Intestinal Microbiota and Relapse After Hematopoietic-Cell Transplantation

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ABSTRACT

Purpose

The major causes of mortality after allogeneic hematopoietic-cell transplantation (allo-HCT) are relapse, graft-versus-host disease (GVHD), and infection. We have reported previously that alterations in the intestinal flora are associated with GVHD, bacteremia, and reduced overall survival after allo-HCT. Because intestinal bacteria are potent modulators of systemic immune responses, including antitumor effects, we hypothesized that components of the intestinal flora could be associated with relapse after allo-HCT.

Methods

The intestinal microbiota of 541 patients admitted for allo-HCT was profiled by means of 16S ribosomal sequencing of prospectively collected stool samples. We examined the relationship between abundance of microbiota species or groups of related species and relapse/progression of disease during 2 years of follow-up time after allo-HCT by using cause-specific proportional hazards in a retrospective discovery-validation cohort study.

Results

Higher abundance of a bacterial group composed mostly of *Eubacterium limosum* in the validation set was associated with a decreased risk of relapse/progression of disease (hazard ratio [HR], 0.82 per 10-fold increase in abundance; 95% CI, 0.71 to 0.95; $P = .009$). When the patients were categorized according to presence or absence of this bacterial group, presence also was associated with less relapse/progression of disease (HR, 0.52; 95% CI, 0.31 to 0.87; $P = .01$). The 2-year cumulative incidences of relapse/progression among patients with and without this group of bacteria were 19.8% and 33.8%, respectively. These associations remained significant in multivariable models and were strongest among recipients of T-cell-replete allografts.

Conclusion

We found associations between the abundance of a group of bacteria in the intestinal flora and relapse/progression of disease after allo-HCT. These might serve as potential biomarkers or therapeutic targets to prevent relapse and improve survival after allo-HCT.

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INTRODUCTION

Greater than 18,000 patients undergo allogeneic hematopoietic-cell transplantation (allo-HCT) yearly with curative intent for hematologic neoplasms.¹ In recent years, major strides have been made in the reduction of its treatment-related toxicity.² Relapse of primary malignancy, however, remains the leading cause of death after allo-HCT.³

The efficacy of allo-HCT in the treatment of hematologic malignancies has been attributed to

both the cytotoxic effects of conditioning chemotherapy and the control or elimination of residual malignant cells through graft-versus-tumor (GVT) activity mediated by the donor immune system. Several risk factors of relapse after allo-HCT have been identified, including somatic genetic alterations, depth of response to pretransplantation treatment,⁴⁻⁶ conditioning intensity,⁷ graft source,^{8,9} mismatch at the human leukocyte antigen (HLA) –DPB1 locus,^{10,11} donor KIR haplotype,¹² graft-versus-host disease (GVHD),¹³ and GVHD prophylaxis modalities.¹⁴

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These factors, however, provide only a partial explanation of relapse risk.

The intestinal microbiota interact with and regulate host immunity.¹⁵ Several important outcomes after allo-HCT—in particular, bloodstream infections,¹⁶ GVHD,¹⁷⁻²² organ toxicity,²³ and overall survival²⁴—have been associated with intestinal bacterial composition. Recent studies have demonstrated that the intestinal microbiota modulate antitumor immunity.²⁵⁻³¹ Whether immune receptors that recognize bacteria can modulate the risk of relapse after allo-HCT is an unresolved question.³²

Because allo-immunity plays an important role in the prevention of posttransplantation relapse, we hypothesized that the composition of the intestinal microbiota might be associated with GVT activity. In this study, we retrospectively examined a single-center population for features of the intestinal microbiota composition that might be associated with post-allo-HCT relapse or progression of disease (POD).

METHODS

Patients and Samples

Stool samples were collected, and genomic 16S ribosomal sequencing was performed as described.^{16,18} The patients were admitted to our center for allo-HCT from 2009 to 2015 after they were prospectively enrolled in a fecal-collection protocol approved by the institutional review board. Patients with at least one sequenced sample collected between days 0 and 21 of a first allo-HCT were included.

