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Intestinal *Blautia* Is Associated with Reduced Death from Graft-versus-Host Disease



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ABSTRACT

The relationship between intestinal microbiota composition and acute graft-versus-host disease (GVHD) after allogeneic blood/marrow transplantation (allo-BMT) is not well understood. Intestinal bacteria have long been thought to contribute to GVHD pathophysiology, but recent animal studies in nontransplant settings have found that anti-inflammatory effects are mediated by certain subpopulations of intestinal commensals. Hypothesizing that a more nuanced relationship may exist between the intestinal bacteria and GVHD, we evaluated the fecal bacterial composition of 64 patients 12 days after BMT. We found that increased bacterial diversity was associated with reduced GVHD-related mortality. Furthermore, harboring increased amounts of bacteria belonging to the genus *Blautia* was associated with reduced GVHD lethality in this cohort and was confirmed in another independent cohort of 51 patients from the same institution. *Blautia* abundance was also associated with improved overall survival. We evaluated the abundance of *Blautia* with respect to clinical factors and found that loss of *Blautia* was associated with treatment with antibiotics that inhibit anaerobic bacteria and receiving total parenteral nutrition for longer durations. We conclude that increased abundance of commensal bacteria belonging to the *Blautia* genus is associated with reduced lethal GVHD and improved overall survival.

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INTRODUCTION

Despite continuing improvements in outcomes of patients undergoing allogeneic blood/marrow transplantation (allo-BMT), acute graft-versus-host disease (GVHD) continues to be a leading cause of mortality [1]. Current immune suppression strategies are only partially effective at preventing GVHD and simultaneously increase the risk for infections and disease recurrence. Strategies that can reduce

GVHD but leave immune function intact may thus potentially significantly improve outcomes. One such strategy is to target the complex community of microbes that reside within our intestinal tracts, collectively termed the intestinal microbiota.

A relationship between the microbiota and GVHD has long been suspected but is still not well understood. Mice transplanted in germ-free conditions [2] or treated with gut-decontaminating antibiotics [3] develop less severe GVHD. Clinical studies initially suggested a benefit from near-total bacterial decontamination [4,5] but later showed no clear benefit [6–8], and this approach was discontinued in the early 1990s [9]. Partial gut decontamination continues to be

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practiced, but little is known regarding optimal selection of antibiotic regimens. One study found the addition of metronidazole to ciprofloxacin led to a significant reduction in acute GVHD, suggesting that anaerobic bacteria may contribute to GVHD pathogenesis [10].

More recent results, however, indicate that this approach may not be ideal. Several studies have found that obligate anaerobes in the intestine, in particular clostridial species, are important mediators of intestinal homeostasis and prevent inflammation by up-regulating intestinal regulatory T cells [11]. In a recent study of 80 allo-BMT recipients at our center, our group reported that increased intestinal bacterial diversity at the time of engraftment was associated with improved overall survival and reduced nonrelapse mortality [12]. Although we did not find a significant association between bacterial diversity and GVHD in this study, this may have been because the population was underpowered to detect a difference in GVHD. Many of the patients had received a T cell–depleted allograft, which confers a much lower risk of developing GVHD [13,14] and may have led to insufficient GVHD events to detect an effect of the microbiota.

In this study we focused on the outcome of GVHD by studying patients who were most at risk. Using a prospectively collected fecal specimen bank, we examined a population of 115 allo-BMT patients from our institution who received T cell–replete allografts. Here, we describe our finding that bacteria in the intestinal tract from the genus *Blautia* are associated with reduced mortality from GVHD.

METHODS

Study Design and Oversight

Our participants were from a subset of patients prospectively enrolled in a fecal collection protocol, where samples were collected during the initial transplant hospitalization and stored in a biospecimen bank. Since 2009, nearly all patients undergoing allo-BMT performed by the adult BMT service at our center (age 18 and older) were approached to enroll, and the vast majority of patients agreed to participate.

Patients who received conventional grafts (non–T cell depleted) and had a fecal sample collected within 4 days of day 12 after allo-BMT were included in this study. Patients who received ex vivo T cell–depleted grafts were specifically excluded, given their historically low rates of grades II to IV acute GVHD of approximately 15%; notably, in this setting patients do not receive any post-transplant GVHD prophylaxis [13,14]. In comparison, in vivo T cell depletion with antithymocyte globulin administration is less effective at reducing the incidence of GVHD in recipients of conventional grafts, estimated at 28% in a large meta-analysis, with most patients still receiving GVHD prophylaxis with calcineurin inhibitors [15]. Thus, in this study we allowed inclusion of patients receiving antithymocyte globulin given their continued substantial risk of developing acute GVHD. No patients in the fecal specimen protocol received alemtuzumab for conditioning or post-transplant cyclophosphamide during the study period.

Stool specimens were requested at least weekly over the course of the transplant hospitalization. After collection, specimens were initially stored at 4°C. Within 24 hours, specimens were aliquoted into cryovials and stored at –80°C. The study was approved by the institutional review board at Memorial Sloan Kettering Cancer Center. All study patients provided written informed consent for biospecimen collection and analysis.

Patients included in this study were divided into cohorts 1 and 2, determined by the sequencing platform used to analyze their fecal samples. Selection of sequencing platform resulted from a combination of BMT date, stool collection days, and number of samples from each patient. From 2009 through 2012, we sequenced using the Roche 454 platform (454 Life Sciences, Branford, CT) samples from consecutive patients who met inclusion criteria regarding number and timing of samples for studies previously published [12,16]; these patients comprised cohort 1. Samples collected from patients who did not meet inclusion criteria were stored but not sequenced at the time. After 2013, we transitioned to the Illumina MiSeq sequencing platform (Illumina, San Diego, CA). Patients with stored samples who met inclusion criteria for the current study as well as additional patients transplanted in 2013 were included in cohort 2. Primary comparisons performed in this study were within each of the 2 cohorts, and thus there were no comparisons across cohort groups.

Study Definitions

Acute GVHD was diagnosed clinically, confirmed pathologically by biopsy whenever possible, and classified according to International Bone Marrow Transplant Registry criteria [17]. Late-onset acute GVHD, occurring more than 100 days after transplantation [18,19], was included in the definition of acute GVHD, whereas chronic GVHD in the absence of acute features was not. Cases of GVHD were further characterized by treatment: (1) topical steroids or budesonide, (2) systemic steroids (prednisone or methylprednisolone, .5 mg/kg daily or higher), and (3) second-line therapy in patients with steroid-refractory GVHD.

