GUT MICROBIOTA DIVERSITY BEFORE ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION AS PREDICTOR OF MORTALITY IN CHILDREN

Supplemental Material

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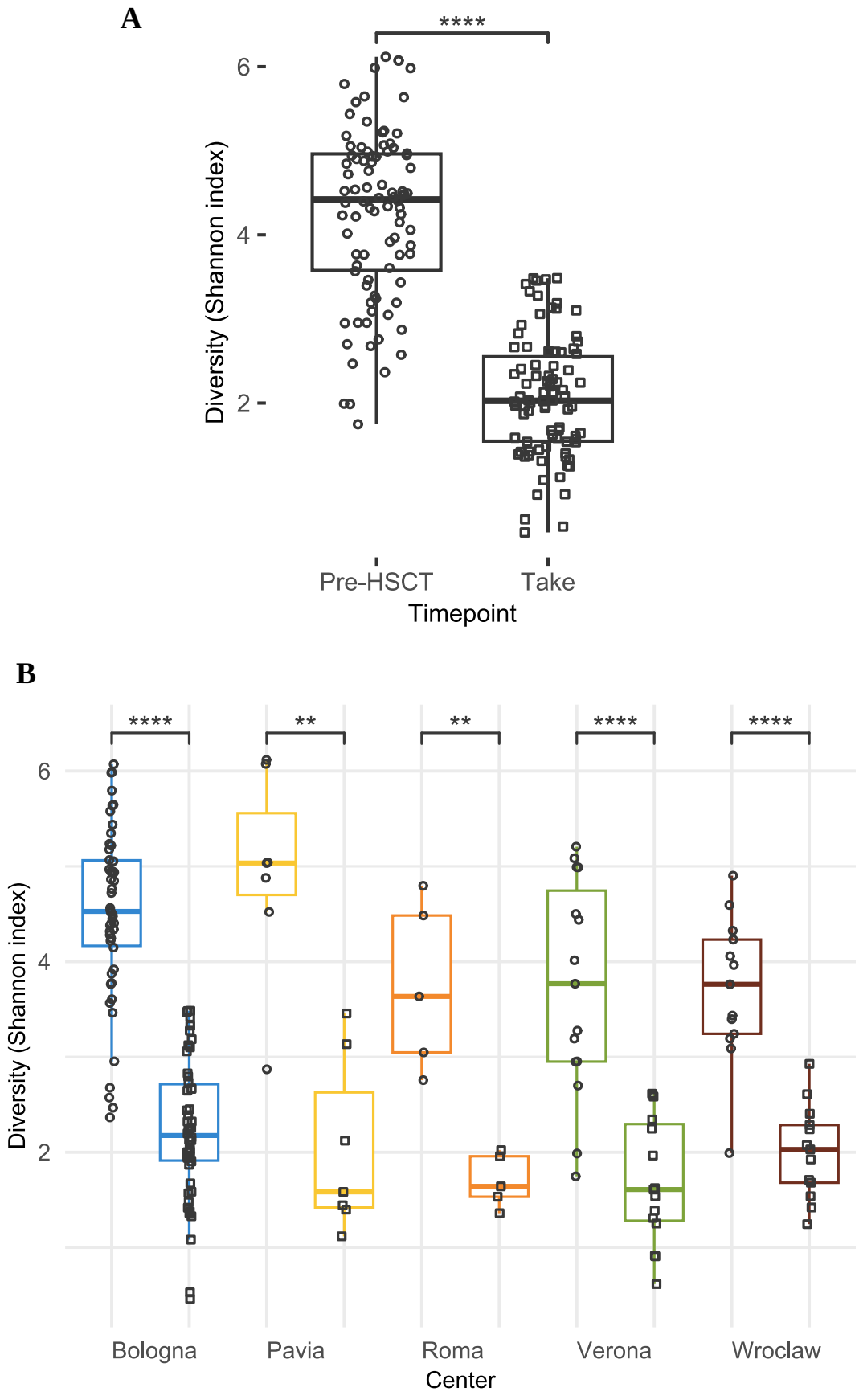
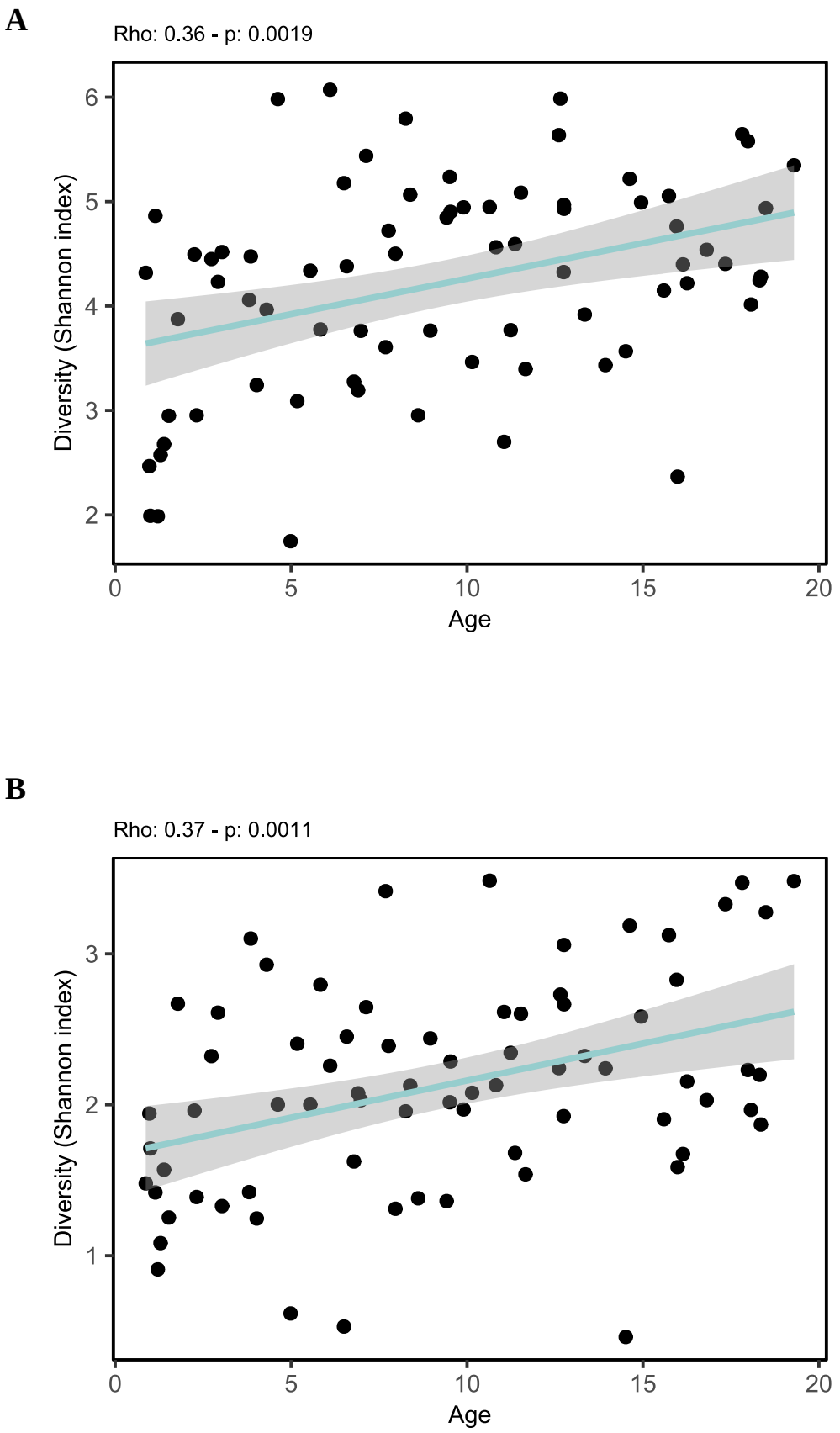
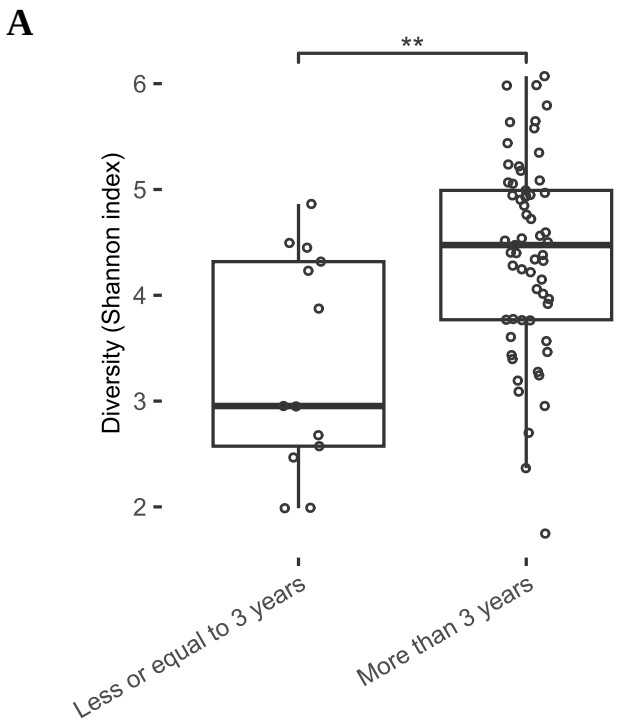
Supplemental Figure 1. Gut microbiota diversity before allo-HSCT and at neutrophil engraftment (take). Panel **A** shows data for the overall cohort and panel **B** for each participating center. Comparisons were performed with FDR-corrected Wilcoxon rank-sum tests and only significant comparisons are reported: ****, $p < 0.0001$; **, $p \leq 0.01$.

