ORIGINAL ARTICLE

Gut Microbiota Predict Pulmonary Infiltrates after Allogeneic Hematopoietic Cell Transplantation

Bianca Harris[1], Sejal M. Morjaria[2], Eric R. Littmann[3], Alexander I. Geyer[1,4], Diane E. Stover[1,4], Juliet N. Barker[4,5], Sergio A. Giralt[4,5], Ying Taur[2,4], and Eric G. Pamer[2,3,4]

[1]Pulmonary Service, [2]Infectious Diseases Service, and [5]Bone Marrow Transplant Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York; [3]Lucille Castori Center for Microbes, Inflammation and Cancer, Sloan Kettering Institute, New York, New York; and [4]Department of Medicine, Weill Cornell Medical College, New York, New York

Abstract

Rationale: Pulmonary complications (PCs) cause significant morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HCT). Shifts in gut microbiota have been linked to HCT outcomes; however, their effect on PCs is unknown.

Objectives: To investigate whether changes in gut microbiota are associated with PCs after HCT.

Methods: A single-center observational study was performed on 94 patients who underwent HCT from 2009 to 2011 and who were previously enrolled in a protocol for 16S ribosomal RNA sequencing of fecal microbiota. The primary endpoint, PC, was defined by new abnormal parenchymal findings on chest imaging in the setting of respiratory signs and/or symptoms. Outcomes were collected up to 40 months after transplant. Clinical and microbiota risk factors for PCs and mortality were evaluated using survival analysis.

Measurements and Main Results: One hundred twelve PCs occurred in 66 (70.2%) subjects. A high comorbidity index (hazard ratio [HR], 2.30; 95% confidence interval [CI], 1.30–4.00; $P = 0.004$),

fluoroquinolones (HR, 2.29, 95% CI, 1.32–3.98; $P = 0.003$), low baseline diversity (HR, 2.63; 95% CI, 1.22–5.32; $P = 0.015$), and γ-proteobacteria domination of fecal microbiota (HR, 2.64; 95% CI, 1.10–5.65; $P = 0.031$), which included common respiratory pathogens, predicted PCs. In separate analyses, low baseline diversity was associated with PCs that occurred preengraftment (HR, 6.30; 95% CI, 1.42–31.80; $P = 0.016$), whereas γ-proteobacteria domination predicted PCs postengraftment (HR, 3.68; 95% CI, 1.49–8.21; $P = 0.006$) and overall mortality (HR, 3.52; 95% CI, 1.28–9.21; $P = 0.016$). Postengraftment PCs were also independent predictors of death (HR, 2.50; 95% CI, 1.25–5.22; $P = 0.009$).

Conclusions: This is the first study to demonstrate prospective changes in gut microbiota associated with PCs after HCT. Postengraftment PCs and γ-proteobacteria domination were predictive of mortality. This suggests an adverse relationship between the graft and lung, which is perhaps mediated by bacterial composition in the gut. Further study is warranted.

Keywords: stem cell transplantation; microbiota; pulmonary disease

Allogeneic hematopoietic stem cell transplantation (HCT) is a potentially curative therapy for many blood disorders and malignancies. Advances in transplant management have greatly improved outcomes (1). However, pulmonary complications (PCs) are associated with high morbidity and mortality. Although PCs have been reported in up to 70% of HCT recipients (2–4), the epidemiology is poorly defined in this heterogeneous population.

Lung injury after HCT can occur early or late, present as acute or chronic, and involve one or more anatomic

At a Glance Commentary

Scientific Knowledge on the Subject: Pulmonary complications are associated with significant morbidity and mortality after allogeneic hematopoietic cell transplantation. It is possible that gut microbiota, which have been associated with adverse transplant outcomes, contribute to lung injury in this setting. In the absence of a clear understanding of infectious and inflammatory disease mechanisms in this population, tools for prediction, diagnosis, and treatment of transplantation-related pulmonary complications are limited.

What This Study Adds to the Field: This is the first study to use high-throughput sequencing of the 16S rRNA gene to identify gut microbiota composition as a risk factor for pulmonary complications and mortality after allogeneic hematopoietic cell transplantation. As transplantation continues to expand as a treatment modality for diverse hematologic diseases, the identification of microbial mechanisms of lung injury is greatly needed to develop predictive risk tools and inform novel mechanistic hypotheses.

compartments of the lower respiratory tract. Risk factors for infectious complications include impairment of host defenses through myeloablative conditioning, T-cell depletion, and the development of graft versus host disease (GVHD) (5). Risk factors for parenchymal inflammation, such as idiopathic pneumonia syndrome (IPS), diffuse alveolar hemorrhage, and organizing pneumonia (OP), are less clear. Host susceptibility to PCs outside of typical engraftment phases may further increase in the setting of critical illness, augmented immunosuppression, and recurrent nosocomial exposures.

Chest imaging is quickly obtained upon clinical suspicion of a respiratory process in transplant patients. Computed tomography (CT) is often preferred because of the low sensitivity of chest X-rays in this population (6, 7). Although nonspecific, abnormal parenchymal patterns such as airspace consolidation, ground glass

opacities, nodules, and reticular changes (8) may help inform the differential diagnosis and guide the next step in clinical decision-making (9). However, the presence of any pulmonary infiltrate, whether focal, multifocal, or diffuse, will commonly trigger broad antimicrobial treatment. Untargeted therapy can lead to additional complications, including antibiotic resistance, and may further confound the diagnostic evaluation when respiratory symptoms persist, progress, or recur.

Recently, associations between features of gut microbiota and bloodstream infection (10), *Clostridium difficile* infection (11), GVHD (12), and mortality (13) were described in allogeneic HCT recipients. Relationships between dysbiosis of gastrointestinal microbiota and allergic airway diseases (14–16), cystic fibrosis (17), and infection-mediated lung inflammation in animal models (18–21) have been suggested. The effects of diet (22) and probiotics (23, 24) on the prevention of pneumonia are also under investigation. The influence of gut microbiota on the development of PCs in HCT recipients, whose gut flora changes markedly upon hospitalization for HCT (10), has not been explored. Because of the likelihood of parallel mucosal changes in the gastrointestinal and respiratory tracts during transplant-related treatments, it is possible that gut–lung cross-talk contributes to PCs after HCT.

The objective of this study was to assess clinical and gut microbiota risk factors for post-transplant PCs, as defined by radiographic parenchymal abnormalities in the setting of respiratory signs and/or symptoms, and associated mortality at a high-volume academic transplantation center. Identification of gut microbiota features and transplant characteristics that may place allogeneic HCT recipients at increased risk for PCs will inform future investigations, with the ultimate goal to advance diagnostic, therapeutic, and prevention practices in this compromised population. Aspects of this research have previously been presented in abstract form (25, 26).

Methods

Study Population and Fecal Sampling Protocol

Chart review was performed on an established cohort of 94 patients who

underwent allogeneic HCT from September 4, 2009, to August 4, 2011, and who were enrolled in a protocol for 16S ribosomal RNA (rRNA) sequencing of stool microbiota during hospitalization. Informed consent, patient enrollment, and sequencing methods have been described (10, 13); a brief summary is available in the online supplement.

Data Sources and Definitions

Clinical data. Results of chest imaging and clinical data were collected up to 40 months after transplant. The primary endpoint, PC, was defined as any new pulmonary infiltrate on chest CT (or X-ray if unable to obtain CT) in the setting of respiratory complaints or abnormal vital signs. Two study physicians independently identified each PC, with adjudication by a third in complex cases, as further described in the online supplement. Specific abnormalities identified on pre-transplant imaging were excluded. PCs did not include imaging findings suggestive of cardiogenic pulmonary edema, atelectasis, or recurrent malignancy unless there was concern for a superimposed process. PCs were defined as "preengraftment" if they occurred before recovery of an absolute neutrophil count $\geqslant 500$ cells/μl for 3 consecutive days and "postengraftment" after the engraftment date (10). PCs were categorized as primarily "infectious" or "inflammatory/other" based on clinical impression in the medical record. PCs were excluded if they developed after a second HCT during the study period.