Cause of death was determined using a standard algorithm where competing causes were prioritized in the following order: (1) primary disease recurrence, (2) graft failure, (3) GVHD, (4) infection, and (5) organ failure. Thus, in patients without disease recurrence or graft failure, those who were being treated for GVHD at the time of death were considered to have succumbed to GVHD-related mortality, including those who died with infections [20]. Deaths classified as non-GVHD treatment-related mortality included patients who succumbed to graft failure, infection (in the absence of acute GVHD therapy), and organ failure.

Disease risk was determined according to the American Society for Blood and Marrow Transplantation Request for Information 2014 disease classification [21]. Conditioning intensity was assigned based on previously established working definitions [22].

Antibiotic Categories

Antibiotics used during the transplant hospitalization were divided into those that included significant activity against anaerobic bacteria (piperacillin-tazobactam, ticarcillin-clavulanate, imipenem-cilastatin, meropenem, metronidazole, oral vancomycin, and clindamycin) and those with reduced anaerobic activity in the intestinal tract (i.v. vancomycin, ceftriaxone, ceftazidime, cefepime, aztreonam, and trimethoprim-sulfamethoxazole) [23].

Transplantation Practices

As per our institutional practice, patients whose conditioning was more intense than nonmyeloablative received prophylaxis with ciprofloxacin (400 mg i.v. or 500 mg orally every 12 hours) and vancomycin (1 g i.v. every 12 hours) starting day –2 relative to BMT infusion [24]. Antibiotic prophylaxis against *Pneumocystis carinii* (trimethoprim-sulfamethoxazole, aerosolized pentamidine, or atovaquone) was given at the discretion of the transplant physician. Administration of total parenteral nutrition (TPN) was considered on day 2 after transplant in patients with poor oral intake and was discontinued upon oral recovery. Treatment of GVHD was at the discretion of the transplant physician and generally involved topical corticosteroids for mild to moderate isolated skin GVHD, oral budesonide for mild gastrointestinal GVHD, and systemic steroids for all other manifestations of GVHD, including liver and more severe skin and gastrointestinal GVHD. Patients were also maintained on a nonsteroid immunosuppressant, typically a calcineurin inhibitor, to allow eventual tapering of systemic steroids. Patients refractory to systemic steroids received second-line therapy at the discretion of the transplant physician.

Analysis of Specimens

For each stool specimen, DNA was purified using a phenol-chloroform extraction technique with mechanical disruption (bead-beating) based on a previously described protocol [25]. Samples from the first 64-patient flora cohort were analyzed using the 454 GS FLX Titanium platform (454 Life Sciences, Branford, CT) to sequence the V1–V3 region of the bacterial 16S rRNA gene. Samples from the second 51-patient flora cohort were subsequently analyzed using the Illumina MiSeq platform to sequence the V4–V5 region of the 16S rRNA gene. Sequence data were compiled and processed using mothur version 1.34 [26], screened and filtered for quality [27], and then classified to the species level [28] using a modified form of the Greengenes reference database [29]. Microbial diversity was quantified using the inverse Simpson index [30] and the Shannon diversity index [31] of operational taxonomic units with 97% similarity [32]. Taxonomic abundance comparisons were performed to identify biomarkers of GVHD-related mortality using linear discriminant analysis effect size analysis [33], using a logarithmic linear discriminant analysis cut-off of 2.0 as described in the original article by the developers [33]. Data from this study have been stored in the NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>). Quantification of 16S rRNA copies was performed using qPCR on DNA extracted from fecal specimens as previously described [34].

Statistical Analysis

The incidence of acute GVHD and GVHD-related mortality was estimated using cumulative incidence functions, treating relapse and death unrelated to GVHD as competing events, and compared across factors using Gray's test. Comparison across *Blautia* abundance was done first in a cohort

Table 1

Clinical Characteristics of Allo-BMT Patients Transplanted at Memorial Sloan Kettering Cancer Center with Stool Samples Collected Day 12 after BMT Included in an Identification and Validation Cohort

	Cohort 1	Cohort 2
Dates of transplant	September 2009 to October 2012	August 2011 to August 2013
Age, yr	25–70, median 53	26–75, median 50
Gender	Female 38%, male 62%	Female 32%, male 69%
Primary malignancy	NHL 38%, AML 38%, ALL 9.4%, Hodgkin disease 6.3%, CLL 6.3%, MDS 3.1%	NHL 35%, AML 37%, ALL 12%, Hodgkin disease 3.9%, CLL 7.8%, MDS 3.9%
Disease risk	High 45%, intermediate 36%, low 19%	High 35%, intermediate 27%, low 37%
Graft source	Peripheral blood 55%, cord blood 42%, bone marrow 3.1%	Peripheral blood 57%, cord blood 39%, bone marrow 3.9%
Donor relationship and HLA	Sibling identical (29%), unrelated identical (22%), unrelated nonidentical (48%)	Sibling identical (24%), unrelated identical (29%), unrelated nonidentical (47%)
Conditioning intensity	Standard intensity myeloablative 23%, reduced-intensity myeloablative 44%, nonmyeloablative 33%	Standard intensity myeloablative 14%, reduced-intensity myeloablative 57%, nonmyeloablative 29%
Stool sample collection day	+8 to +16, median +12	+8 to +16, median +12

NHL indicates non-Hodgkin lymphoma; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphoblastic leukemia; MDS, myelodysplastic syndrome.

of patients and then confirmed in a second cohort. Overall survival probabilities were estimated using Kaplan-Meier methodology and compared using the log rank test. Incidence and overall survival data are presented relative to the time of BMT, but patient cohorts were analyzed relative to the

landmark of day 12, the median time of stool collection. We note that no patients in the study died or developed GVHD before day 16. Comparison of the risk of acute GVHD and GVHD-related mortality after adjusting for clinical factors was performed using Cox regression. A receiver operating