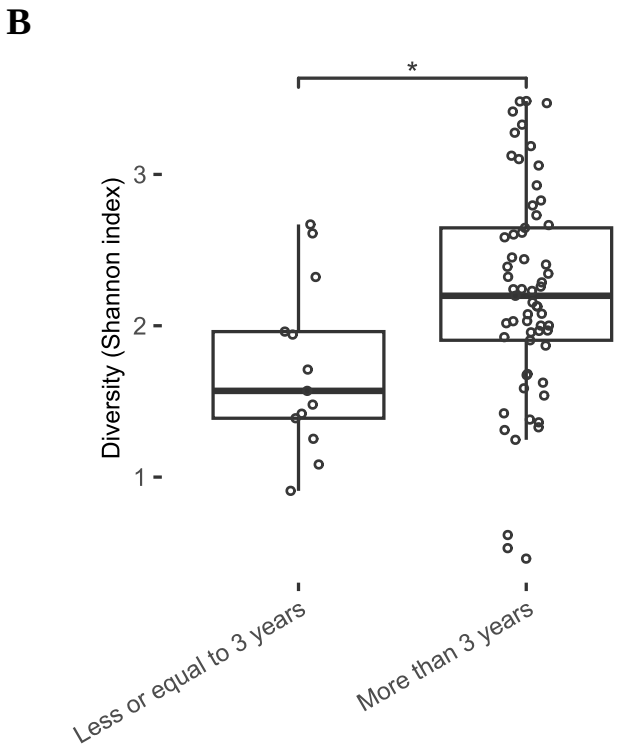


Supplemental Figure 2. Distribution of gut microbiota diversity by patient age (in years). Panel **A** shows data for samples collected before allo-HSCT and panel **B** for those collected at neutrophil engraftment.