The endpoint of mortality was defined as the percentage of patients who died because of any cause, and was considered transplant-related in subjects who had not experienced disease relapse or progression (27).

Potential clinical predictors included demographic characteristics, underlying disease, allogeneic stem cell source, donor status, conditioning regimen intensity (28), disease risk score (based on the 2014 American Society for Blood and Marrow Transplantation Request for Information Disease Classifications schema) (29), the HCT comorbidity index (HCTCI; a weighted score of pre-transplantation comorbidity) (30), and antibiotic use during the transplantation hospitalization. The examined pulmonary risk factors included history of smoking, chronic lung disease, abnormal baseline pulmonary function (impaired diffusing capacity of the

lung for carbon monoxide adjusted for hemoglobin, obstructive ventilatory defect, restrictive ventilatory defect, and/or oxygen desaturation, defined according to American Thoracic Society/European Respiratory Society guidelines [31]), and the use of a fixed-dose busulfan-containing conditioning regimen.

Microbiota data. Phylogenetic classification for each of the 16S sequences was achieved using a naïve Bayesian classification approach and the Greengenes reference database (13, 32) for this analysis. Based on previous findings in this population, in whom intestinal composition frequently shifted from a diverse microbiota to one largely occupied by a single taxon soon after transplantation (10), we established two microbiota "states" as predictors. A state of low diversity, estimated by calculating the Shannon diversity index (SDI) (33), was defined by a SDI $\leqslant 1.5$ (10, 34). A state of taxon domination was defined as 30% or greater relative abundance of the most dominant taxon identified in a stool specimen collected until Transplantation Day 35, hospital discharge, or death (10). Using this threshold, domination states by bacteria from the taxa proteobacteria (phylum), *Enterococcus* (genus), and *Streptococcus* (genus) were included based on previous findings as the most abundant and clinically relevant taxa in this allogeneic HCT population (10).

The microbiota of these patients were further described by grouping sequences into operational taxonomic units of 97% similarity and calculating α- and β-diversity measures. The SDI was calculated as a measure of α-diversity, and Bray-Curtis dissimilarity distances were calculated and plotted by principal components analysis as a measure of β-diversity.

Statistical Analysis

Fisher's exact test was used for comparison of characteristics of HCT subjects by PC status. Kaplan-Meier analysis and Cox proportional hazards modeling were used to identify risk factors for PCs and adjusted for potential confounders. Dynamic predictors occurring after HCT, such as acute GVHD, bloodstream infection, disease relapse, antibiotic regimens, and microbiota states, were coded as time-dependent variables (assessed from HCT infusion, Day 0, forward) consistent with evaluation of the primary endpoint.

Table 1. Baseline Transplant and Pulmonary Characteristics of Hematopoietic Stem Cell Transplantation Patients with and without Pulmonary Complications

Variable	At Least 1 PC (*n* = 66; 70.2%)	No PC (*n* = 28; 29.8%)
Pretransplant characteristic		
Age, yr		
≤29	6 (9.1)	1 (3.6)
30–39	10 (13.8)	3 (10.7)
40–49	10 (15.2)	9 (32.1)
50–59	19 (28.8)	9 (32.1)
≥60	21 (31.8)	6 (21.4)
Sex, male	37 (56.1)	16 (57.1)
Race		
White	52 (78.8)	23 (82.1)
Black	3 (4.5)	3 (10.7)
Asian/Southeast Asian	9 (13.6)	1 (3.6)
Disease		
Leukemia	30 (45.5)	14 (50.0)
Lymphoma	17 (25.8)	10 (35.7)
Multiple myeloma	7 (10.6)	1 (3.6)
Myelodysplastic syndrome	9 (13.6)	3 (10.7)
Donor		
Matched-related	20 (30.3)	11 (39.3)
Matched-unrelated	20 (30.3)	8 (28.6)
Mismatched-unrelated	10 (15.2)	2 (7.1)
Umbilical cord	16 (24.2)	7 (25.0)
Transplant type		
T cell–depleted peripheral blood	29 (43.9)	13 (46.4)
Unmodified peripheral blood	21 (31.8)	8 (28.6)
Double umbilical cord	16 (24.2)	7 (25.0)
Conditioning intensity		
Non-myeloablative	12 (18.2)	6 (21.4)
Reduced intensity	21 (31.8)	9 (32.1)
Myeloablative	33 (50.0)	13 (46.4)
Disease risk		
Low	14 (21.2)	13 (46.4)
Intermediate	16 (24.2)	7 (25.0)
High	**36 (54.5)**	**8 (28.6)**
HCT comorbidity index		
≤1	19 (28.8)	15 (53.6)
2–3	22 (33.3)	10 (35.7)
≥4	**25 (37.9)**	**3 (10.7)**
Antibiotics administered*		
Vancomycin[†]	61 (92.4)	28 (100.0)
β-Lactam[†]	61 (92.4)	25 (89.3)
Fluoroquinolone	**51 (77.3)**	**14 (50.0)**
Flagyl	36 (54.5)	12 (42.9)
Azithromycin	**39 (59.1)**	**7 (25.5)**
Pretransplant pulmonary history		
Smoking (former/current)	33 (50.0)	14 (50.0)
≥20 pack-years[‡]	11 (33.3)	4 (28.6)
Previous or chronic lung disease[§]	11 (16.7)	3 (10.7)
Fungal risk factors[‖]	43 (74.1)	19 (86.4)
Abnormal chest imaging[¶]	37 (56.1)	13 (46.4)
Abnormal PFT**	**44 (66.7)**	**11 (39.3)**
Impaired D_{LCO} [Hb]	37 (84.1)	9 (81.8)
OVD	5 (11.4)	1 (9.1)
RVD	8 (18.2)	4 (36.4)
Busulfan-containing regimen	25 (37.9)	4 (14.3)

Definition of abbreviations: CT = computed tomography; D_{LCO} [Hb] = diffusing capacity of the lung for carbon monoxide adjusted for hemoglobin; HCT = hematopoietic stem cell transplantation; OVD = obstructive ventilatory defect; PC = pulmonary complication; PFT = pulmonary function test; RVD = restrictive ventilatory defect.
Data are shown as n (%). *P* values are two-sided and based on Fisher's exact test; significant values in bold if $P < 0.05$.
*Antibiotics were assessed only during in-patient hospitalization for allogeneic HCT; variables are not mutually exclusive and do not sum to 100%.
[†]Intravenous vancomycin only; β-lactams include cephalosporins, β-lactam–β-lactamase combinations, and carbapenems.
[‡]Two former smokers without pack-year data; percent of smokers shown in parentheses.
[§]Asthma, chronic obstructive pulmonary disease, interstitial lung disease, or previous pneumonia.
[‖]HCT provider assessment of fungal risk pre-transplant (e.g., history of extensive neutropenia, prolonged steroid use, presumed or confirmed fungal pneumonia); 14 with missing data.
[¶]Abnormal parenchymal finding (nodule, consolidation, ground glass, interstitial changes) on baseline chest CT, or infiltrate on chest X-ray if CT is unavailable.
**As per American Thoracic Society/European Respiratory Society guidelines (31); seven with missing lung volumes; percent of abnormal PFTs shown in parentheses do not add up to 100% because of the presence of multiple abnormalities per PFT.

Covariates associated with PCs at a significance level of $P < 0.2$ on univariate analysis were included in the multivariate model.

Similar calculations were performed using mortality as the outcome of interest. The impact of pre- and postengraftment PCs on mortality was also examined by incorporating an interaction term for PCs and engraftment, both coded as time-dependent predictors. All analyses were performed using the machine learning package R, version 3.2 (R, Vienna, Austria).

