ORIGINAL ARTICLE

Lung Allograft Microbiome Association with Gastroesophageal Reflux, Inflammation, and Allograft Dysfunction

Pierre H. H. Schneeberger[1,2,3,4*], Chen Yang Kevin Zhang[1,2,5*], Jessica Santilli[1,2,3], Bo Chen[6], Wei Xu[6], Youngho Lee[1,2,3], Zonelle Wijesinha[1,2,3], Elaine Reguera-Nuñez[1,2,3], Noelle Yee[1,2,3], Musawir Ahmed[3,5], Kristen Boonstra[5], Rayoun Ramendra[5], Courtney W. Frankel[7], Scott M. Palmer[7], Jamie L. Todd[7], Tereza Martinu[1,2,3,5‡], and Bryan Coburn[1,2,3‡]

[1]Department of Medicine, [2]Department of Laboratory Medicine & Pathobiology, and [6]Department of Biostatistics, Princess Margaret Cancer Centre, University of Toronto, Toronto, Ontario, Canada; [3]Department of Medicine and [5]Toronto Lung Transplant Program, University Health Network, Toronto, Ontario, Canada; [4]Swiss Tropical and Public Health Institute, University of Basel, Allschwil, Switzerland; and [7]Department of Medicine, Duke University Medical Center, Durham, North Carolina

ORCID IDs: 0000-0002-8586-4937 (P.H.H.S.); 0000-0003-4247-3693 (J.L.T.); 0000-0003-0150-4510 (B.C.).

Abstract

Rationale: It remains unclear how gastroesophageal reflux disease (GERD) affects allograft microbial community composition in lung transplant recipients and its impact on lung allograft inflammation and function.

Objectives: Our objective was to compare the allograft microbiota in lung transplant recipients with or without clinically diagnosed GERD in the first year after transplant and assess associations between GERD, allograft microbiota, inflammation, and acute and chronic lung allograft dysfunction (ALAD and CLAD).

Methods: A total of 268 BAL samples were collected from 75 lung transplant recipients at a single transplant center every 3 months after transplant for 1 year. Ten transplant recipients from a separate transplant center provided samples before and after antireflux Nissen fundoplication surgery. Microbial community composition and density were measured using 16S ribosomal RNA gene sequencing and quantitative polymerase chain reaction, respectively, and inflammatory markers and bile acids were quantified.

Measurements and Main Results: We observed a range of allograft community composition with three discernible types (labeled community state types [CSTs] 1–3). Transplant recipients with GERD were more likely to have CST1, characterized by high bacterial density and relative abundance of the oropharyngeal colonizing genera *Prevotella* and *Veillonella*. GERD was associated with more frequent transitions to CST1. CST1 was associated with lower inflammatory cytokine concentrations than pathogen-dominated CST3 across the range of microbial densities observed. Cox proportional hazard models revealed associations between CST3 and the development of ALAD/CLAD. Nissen fundoplication decreased bacterial load and proinflammatory cytokines.

Conclusions: GERD was associated with a high bacterial density, *Prevotella*- and *Veillonella*-dominated CST1. CST3, but not CST1 or GERD, was associated with inflammation and early development of ALAD and CLAD. Nissen fundoplication was associated with a reduction in microbial density in BAL fluid samples, especially the CST1-specific genus, *Prevotella*.

Keywords: lung microbiota; gastroesophageal reflux disease; bronchoalveolar lavage; chronic lung allograft dysfunction; lung allograft inflammation

Gastroesophageal reflux disease (GERD) is common after lung transplantation and is characterized by reflux of gastric contents, including gastric acid, mucus, digestive enzymes, and bile acids. This refluxate can be aspirated, leading to lung allograft injury and inflammation. We recently reported an association between GERD, concentrations of taurocholic acid (TCA; a bile acid) and inflammatory markers in the BAL fluid (BALF), and acute lung allograft dysfunction (ALAD) at 3 months after transplant (1).

(Received in original form October 25, 2021; accepted in final form July 22, 2022)

*These authors contributed equally to this work.

‡These authors contributed equally to this work.