The genomic 16S ribosomal RNA V4/V5 variable region was amplified and sequenced on the Illumina MiSeq (San Diego, CA) platform, as previously described.^{18,24} Quality-filtered sequences with > 97% identity were grouped into operational taxonomic units (OTUs).³³ Time-weighted average abundances (Data Supplement) were calculated for each OTU. Similar metrics of area-under-the-curve of concentration over time have been used in other biomarker time-series studies.^{34,35} A phylogenetic tree was constructed from a sequence alignment of all of the OTUs. The branch points (nodes) of the phylogenetic tree were considered to be clusters of related OTUs (crOTUs; Fig 1A; Data Supplement). The abundance of each crOTU was calculated as the sum of the abundances of its member OTUs. Additional details are in the Data Supplement.

Statistical Analysis

The primary outcome was time to relapse or time to POD by disease-specific criteria. Detection of minimal residual disease was scored as a relapse/POD event when flow cytometry, radiographic, or molecular results were acted upon clinically by initiation of therapy, infusion of donor lymphocytes, or withdrawal of immunosuppression. The 541-patient cohort was partitioned temporally into discovery (n = 271) and validation (n = 270) sets. Cause-specific Cox proportional hazards multivariable regression models were used to assess associations between microbiota and outcomes. The cumulative incidences of relapse/POD, transplant-related mortality, and GVHD were determined with the competing-risks method. Additional details are in the Data Supplement.

RESULTS

Patient Characteristics

Among patients who underwent transplantation at our center between 2009 and 2015, a cohort of 541 patients met the inclusion criteria and had at least one sample collected in the 3 weeks after

Fig 1. (A) Phylogenetic tree of operational taxonomic units (OTUs) and clusters of related OTUs (crOTUs). Each black point is a crOTU. Phylum is color coded along the circumference. A schematic of how the tree was constructed and a detailed tree are presented in the Data Supplement. (B) Volcano plot of multivariable *P* values of crOTUs against the multivariable hazard ratios for relapse/progression of disease (POD) in the discovery set. crOTUs are color coded by *P* value. Multivariable adjustment was performed for refined disease risk index, graft source, and conditioning intensity. The most abundant species in each of the labeled crOTUs are as follows: *Eubacterium limosum* in crOTU 1614; *Streptococcus sinensis* in crOTU 2022-3; *E. limosum* in crOTU 1638; *E. limosum* in crOTU 1630-1; *Parvimonas micra* in crOTU 1790; *Leptotrichia hongkongensis* in crOTU 0951-3; *Flavonifractor plautii* in crOTU 2986; and *Actinomyces odontolyticus* in crOTU 1439. OTU-level analysis and univariable analysis results are presented in the Data Supplement. crOTU composition at the species level and numeric values are tabulated for the top candidates and for all candidates in the Data Supplement.

allo-HCT (Data Supplement). This was a heterogeneous population with a variety of underlying primary hematologic malignancies (Table 1). The patients had a range of pretransplantation

Table 1. Baseline Characteristics of the Patients

Characteristic	No. (%) of Patients		
	Combined (N = 541)	Discovery (n = 271)	Validation (n = 270)
Disease			
AML*	195 (36.0)	97 (35.8)	98 (36.3)
MDS	85 (15.7)	42 (15.5)	43 (15.9)
NHL	68 (12.6)	42 (15.5)	26 (9.6)
Multiple myeloma	61 (11.3)	21 (7.7)	40 (14.8)
ALL†	44 (8.1)	24 (8.9)	20 (7.4)
T-cell malignancies	24 (4.4)	14 (5.2)	10 (3.7)
CLL	16 (3.0)	10 (3.7)	6 (2.2)
Hodgkin lymphoma	15 (2.8)	8 (3.0)	7 (2.6)
CML	12 (2.2)	7 (2.6)	5 (1.9)
MPN	12 (2.2)	2 (0.7)	10 (3.7)
CMML	8 (1.5)	3 (1.1)	5 (1.9)
MDS/MPN	1 (0.2)	1 (0.4)	0 (0.0)
Conditioning intensity			
Myeloablative	317 (58.6)	162 (59.8)	155 (57.4)
Reduced intensity	162 (29.9)	63 (23.2)	99 (36.7)
Nonmyeloablative	62 (11.5)	46 (17.0)	16 (5.9)
Graft source			
Unmodified PBSC/BM‡	172 (31.8)	83 (30.6)	89 (33.0)
Cord blood	95 (17.6)	54 (19.9)	41 (15.2)
T-cell depleted§	274 (50.6)	134 (49.4)	140 (51.9)
Donor			
Related	162 (29.9)	83 (30.6)	79 (29.3)
Unrelated	379 (70.1)	188 (69.4)	191 (70.7)
DRI			
Low	63 (11.6)	39 (14.4)	24 (8.9)
Intermediate	353 (65.2)	171 (63.1)	182 (67.4)
High	125 (23.1)	61 (22.5)	64 (23.7)
Age, years			
Mean (SD)	53.8 (12.1)	53.2 (11.8)	54.3 (12.5)
Range	19-75	19-75	21-75
Male sex	323 (59.7)	159 (58.7)	164 (60.7)
Mean (SD) follow-up time, months	21.5 (16.6)	28.8 (19.6)	14.2 (7.8)