Results

Cohort Characteristics

Clinical. Of 94 subjects, 66 (70.2%) had at least one post-transplant PC, with a median time of onset 50 days after HCT. Table 1 shows baseline transplant and pulmonary characteristics of participants by PC. Compared with subjects without PCs, the PC group had significantly lower abnormal baseline pulmonary function tests (PFTs) ($P = 0.021$), a higher disease risk profile ($P = 0.032$), a higher HCT cardiac index score ($P = 0.013$), previous fixed-dose busulfan ($P = 0.028$), and fluoroquinolone ($P = 0.014$) or azithromycin ($P = 0.003$) use during hospitalization. Pretransplant characteristics of this cohort were similar to the overall allogeneic HCT case mix at our institution during the same period, as reported (13).

Microbiota. As previously stated by Taur and colleagues (10), 439 fecal specimens were collected from 94 subjects (3–8 per patient) during the transplant hospitalization, yielding 1,838,205 high-quality 16S rRNA-encoding sequences (mean 4,187; range 852–9,862 per specimen). In this study, 71.3% (67 of 94) of subjects developed at least one state of low diversity, and 67.0% (63 of 94) developed at least one taxon-dominated state: 38 (40.4%) *Enterococcus*-dominated, 29 (30.9%) *Streptococcus*-dominated, and 11 (11.7%) proteobacteria-dominated (all sequences within this phylum belonged to the class γ-proteobacteria, heretofore referred to as a "γ-proteobacteria" domination state). Table 2 describes these microbiota states in subjects according to their PC status, and Table E1 in the online supplement shows microbiota features by mortality status. There were no significant differences between subjects with low diversity at baseline, which was defined as the specimen meeting the defined criteria closest to Day 0 (mean Day −3; range −8 to −1), at engraftment, or by domination states. Ten of 11 subjects who developed γ-proteobacteria domination also developed PCs; however, not all states preceded the PC. There was a trend toward a significant difference in γ-proteobacteria domination in subjects who died compared with those who were alive at study end (9 vs. 2; $P = 0.051$).

Microbiota domination states were each largely defined by single species, including *Enterococcus faecium* in 34 of 38 subjects (89.5%; mean relative abundance, 76.4% per specimen; range, 32.0–99.9%) and *Streptococcus thermophilus* in 26 of 29 (89.7%; mean relative abundance, 73.2%; range, 24.0–99.9%). The species driving γ-proteobacteria domination states were *Escherichia coli* in 4 of 11 subjects (36.4%; mean relative abundance, 75.8%; range, 56.9–94.8%), *Klebsiella* spp. in 3 (27.3%; relative abundance of *K. oxytoca* 89.9%, *K. variicola* 59.3%, and *K. pneumoniae* 40.25%), *Enterobacter asburiae* in 2 (18.2%; mean relative abundance 50.7%; range, 39.3–62.1%), and *Leclercia adecarboxylata* (relative abundance 47.5%), and *Haemophilus parainfluenzae* (relative abundance 31.9%) in one subject each. Figure 1 is a heat map that shows the relative abundance of all species identified in 11 subjects with specimens meeting criteria for γ-proteobacteria domination. Figure E1 is a heat map depicting the maximum relative abundance of all γ-proteobacteria species identified in all study specimens by PC status. Plots of α-diversity by PC status and β-diversity by domination state and PC status are shown in Figures E2 and E3, respectively.

Pulmonary Complications, Mortality, and Other Clinical Outcomes

There were 112 total PC events in 66 subjects during the study period, including 2 PCs in 31 subjects and 3 PCs in 15. Ninety-four of 112 (83.8%) PCs were first diagnosed by chest CT, and 71.4% (80 of 112) of radiographic findings were described as multifocal (35) or diffuse (20). Thirty-three PCs (29.2%) occurred during the first 30 days, and 86 (76.8%) occurred by the end of the first post-transplantation year. PCs developed preengraftment in 19 subjects (28.8%), postengraftment in 56 (84.9%), and during both intervals in 9 patients (13.6%). Forty-eight subjects (51.1%) died during the study, and one-half of the deaths were considered transplant-related. Median time to death was 218 days. Subjects who died were more likely to have abnormal baseline PFTs ($P = 0.006$) and exposure to azithromycin ($P = 0.026$) compared with those who were alive at the end of the study period. Figure 2 shows a timeline of PCs and mortality events across all subjects, and highlights subjects who developed fecal domination by *Enterococcus*

Table 2. Gut Microbiota States during Transplant Hospitalization and Clinical Outcomes

Microbiota State	At least 1 PC (n = 66; 70.2%)	No PC (n = 28; 29.8%)
Taxon domination*		
γ-Proteobacteria	10 (15.2)	1 (3.6)
Enterococcus	30 (45.5)	8 (28.6)
Streptococcus	18 (27.3)	11 (39.3)
Low diversity†		
Baseline‡	12 (18.2)	3 (10.7)
Engraftment§	33 (53.2)	11 (42.3)

Definition of abbreviation: PC = pulmonary complication.
Data are shown as n (%). Using two-sided P values based on Fisher's exact test with a significance threshold of $P < 0.05$, there were no statistically significant differences in the absence of time-dependent analysis.
*Defined as relative abundance greater than 30% and the most dominant taxon in a stool specimen collected during initial transplantation hospitalization (until Day 35 or death) (10). Seven of 94 patients had a domination state at the time of stem cell infusion (Day 0).
†Low diversity as defined by Shannon diversity index <1.5 (33, 34).
‡Baseline defined as the pretransplant specimen collected most proximal to Day 0 (mean Day −3, range −8 to −1).
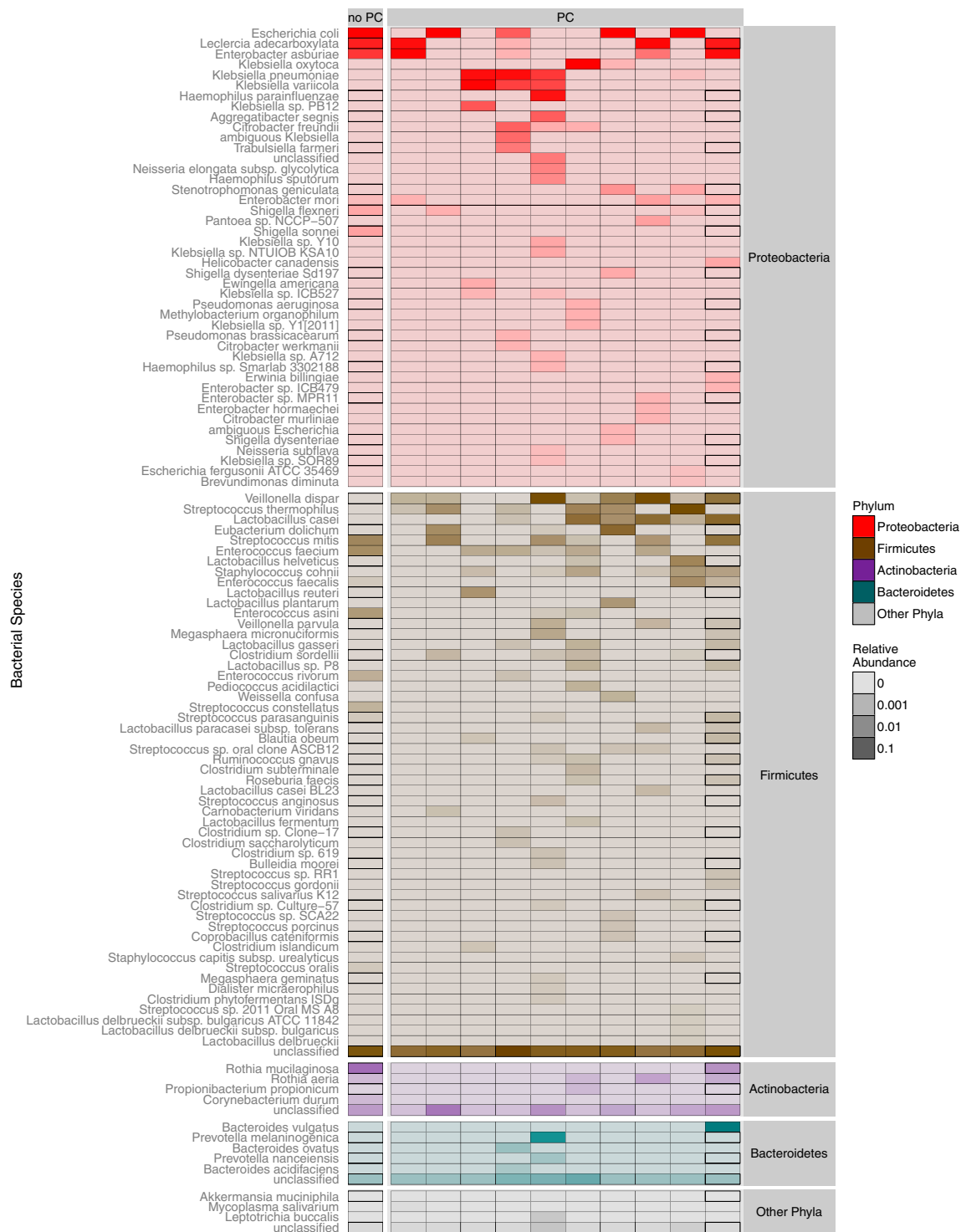§Two individuals did not engraft.