Supported by Cystic Fibrosis Canada (B.C. and T.M.); Comprehensive Research Experience for Medical Students (CREMS) (C.Y.K.Z.); Clinical Trials in Organ Transplantation (CTOT-20) (T.M.); Institute of Circulatory and Respiratory Health (PJT149057 [B.C. and T.M.]); National Institute of Allergy and Infectious Diseases; and the Multi-Organ Transplant Program at the University Health Network (B.C. and T.M.).

Am J Respir Crit Care Med Vol 206, Iss 12, pp 1495–1507, Dec 15, 2022

Originally Published in Press as DOI: 10.1164/rccm.202110-2413OC on July 23, 2022
Internet address: www.atsjournals.org

The association between GERD and chronic lung allograft dysfunction (CLAD) (the leading cause of death in the late posttransplant period [2]) is inconsistent, with some studies indicating GERD is a risk factor for CLAD (3, 4).

The composition of microbial communities in the lung allograft after transplantation varies between individuals and is associated with CLAD (5, 6) and acute inflammation (7, 8). The composition of the allograft microbial community may follow dynamics similar to those presented in the ecological concept of island biogeography (9), in which community composition is the product of immigration and extinction rates (10, 11). These rates, as well as the relative rates of proliferation of different bacterial species, may be affected by GERD because of factors such as influx rates or variation in airway nutrient composition or pH.

In BALF, microbial communities dominated by *Proteobacteria* or *Firmicutes* are associated with inflammation, and *Bacteroidetes* domination is associated with markers of airway remodeling (7, 8). We reasoned that allograft microbial community composition, GERD, and inflammation may be associated in the posttransplant period and that models incorporating both GERD and community composition may predict inflammation, ALAD, and CLAD better than models including only individual predictors.

To address this, we compared lung allograft microbial community composition from BALF between individuals with and without GERD in the first year after transplant and assessed GERD/microbial community/inflammation associations, including longitudinal comparisons. We assessed GERD and microbial community composition as predictors of inflammation, ALAD, and CLAD in this cohort. Finally, we measured changes in microbial density and inflammatory cytokine concentrations before and after Nissen fundoplication in a second cohort of lung transplant recipients to determine whether surgical treatment of GERD altered microbial density and inflammation.Some of the results of these studies have been previously reported in the form of a preprint (bmedRxiv, [10 September 2021] https://doi.org/10.1101/2021.09.03.21263067).

Methods

Cohort Design

This study was approved by the Toronto University Health Network Research Ethics Board (15–9698-AE) and the Duke University Internal Review Board (Pro00013378).

GERD cohort (Figure E1 in the online supplement). Patients were selected from a previously-published GERD cohort (1), which drew from subjects who underwent lung transplantation between 2010 and 2015 and GERD testing 1.5–12 months after transplant and included 25 patients with GERD (48 or more reflux episodes per 24 h) and 51 no-GERD control subjects (23 or fewer reflux episodes per 24 h). A total of 24 patients with GERD and 51 no-GERD patients were included in this present study on the basis of having at least one available raw BALF sample obtained in the first year after transplant.

Nissen cohort. Patients were selected from a previously published Nissen cohort of 18 patients who underwent lung transplantation between 2005 and 2008 and Nissen fundoplication within 6 months after transplant (1). Ten patients were included on the basis of having sufficient before and after Nissen BALF supernatant remaining for analysis.

Clinical Standards of Care and Definitions

Standard of care for lung transplant recipients was delivered as described previously by the Toronto and Duke programs (1, 12, 13). ALAD was defined as a $\geqslant$10% decline in measured FEV_1 compared with the higher of the two preceding FEV_1 measurements (1), consistent with prior definitions of spirometric stability (14, 15). CLAD was defined as per the latest consensus report from the International Society for Heart and Lung Transplantation (2). Outcomes were censored on February 28, 2019 (range of follow-up, 4–9 yr).

BALF Sample Processing

In Toronto, raw (unprocessed) BALF samples were aliquoted and stored at −80°C. The remaining BALF was centrifuged at 3,184 g for 20 minutes, and the supernatant was also stored at −80°C. At Duke, BALF samples were centrifuged at 1,750 g for 10 minutes at 4°C, and the supernatant was stored at −80°C.