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; CMML, chronic myelomonocytic leukemia; CR, complete remission; DRI, refined disease risk index; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm. NHL, non-Hodgkin lymphoma; PBSC, peripheral-blood stem cell; SD, standard deviation.

*AML disease stage: CR1 (n = 140); > CR1 (n = 26); refractory (n = 29).

†ALL disease stage: CR1 (n = 27); > CR1 (n = 16); refractory (n = 1).

‡Unmodified PBSC/BM: unmodified BM grafts (n = 18) and unmodified PBSC grafts (n = 154).

§T-cell depleted: T-cell depleted BM grafts (n = 6) and T-cell depleted PBSC grafts (n = 268).

relapse/POD risks, as assessed by the refined disease risk index (DRI), a validated disease-focused prognostic instrument.⁴ The conditioning regimens and types of allografts administered varied: 50.6% of the patients received T-cell–depleted grafts, 31.8% received unmodified grafts of peripheral-blood stem cells (PBSCs) or bone marrow (BM) stem cells, and 17.6% received double umbilical cord blood transplantations. Relapse/POD events occurred in 138 of the patients (incidence, 25.5%) during the 2-year period of analysis. Characteristics of these 138 patients are tabulated in the Data Supplement.

Microbiota Features

Genomic 16S ribosomal RNA sequences were obtained from 2,303 stool samples from 541 patients (mean, 4.3 per patient). Of these, 1,186 had been collected in the 3 weeks after stem-cell infusion (Data Supplement). According to a standard approach in microbiota studies, 16S sequences > 97% identical were grouped into bins known as OTUs.³³ The relative abundance of each OTU was determined for each sample. To accommodate variegated

collection time points and numbers of samples for each patient, the cumulative exposure of each patient to each OTU was calculated as a time-weighted average abundance during the 3-week period after stem-cell infusion (Data Supplement).

Intestinal microbial diversity, as assessed by the inverse Simpson index, was not associated with time to relapse/POD ($P = .16$; Data Supplement). This is in keeping with our prior observation of no association between diversity and relapse-related mortality.²⁴ Next, the association of abundance of particular bacterial subsets with time to relapse/POD was considered. We hypothesized that evolutionarily related species exhibit functional similarities. Therefore, OTUs were grouped by evolutionary distance by the construction of a phylogenetic tree empirically from a sequence alignment of all OTUs identified in the whole cohort. Members of the same phyla were grouped together (Fig 1A; Data Supplement), which indicated that the tree was broadly concordant with standard taxonomy. The branch points (nodes) of the tree were defined as clusters of related OTUs (crOTUs; Data Supplement). The crOTUs were then evaluated for associations with clinical outcomes.

Association of Microbiota With Time to Relapse/POD in the Discovery Set

The analysis was limited to taxa that met an abundance threshold of $> 0.01\%$ in $> 10\%$ of patients (Data Supplement). Associations between abundance and time to relapse/POD in the discovery set were evaluated for 208 OTUs and 1,343 crOTUs (Fig 1B; Data Supplement). Abundance was considered as a $\log_{10}$ -transformed continuous variable (Data Supplement). Multivariable models were adjusted for DRI, conditioning intensity, and graft source. The candidate most closely associated with relapse/POD risk in the discovery set was crOTU 1614 (multivariable hazard ratio [HR], 0.84; 95% CI, 0.73 to 0.96; $P = .01$). This HR can be interpreted as a 0.84-fold change (reduction) in risk of relapse/POD for every 10-fold increase in relative abundance of the crOTU. crOTU 1614 was composed mostly of *Eubacterium limosum* as well as other related species (Fig 1B; Data Supplement).