Figure 1. Heat map of bacterial species identified in subjects with γ-proteobacteria domination of fecal microbiota during the transplant hospitalization. The heat map shows the log-transformed relative abundance of all species identified in subjects with fecal specimens meeting criteria for γ-proteobacteria domination (i.e., the first sample with at least 30% relative abundance of γ-proteobacteria and the most abundant taxon identified) between hematopoietic stem cell transplantation infusion and Day 35 or hospital discharge. Based on this definition, each specimen was collected at a different time point (median Day 14 in subjects with pulmonary complications [PCs]; range Days 4–31; and Day 0 in the subject without PCs). Each *column* represents one patient, stratified by PC status. Each *row* represents bacterial taxa present in the γ-proteobacteria dominant specimen resolved to the species level, which are clustered by phylum, the highest taxonomic rank to which the species belongs, according to the color legend at the *right* of the plot. The most abundant species within each sample are from the phylum proteobacteria, by definition, as shown at the *top* of the heat map. In all subjects, 16S sequences were dominated by only one or two γ-proteobacteria species, including common respiratory pathogens such as *Klebsiella pneumoniae, K. oxytoca,* and *Escherichia coli.*
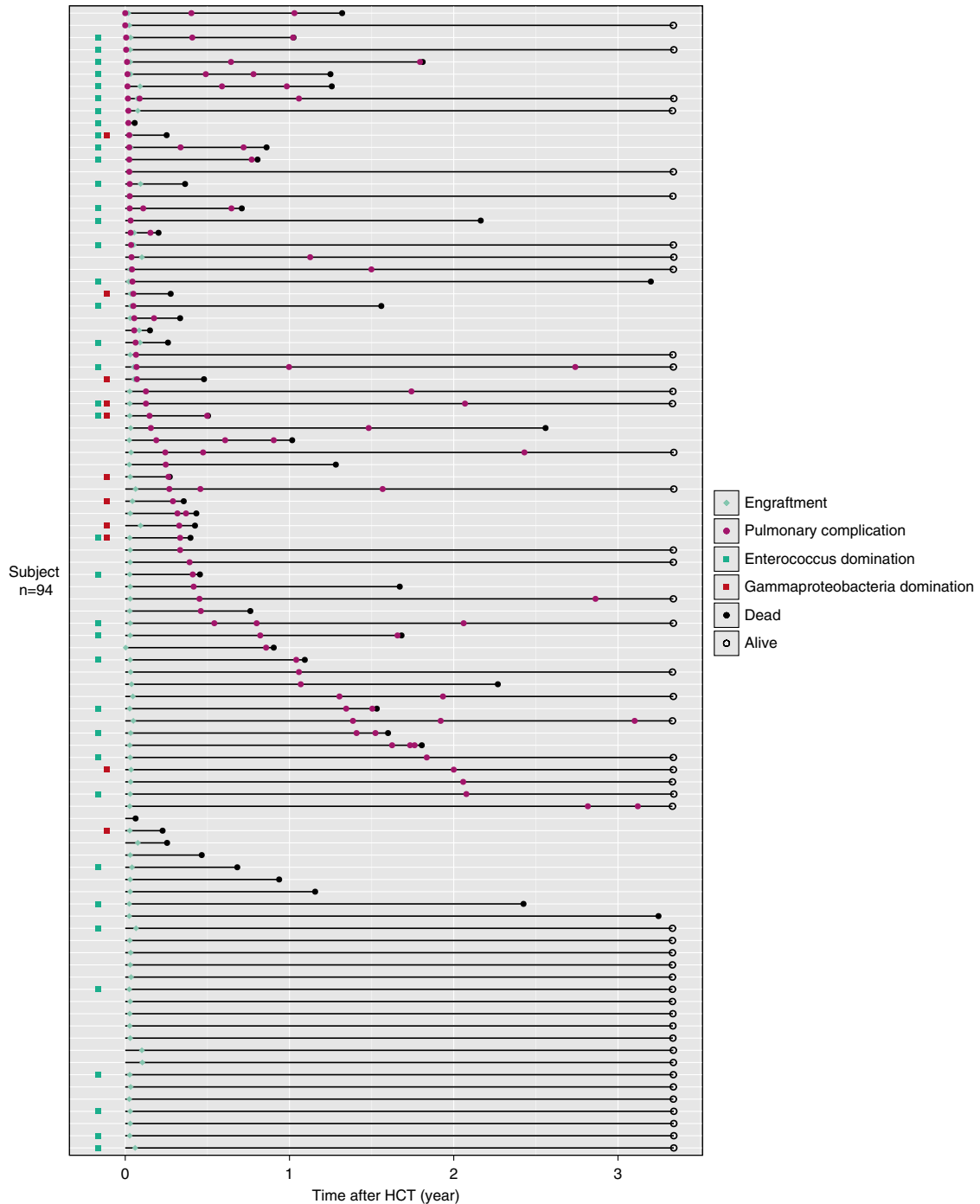
Figure 2. Time to post-transplant pulmonary complications and survival in all subjects. Time to development of pulmonary complications (PCs) after hematopoietic stem cell transplantation (HCT) during the study period, or until death or loss to clinical follow-up is depicted. Each *row* represents a subject, and the occurrence of a microbiota domination state by *Enterococcus* or γ-proteobacteria during transplant hospitalization is indicated at the *left* of the plot (note that time to domination is not indicated). Most of the subjects who died during study follow-up experienced at least one PC before death, and many PCs directly preceded the death event. Median time to first PC was 50 days, and median time to death was 218 days after HCT. Median follow-up was 3.3 years for subjects who remained alive at the end of the study period. There were 14 subjects with multiple domination events during the transplant hospitalization, including 10 in the PC group. Four of these subjects with PCs developed more than one domination event before the occurrence of the PC. For the time-dependent analysis, the specimen defined as having a domination state most proximal to the PC endpoint was analyzed, including the γ-proteobacteria dominant specimen in three subjects and the *Enterococcus*-dominant specimen in one patient. In the four subjects with multiple domination events and without PCs, the first specimen collected after time 0 was used in the time-dependent analysis, including γ-proteobacteria domination in one subject, *Enterococcus* domination in one patient, and *Streptococcus* domination in two subjects (not shown). *Enterococcus* domination (*green square*); γ-proteobacteria domination (*red square*); engraftment (*light green diamond*); PC (*purple circle*); dead (*closed circle*); and alive (*open circle*).

and/or γ-proteobacteria during that transplant hospitalization.