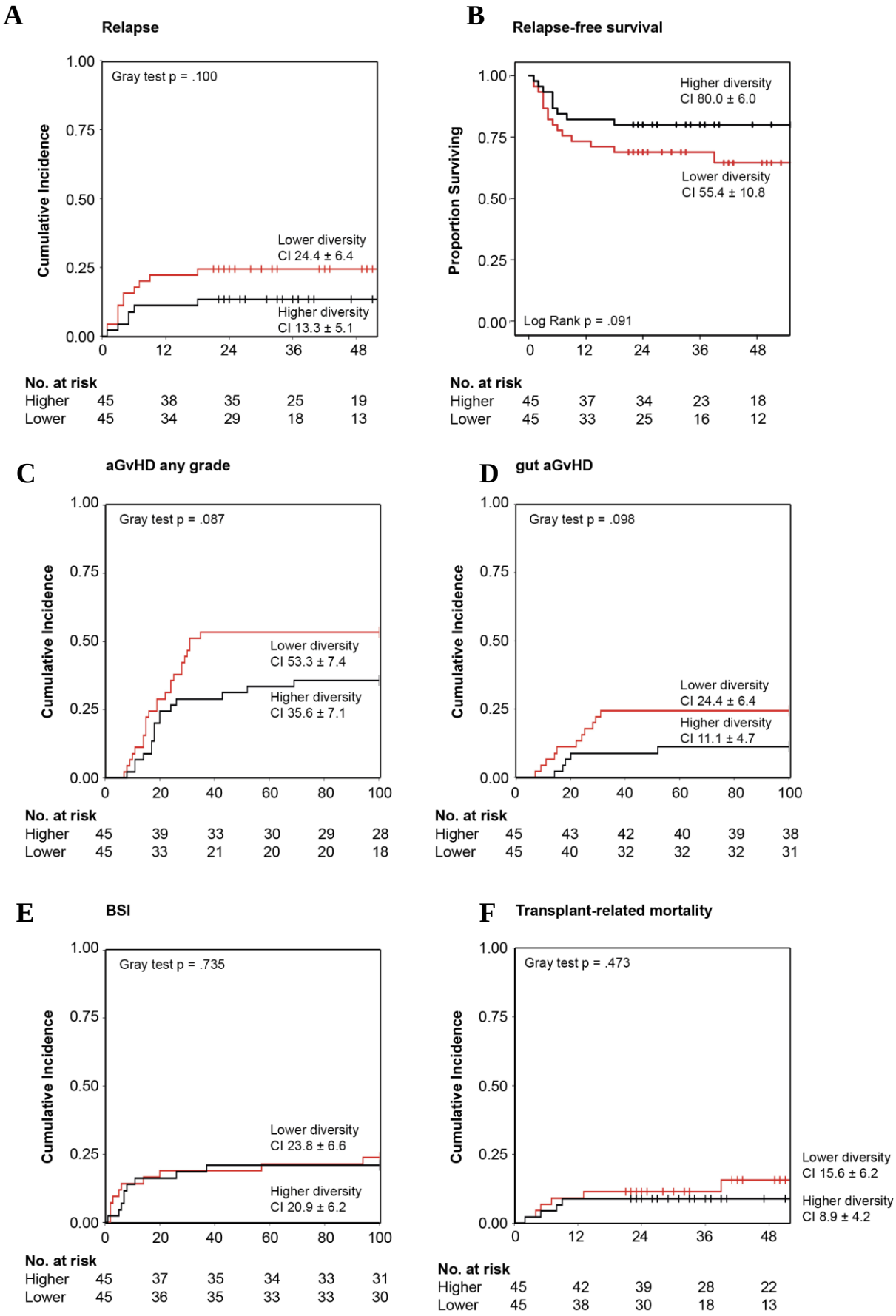


Supplemental Figure 3. Gut microbiota diversity in allo-HSCT patients aged less than (or equal to) or over three years. Panel **A** shows data for samples collected before allo-HSCT and panel **B** for those collected at neutrophil engraftment. Comparisons were performed with Wilcoxon rank-sum tests: **, $p = 0.0021$; * $p = 0.013$.

