool samples and BSI isolates were assembled using SPAdes v3.15.2 (68). Stool metagenomic sequences were subsequently binned using CONCOCT v1.1.0 (69), Metabat2 v2.15 (70), and Maxbin v2.2.7 (71), aggregated using DASTool v1.1.1 (72) and de-replicated using dRep v2.5.4 (73). Bins were evaluated for completeness and contamination using QUAST (74). Metagenome assembled genome (MAG) quality was assessed using previously established standards by Bowers, \textit{et al.} (75) and Nayfach, \textit{et al.} (76). Workflows are available at: https://github.com/bhattlab/bhattlab_workflows/blob/master/manual/assembly.md (59).

Antibiotic resistance gene detection

Assembled BSI contigs and binned contigs from stool metagenomic sequences were profiled for antibiotic resistance genes (ARGs) with the Comprehensive Antibiotic Resistance Database (CARD) and the Resistance Gene Identifier (RGI) using default parameters (77).
Determining strain specificity of BSI isolates and stool metagenome assemblies

To compare bacterial strains and gut metagenomes in multiple samples, we used inStrain v1.0.0 (30). Sequencing reads were mapped against assembled BSI genomes using BWA (62). Pairs of samples with >50% coverage breadth at a depth of at least five reads were compared to analyze SNPs and determine average nucleotide identity (ANI) between the samples.

Data availability

All sequencing data sets from the current study have been deposited in the Sequence Read Archive under the National Center for Biotechnology Information (NCBI) BioProject ID PRJNA787952 at http://www.ncbi.nlm.nih.gov/bioproject/787952

Statistics

Statistical analysis and graphical presentation methods

Taxonomic abundance plots, antibiotic time course, and vancomycin-polymyxin B dosage graphs were created using GraphPad Prism version 9.1.2 for macOS, GraphPad Software, San Diego, California USA, www.graphpad.com, and the ggplot2 package v3.3.3 (78) with code modified from (19, 26, 79). Comparisons by treatment group were performed using Fisher's exact test (for binary variables), Wilcoxon rank-sum test (for continuous variables), or Wilcoxon signed-rank test (for paired continuous variables) for comparison of baseline versus two-weeks post-HSCT within patients. The Wilcoxon rank-sum and Wilcoxon signed-rank tests were adjusted using a false-discovery rate (FDR) \( \leq 0.05 \) or Bonferroni correction. Cumulative incidence curves of BSI were compared using the Gray's test with adjustment for the competing risk of death. Alpha and beta diversity were calculated using the vegan package v2.5-7 (67), and compared with the Wilcoxon rank-sum test and corrected using an FDR of \( \leq 0.05 \). Analysis of Similarity (ANOSIM) statistic after
999 permutations was done for comparison of beta-diversity for patients with healthy sibling samples to compare. Figure 2 and graphical abstract created with BioRender.com

**Study approval**

The trial was approved by the institutional review board (IRB) under the IRB of Dana-Farber Cancer Institute (DFCI) (Protocol #15-394 approved Oct. 2015; principal investigator: J.S.W.), was performed at Boston Children’s Hospital (BCH) and DFCI. IRB protocol was open to patient entry March 2016 through September 2019. Informed consent for the patient (if ≥18 years), parent (if <18 years), or legally authorized representative, was obtained prior to any specimen collection. Full protocol available at DFCI. Trial is registered under ClinicalTrials.gov Identifier: NCT02641236.
AUTHOR CONTRIBUTIONS

J.S.W., A.S.B., and J.R. conceived of the original study.

W.B.L and J.S.W. designed the clinical trial.

L.E.L., S.P.M., and C.N.D. contributed to the clinical protocol, assisted in enrollment and acquisition of clinical samples.

S. Silverstein, S.K., and O.B. collected the clinical samples.

Survey Core at DFCI https://surveycore.org/ conducted the informal survey of gut decontamination practice at pediatric HCT centers.

C.J.S., J.S.W., W.B.L., and N.C. organized, generated figures, and analyzed the clinical data.

C.J.S., M.M.L., extracted DNA, and prepared short-read sequencing libraries, selected samples for sequencing.

C.J.S., B.A.S., and A.S.B. analyzed the sequencing quality, conceived of the assembly approach and performed the InStrain analysis.

N.B. and A.L. performed and analyzed antibiotic resistance of gram-negative BSI’s to polymyxin B / colistin.

C.J.S. and S.TJK. wrote and modified code and generated figures for the clinical data.

C.J.S. and T.M.A. analyzed the infectious disease data.

S. Sun and A.A.F. modeled and considered alternative analysis paths for the microbial sequence datasets.

C.J.S. wrote the original manuscript, and generated figures & tables.