Table 3 shows results of diagnostic tests that led to a working PC diagnosis. Although most of the events (97; 86.6%) were considered primarily infectious, only 39 (34.8%) of diagnoses had supportive microbiologic or histopathologic data that identified a causative agent. The remainder (58; 59.8%) was attributed to nonspecific infectious processes, such as community-acquired or health care–acquired pneumonia. There were 15 noninfectious primary diagnoses, including 7 (46.6%) within the IPS spectrum (36) and 4 (26.7%) with biopsy-proven interstitial lung disease. In several cases, two processes were treated concomitantly, such as bacterial pneumonia and engraftment syndrome, or viral pneumonitis and alveolar hemorrhage. Of nine subjects who underwent autopsy, seven (77.8%) had histopathologic evidence of lung injuries deemed contributory to death, including bronchopneumonia, acute lung injury, diffuse alveolar damage, and OP.

Other post-transplant complications included disease relapse and/or progression in 21.3% (24), bloodstream infection in 37.2% (36), and acute GVHD in 38.6% (34 of 88 evaluated). Differences between PC groups were not statistically significant.

Risk Factors for Pulmonary Complications

In the Cox hazards model, associations were found between the occurrence of PCs and high HCTCI, fluoroquinolone use, low baseline diversity, and γ-proteobacteria domination of fecal microbiota (Table 4). γ-Proteobacteria domination was a strong, independent predictor of PCs; subjects with this microbiota composition were two to three times more likely to develop a PC, independent of other factors. Low baseline diversity was also an independent predictor, although only in the multivariate model after adjusting for antibiotics during the transplant hospitalization. In separate pre- and postengraftment analyses, low baseline diversity was solely predictive of PCs occurring preengraftment (hazard ratio [HR], 6.30; 95% confidence interval [CI] 1.42–31.80; $P = 0.016$) (see Table E2). The relationship between Enterococcus-domination and preengraftment PCs (HR, 5.72; 95% CI, 2.12–15.45; $P = 0.001$) was attenuated after adjusting for antibiotics (HR, 1.38; 95% CI, 0.38–4.95; $P = 0.625$), whereas

Table 3. Infectious and Noninfectious Etiologies of Primary Pulmonary Complication Diagnoses

Pulmonary Complication (n = 112)	Specimen/Evaluation
Infectious* (n = 97)	
Bacterial pneumonia (n = 20)	
Legionella pneumophilia (n = 2)	Lung tissue FNA/culture[†]
	Urine/serotype 1 urine antigen
Legionella micdadei (n = 1)	Pleural fluid/culture[†]
Pseudomonas aeruginosa (n = 4)	BAL/culture
	Sputum/culture
	TA/culture
	Blood/culture
Klebsiella pneumoniae (n = 4)	Sputum/culture (n = 2)
	TA/culture
	Blood/culture
Klebsiella oxytoca (n = 3)	Sputum/culture
	Blood /culture (n = 2)
Escherichia coli (n = 2)	Blood/culture
Moraxella catarrhalis (n = 2)	Sputum/culture
	BAL/culture
Stenotrophomonas maltophilia (n = 1)	Blood/culture
Vancomycin-resistant *Enterococcus*	Blood/culture
faecium (n = 1)	Endobronchial tissue biopsy/pathology
Viral pneumonia (n = 16)	
Human herpes virus 6 (n = 2)[‡]	BAL/PCR
Cytomegalovirus (n = 2)[‡]	BAL /culture
	Blood/viral antigen, culture
Adenovirus (n = 1)[‡]	Blood/PCR
Respiratory syncytial virus (n = 5)	NP swab/PCR
Parainfluenza (n = 3)	NP swab/PCR
Influenza (n = 2)	BAL/PCR (influenza B)
	NP swab/PCR (influenza A)
Metapneumovirus (n = 1)	NP swab/PCR
Fungal pneumonia (n = 3)	
Pneumocystis jirovecii (n = 1)	BAL/PCR, cytology
Aspergillus spp (n = 1)	BAL/*Aspergillus galactomannan* antigen
Rhizopus (n = 1)	Lung tissue (transbronchial biopsy)/culture
Unspecified (n = 58)[§]	N/A
Inflammatory/other (n = 15)[‖]	
Diffuse alveolar hemorrhage* (n = 2)	BAL
Engraftment syndrome (n = 2)	N/A
Idiopathic pneumonia syndrome (n = 3)	N/A
Organizing pneumonia (n = 3)	Lung tissue: transbronchial, CT-guided and surgical biopsies)/pathology (n = 3)
Desquamative interstitial pneumonitis (n = 1)	Lung tissue (surgical biopsy)
Drug toxicity (n = 3)	N/A
Transfusion reaction (IVIG) (n = 1)	N/A

Definition of abbreviations: BAL = bronchoalveolar lavage; CT = computed tomography; FNA = fine needle aspirate; IVIG = intravenous immunoglobulin; N/A = not applicable (negative workup); NP = nasopharyngeal; PCR = polymerase chain reaction; TA = tracheal aspirate.
*Infectious diagnoses on the basis of diagnostic evaluation of respiratory or peripheral specimens in the appropriate clinical context; other positive results of microbiological testing (e.g., isolation of *Staphylococcus aureus* [n = 2], rhinovirus [n = 1], and atypical mycobacteria [n = 3] in BAL or sputum specimens were not considered to be likely causes of pulmonary complications [PCs]). Furthermore, two cases of vancomycin-resistant *Enterococcus* were identified in clinical specimens; however, only one was considered a likely cause of PC (positive blood culture with chest imaging suggestive of septic emboli); the other was derived from pathological examination of an endobronchial lesion biopsied during bronchoscopy; further information about these cases is available in the online supplement.
[†]Culture performed at the New York City Department of Health after negative diagnostic workup at Memorial Sloan Kettering Cancer Center (both BAL and urine antigen were negative for *L. pneumophilia* in the case of *L. micdadei*).
[‡]Significantly elevated viral titres in lung specimens compared with blood, and/or blood with disseminated disease and supportive clinical and radiographic data suggestive of viral pneumonitis.
[§]Includes community-acquired pneumonia, health care–acquired pneumonia, aspiration pneumonia, and nonspecific fungal pneumonia (e.g., elevated β-D-glucans with nodular opacities on CT).
[‖]One case of alveolar hemorrhage in setting of cytomegalovirus pneumonitis; two cases of idiopathic pneumonia syndrome treated with etanercept after negative infectious workup; drug toxicity was presumed to be due to radiation, sirolimus, and busulfan toxicity, respectively.

Table 4. Predictors of Pulmonary Complications after Allogeneic Hematopoietic Stem Cell Transplantation

Clinical Predictor	Univariate		Multivariate	
	Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value
Age, ≥60 yr	1.22 (0.71–2.05)	0.462		
Sex, female	1.24 (0.75–2.02)	0.398		
Race, white vs. other	0.74 (0.42–1.43)	0.355		
Disease, leukemia vs. other	1.13 (0.69–1.85)	0.622		
Donor stem cell type, matched related vs. other	0.68 (0.41–1.13)	0.136	0.63 (0.35–1.11)	0.110
Double umbilical cord	1.43 (0.79–2.48)	0.230		
T-cell depletion	0.89 (0.54–1.46)	0.653		
Conditioning intensity, myeloablative vs. other	1.20 (0.73–1.97)	0.464		
Busulfan	1.59 (0.94–2.63)	0.082	1.72 (0.96–3.04)	0.068
HCT comorbidity index, ≥4 vs. other*	**2.40 (1.41–3.98)**	**0.001**	**2.30 (1.30–4.00)**	**0.004**
Disease risk, high vs. other[†]	1.43 (0.87–2.36)	0.082	1.72 (0.96–3.04)	0.473
Abnormal baseline PFTs	**1.93(1.12–3.32)**	**0.012**	1.73 (0.99–3.14)	0.056
Acute GVHD, Day 0 to 100[‡]	1.44 (0.66–2.94)	0.344		
Bloodstream infection[‡]	1.35 (0.77–2.29)	0.286		
Shannon diversity index <1.5 at baseline[§]	1.69 (0.83–3.11)	0.138	**2.63 (1.22–5.32)**	**0.015**
γ-Proteobacteria domination[‡]	2.30 (1.00–4.66)	0.051	**2.64 (1.10–5.65)**	**0.031**
Enterococcus domination[‡]	1.33 (0.77–2.24)	0.303		
Streptococcus domination[‡]	0.83 (0.43–1.48)	0.535		
Vancomycin[‡||]	0.75 (0.35–1.97)	0.532		
β-Lactam[‡¶]	1.12 (0.582.39)	0.752		
Fluoroquinolone[‡]	**1.99 (1.20–3.32)**	**0.008**	2.29 (1.32–3.98)	0.003
Metronidazole[‡]	1.19 (0.69–2.00)	0.515		
Azithromycin[‡]	1.42 (0.632.86)	0.374		