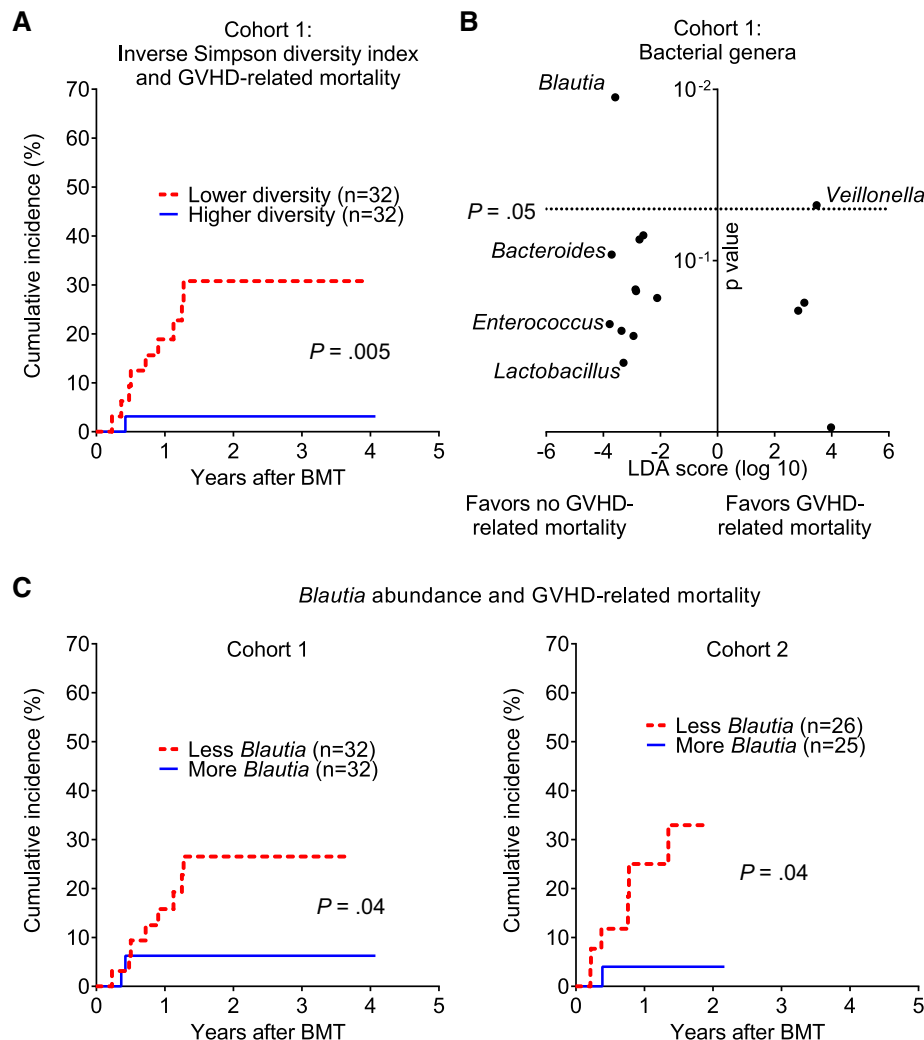


Figure 1. Changes in the intestinal flora are associated with differences in GVHD-related mortality. (A) Bacterial diversity was quantified using the inverse Simpson index after composition analysis of stool samples from cohort 1 performed by 16S gene sequencing. Patients were stratified by the median diversity index value and analyzed for cumulative incidence of GVHD-related mortality. (B) Associations of bacterial genera with GVHD-related mortality outcomes were quantified by linear discriminant analysis effect size analysis. Position along the vertical axis indicates statistical significance. (C) Patients from cohorts 1 and 2 were stratified by median *Blautia* abundance (.05% in both) and analyzed for incidence of GVHD-related mortality.

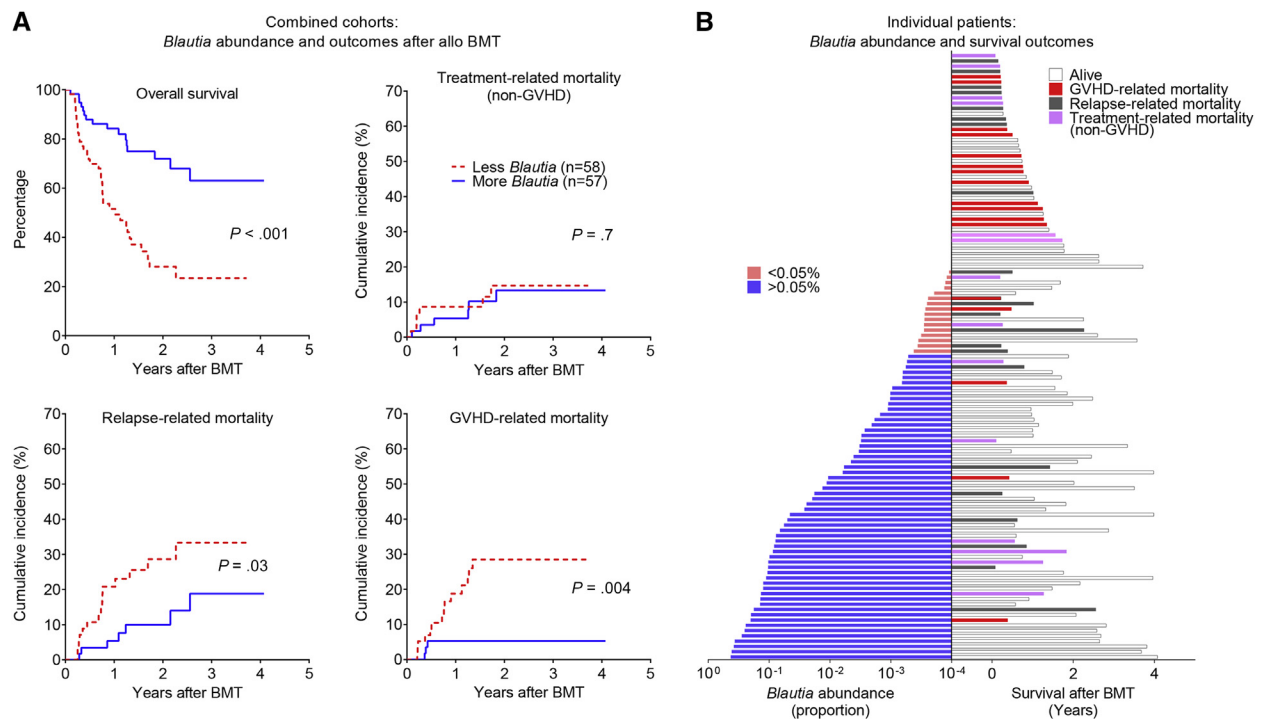


Figure 2. (A) Association of *Blautia* abundance and outcomes after allo-BMT. Patients from the 2 flora cohorts were combined and stratified by *Blautia* abundance below or above .05% and evaluated for the indicated outcomes; similar results were seen for each individual cohort (not shown). (B) *Blautia* abundance (left), survival, and cause of death (right) are indicated for individual patients from 2 cohorts combined, ranked by *Blautia* abundance.