Association of Microbiota With Time To Relapse/POD in the Validation Set

The top candidate crOTU identified in the discovery set was evaluated for the reproducibility of its association with the outcome in the validation set. The abundance of crOTU 1614 was significantly associated with reduced risk of relapse/POD in the validation set (HR, 0.82; 95% CI, 0.71 to 0.95; $P = .009$; Table 2). This HR can be interpreted as a 0.82-fold change (reduction) in risk of relapse/POD for every 10-fold increase in relative abundance of the crOTU. This association remained significant after multivariable adjustment for conditioning intensity, graft source, and DRI (HR, 0.82; 95% CI, 0.70 to 0.96; $P = .01$; Table 2). Of note, the second-best candidate from the discovery set (crOTU 222; Fig 1B; Data Supplement) was

associated with an increased risk of relapse/POD in the discovery set but did not have any association with the outcome in the validation set (HR, 0.96; 95% CI, 0.8 to 1.16; $P = .66$).

We next stratified the patients in the discovery and validation sets according to presence or absence of crOTU 1614. Presence was defined as any detectable amount, as detailed in the Data Supplement. Intestinal presence of crOTU 1614 was associated with reduced risk of relapse/POD in both the discovery (HR, 0.49; 95% CI, 0.30 to 0.82; $P = .006$) and the validation (HR, 0.52; 95% CI, 0.31 to 0.87; $P = .01$) sets (Fig 2A). In the validation set, the 2-year cumulative incidence of relapse/POD in patients with intestinal presence of crOTU 1614 was 19.8%; in patients without crOTU 1614, it was 33.8%. This association remained significant after adjustment for DRI, graft source, and conditioning intensity in both the discovery set (HR, 0.46; 95% CI, 0.27 to 0.78; $P = .004$; Data Supplement) and the validation set (HR, 0.54; 95% CI, 0.31 to 0.92; $P = .03$; Table 2).

Discriminatory Ability of the Biomarker Relative to Clinical Predictors: Combined Cohorts

After an association of crOTU 1614 with a lower risk of relapse/POD was reproducibly observed, the discovery and validation sets were combined for additional exploratory analyses. crOTU 1614 was present in 422 (78%) of the 541 patients in the combined cohort; the mean abundance was 0.16%, and the maximum abundance was 8%. A progressively lower risk of relapse/POD was observed across the combined cohort when it was stratified into four abundance bins ($P = .001$; Figs 2B and 2C), and this association remained significant after multivariable adjustment ($P = .004$; Data Supplement).

Table 2. Univariable and Multivariable Associations of crOTU 1614 With the Risk of Relapse/POD After Allo-HCT

Validation cohort (N = 270)	Univariable		Multivariable I (crOTU log transformed)		Multivariable II (crOTU present v absent)		Multivariable III (crOTU abundance bins)	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
crOTU 1614, log transformed	0.82 (0.71 to 0.95)	.009	0.82 (0.7 to 0.96)	.01				
OTU 1614, present	0.52 (0.31 to 0.87)	.01			0.54 (0.31 to 0.92)	.03		
crOTU 1614, abundance bins		.05						.09
Absent	(reference)						(reference)	
Present, tertile 1	0.68 (0.37 to 1.25)						0.67 (0.36 to 1.26)	
Present, tertile 2	0.43 (0.21 to 0.89)						0.48 (0.23 to 1.01)	
Present, tertile 3	0.43 (0.2 to 0.91)						0.4 (0.18 to 0.88)	
Intensity		.79		.71		.72		.66
Myeloablative	(reference)		(reference)		(reference)		(reference)	
Reduced intensity	0.87 (0.51 to 1.49)		1.36 (0.61 to 3.04)		1.33 (0.6 to 2.99)		1.41 (0.63 to 3.18)	
Nonmyeloablative	0.72 (0.22 to 2.34)		1.03 (0.25 to 4.24)		0.95 (0.23 to 3.87)		1.02 (0.25 to 4.21)	
Source		.10		.03		.03		.03
Unmodified PBSC/BM	(reference)		(reference)		(reference)		(reference)	
Cord blood	0.32 (0.11 to 0.91)		0.23 (0.07 to 0.69)		0.24 (0.08 to 0.72)		0.23 (0.07 to 0.69)	
T-cell depleted	0.79 (0.47 to 1.32)		0.76 (0.37 to 1.56)		0.75 (0.37 to 1.56)		0.76 (0.37 to 1.58)	
DRI		.03		.07		.08		.09
Low	0.73 (0.26 to 2.04)		0.76 (0.24 to 2.36)		0.7 (0.23 to 2.16)		0.79 (0.25 to 2.49)	
Intermediate	(reference)		(reference)		(reference)		(reference)	
High	1.91 (1.13 to 3.25)		1.81 (1.06 to 3.11)		1.78 (1.04 to 3.05)		1.79 (1.04 to 3.08)	