Definition of abbreviations: CI = confidence interval; HCT = hematopoietic stem cell transplantation; PFT = pulmonary function test;
GVHD = graft-versus-host disease.
Bold values indicate statistically significant P value <0.05.
*HCT comorbidity index groups: low (0–1), intermediate (2–3) or high (≥4) (30).
[†]Disease risk: American Society for Blood and Marrow Transplantation Request for Information Classification as low, intermediate, or high (29).
[‡]Time-dependent variables.
[§]Shannon diversity index takes into account number of species and relative abundance (33).
[||]Vancomycin includes intravenous mode of administration only.
[¶]β-Lactams include cephalosporins, β-lactam–β-lactamase combinations, and carbapenems.

γ-proteobacteria domination remained a strong predictor of postengraftment PCs in the fully adjusted model (HR, 3.68; 95% CI, 1.49–8.21; P = 0.006) (*see* Table E3). Relationships between microbiota domination states and PCs using Kaplan-Meier analysis are shown in Figure 3, including associations between γ-proteobacteria domination and postengraftment PCs (log-rank P = 0.006) (Figure 3A), as well as *Enterococcus*-domination and preengraftment PCs (log-rank P ⩽ 0.001) (Figure 3B). Figure 4 presents a timeline for a case of pneumonia caused by *K. pneumoniae*, a known respiratory pathogen from the γ-proteobacteria class, which developed after the transition to fecal domination by *K. pneumoniae* in serial stool samples, raising the possibility of translocation from the gut to the lung.

Risk Factors for Mortality
In the Cox hazards model, a strong association was found between overall mortality and PCs occurring postengraftment, but not preengraftment (Table 5), which remained after evaluating for a potential interaction between PCs and engraftment. Similarly, Kaplan-Meier analysis demonstrated significant associations between mortality and PCs occurring postengraftment (log-rank P ⩽ 0.001), but not preengraftment (log-rank P = 0.131). Other independent predictors of death in the multivariate model included disease relapse, azithromycin use, and γ-proteobacteria domination. Relationships between transplant-related mortality and γ-proteobacteria domination (HR, 4.49; 95% CI, 1.54–11.08; P = 0.008) and with postengraftment PCs (HR, 4.77; 95% CI, 2.23–10.98 ; P ⩽ 0.001) were attenuated after adjusting for antibiotics in the full model.

Discussion

We examined clinical and microbiota predictors of PCs and mortality in a cohort of 94 allogeneic HCT recipients, in whom serial changes in gut flora had been previously evaluated using high-throughput sequencing methods. The composition of gut microbiota, including low baseline diversity and γ-proteobacteria domination, during the early transplant period was associated with the development of PCs. γ-Proteobacteria domination and postengraftment PCs were also independent predictors of death after transplant. To our knowledge, this is the first study to demonstrate a relationship between gut microbiota and lung injury, which may, in turn, increase the risk of death in this population. These findings present an opportunity for further investigation into potential biomarkers and mechanisms of poorly understood pulmonary processes.