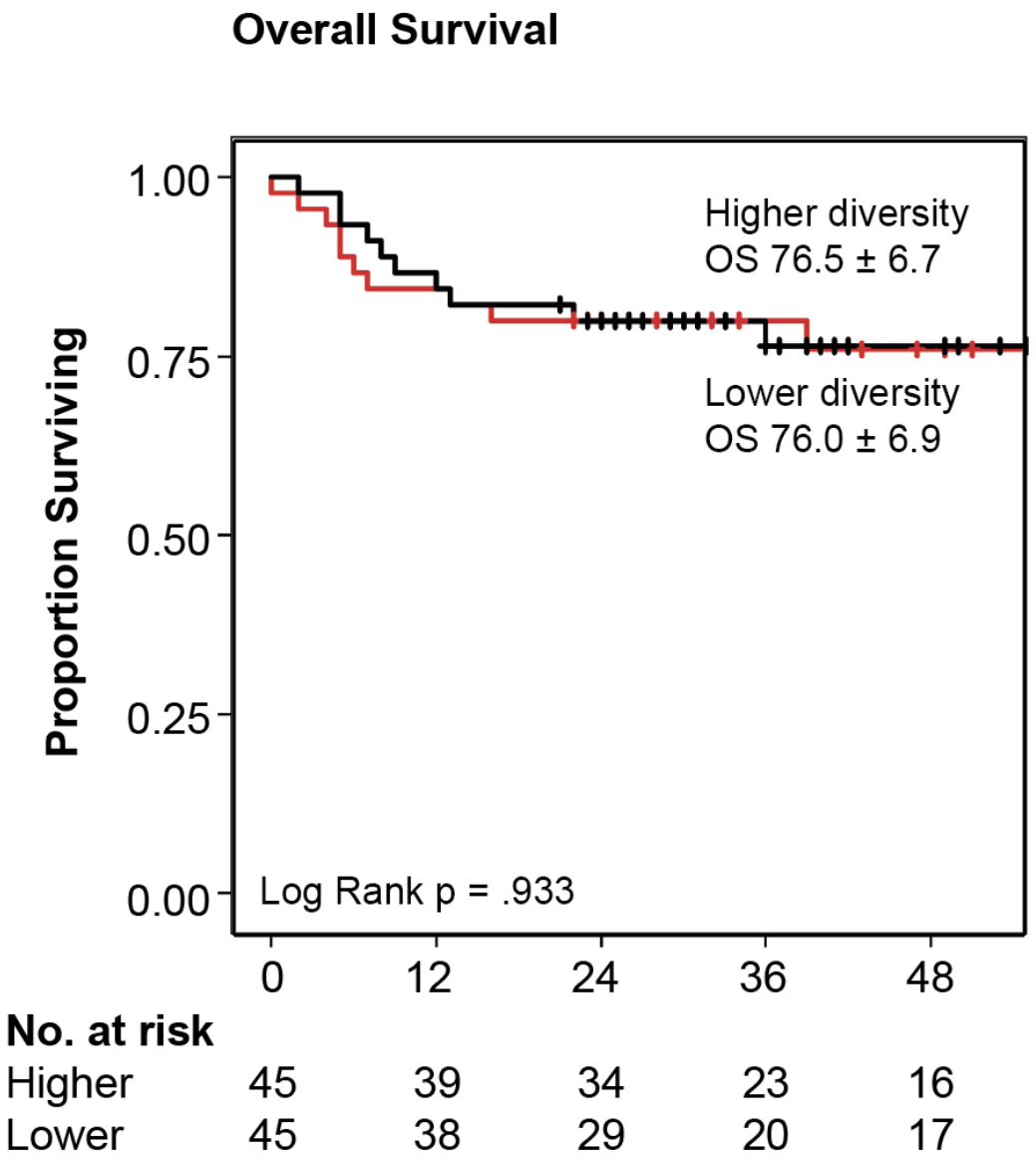




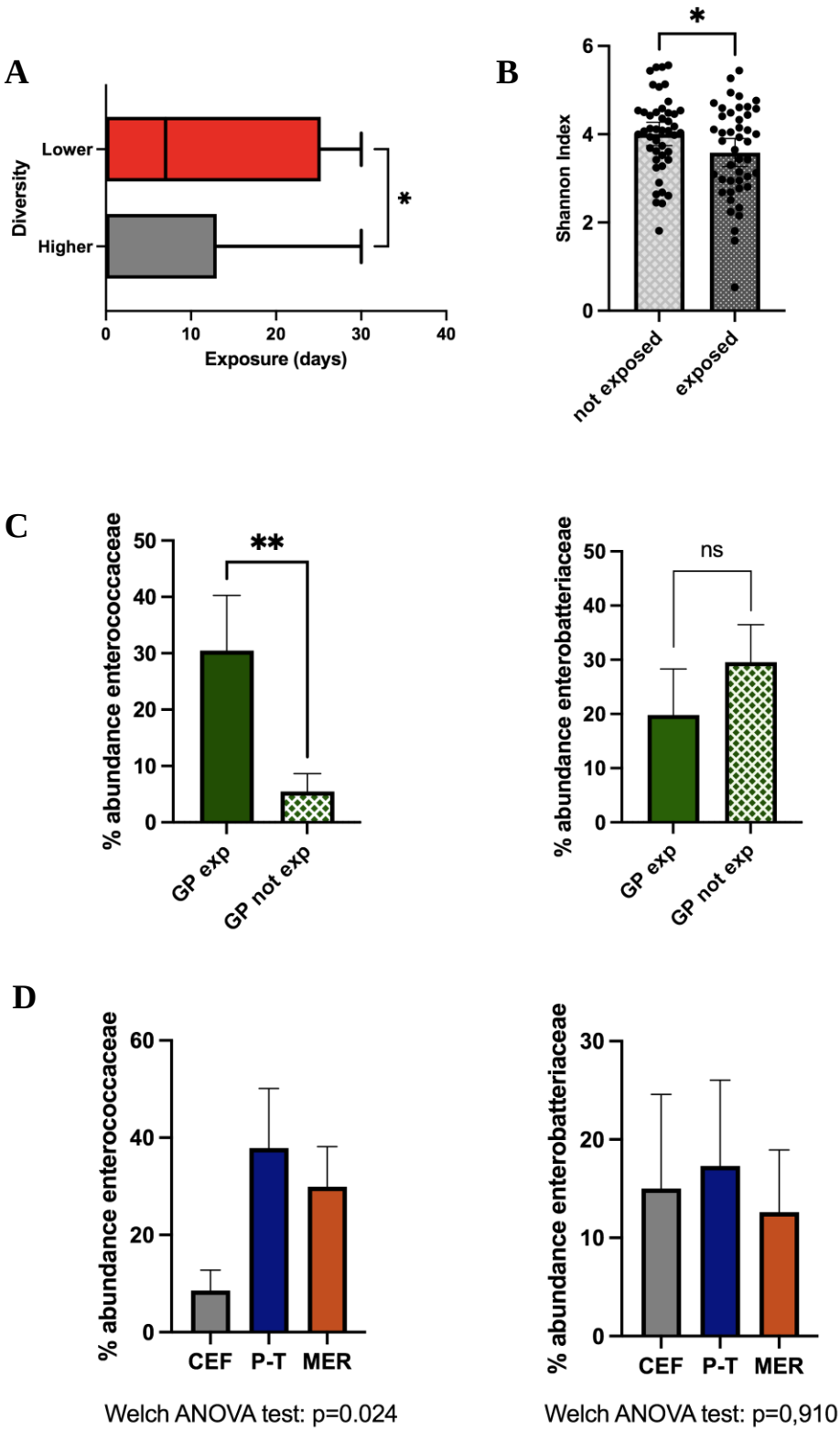
Supplemental Figure 4. Association between pre-allo-HSCT gut microbiota diversity and clinical outcomes. Cumulative incidence in the higher- and lower-diversity groups before transplantation for relapse (**A**), any grade acute graft-versus-host disease (aGvHD, **C**), gut aGvHD (**D**), bloodstream infection (BSI, **E**) and transplant-related mortality (**F**). Kaplan-Meier estimate for relapse-free survival for the two diversity groups (**B**).



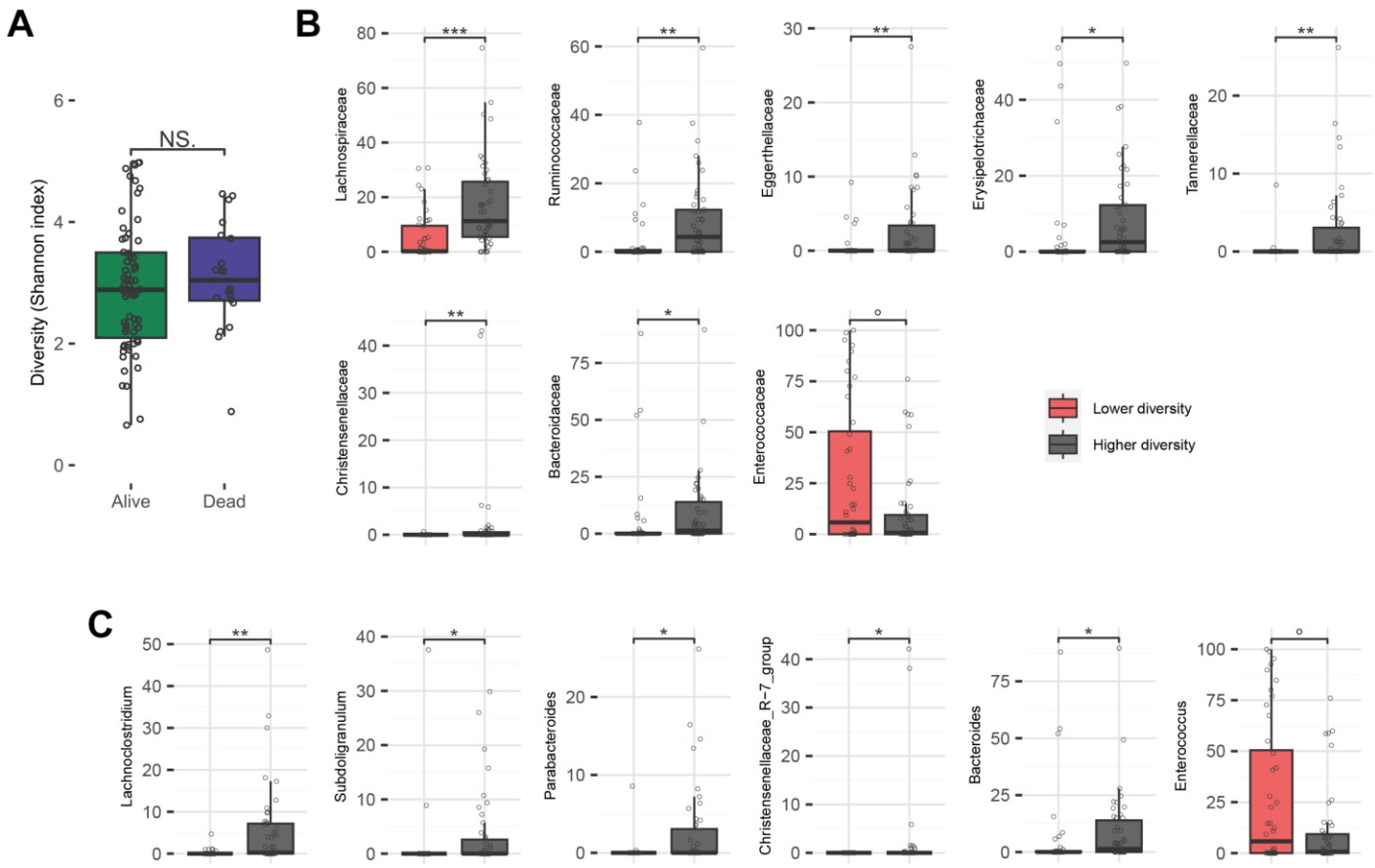
Supplemental Figure 5. Kaplan-Meier estimate for overall survival for the higher- and lower-diversity groups based on gut microbiota diversity at neutrophil engraftment.