characteristic curve was used to explore other thresholds of *Blautia* as discriminators for 1-year GVHD-related mortality [35]. A *Blautia* abundance trend curve was constructed using LOESS (locally weighted scatter plot smooth) local regression to estimate the marginal trend of *Blautia* abundance over time with a corresponding 95% confidence interval (CI). Comparisons of bacterial abundance were performed using the Mann-Whitney U for unpaired 2-way tests, Kruskal-Wallis for 3-way tests, and Wilcoxon signed-rank for paired tests. In all analyses statistical significance was defined as $P < .05$ based on a 2-sided test. Statistical analyses were performed using R version 3.1.0 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Intestinal Flora Diversity Near Day 12 Is Associated with Reduced GVHD-Related Mortality

Our group recently reported that increased bacterial diversity at the time of engraftment after allo-BMT was associated with improved overall survival and reduced transplant-related mortality, but in that heterogeneous patient population we did not find an association between diversity and GVHD [12]. For the current study, we began by asking if bacterial flora diversity was associated with lethal GVHD in a more uniform population of patients at higher risk for developing GVHD. We used prospectively banked stool samples collected from patients who underwent allo-BMT at our center. We chose to target day 12, reasoning that this time point was late enough for stool specimens to reflect changes induced by the transplant hospitalization, including antibiotic exposures, and still preceded the development of GVHD in all patients. We identified 64 patients in cohort 1 who, after conventional allo-BMT without T cell depletion, had provided a stool sample after BMT infusion and before hospital discharge (collected days, 8 to 16; median, day 12; clinical characteristics summarized in Table 1). We analyzed the flora composition of these stool samples using the

454 platform and followed patients clinically for development of GVHD-related mortality. Importantly, our institutional practice regarding bacterial prophylaxis is to administer ciprofloxacin and i.v. vancomycin prophylaxis starting day -2 . We have found that this antibiotic prophylaxis practice has a relatively modest impact on intestinal flora diversity [12], which is in agreement with the pharmacokinetics and biodistribution of i.v. vancomycin [36].

To evaluate the association between flora diversity and GVHD-related mortality, we used a typical approach to evaluate potential biomarkers, where patients are stratified by the median value into 2 equal-sized groups and the incidence of the outcome of interest is compared [37]. We found that increased bacterial diversity, quantified by the inverse Simpson index, was associated with reduced GVHD lethality (Figure 1A, $P = .005$).

Table 2

Association of Various Bacterial Taxa with GVHD-Related Mortality in Patients from the Cohorts Combined

Classification Level	Bacterial Taxon	Median Abundance	GVHD-Related Mortality Log Rank P
Genus	<i>Blautia</i>	.05%	.004
Genus	<i>Enterococcus</i>	1.32%	.8
Genus	<i>Bacteroides</i>	.02%	.5
Genus	<i>Lactobacillus</i>	.23%	.3
Genus	<i>Veillonella</i>	.03%	.8
Family	<i>Lachnospiraceae</i>	.21%	.02
Order	<i>Clostridiales</i>	2.92%	.02
Class	<i>Clostridia</i>	3.51%	.02
Phylum	<i>Firmicutes</i>	95.39%	.7
Suborder	Clostridial clusters IV, XIVa, and XVIII	.31%	.09

$P < .05$ are depicted in bold.