From pretransplant conditioning through early engraftment, the gastrointestinal tract bears the brunt of damage to immune and mucosal defenses, leading to systemic translocation of bacteria
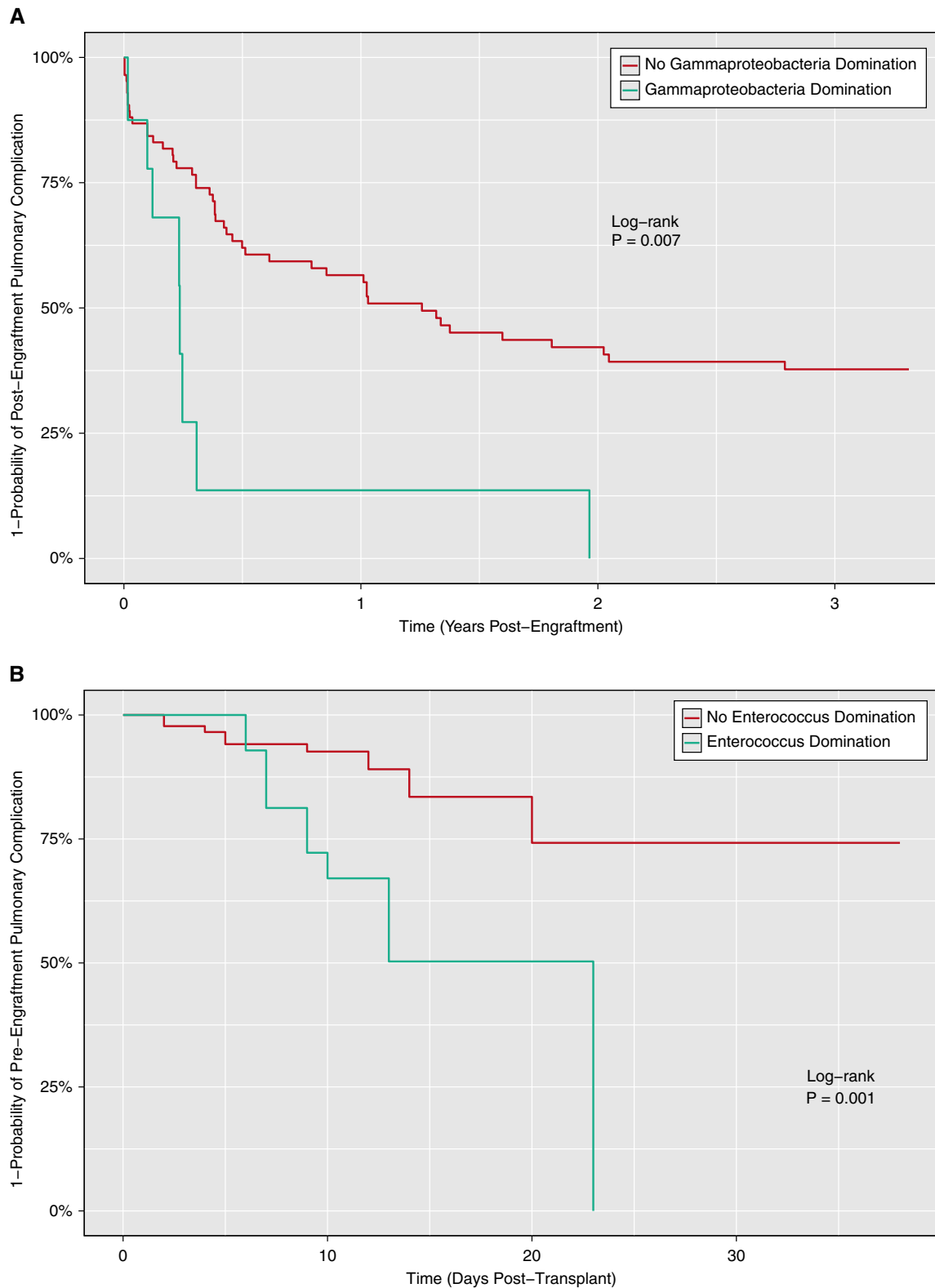
Figure 3. (*A*) Association between γ-proteobacteria domination of gut microbiota and the development of a pulmonary complication (PC) after engraftment. The relationship between γ-proteobacteria domination of fecal microbiota and the development of a postengraftment PC on Kaplan-Meier analysis is shown. Log-rank *P* values are significant if *P* < 0.05. (*B*) Association between *Enterococcus* domination of gut microbiota and the development of a PC before engraftment. The relationship between the *Enterococcus* domination state and the development of a preengraftment PC on Kaplan-Meier analysis is shown. Log-rank *P* values are significant if *P* < 0.05.
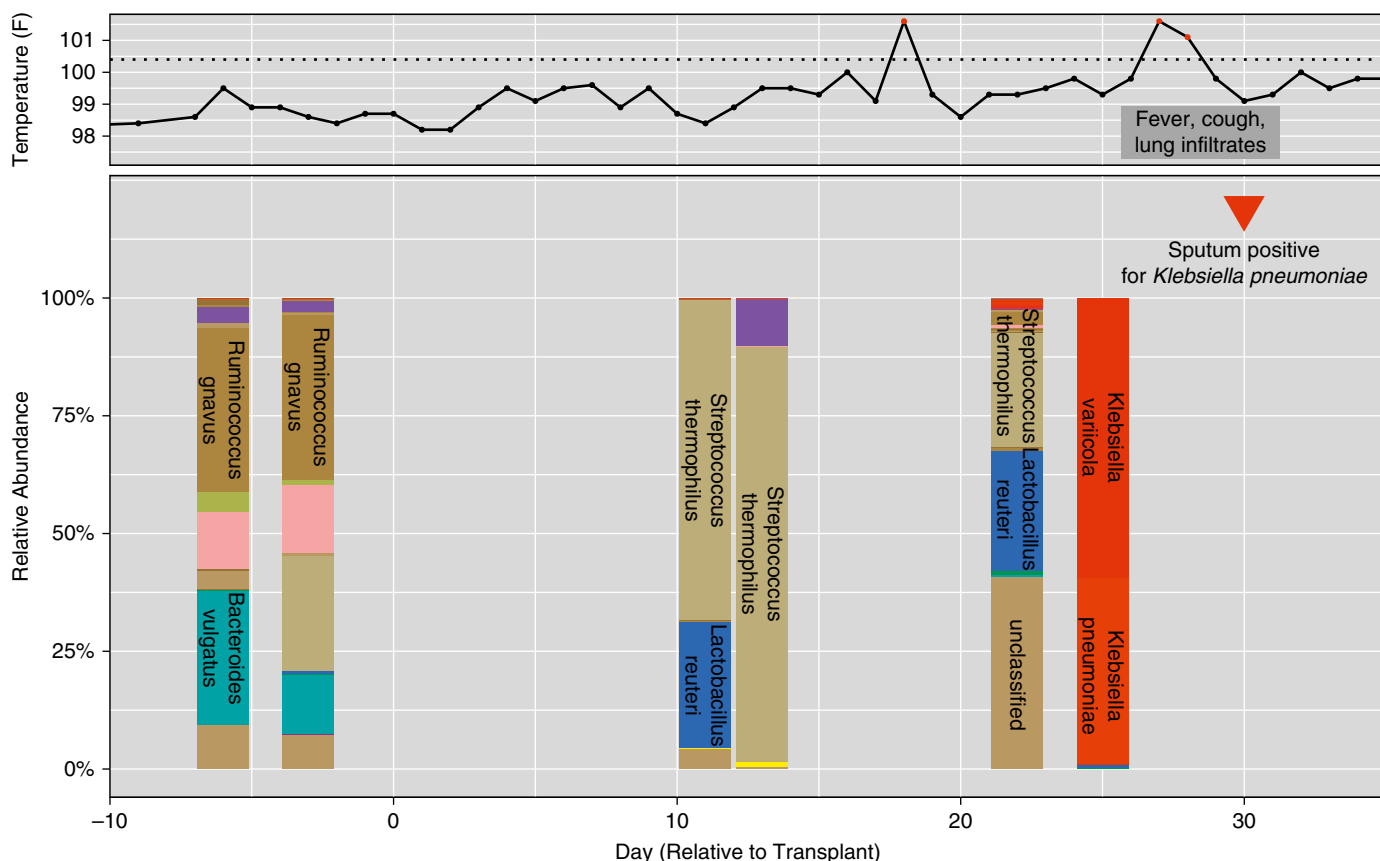
Figure 4. Shift to dominant *Klebsiella pneumoniae* in the gastrointestinal tract precedes clinical diagnosis of culture-proven *K. pneumoniae* pneumonia. Serial changes in gastrointestinal microbiota in a patient who received a double umbilical cord transplant for non-Hodgkin's lymphoma. On Transplantation Day 29, there was a shift in the composition of stool microbiota to *K. pneumoniae* gut domination. The patient soon thereafter developed a fever, cough and infiltrates on chest X-ray, and was empirically started on broad-spectrum antibiotics. Sputum culture was significant for *K. pneumoniae*, with interval chest CT showing multifocal pneumonia. Although it is possible that there was colonization of the respiratory tract that preceded gut domination, because the temporality of the clinical presentation and clinical findings, it is plausible that *K. pneumoniae* translocated from the gastrointestinal tract to the lungs. Expansion of this taxon in stool specimens likely began several days before observation in the dominant specimen, which cannot be appreciated in this figure (only taxa with relative abundance >30% are depicted). Each *stacked bar* represents the microbial composition of a single fecal specimen based on taxonomic classification to species level, with percent relative abundance of 16S sequences to 100% shown on the *y-axis*. Colors denote taxa from distinct phyla, with gradations in the color representing abundant species within that phylum. Sequences from the phylum proteobacteria are *red* (all are from the class γ-proteobacteria). Species are labeled within the *bars* representing taxon abundance when there was at least 30% relative abundance, as with *K. pneumoniae* and *K. variicola* in the stool specimen preceding the clinical diagnosis of pneumonia. The clinical microbiology result of the sputum culture is shown under the *red triangle* following the last stool specimen on the timeline. The *solid line* at the *top* of the plot depicts the fever trend during transplantation hospitalization, with a *red peak* indicating temperature higher than 100.4°F (*dotted line*), concurrent with respiratory signs and symptoms.

and subsequent infection (10, 37). By identifying features of fecal microbiota that may predict PCs, we were able to extend the impactful scope of these treatment-related shifts in gastrointestinal flora on clinically relevant endpoints. Early gut domination by γ-proteobacteria, a class of bacteria that includes respiratory pathogens such as *K. pneumoniae* and *K. oxytoca*, was associated with the development of postengraftment PCs, which, in turn, more than doubled the risk of mortality. Interestingly, the identification of abundant *K. variicola,* a recently named and virulent phylogroup of *K. pneumoniae* (38), in several specimens

from subjects with PCs may highlight missed opportunities for isolating potential pathogens in clinical specimens using traditional diagnostic methods. The same may be true for *E. asburiae* and *L. adecarboxylata*, formerly known as *E. adecarboxylata*, which have also been isolated from clinical specimens in patients with pneumonia and bacteremia (39,40).