NOTE. Cox proportional hazard regression in the validation cohort. crOTU 1614 is considered in three ways: as a log-transformed continuous variable (multivariable I), as a binary variable of present versus absent (multivariable II), or as an ordered categorical variable of abundance bins (multivariable III). Patients who had very-high-risk DRI were grouped together with patients who were high-risk DRI. The multivariable analyses of the discovery and combined cohorts are in the Data Supplement.

Abbreviations: BM, bone marrow; crOTU, clusters of related OTUs; HCT, hematopoietic-cell transplantation; HR, hazard ratio; PBSC, peripheral-blood stem cell; OTU, operational taxonomic unit; POD, progression of disease.

Fig 2. Cluster of related operational taxonomic units (crOTU) 1614, which includes members of the family *Eubacteriaceae*, is associated with decreased relapse/progression of disease (POD) after allogeneic hematopoietic-cell transplantation (allo-HCT). (A) Cumulative incidence of relapse/POD in the discovery (n = 271) and validation (n = 270) sets stratified by presence or absence of crOTU 1614. (B) Cumulative incidence of relapse/POD in the whole cohort (N = 541) stratified by crOTU 1614 abundance. (C) Upper panel, mean abundance of crOTU 1614 in the four strata (error bars are standard error of the mean) and, lower panel, cumulative incidence of relapse/POD at 2 years in the four strata (error bars are 95% CIs). (D) Far left: refined disease risk index (DRI) alone stratifies the relapse/POD risk in this cohort. Three right panels: crOTU 1614 presence further stratifies relapse among patients with high-risk and intermediate-risk DRIs. Patients with very-high-risk DRIs were grouped together with those who had high-risk DRIs. HR, hazard ratio.

Examination of the clinical features of patients according to presence or absence of crOTU 1614 (Data Supplement) demonstrated no significant differences in disease type, conditioning intensity, or graft source. There was a moderate preponderance of

higher-risk DRI scores in patients who had an absence of intestinal crOTU 1614 ($P = .02$; Data Supplement), but the association remained significant when the analysis was adjusted for DRI in a multivariable model (HR, 0.54; 95% CI, 0.38 to 0.78; $P < .001$;

Data Supplement). Patients who had a presence of crOTU 1614 were slightly older (mean age, 54.4 years ν 51.7 years; $P = .04$). We also investigated the association between crOTU 1614 and relapse/POD risk within each of the three DRI categories. Intestinal presence of crOTU 1614 was associated with less relapse/POD risk among patients with high DRIs (HR, 0.45; 95% CI, 0.25 to 0.81; $P = .008$) and intermediate DRIs (HR, 0.52; 95% CI, 0.33 to 0.83; $P = .006$; Fig 2D). There was no association, however, between crOTU 1614 presence and relapse/POD risk in patients with low DRIs (HR, 2.16; 95% CI, 0.28 to 16.95; $P = .46$).

To evaluate the discriminatory ability of crOTU 1614 in relation to known clinical risk factors of relapse/POD, we used a concordance index (c-index), in which a value closer to 1 indicates greater accuracy (Data Supplement).³⁶ The discriminatory ability of crOTU 1614 (c-index, 0.572) was comparable to that of the DRI (c-index, 0.569). The combination of crOTU 1614, DRI, graft source, and conditioning intensity produced a moderately stronger discriminatory power (c-index, 0.650) than the three clinical factors alone (c-index, 0.619). This degree of predictive power is comparable to established models for other outcomes after allo-HCT³⁷ and indicates that an intestinal microbiota biomarker can add to currently known clinical risk assessments of relapse/POD.