Supplemental Figure 6. Impact of antibiotic administration before allo-HSCT over gut microbiota diversity and composition. Antibiotic exposure duration (in days) prior to allo-HSCT in the higher- and lower-diversity groups (**A**); Pre-allo-HSCT diversity values in patients exposed or not to antibiotic therapy (**B**); relative abundance of *Enterococcaceae* and *Enterobacteriaceae* at engraftment time-point in patients exposed or not glycopeptides (GP, **C**) and other classes of antibiotics (ceftazidime or cefepime - CEF, piperacillin-tazobactam – P-T, and meropenem – MER, **D**) during the pre-engraftment period *(i.e.*, from HSCT to engraftment). Student T-test/Mann-Whitney test: **, $p < 0.01$; *, $p < 0.05$.

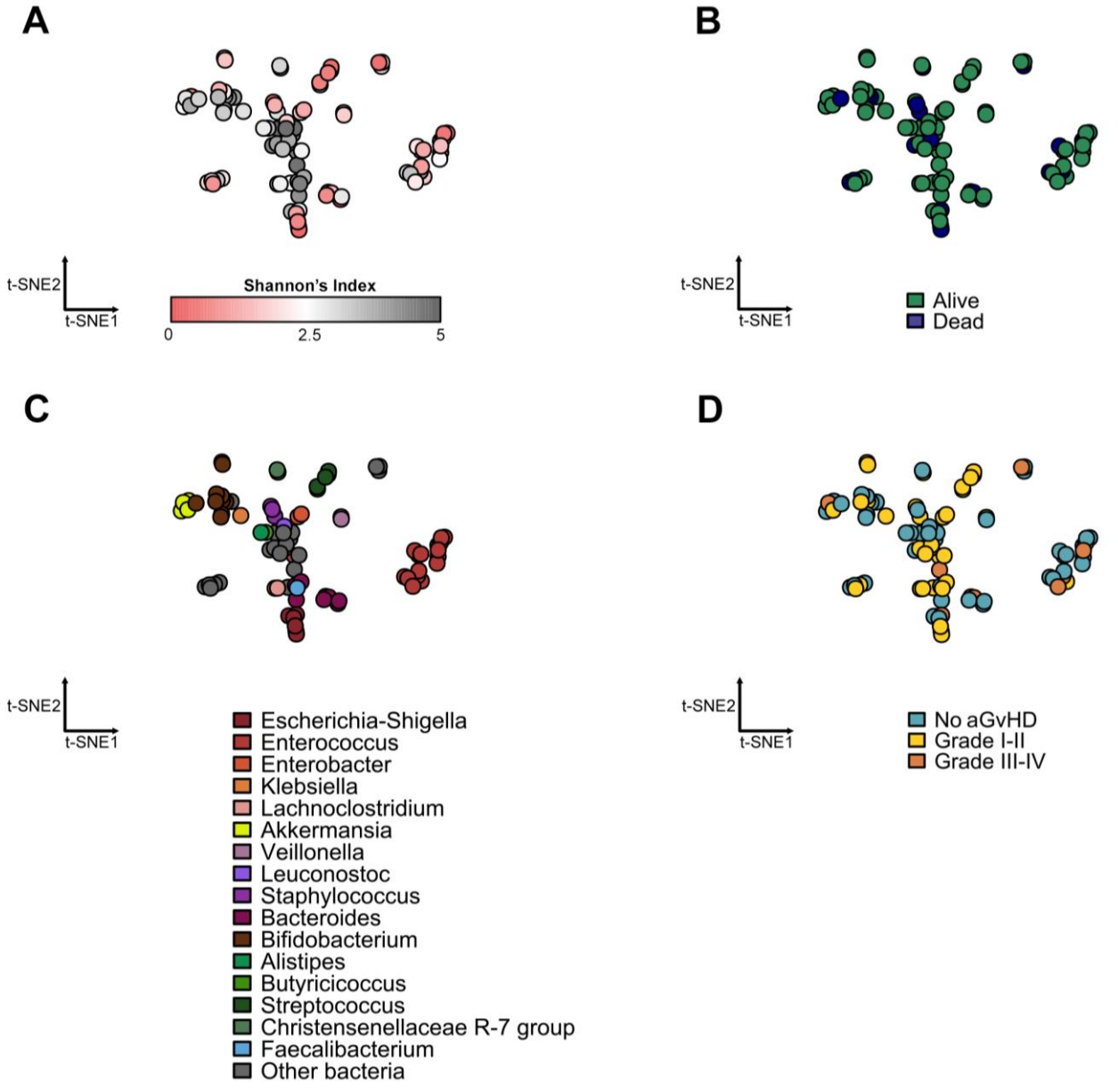


Supplemental Figure 7. Gut microbiota diversity and composition at neutrophil engraftment. (**A**) Boxplots showing the distribution of alpha diversity estimated with the Shannon index according to patient survival outcome (alive vs dead). Significant differences in the gut microbiota composition at the family (**B**) and genus (**C**) level between the higher- and lower-diversity groups. FDR-corrected Wilcoxon rank-sum tests. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; °, $p < 0.1$ (considered as a trend); NS, not significant ($p \geq 0.1$).