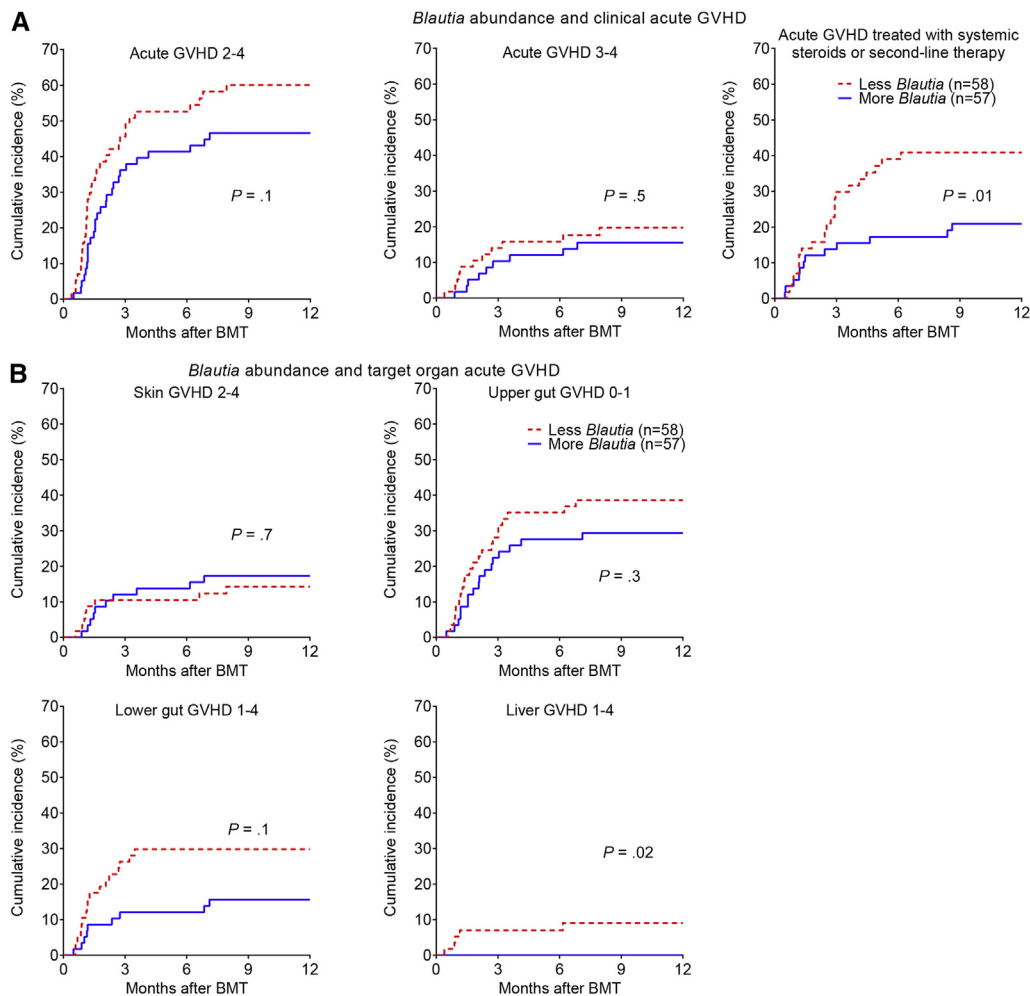


Figure 3. Association of *Blautia* abundance with clinical acute GVHD. (A) Patients were stratified by *Blautia* abundance below or above .05% and evaluated for development of the indicated severity grades of acute GVHD as well as acute GVHD that required systemic therapy with corticosteroids. (B) Patients were evaluated for development of acute GVHD in typical target organs.

Abundance of Bacteria from the Genus *Blautia* Near Day 12 Is Associated with Reduced Acute GVHD Requiring Systemic Immune Suppression

To identify bacterial subsets associated with GVHD-related mortality, we focused on bacterial phylogeny at the genus level, which is generally the most specific level at which 16S deep sequencing still provides reliable classification. Our approach involved first identifying bacterial genera potentially associated with GVHD-related mortality using a taxonomic discovery analysis, then evaluating candidate genera for their association with the incidence of GVHD-related mortality, and finally evaluating for reproducibility of observed associations in an additional independent cohort of patients from the same institution. Beginning with taxonomic discovery analysis of bacterial genera, we found that bacteria belonging to the genus *Blautia* were most significantly associated with reduced GVHD-related mortality (Figure 1B, $P = .01$). The *Blautia* genus notably includes anaerobic intestinal commensal organisms within the bacterial class Clostridia [38,39]. The genus *Veillonella*, in turn, was associated with increased GVHD-related mortality ($P = .047$).

We then evaluated the association of *Blautia* and *Veillonella* abundance with GVHD-related mortality, stratifying patients by the median abundance (.05% and .04%, respectively). We found that patients with higher *Blautia* abundance had reduced GVHD-related mortality (Figure 1C,

Table 3

Subgroup Analysis of Patients from the Cohorts Combined Examining the Relationship Between *Blautia* Abundance and GVHD-Related Mortality

Subgroup	No. of Cases (<i>Blautia</i> Low)	No. of Cases (<i>Blautia</i> High)	GVHD-Related Mortality Log Rank P
Myeloablative conditioning	13	9	.1
Reduced-intensity conditioning	27	30	.3
Nonmyeloablative conditioning	18	18	.02
PBSC graft	31	33	.006
Cord blood graft	24	23	.2

PBSC indicates peripheral blood stem cell.

$P < .05$ are depicted in bold.