Other Clinical Outcomes and Subset Analyses: Combined Cohorts

Intestinal presence of crOTU 1614 was associated with an increase in overall survival (HR, 0.65; 95% CI, 0.47 to 0.90; $P = .008$; Fig 3A). The crOTU was not significantly associated with acute GVHD (grades 2 to 4; HR, 0.81; 95% CI, 0.56 to 1.17; $P = .27$) or with transplantation-related mortality (HR, 1.0; 95% CI, 0.63 to 1.59; $P = .99$; Fig 3A). In light of the heterogeneity of the population under study, we explored the association of crOTU 1614 with relapse/POD in patient subsets according to graft source, conditioning intensity, extent of HLA match, DRI, and disease type (Fig 4). With respect to disease type, the association of crOTU 1614 with a reduced risk of relapse/POD was significant among patients with acute myeloid leukemia (HR, 0.56; 95% CI, 0.32 to 0.96; $P = .04$) and multiple myeloma (HR, 0.29; 95% CI, 0.12 to 0.67; $P = .004$) and not statistically significant for other disease types. For graft source (Fig 4; Data Supplement), the association of crOTU 1614 with reduced risk of relapse/POD was significant in recipients of T-cell-replete grafts (HR, 0.40; 95% CI, 0.24 to 0.65; $P < .001$), particularly among recipients of unmodified PBSC/BM grafts (HR, 0.40; 95% CI, 0.23 to 0.69; $P = .001$). No significant association was observed in recipients of T-cell-depleted grafts (HR, 0.66; 95% CI, 0.39 to 1.11; $P = .12$) or in recipients of cord-blood grafts (HR, 0.38; 95% CI, 0.11 to 1.32; $P = .13$; Data Supplement).

Biomarker Performance in Pretransplantation Samples

A biomarker of relapse risk might have utility if it could be assessed prior to transplantation while treatment decisions are being made. Among the 172 recipients of unmodified PBSC/BM (T-cell-replete) grafts in whom the association of crOTU 1614 was most clear, 143 patients (83%) also had a sample collected in the 3 weeks before stem-cell infusion. These patients were stratified into four bins on the basis of the abundance of crOTU 1614 in

a single stool sample collected during the 3 weeks before allo-HCT. The patients in the highest abundance bin had a lower risk of relapse/POD than the combined three lower-abundance groups (HR, 0.28; 95% CI, 0.10 to 0.80; $P = .02$; Fig 3B). Thus, intestinal presence of crOTU 1614 either before or after allo-HCT can be used as a biomarker of posttransplantation relapse/POD risk.

Bacterial Composition of the Biomarker

The composition of crOTU 1614 (Data Supplement) includes 30 OTUs, of which 5 OTUs, 7 OTUs, and a single OTU were identified as, respectively, *E. limosum*, *Anaerofustis stercorihominis*, and *Pseudoramibacter alactolyticus*. These are all members of the family *Eubacteriaceae*. An additional 15 OTUs were identified as *Peptococcus niger*, a member of the related family *Peptococcaceae*. In the whole cohort, the majority (67%) of the abundance of crOTU 1614 was attributable to *E. limosum*, with lesser contributions from *A. stercorihominis* and *P. niger* (15% each; Data Supplement). The OTU sequences that define the composition of this cluster are presented in FASTA format (Data Supplement). Thus, in this cohort of allo-HCT recipients, a cluster of species predominantly comprising *E. limosum* and other related bacteria was associated with a decreased risk of relapse/POD.

DISCUSSION

In this retrospective, observational, single-center study, the intestinal microbiota composition of patients who underwent allo-HCT was analyzed. To our knowledge, this is the largest cohort of allo-HCT patients assembled for this type of analysis to date. A discovery-validation approach was used to identify an association (Fig 2) between the abundance of a particular subset of intestinal bacteria and a decreased risk of relapse/POD after allo-HCT. In contrast, the overall diversity of the intestinal microbiota was not associated with relapse/POD. (Data Supplement). This is consistent with our previous observation in a much smaller cohort that diversity of the intestinal flora measured at the time of posttransplantation neutrophil engraftment was not associated with relapse-related mortality.²⁴

The cohorts in which this biomarker was identified and validated were heterogeneous with respect to graft source and disease type. In an exploratory subset analysis, the association of the microbial biomarker with a lower risk of relapse/POD was strongest among recipients of grafts containing T cells and other mature lymphocytes (Fig 4). In these patients, there is a greater role for donor-cell-mediated GVT activity compared with recipients of T-cell-depleted grafts, which suggests that the composition of the intestinal microbiota could modulate GVT activity. This interpretation is interesting in light of an emerging appreciation of interactions between the intestinal microbiota and systemic immunity,³⁸ which include antitumor immune reactions.²⁵⁻³¹