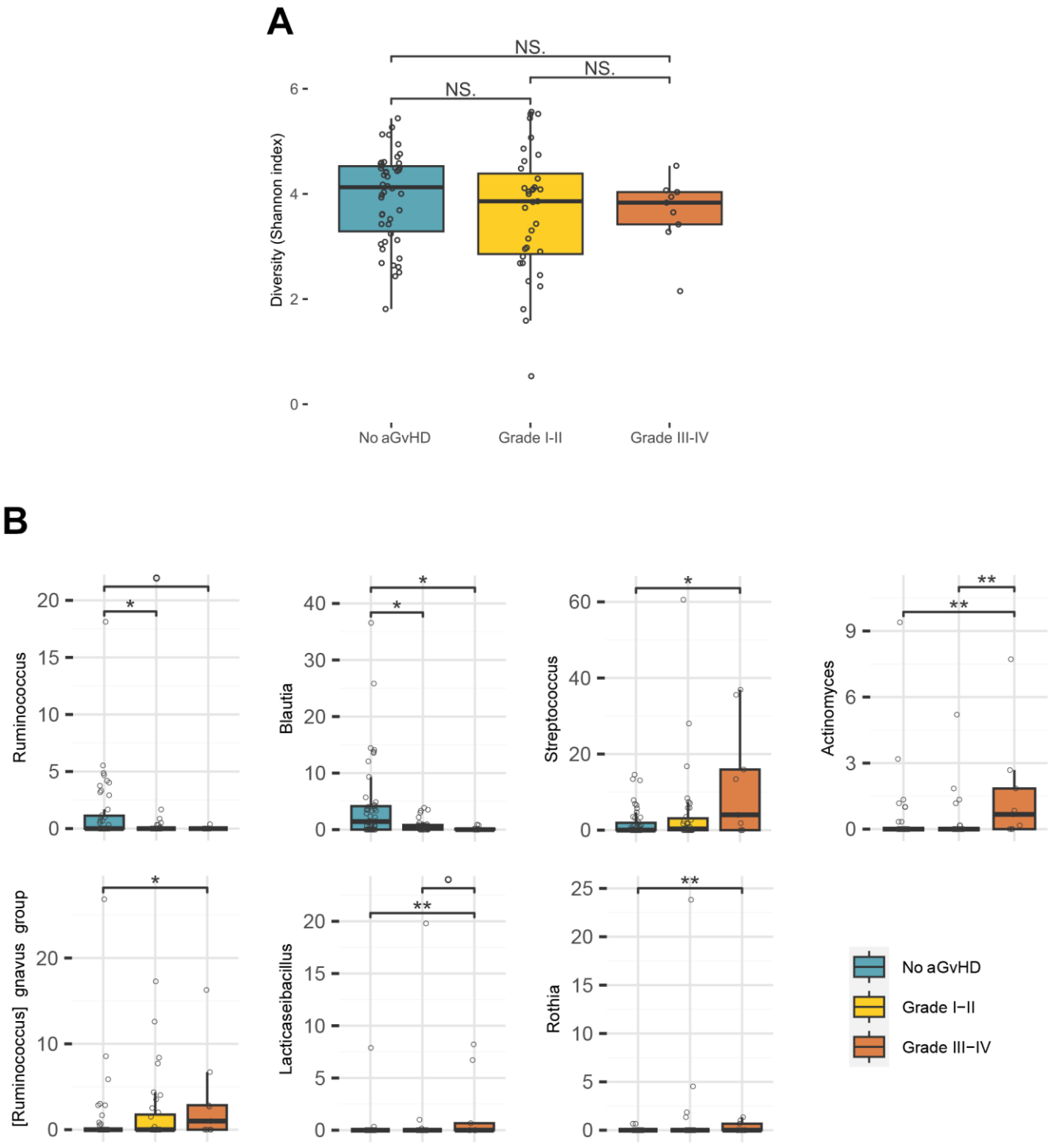


Supplemental Figure 8. Differences in gut microbiota at neutrophil engraftment represented using t-SNE plots.

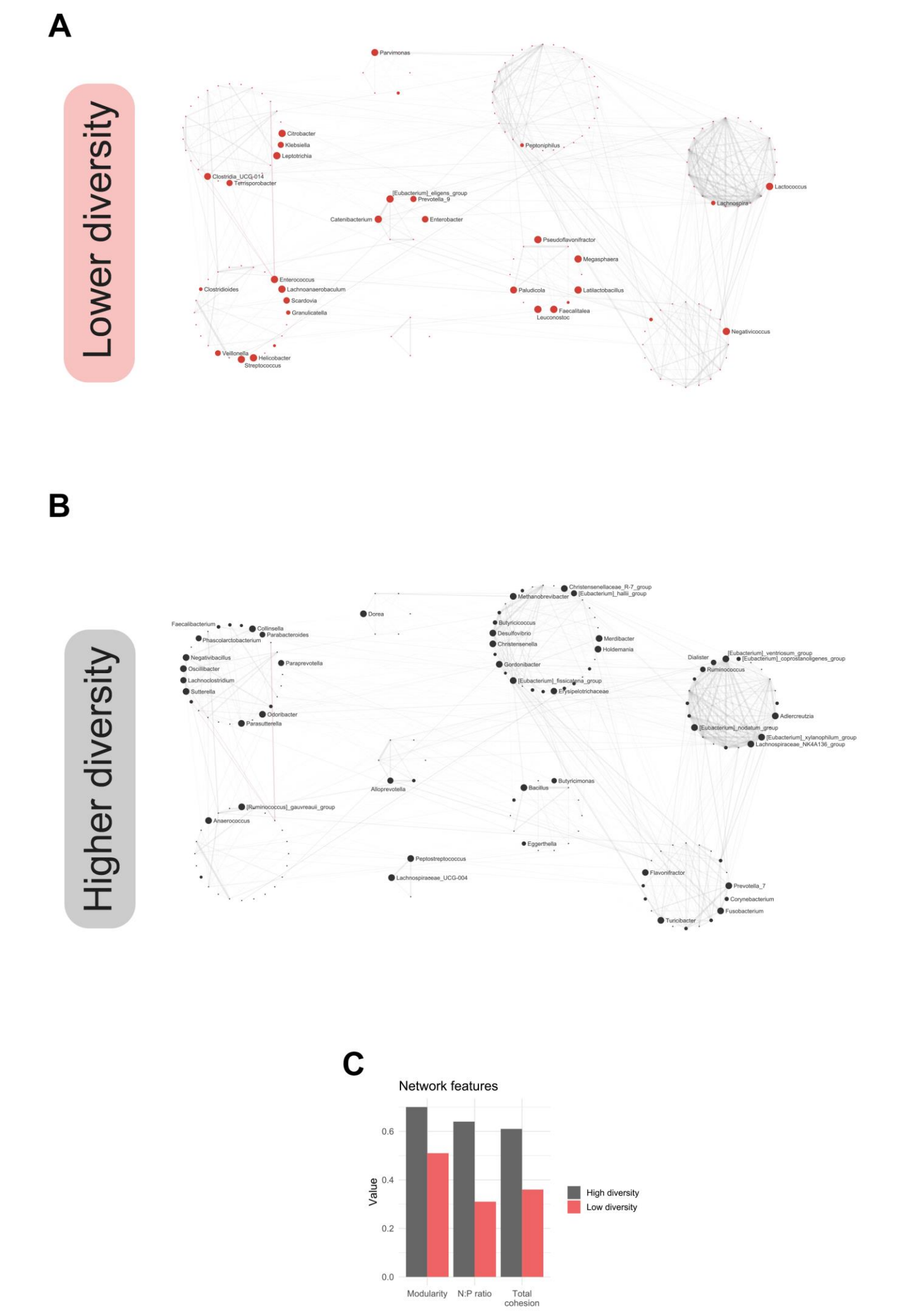
The gut microbiota composition at the genus level of the 90 patients at neutrophil engraftment is represented according to the t-distributed stochastic neighbor embedding (t-SNE) algorithm. The more similar the samples are in microbiota composition, the closer they appear on the t-SNE plot. Higher-diversity samples (**A**) congregate in the center, but do not co-localize with favorable survival outcome (**B**). The upper and lower part of the t-SNE space is mostly composed of lower-diversity samples, which show higher presence of *Escherichia-Shigella*, *Enterococcus*, and *Streptococcus* when color-coded for the most abundant taxon detected (**C**). No evident trends can be appreciated for the onset of acute graft-versus-host disease (aGvHD) (**D**).