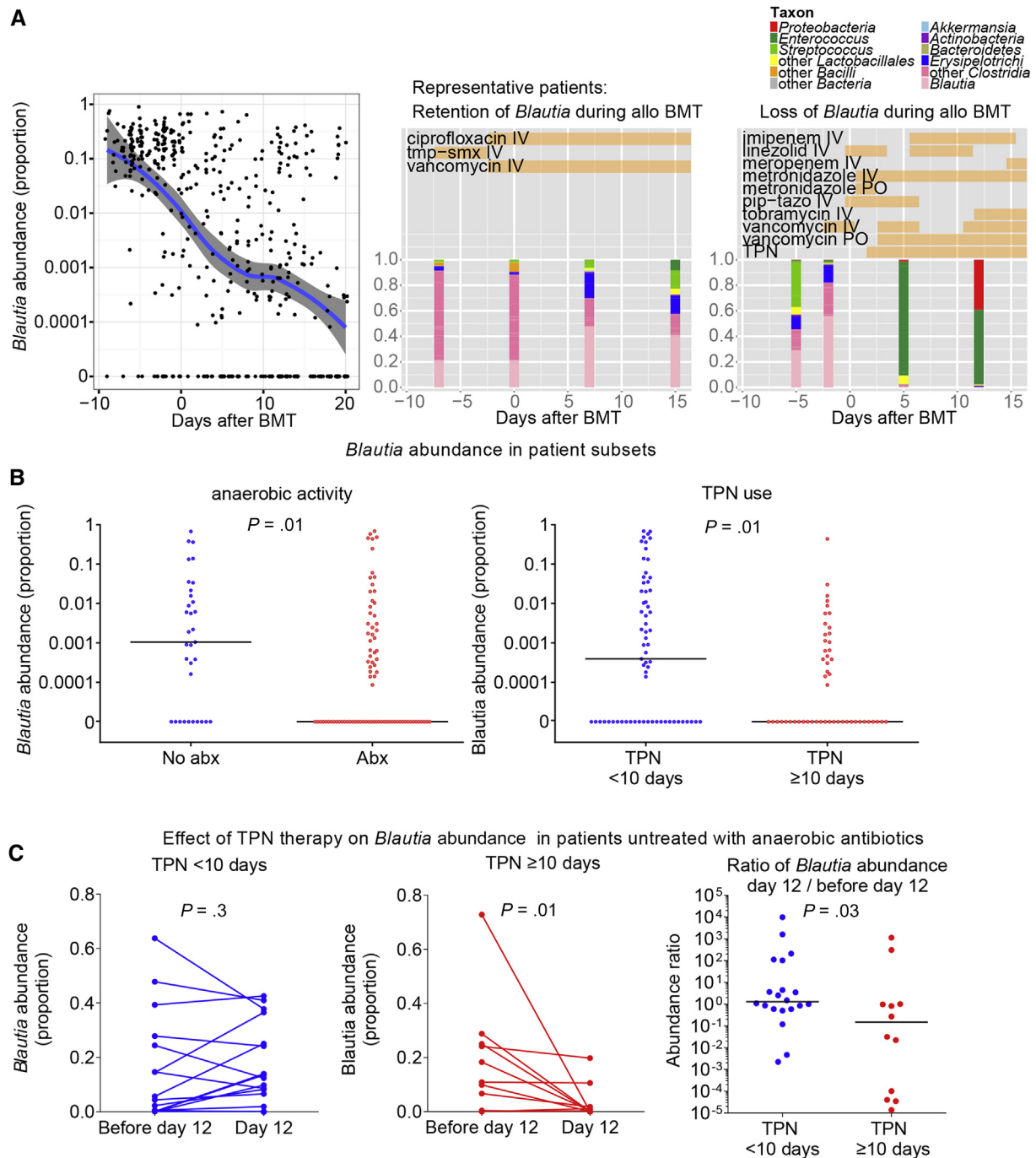


Figure 4. Identifying potential determinants of *Blautia* abundance in allo-BMT patients. (A) Left: *Blautia* abundances in all available stool samples from both flora cohorts. An abundance trend was constructed using moving average filtering (solid blue line; 95% confidence intervals shown in gray). Middle and Right: Serial fecal sample composition analysis from 2 representative patients. Vertical bars indicate composition of fecal microbiota by the indicated taxa. Horizontal bars indicate antibiotic and TPN exposures. Flora composition and time courses of antibiotic and TPN exposure for all patients are shown in Supplemental Figure 2. IV indicates intravenous; PO, oral; pip-tazo, piperacillin-tazobactam; tmp-smx, trimethoprim-sulfamethoxazole. (B) Patients were subsetted by exposure to antibiotics with anaerobic coverage before sample collection and duration of TPN therapy, and *Blautia* abundance was evaluated. Horizontal bars indicate median *Blautia* abundance. (C) A subset of patients who were not exposed to antibiotics with anaerobic coverage before sample collection were further subsetted by duration of TPN therapy and evaluated for *Blautia* abundance in a stool sample before day 12 and from day 12.

$P = .04$), whereas *Veillonella* was not significantly associated with GVHD-related mortality (data not shown, $P = .16$). We repeated this analysis in cohort 2, which included 51 independent patients from the same institution who provided fecal samples analyzed on the MiSeq deep-sequencing platform (clinical characteristics summarized in Table 1). This

cohort reproduced our prior results, where a *Blautia* abundance above the median (which, similar to cohort 1, was .05%) was associated with less GVHD lethality (Figure 1C, $P = .04$). In this second cohort, *Veillonella* again was not associated with GVHD-related mortality (data not shown, $P = .5$).

Abundance of Bacteria from the Genus *Blautia* Near Day 12 Is Associated with Improved Overall Survival

By evaluating the combined cohorts, we found that *Blautia* abundance was strongly associated with improved overall survival after allo-BMT ($P < .001$). This could largely be explained by reduced GVHD-related mortality ($P = .004$) and to a lesser degree reduced relapse-related mortality ($P = .03$), with no difference in non-GVHD treatment-related mortality (Figure 2A). Similar results were observed when cohorts were analyzed separately (data not shown). *Blautia* abundance, survival, and cause of death are depicted for each individual patient in Figure 2B, demonstrating that many of the GVHD-related deaths occurred in patients with lower *Blautia* abundance.

Some of the GVHD-related deaths in this patient population occurred somewhat later than would be expected after allo-BMT, with 9 of 17 events occurring after day 180. In these patients, most developed acute GVHD during the taper of immune suppression (median day after BMT, 41; range, 21 to 80). Donor lymphocyte infusion, commonly performed for disease relapse and a potential risk factor for late acute GVHD, was administered to 3 patients but did not contribute to acute GVHD in these patients. Chronic GVHD, which can occur after acute GVHD or develop de novo, was diagnosed in only 1 patient who experienced GVHD-related mortality and thus was also not a significant contributor to late GVHD-related mortality in this study.

Having demonstrated an association of *Blautia* abundance with GVHD-related mortality using median *Blautia* abundance to stratify patients, we next sought to compare this threshold with other potential cut-offs of *Blautia* abundance. We generated a receiver operating characteristic curve (Supplemental Figure 1A), which had an estimated area under the curve of .72 (95% CI, .52 to .93), further suggesting that *Blautia* abundance may have utility as a biomarker of GVHD-related mortality. With a sensitivity of .51 and specificity of .84, the receiver operating characteristic curve supported the use of the median to stratify patients at high risk for GVHD-related mortality.

A potential limitation of 16S rRNA deep sequencing is the lack of absolute abundance information regarding bacterial subtypes. To determine the absolute abundance of *Blautia* 16S rRNA genes in fecal samples, we normalized the relative-abundance measurements from deep sequencing to the absolute abundance of 16S rRNA quantified by real-time PCR. We had adequate DNA to perform this for most patients (84 of 115, Supplemental Table 1) and found that absolute *Blautia* abundance, similar to relative *Blautia* abundance, was associated with reduced GVHD-related mortality ($P = .01$, Supplemental Figure 1B).

We also searched for an association of GVHD-related mortality with other bacterial genera. Because increased *Enterococcus* may be associated with GVHD [40], we evaluated if *Enterococcus*, or potentially beneficial bacterial genera (*Lactobacillus* [41,42] and *Bacteroides* [43]), were associated with GVHD-related mortality in our patient population. Our results indicated that none of these bacterial genera was associated with GVHD-related mortality in the combined cohorts ($P = .8$, $P = .5$, and $P = .3$, respectively, Table 2).

We next asked if bacterial subtypes related to *Blautia* were associated with reduced lethal GVHD. Bacteria from the genus *Blautia* are classified as follows: family Lachnospiraceae, order Clostridiales, class Clostridia, and phylum Firmicutes [38]. Stratification of patients by abundance of bacteria from

Lachnospiraceae, Clostridiales, and Clostridia all demonstrated associations with a reduced incidence of lethal GVHD ($P = .02$, $P = .02$, and $P = .02$, respectively, Table 2), suggesting that members of *Blautia*, and potentially its close relatives, contribute a protective effect against lethal GVHD. Inclusion of more distant relatives by examining all Firmicutes, however, resulted in loss of the association ($P = .7$, Table 2).