E. limosum is the most abundant species in this group of bacteria identified as a biomarker of GVT. This bacterium is an anaerobic, non-spore-forming Gram-positive rod that is a common member of the human intestinal microbiota.³⁹ It can ameliorate an experimental model of colitis, perhaps through production of immunomodulatory short-chain fatty acids,⁴⁰ and it

Fig 3. (A) Presence of cluster of related operational taxonomic units (crOTUs) 1614 in the 3 weeks after allogeneic hematopoietic-cell transplantation (allo-HCT) is associated with increased overall survival and decreased cumulative incidence of relapse/progression of disease (POD), but it is not associated with cumulative incidence of acute grades 2 to 4 graft-versus-host disease (GVHD) or with transplantation-related mortality (TRM). (*)Seventeen patients developed grades 2 to 4 GVHD before landmark day 21 and were excluded from this panel. (B) Among 143 recipients of unmodified peripheral-blood stem-cell (PBSC)/bone marrow (BM) stem-cell grafts with available pretransplantation microbiota data, those with the highest abundance of crOTU 1614 in a single sample collected before allo-HCT had a reduced risk of relapse/POD ($P = .02$; for all four groups $P = .10$). The pretransplantation analysis for recipients of all graft types is plotted in the Data Supplement. HR, hazard ratio.

has been reported to characterize a microbiota signature prevalent in centenarians.⁴¹

Two aspects of the methodologic approach were particularly helpful in this analysis. First, consideration of cumulative microbial exposures as time-weighted averages of abundance allowed inclusion of many more samples per patient and potentially reduced biases that could have occurred by sampling only single time points. This is particularly relevant in the setting of the rapid shifts in microbiota composition that can occur in the weeks after allo-HCT.^{16,18}

Another useful technique was the empirical derivation of crOTUs from the branch points (nodes) of a phylogenetic tree (Fig 1A; Data Supplement). One limitation of a traditional OTU-level analysis is that the association strength of a single species can be distributed among multiple OTUs. Conversely, in an analysis of higher taxonomic levels, such as genus or family, potential associations may be lost when dozens or hundreds of OTUs are grouped together. The approach used in this study overcame these issues by combining the abundances of evolutionarily related OTUs, as determined by 16S sequence similarity.

Fig 4. Association of the presence of cluster of related operational taxonomic units (crOTU) 1614 with relapse/progression of disease (POD) in patient subsets according to graft source, conditioning intensity, degree of HLA match, refined disease index (DRI), and disease type. The size of the gold box is proportional to number of patients in the subgroup. Cumulative incidence curves by graft source are plotted in the Data Supplement. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow stem cells; CLL, chronic lymphocytic leukemia; HR, hazard ratio; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; NHL, non-Hodgkin lymphoma; PBSC, peripheral-blood stem cells.

Most of the bacteria in the crOTU biomarker identified in this study are members of the family *Eubacteriaceae* (Data Supplement). There are, however, an additional 15 species in the data set that belong to the family *Eubacteriaceae* that are not part of crOTU 1614. In fact, when standard taxonomic classifications were evaluated, neither the family *Eubacteriaceae* as a whole, nor the genus *Eubacterium*, had a significant association with relapse/POD (data not shown). Of note, the main OTU that represented *E. limosum* was itself associated with

reduced risk of relapse (Data Supplement). This illustrates the utility of this approach in the study of associations between microbiota and clinical outcomes. Moreover, it is in line with an increasing appreciation of 16S rRNA sequence similarity as a better measure of evolutionary proximity than phenotypic traits.⁴²

Limitations of this study include its retrospective nature and the collection of samples from patients at a single center. Prospective and multicenter approaches in strictly defined patient

populations would be important next steps to confirm this association between microbiota and relapse after allo-HCT. In this study, a feature of the microbiota was identified as a potential biomarker; whether components of the microbiota have the potential to become modifiable risk factors for relapse after allo-HCT remains to be seen. With a detailed understanding of how the microbiota and the immune system interact, microbiota-based strategies⁴³ might be designed to improve patient outcomes after allo-HCT.

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Disclosures provided by the authors are available with this article at jco.org.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Intestinal Microbiota and Relapse After Hematopoietic-Cell Transplantation

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