Supplemental Figure 9. Pre-allo-HSCT gut microbiota signatures related to aGvHD onset. Boxplots showing the distribution of alpha diversity estimated with the Shannon index in patients not developing aGvHD vs those developing grade I-II or grade III-IV aGvHD (**A**). Significant differences in the gut microbiota composition related to aGvHD (**B**). FDR-corrected Wilcoxon rank-sum tests. **, $p < 0.01$; *, $p < 0.05$; °, $p < 0.1$ (considered as a trend); NS, not significant ($p \geq 0.1$).



Supplemental Figure 10. Gut microbiota networks of the higher- and lower-diversity groups at neutrophil engraftment. (**A**) Correlation networks of interactions reconstructed from genus-level compositional data in the lower- and higher-diversity groups at neutrophil engraftment. Violet lines represent negative interactions, whilst solid grey lines stand for positive ones. Node size is proportional to the overabundance value of the corresponding genus in the gut microbiota configuration of the diversity group considered. Only nodes corresponding to genera showing overabundance ≥1.4 were displayed. Labels were displayed only for genera showing overabundance of at least 1.5. Nine different modules were detected according to a statistical mechanics spin-glass model and simulated annealing. (**B**) Values of computed network features (*i.e.*, modularity, ratio of negative to positive cohesions – N:P ratio, and total cohesion).


A
Lower diversity
Parvimonas
Citrobacter
Klebsiella
Leptotrichia
Clostridia_UCG-014
Terrisporobacter
Peptoniphilus
Lactococcus
Lachnospira
[Eubacterium]_eligens_group
Prevotella_9
Catenibacterium
Enterobacter
Pseudoflavonifractor
Megasphaera
Enterococcus
Clostridioides
Lachnoanaerobaculum
Scardovia
Granulicatella
Paludicola
Latilactobacillus
Faecalitalea
Leuconostoc
Negativicoccus
Veillonella
Helicobacter
Streptococcus
B
Higher diversity
Christensenellaceae_R-7_group
Methanobrevibacter
[Eubacterium]_hallii_group
Dorea
Faecalibacterium
Collinsella
Parabacteroides
Phascolarctobacterium
Butyricicoccus
Desulfovibrio
Christensenella
Merdibacter
Holdemania
Negativibacillus
Oscillibacter
Lachnoclostridium
Sutterella
Paraprevotella
Gordonibacter
[Eubacterium]_fissicatena_group
Erysipelotrichaceae
Dialister
[Eubacterium]_ventriosum_group
[Eubacterium]_coprostanoligenes_group
Ruminococcus
Adlercreutzia
[Eubacterium]_nodatum_group
[Eubacterium]_xylanophilum_group
Lachnospiraceae_NK4A136_group
Odoribacter
Parasutterella
Alloprevotella
Butyricimonas
Bacillus
[Ruminococcus]_gauvreauii_group
Anaerococcus
Eggerthella
Peptostreptococcus
Lachnospiraceae_UCG-004
Flavonifractor
Prevotella_7
Corynebacterium
Fusobacterium
Turicibacter
C
Network features
Value
0.0
0.2
0.4
0.6
Modularity
N:P ratio
Total cohesion
High diversity
Low diversity

Supplemental Table 1. Clinical characteristics of patients in the higher- and lower-diversity groups based on diversity before allo-HSCT. Wilcoxon rank-sum test and Fisher's exact test were used to compare continuous and categorical clinical and GM features, respectively (HSCT = hematopoietic stem cell transplantation; MDS = myelodysplastic syndrome; JMML = juvenile myelomonocytic leukaemia; PBSC = peripheral blood stem cell).

	Lower diversity (N = 45)	**Higher diversity (N = 45)**	**p**
Age at HSCT – y (± SD)	8.00 ± 6.1	10.06 ± 4.8	0.080
Male sex - no. (%)	30 (67)	23 (53)	0.134
Disease - no. (%)			0.431
Acute lymphoblastic leukemia	20 (44)	13 (29)	
Acute myeloid leukemia	7 (16)	12 (27)	
Non-Hodgkin lymphoma	1 (2)	1 (2)	
MDS or JMML	6 (13)	4 (9)	
Non-malignant disease	11 (24)	15 (33)	
Type of donor (%)			0.592
Unrelated	28 (62)	27 (60)	
Familiar haploidentical	8 (18)	10 (22)	
Identical sibling	9 (20)	8 (18)	
Stem cell source (%)			0.861
Bone marrow	24 (69)	34 (76)	
PBSC	10 (29)	11 (24)	
Cord blood	1 (3)	0 (0)	
Intensity of conditioning (%)			
Ablative	43 (96)	40 (89)	0.237
Reduced intensity	2 (4)	5 (11)	
Follow-up of survivors — mo.			
Median	46.3	57.0	0.145
Interquartile ragne	23.5-65.5	31.0-79.0	
Pre-HSCT diversity (Shannon) (± SD)	2.15 ± 0.52	3.00 ± 0.31	0.002
Center – no. (%)			
Bologna	21 (47)	29 (64)	0.011
Wroclaw	10 (22)	5 (11)	
Verona	2 (4)	3 (7)	
Roma	1 (2)	6 (13)	
Pavia	11 (24)	2 (4)	

Supplemental Table 2. Clinical characteristics of patients in the higher- and lower-diversity groups based on diversity at neutrophil engrafment. Wilcoxon rank-sum test and Fisher's exact test were used to compare continuous and categorical clinical and GM features, respectively (HSCT = hematopoietic stem cell transplantation; MDS = myelodysplastic syndrome; JMML = juvenile myelomonocytic leukaemia; PBSC = peripheral blood stem cell).