Abundance of Bacteria from the Genus *Blautia* Near Day 12 Is Associated with Reduced Need for Acute GVHD Treatment

We then asked if *Blautia* abundance was associated with a reduction in the clinical severity of acute GVHD. We found that the association between *Blautia* abundance and reduced incidence of acute GVHD grades II to IV did not reach statistical significance ($P = .1$, Figure 3A), and there was no association with acute GVHD grades III to IV ($P = .5$, Figure 3A). *Blautia* abundance, however, was associated with reduced development of acute GVHD that required treatment with systemic corticosteroids or was steroid refractory ($P = .01$, Figure 3A), suggesting that loss of *Blautia* is associated with acute GVHD that will not respond to topical corticosteroids or budesonide alone. Regarding classical acute GVHD target organs, increased *Blautia* abundance was not associated with skin GVHD or upper gut GVHD (Figure 3B). The association with reduced lower gut GVHD was not statistically significant ($P = .1$, Figure 3B), whereas that with reduced liver GVHD was significant ($P = .02$, Figure 3B), although the number of events was small.

Abundance of Bacteria from the Genus *Blautia* Is Most Associated with Reduced GVHD Lethality in Patients Receiving Nonmyeloablative Conditioning and Peripheral Blood Stem Cell Allografts

We then evaluated the association between *Blautia* and lethal GVHD in patients divided into subgroups by conditioning intensity or by graft source. *Blautia* was most associated with reduced lethal GVHD in patients who received nonmyeloablative conditioning ($P = .02$, Table 3), whereas the association in patients receiving myeloablative and reduced-intensity conditioning did not reach statistical significance ($P = .1$ and $P = .3$, Table 3). In patients divided by graft source, those who received peripheral blood stem cell allografts showed an association between *Blautia* abundance and reduced lethal GVHD ($P = .006$, Table 3), whereas in those receiving umbilical cord blood stem cell grafts the association did not reach statistical significance ($P = .2$, Table 3).

***Blautia* Abundance Is Independent of Known Clinical Acute GVHD Risk Factors**

To determine if *Blautia* abundance provides additional prognostic information to GVHD outcomes, we investigated potential associations between *Blautia* abundance and known risk factors for acute GVHD [44–46]. We found that patient age, performance status, disease risk, cytomegalovirus status, graft source, donor/patient gender, conditioning intensity, antithymocyte globulin administration, or GVHD prophylactic regimen were not associated with *Blautia* abundance (Supplemental Table 2). Although limited by small numbers, patients of an Asian or Hispanic background appeared to have lower abundance of *Blautia*.

Identifying Potential Clinical Determinants of *Blautia* Abundance During Allo-BMT Hospitalization

To better understand the heterogeneity in *Blautia* abundance in our patient population, we attempted to identify determinants of *Blautia* abundance. An analysis of all stool samples collected from both flora cohorts showed that most patients had relatively abundant amounts of *Blautia* upon admission for transplant hospitalization, with a median abundance $> .1$ (10%) (Figure 4A). Some patients retained their *Blautia* throughout their hospital course, whereas others rapidly dropped *Blautia* levels during hospitalization (Figure 4A and Supplemental Figure 2). We found that patients not exposed to antibiotics containing increased anaerobic coverage were more likely to have increased levels of *Blautia* ($P = .01$, Figure 4B), a finding that was expected given that *Blautia* is an obligate anaerobe. Intestinal flora composition analyses for individual patient samples along with antibiotic exposures are provided in Supplemental Figure 2.

We then asked if conditioning intensity was also associated with *Blautia* abundance but found this was not the case ($P = .5$, Supplemental Table 2). Interestingly, however, use of TPN was associated with changes in *Blautia* abundance. Because of nausea and mucositis after conditioning, allo-BMT patients commonly experience a prolonged period of significantly reduced oral intake and are treated with supplementary TPN. Because initiation of TPN at our institution is considered at the earliest on day 2 and stool samples were on average collected on day 12, we compared patients with TPN use of less than 10 days duration (indicating delayed, interrupted, or discontinued TPN therapy) with those with TPN use of 10 days or more. We found increased levels of *Blautia* in patients who received shorter courses of TPN therapy (Figure 4B), and TPN duration was associated with loss of *Blautia* even in patients who avoided treatment with anaerobe-active antibiotics (Figure 4C). TPN exposures for individual patients are provided in Supplemental Figure 2.

Blautia Abundance Remains Associated with GVHD-Related Mortality after Adjusting for Potential Clinical Confounders

We finally performed univariate and multivariate analyses to determine if the association of *Blautia* abundance with GVHD outcomes was driven by potential clinical confounders. Given the limited numbers of GVHD-related mortality events, we restricted our multivariate analysis to 2 factors, in addition to *Blautia* abundance. To identify potential confounders, we examined a variety of clinical factors previously shown to be linked to GVHD. This approach identified the GVHD prophylaxis regimen as the only statistically significant factor for GVHD-related mortality; specifically, patients who received cyclosporine and mycophenolate had higher event rates ($P = .044$). This regimen is the standard GVHD prophylaxis for patients receiving cord blood grafts at our center, and indeed, recipients of cord grafts showed a similar trend toward higher GVHD-related mortality ($P = .062$). An additional factor that almost reached statistical significance was exposure to antibiotics with anaerobic coverage ($P = .055$). We thus generated a model adjusting for 2 factors, graft source and exposure to anaerobic antibiotics, and found that *Blautia* abundance remained associated with both GVHD-related mortality (hazard ratio, .18; 95% CI, .05 to .63; $P = .007$) and clinical GVHD treated with systemic steroids (hazard ratio, .3; 95% CI, .14 to .64; $P = .002$). Finally, we tested the

association of *Blautia* abundance with relapse-related mortality after adjusting for 2 factors, disease risk and graft source, and found that the association with *Blautia* abundance no longer reached statistical significance ($P = .055$).

DISCUSSION

In this study we found that in allo-BMT recipients' intestinal flora diversity was associated with reduced GVHD lethality. Furthermore, the bacterial genus from stool samples most associated with reduced GVHD-related mortality was *Blautia* in 2 independent cohorts from the same institution. Patients with more *Blautia* also showed a reduced incidence of acute GVHD that required treatment with systemic corticosteroids or was steroid refractory, as well as improved overall survival. Surprisingly, despite the association with GVHD-related mortality, *Blautia* abundance did not distinguish the incidence of acute GVHD grades II to IV or even grades III to IV, which has been shown to identify patients less likely to respond to steroids, leading to poorer survival [47]. However, a subpopulation of patients who initially presented with grade II acute GVHD but nevertheless fared poorly has been described, and these patients may be better identified by novel GVHD grading systems [47] or by novel biomarkers [48]. Further investigations in additional larger patient cohorts may determine if *Blautia* abundance can similarly supplement the prognostic utility of clinical acute GVHD grading.