These findings may suggest direct translocation of bacteria to the lungs during early transplant (as seen in Figure 4) or indirect lung injury caused by microbiota-stimulation of a systemic or local inflammatory response as possible

mechanisms; alternatively, associations might reflect overall microbiota status or antimicrobial use. The latter may best explain the attenuation of the relationship between gut domination by bacteria belonging to the genus *Enterococcus* (where most of the sequences were identified as vancomycin-resistant *E. faecium* [10]) and preengraftment PCs, because antibiotic use for prophylaxis and/or empiric treatment is heaviest at this time and may foster its aberrant colonization (10). However, although *E. faecium* is infrequently considered a true lung pathogen in the clinical setting, perhaps because of its

Table 5. Predictors of Overall Mortality after Allogeneic Hematopoietic Stem Cell Transplantation

Clinical Predictor	Univariate		Multivariate	
	Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value
Age, ≥60 yr	1.25 (0.67—2.23)	0.465		
Sex, female	1.28 (0.73–2.25)	0.388		
Race, white vs. other	0.80 (0.42–1.68)	0.542		
Disease, leukemia vs. other	1.09 (0.62–1.91)	0.772		
Donor stem cell type, matched related vs. other	0.66 (0.37–1.18)	0.155	0.69 (0.34–1.40)	0.300
Double umbilical cord	1.41 (0.71–2.61)	0.311		
T-cell depletion	0.94 (0.53–1.64)	0.816		
Conditioning intensity, myeloablative vs. other	1.04 (0.59–1.83)	0.891		
HCT comorbidity index, ≥4 vs. other*	**2.24 (1.25–3.95)**	**0.007**	0.91 (0.47–1.76)	0.785
Disease risk, high vs. other[†]	1.07 (0.61–1.88)	0.815		
Abnormal baseline PFTs	**2.45 (1.34–4.77)**	**0.003**	1.79 (0.90–3.72)	0.097
Preengraftment PC[‡]	1.74 (0.88–3.22)	0.107	1.19 (0.54–2.53)	0.652
Postengraftment PC[‡]	**4.29 (2.34–8.28)**	**<0.001**	**2.50 (1.25–5.22)**	**0.009**
Acute GVHD[‡]	1.27 (0.68–2.32)	0.443		
Bloodstream infection[‡]	1.69 (0.95–2.97)	0.071	1.62 (0.86–3.06)	0.132
Relapse or progression[‡]	**2.92 (1.60–5.20)**	**0.001**	**2.68 (1.29–5.48)**	**0.008**
Shannon diversity index <1.5 at baseline[§]	0.96 (0.40–1.96)	0.907		
γ-Proteobacteria domination[‡]	**4.34 (1.98–8.60)**	**0.001**	**3.52 (1.28–9.21)**	**0.016**
Enterococcus domination[‡]	1.44 (0.82–2.53)	0.207		
Streptococcus domination[‡]	1.35 (0.74–2.39)	0.323		
Vancomycin[‡ǁ]	1.67 (0.57–8.10)	0.393		
β-Lactam[‡¶]	2.42 (0.82–11.74)	0.119	1.67 (0.51–8.53)	0.435
Fluoroquinolone[‡]	**1.90 (1.05–3.65)**	**0.035**	1.12 (0.53–2.43)	0.767
Metronidazole[‡]	**1.88 (1.06–3.37)**	**0.029**	1.44 (0.73–2.87)	0.290
Azithromycin[‡]	**3.53 (1.99–6.43)**	**<0.001**	**2.84 (1.49–5.51)**	**0.001**

Definition of abbreviations: CI = confidence interval; HCT = hematopoietic stem cell transplantation; PC = pulmonary complication; PFT = pulmonary function test; GVHD = graft-versus-host disease.
Bold values indicate statistically significant *P* value <0.05.
*HCT comorbidity index groups: low (0–1), intermediate (2–3), or high (≥4) (30).
[†]Disease risk: American Society for Blood and Marrow Transplantation Request for Information Classification as low, intermediate, or high (29).
[‡]Time-dependent variables.
[§]Shannon diversity index takes into account number of species and relative abundance (33).
[ǁ]Vancomycin includes intravenous mode of administration only.
[¶]β-Lactams include cephalosporins, β-lactam–β-lactamase combinations, and carbapenems.

diminished virulence compared with *K. pneumoniae* (41), infection and direct lung injury may occur, especially in the compromised host (42). Conversely, the emergence of low diversity as an independent predictor of PCs only once antibiotics were included in the model is consistent with previous findings in this population (13) and may have biological relevance. Although diversity and domination states did not always occur in close proximity to the PC (an unavoidable limitation to our study design), changes in gut microbiota composition early during HCT might have affected long-term pulmonary health, as shown in association with other post-transplant outcomes (43).

We also observed a correlation between specific antibiotics and PCs, which is difficult to interpret and likely confounded by the context in which antibiotics were administered. For example, subjects who were considered to be at higher risk for

pneumonia might have received early empiric coverage of typical and atypical bacteria with fluoroquinolones and/or azithromycin before chest imaging or further diagnostic workup. It is also possible that the relationship between antibiotics and PCs was confounded by their associations with other microbiota taxa or characteristics that were not examined in this study.

Disordered respiratory microbiota, often dominated by γ-proteobacteria, have been associated with several chronic lung diseases (44–50) and complications of lung transplantation (51, 52). The origin of some lung communities has been speculated to derive from nonpulmonary sources, including the oral cavity, upper respiratory tract, and gastrointestinal system. In the absence of lung colonization or infection, several studies have shown that gut dysbiosis triggers inflammation in asthma and allergic airways diseases (14–16), cystic

fibrosis (17), and infection-mediated lung inflammation (18–21). Intestinal permeability may be implicated in the translocation of bacteria and/or microparticles, such as lipopolysaccharide, on the pathway to acute lung injury and sepsis-associated acute respiratory distress syndrome (53–55). Because of the emerging recognition of shared mucosal immune responses in these parallel systems, it is possible that changes in gut microbiota associated with treatment-induced mucosal damage elicit injurious lung inflammation, which may persist or recur after engraftment. Further investigation into these relationships will be important to inform novel mechanistic hypotheses and develop predictive risk tools.

Finally, we observed expected clinical risk factors to predict PCs and mortality, such as underlying disease relapse and/or progression and having a high HCTCI score. The latter is a validated summary

measurement of multisystem pretransplant comorbidities, including moderate or severe pulmonary impairment, which has been shown to predict mortality (30, 56). PCs have also been associated with HCT-related mortality throughout the literature (57–60), albeit using varied definitions and study designs. Although it is difficult to determine whether PCs proximal to death were causal in our study, their contribution is likely underestimated based on autopsy studies (59, 61). The strength of association between mortality and postengraftment PCs was also interesting. In a study of HCT recipients with bloodstream infections, postengraftment events were associated with worse outcomes, including death (62). "Late onset" noninfectious PCs, which may be subjected to diagnostic inaccuracy (63), have been associated with acute and chronic GVHD (35, 64, 65) and mortality (66). Many subjects in our study had multiple PCs, suggesting susceptibility to recurrent lung injury, incomplete resolution, and/or misdiagnosis and treatment of a previous PC. Most postengraftment PCs were empirically treated as infectious, but they may represent an infection-mediated alloimmune or inflammatory mechanism (67, 68). However, it is reassuring that a preengraftment PC may not be a harbinger of death, as supported by survival rates of HCT recipients in the intensive care unit with acute respiratory distress that developed prior to engraftment who are comparable to noncancer patients in that setting (69).

There are several limitations to this study. First, we used a broad clinical–radiographic definition of post-transplant PCs to maximize capture of poorly defined events. We used commonly accepted abnormal radiographic patterns (8, 9) in conjunction with clinical information, similar to the approach taken by Garcia and colleagues, to identify a "pneumonia syndrome" in patients with acute leukemia (70), and our PC yield compared well with reported estimates (3). However, the inclusion of all PCs into one primary endpoint, regardless of severity, distribution or diagnosis, could inflate or dilute the impact of PCs on mortality. Prospective characterization of clinical and radiographic features of PCs will improve insight into which processes portend the greatest risk of death.

Second, microbiota data were collected solely during transplantation admission, and therefore, they were not contemporaneous with later clinical events. Although PC data were identified retrospectively, the microbiota protocol was prospective in nature. We thus used time-dependent variables to address this issue.

Third, the small sample size limited the power to analyze specific measures of baseline pulmonary dysfunction (e.g., impaired diffusing capacity in predicting PCs), and overlapping variables within PFT and HCTCI assessments may have contributed. Lastly, although this cohort was representative of the HCT population at our center (10, 13), it might not reflect patient characteristics or clinical practice elsewhere.

In conclusion, gut microbiota composition, including low diversity and domination by γ-proteobacteria, during the early HCT process is important in determining post-transplant trajectory, including the occurrence of PCs and/or death. The interaction between PCs and engraftment suggests that the graft–lung relationship may play an important role, which may be mediated either directly or indirectly by gut microbiota composition. Further study is warranted to delineate mechanisms, identify risk factors, and design interventions that could improve outcomes for at-risk patients. ∎

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