	Lower diversity (N = 45)	**Higher diversity (N = 45)**	**p**
Age at HSCT – y (± SD)	7.54 ± 5.8	10.52 ± 4.9	0.010
Male sex - no. (%)	22 (49)	31 (69)	0.054
Disease - no. (%)			0.543
Acute lymphoblastic leukemia	18 (40)	16 (36)	
Acute myeloid leukemia	7 (16)	10 (22)	
Non-Hodgkin lymphoma	0 (0)	1 (2)	
MDS or JMML	7 (16)	4 (9)	
Non-malignant disease	13 (29)	14 (31)	
Type of donor (%)			0.488
Unrelated	27 (60)	28 (62)	
Familiar haploidentical	11 (24)	7 (16)	
Identical sibling	7 (16)	1 (22)	
Stem cell source (%)			0.257
Bone marrow	31 (69)	37 (82)	
PBSC	13 (29)	8 (18)	
Cord blood	1 (2)	0 (0)	
Intensity of conditioning (%)			
Ablative	42 (93)	41 (91)	0.694
Reduced intensity	3 (7)	4 (9)	
Follow-up of survivors — mo.			
Median	41.5	44.8	0.799
Interquartile ragne	23-78	24.0-77	
Diversity at engraftment (Shannon) (± SD)	1.50 ± 0.40	2.64 ± 0.44	<0.001
Center – no. (%)			
Bologna	19 (42)	31 (69)	0.051
Wroclaw	10 (22)	5 (11)	
Verona	5 (11)	0 (0)	
Roma	4 (9)	3 (7)	
Pavia	7 (16)	6 (13)	

Supplemental Table 3. Association of transplant characteristics with overall survival. CI = confidence interval; OS = overall survival; PBSC = peripheral blood stem cell; SE = standard error.

Variable	Hazard Ratio (95% CI)	p
Age (continuous)	1.07 (0.99-1.16)	0.090
Type of donor	**OS (at 52 months)**	0.539
Unrelated	74.6 ± 6.3 (SE)	
Familiar haploidentical	70.0 ± 11.5 (SE)	
Identical sibling	88.2 ± 7.8 (SE)	
Stem cell source		0.862
Bone marrow	76.1 ± 5.6 (SE)	
PBSC	76.2 ± 9.3 (SE)	
Cord blood	100.0 ± 7.8 (SE)	
Intensity of conditioning		0.714
Ablative	75.7 ± 5.0 (SE)	
Reduced intensity	85.7 ± 13.2 (SE)	

Supplemental Table 4. Association of pre-allo-HSCT gut microbiota diversity with relevant clinical variables. aGvHD = acute graft-versus-host disease; BSI = bloodstream infection; TRM = transplant-related mortality.

Variable	No. of events	No. of patients	Univariate analysis	Multivariate analysis*
Death	20	90		
Lower diversity			Reference	
Higher diversity			0.29 (0.11-0.80)	0.26 (0.09-0.75)
TRM	11	90		
Lower diversity			Reference	
Higher diversity			0.51 (0.15-1.75)	
aGvHD any grade	44	90		
Lower diversity			Reference	
Higher diversity			0.56 (0.30-1.06)	
aGvHD grade II-IV	29	90		
Lower diversity			Reference	
Higher diversity			0.38 (0.17-0.83)	0.31 (0.14-0.71)
aGvHD grade III-IV	14	90		
Lower diversity			Reference	
Higher diversity			0.10 (0.01-0.77)	0.06 (0.08-0.53)
Gut aGvHD	16	90		
Lower diversity			Reference	
Higher diversity			0.40 (0.14-1.16)	
BSI	24	90		
Lower diversity			Reference	
Higher diversity			0.81 (0.36-1.81)	
Relapse	25	90		
Lower diversity			Reference	
Higher diversity			0.45 (0.17-1.20)	

*****Multivariate Cox proportional-hazards were calculated with multivariate models adjusted for age, graft source, donor type, intensity of conditioning regimen, center, and oncological versus non-oncological disease.

Supplemental Table 5. Association of gut microbiota diversity at neutrophil engraftment with relevant clinical variables. aGvHD = acute graft-versus-host disease; BSI = bloodstream infection; CI = cumulative incidence; OS = overall survival; RFS = relapse-free survival; SE = standard error; TRM = transplant-related mortality.

Variable	Proportion Surviving/ Cumulative incidence	p
OS		0.931
Lower diversity	76.0 ± 6.9 (SE)	
Higher diversity	76.5 ± 6.7 (SE)	
RFS		0.678
Lower diversity	73.9 ± 7.0 (SE)	
Higher diversity	68.9 ± 6.9 (SE)	
CI of any grade aGvHD		0.490
Lower diversity	40.0 ± 7.3 (SE)	
Higher diversity	48.9 ± 7.5 (SE)	
CI of aGvHD grade II-IV		0.483
Lower diversity	28.9 ± 6.8 (SE)	
Higher diversity	35.6 ± 7.1 (SE)	
CI of aGvHD grade III-IV		0.485
Lower diversity	13.3 ± 5.1 (SE)	
Higher diversity	8.9 ± 4.2 (SE)	
CI of Gut aGvHD		1.000
Lower diversity	17.8 ± 5.7 (SE)	
Higher diversity	17.8 ± 5.7 (SE)	
CI of BSI		0.747
Lower diversity	20.9 ± 6.2 (SE)	
Higher diversity	23.8 ± 6.6 (SE)	
CI of TRM		0.526
Lower diversity	10.9 ± 5.4 (SE)	
Higher diversity	13.3 ± 5.0 (SE)	
CI of Relapse		0.616
Lower diversity	15.6 ± 5.4 (SE)	
Higher diversity	22.2 ± 6.2 (SE)	