This study has several important limitations. First, because our results are from a single center, results may not be generalizable to other centers with different patient populations, in particular, different antibiotic practices. Additionally, although we have found that *Blautia* is associated with GVHD-related mortality, this was identified in an exploratory manner and confirmed in a relatively small validation cohort. Furthermore, we have not demonstrated causality, that is, that the presence of *Blautia* directly protects against lethal GVHD. It is possible that *Blautia* is predominant member of a larger group of bacteria that can mediate beneficial anti-inflammatory effects and disappear as a consortium. Atarashi et al. [11] reported that administering a mixture of 17 Clostridia isolates can reduce intestinal inflammation in mice by increasing infiltration of the large intestine with regulatory T cells. Consistent with this, we found that the abundance of bacteria from the class Clostridia, which includes *Blautia*, was associated with reduced GVHD-related mortality in our patients. A beneficial anti-inflammatory association of *Blautia* has also been observed in other clinical settings, including colorectal cancer [49], inflammatory pouchitis after ileal pouch-anal anastomosis [49], and liver cirrhosis [50], where *Blautia* was one of several subtypes of bacteria associated with improved outcomes.

One question that arises is how a patient's *Blautia* abundance on day 12 after allo-BMT could biologically have an impact on whether months later he or she develops symptoms of acute GVHD or as late as a year later succumbs acute GVHD-related mortality. There are precedents, however; low serum cyclosporine concentrations in the first week after allo-BMT were found to be associated with later development of acute GVHD with onset largely occurring after day 30 [51]. Additionally, serum levels of the biomarker ST2 levels on day 14 were found to be associated with a 6-month nonrelapse mortality [48]. Together, these studies suggest that clinical conditions early post-BMT may affect the initiation of GVHD and modulate its eventual severity, although this can in some cases take months to fully manifest, perhaps

because of partial containment of inflammation by ongoing administration of immune suppressants in the forms of GVHD prophylaxis and therapy.

Interestingly, although our results indicate that *Blautia* is associated with reduced GVHD suggesting a link with immune suppression, *Blautia* was not associated with increased relapse-related mortality. Indeed, we detected an association of *Blautia* with a reduction in relapse-related mortality, although this no longer reached significance in our multivariate analysis. One explanation is that *Blautia* may be associated with a localized anti-inflammatory effect, primarily in the intestinal tract, without systemic immune suppression that could hinder a graft-versus-tumor effect. This possibility is supported by the absence of an association with skin GVHD. Together, these data suggest that targeting the intestinal microbiota may allow for reduced intestinal GVHD without simultaneously increasing the risk for malignant relapse.

In our subgroup analyses, we found associations between *Blautia* and GVHD outcomes in patients who received non-myeloablative conditioning and in patients who received peripheral blood stem cell allografts, but these associations did not reach significance in other patient subgroups who received more intense conditioning or received cord blood grafts. Limitations in sample size may have contributed to this, and larger studies are likely required to evaluate the association of *Blautia* with GVHD outcomes in allo-BMT subgroups. In this study, patients undergoing increased conditioning intensity experienced higher rates of treatment with antibiotics and TPN, as did patients receiving cord blood grafts. It is possible that an accumulation of risk factors for microbiota injury led to reduced heterogeneity in certain subgroups, and as a result the ability of *Blautia* abundance to discriminate patients with GVHD outcomes was reduced.

By characterizing the abundance of *Blautia* in our patients over the course of their transplant hospitalization, we found that most patients initially had relatively large amounts of *Blautia* that were dramatically lost during their hospital course. Established risk factors for GVHD were not associated with changes in *Blautia* abundance, but 2 clinical risk factors were associated with loss of *Blautia*: treatment with antibiotics with increased anaerobic coverage and prolonged administration of TPN. Although conditioning intensity and duration of TPN necessity are known to be associated [52], we found no significant association between conditioning intensity and *Blautia* abundance. This suggests that mucositis and/or poor oral nutrition may contribute to loss of *Blautia* more directly than intense conditioning. Corroborating this are findings in mouse models that myeloablative conditioning is associated with only mild perturbations in flora composition, in comparison with larger perturbations characterized by loss of Clostridiales seen in both mice and humans with the onset of GVHD, a potent inducer of anorexia [42]. A pattern of loss of members of Clostridiales, including *Roseburia*, *Faecalibacterium*, *Ruminococcus*, and *Blautia* species, can similarly be observed in volunteers placed on high-protein and low-carbohydrate diets [53] or on diets derived entirely from animal products [54]. Alternatively, epithelial damage due to conditioning-induced mucositis could also potentially result in changes in flora composition via changes in expression of antimicrobial molecules produced by intestinal epithelium [55].

A possibility raised by this study is that clinical strategies to prevent damage to the intestinal microbiota, in particular to preserve obligate anaerobic bacteria such as *Blautia*, may

be worth investigating in an effort to improve clinical outcomes for allo-BMT patients. Potential strategies include avoidance of antibiotics that suppress anaerobic members of the commensal flora or encouraging oral nutrition to supporting anaerobic populations. Interestingly, a study from Seguy et al. [56] in 2006 reported that treating patients with enteral feeding led to a benefit in overall survival and reduced rates of acute GVHD grades III to IV and deaths due to infection. In the context of our findings, these results may indicate that intestinal commensals could mediate some of the benefits of enteral nutrition after allo-BMT. Choosing less intense conditioning regimens may also help patients maintain oral nutrition and boost *Blautia* abundance; indeed, multicenter registry data indicate that less intense conditioning regimens are associated with reduced acute GVHD [45].

In conclusion, we have shown that the composition of the intestinal flora, in particular the abundance of anaerobic bacteria from the genus *Blautia*, can be used as a novel prognostic indicator for GVHD-related mortality. Further studies will need to be performed to determine if this finding holds true at other institutions. Additionally, studies to evaluate the potential impact of *Blautia* and related bacteria on the pathophysiology of GVHD are warranted, as are efforts to develop strategies to support anaerobic intestinal commensals in patients who undergo allo BMT.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbmt.2015.04.016>.

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