All authors reviewed, commented on, and approved the manuscript.
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Interim analyses have been previously reported in abstract form:

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**Figure 1. Study flow diagram.** ClinicalTrials.gov Identifier NCT02641236. Self-reported racial and ethnic categories in Supplemental Table 11. Absolute neutrophil count (ANC), gut decontamination (GD), hematopoietic cell transplantation (HCT).
Figure 2. Study design. Twenty patients undergoing allo-HCT were randomized to two arms, 10 patients with Gut Decontamination (GD) and 10 patients with no-GD. The GD arm received vancomycin-polymyxin B starting day -5 through engraftment (median neutrophil engraftment day +25, see Supplemental Figure 1), and was analyzed as intention-to-treat (Supplemental Table 1). The no-GD arm had the same stool and blood collection time points and did not receive oral vancomycin-polymyxin B. Black circles are time of stool collections including pre-transplant, weekly until engraftment, and monthly until day +100. An additional cohort of two healthy sibling donors serve as a stool control comparison group (Supplemental Figure 8). For immune reconstitution studies, blood samples (red circles) were collected at pre-transplant, 2 weeks, monthly for the first 3 months, then months 6, 9, and 12.
Figure 3. Shannon diversity is similar between the GD and no-GD groups based on intention-to-treat analysis at the species taxonomic level. Samples from patients undergoing GD (red) and no-GD (blue). (A) Shannon diversity over time analyzed at the species level using local polynomial regression fitting (LOESS-locally estimated scatterplot smoothing of the mean Shannon diversity) showing similarity between the two groups. N=48 samples from 10 patients in GD arm, N= 51 samples from 10 patients in no-GD arm. (B) Shannon diversity of individual patients from pre-transplant (before GD antibiotics) to 2 weeks post HCT connected with a line. Boxes shown are the median with hinges at the 25% and 75%. All comparisons not significant (see Supplemental Table 4 for details) using Wilcoxon rank-sum test. N=10 subjects GD arm, N=10 subjects no-GD arm.
Figure 4. Immune reconstitution. Peripheral blood samples at pre-transplant, monthly for the first 3 months, then months 6, 9, 12. Shown are the median values ± interquartile range, along
with the number of subjects sampled at each time point below each graph. (A) CD4+ T helper cells, (B) T_{reg}:T_{conv} (C) CD8+ cytotoxic T-cells, (D) CD4+ T_{conv} naïve cells, (E) CD19+ B-cells, (F) CD56+CD3- natural killer (NK) cells. Gut decontamination (GD). For CD19+ B-cells at 12 months, an uncorrected $p=0.016$ with a Wilcoxon rank-sum test was not significant (ns) when tested against a stringent Bonferroni-adjusted alpha level of 0.0045 (0.05/11 biomarkers tested).
Figure 5. Cumulative incidence of BSI during the first 100 days of transplant. Patients are separated by treatment group with GD (dashed red line, N=10 subjects) and no-GD (solid blue line, N=10 subjects). Of the 6 patients with a BSI, 5 occurred within the first 31 days; one patient in the no-GD arm had BSI on day +85 relative to their first transplant (on day +6 of their second transplant). $p=0.0483$, Gray’s test.
Figure 6. Bacterial abundance in the gut around the time of BSI. Multiple pathogens are present in the gut around the time of the BSI. Days relative to the course of the transplant on shown on the X-axis,
(from top to bottom on the Y-axis) antibiotic administration, alpha (Shannon) diversity, relative abundance of microbes in the stool samples at the genus taxonomic level (with organisms listed by color according to the key at the lower left), and relative abundance in the gut of the BSI-causing organism with the date of the BSI shown as an asterisk (*). Note: Y-axis is a different scale between abundance plots for focused organisms found in the BSI. (A) Subject C04 had two *Staphylococcus aureus* BSI 89 days apart (day +5 and Day +94), and a *Klebsiella oxytoca* BSI on day +18. (B) *Escherichia coli* BSI on day +8 in Subject C10. (C) *Staphylococcus epidermidis* BSI on day +23 in Subject C20. (D) Subject C22 received two transplants and had low abundance of *Rothia dentrocariosa* in the gut during the first transplant; *Rothia* was not detectable in the gut after day +15 of the first transplant, with a *Rothia* BSI on day +6 of the second transplant (day +85 relative to their first transplant). An *Enterococcus faecium* BSI occurred on day +20 of the second transplant (day +99 relative to their first transplant).

Antifungal and antiviral medications shown in supplemental Figure 13. Information on patients C03 and C11 may be found in Supplemental Figure 11. Azithromycin (Azithro), Ciprofloxacin (Cipro), Clindamycin (Clinda), Levofloxacin (Levo), Meropenem (Mero), Piperacillin/Tazobactam (PipTazo), Trimethoprim/Sulfamethoxazole (TMP/SMX, cotrimoxazole)
### Table 1. Patient baseline characteristics

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<td>Patient or Donor CMV serostatus</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>50</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>Patient/Donor Sex Mismatch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F → M</td>
<td>2</td>
<td>20</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Graft Source</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>9</td>
<td>90</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Cord</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GVHD Prophylaxis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CsA/MTX +/- methylprednisone</td>
<td>8</td>
<td>80</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>CsA/MMF</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

Gut decontamination (GD), acute lymphoid leukemia (ALL), acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), human leukocyte antigen (HLA), cytomegalovirus (CMV), graft-versus-host disease (GVHD), cyclosporine (CsA), methotrexate (MTX), mycophenolate (MMF)
<table>
<thead>
<tr>
<th></th>
<th>GD (N=10)</th>
<th>No GD (N=10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diarrhea in first 7 days post-HCT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>%</td>
<td>40</td>
<td>40</td>
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</tr>
<tr>
<td><strong>Grade ≥ 2 Acute GVHD</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Grade 2</td>
<td>1</td>
<td>3</td>
<td>0.58</td>
</tr>
<tr>
<td>Grade 3-4</td>
<td>0</td>
<td>3</td>
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<tr>
<td><strong>Bacteremia in first 100 days post-HCT</strong></td>
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<tr>
<td>BSI episodes:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gram positive episodes</td>
<td>1</td>
<td>8</td>
<td>0.09</td>
</tr>
<tr>
<td>Gram negative episodes</td>
<td>0</td>
<td>3</td>
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<tr>
<td><strong>Graft failure</strong></td>
<td>1</td>
<td>1</td>
<td>1.0</td>
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<tr>
<td><strong>Relapse of malignant disease ^</strong></td>
<td>3 of 7</td>
<td>2 of 8</td>
<td>ND</td>
</tr>
<tr>
<td><strong>1- year RFS: 73±11.4 (n=15 total)</strong></td>
<td></td>
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<tr>
<td><strong>OS at 1 year</strong></td>
<td>10</td>
<td>10</td>
<td>ND</td>
</tr>
</tbody>
</table>

^ For malignant diseases at 1-year post-HCT, N=7 for GD arm, N=8 for no-GD arm.

p-value based on Wilcoxon rank-sum test.
Diarrhea defined as >3 stools/day.
Bloodstream infection (BSI), methicillin resistant (MRSA), relapse-free survival (RFS), overall survival (OS)

ND- Not done, as study was not designed or powered for comparison of OS and PFS
Table 3. InStrain results comparing assembled BSI isolates to gut metagenomic samples

<table>
<thead>
<tr>
<th>Subject</th>
<th>Arm</th>
<th>Organism</th>
<th>Day of BSI</th>
<th>Day of stool sampling</th>
<th>Coverage (%)</th>
<th>popANI (%)</th>
<th>population SNPs</th>
<th>conANI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C03</td>
<td>B</td>
<td>Leclercia adecarboxylata</td>
<td>22</td>
<td>A</td>
<td></td>
<td></td>
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<tr>
<td>C04</td>
<td>B</td>
<td>MRSA (Staphylococcus aureus) *</td>
<td>5</td>
<td>12</td>
<td>99.996</td>
<td>100</td>
<td>0</td>
<td>99.9988</td>
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<tr>
<td></td>
<td></td>
<td>Klebsiella oxytoca</td>
<td>18</td>
<td>18</td>
<td>98.4936</td>
<td>100</td>
<td>0</td>
<td>99.9966</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MRSA (Staphylococcus aureus) *</td>
<td>94</td>
<td>12</td>
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<td>99.9999</td>
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<td>99.9993</td>
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<tr>
<td>C10</td>
<td>B</td>
<td>E. coli</td>
<td>8</td>
<td>-4</td>
<td>94.79</td>
<td>99.7124</td>
<td>13303</td>
<td>99.7124</td>
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<td></td>
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<td>99.86</td>
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<td>0</td>
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<td>99.9954</td>
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<td>99.75</td>
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<td>20</td>
<td>96.87</td>
<td>99.9996</td>
<td>21</td>
<td>99.987</td>
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<td>32</td>
<td>99.96</td>
<td>100</td>
<td>0</td>
<td>99.9947</td>
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<tr>
<td>C11</td>
<td>A</td>
<td>Lysinibacillus fusiformis (also called Bacillus fusiformis)</td>
<td>31</td>
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<tr>
<td>C20</td>
<td>B</td>
<td>Staphylococcus epidermidis *</td>
<td>23</td>
<td>BSI isolate contaminated; unable to perform analysis</td>
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<tr>
<td>C22</td>
<td>B</td>
<td>Rothia dentocariosa</td>
<td>6 a</td>
<td>A</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Enterococcus faecium</td>
<td>20 a</td>
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<td>3 a</td>
<td>99.97</td>
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<td>20 a</td>
<td>99.9747</td>
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<td>60 a</td>
<td>99.9763</td>
<td>99.9999</td>
<td>2</td>
<td>99.8463</td>
</tr>
</tbody>
</table>

Non-mucosal barrier injury (non-MBI) pathogens, (defined in (38)) including S. aureus (twice in the same patient C04 separated by 89 days) and S. epidermidis in patient C20, are labeled with an asterisk (*). A Not enough coverage. a Day relative to second transplant.
Population average nucleotide identity (popANI), single nucleotide polymorphisms (SNPs), and consensus ANI (conANI).