Analysis of BALF Supernatant Samples

Markers of innate immune activation (IL-1α, IL-1β, IL-6, and IL-8) were measured in the BALF supernatant using a custom-designed multiplex assay (R&D), as reported previously (1). TCA, glycocholic acid (GCA), and cholic acid, some of the most abundant bile acids, were measured using liquid chromatography with tandem mass spectrometry, as reported previously (1).

Author Contributions: P.H.H.S.: research design, experiments, statistical analyses, figure generation, writing of the initial manuscript, and manuscript editing; C.Y.K.Z.: cohort design, cohort organization, clinical data collection, patient phenotyping, sample organization, multiplex data analysis, and writing and editing the manuscript; J.S. statistical analyses, figure generation, writing of the initial manuscript, and manuscript editing; B.C. and W.X.: statistical analyses and manuscript editing; Y.L.: experiments (presequencing workup, DNA isolation, and qPCR) and manuscript editing; Z.W.: sample organization, experiments for Nissen cohort (DNA isolation and 16S qPCR quantification), and qPCR data analysis; E.R.-N. and N.Y.: sample processing and manuscript editing; M.A.: cohort design, cohort organization, clinical data collection, patient phenotyping; K.B.: multiplex cytokine assays; R.R.: clinical data collection and curation; C.W.F.: cohort organization, clinical data collection, and sample organization (for Nissen cohort); S.M.P. and J.L.T.: cohort design, clinical data collection, and patient phenotyping; T.M.: research design, project supervision, cohort design, data analysis, and manuscript writing and editing; and B.C.: research design, project supervision, data analysis, and manuscript writing and editing.

Sequence data that support the findings of this study have been deposited in the NCBI Short Read Archive with the primary accession code PRJNA754787.

Correspondence and requests for reprints should be addressed to Tereza Martinu, M.D., Department of Medicine, University Health Network, 585 University Avenue, Toronto, ON, M5G 2N2 Canada. E-mail: tereza.martinu@uhn.ca.

This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the Subject: Compositional differences in the lung microbiota have been associated with outcomes of lung transplantation. While our understanding of microbiome–allograft–host interactions is progressing, there is little knowledge regarding parameters that can affect the composition of lung microbial communities. Gastroesophageal reflux disease (GERD) may modulate the immigration and/or proliferation of microbes in the lung, thus influencing pulmonary microbiome composition and microbe–allograft interactions.

What This Study Adds to the Field: In this study, we performed a longitudinal assessment of the lung microbiota in a cohort of lung transplant recipients with and without GERD. We identified three main microbial community structure types similar to those reported previously. We found that patients with GERD were more likely to have a lung microbial community enriched in oropharyngeal taxa, including *Prevotella* and *Veillonella*. Pathogen-dominated communities observed in this cohort were associated with increased concentrations of proinflammatory markers, while the GERD-associated community type was not associated with higher degrees of inflammation. Finally, we found that patients with GERD had increased bacterial variability over the first year after transplant and that antireflux surgery was associated with decreased bacterial load in the lungs. Overall, these data suggest that GERD is an important modulator of the lung allograft microbiome and host responses after transplantation.

DNA Isolation and Quantification from BALF Samples

Nucleic acids were isolated from 250 μl of raw BALF samples (Toronto) or BALF supernatant (Duke) using a PowerSoil DNA isolation kit (MO-BIO) following the manufacturer's instructions. Bacterial density was measured using a 16S quantitative polymerase chain reaction (qPCR) (16).

16S Ribosomal RNA (rRNA) Gene Sequencing of DNA from Raw BALF Samples

The V4 hypervariable region of the 16S rRNA gene is amplified using a universal forward sequencing primer and a uniquely barcoded reverse sequencing primer to allow for multiplexing (17). Sequencing was performed using the V2 (150 bp × 2) chemistry on an Illumina Miseq sequencer.

Analysis of the Bacterial Microbiome

The UNOISE pipeline was used for sequence analysis (18–20). Taxonomy assignment was executed using SINTAX (21) available through USEARCH v11.0.667 and the UNOISE compatible Ribosomal Database Project database version 16, with a minimum confidence cutoff of 0.8 (22). Operational taxonomic unit sequences were aligned using align_seqs.py v.1.9.1 through Quantitative Insights Into Microbial Ecology (23).

Postprofiling Filtering Approaches

16S rRNA gene sequences from contaminants is a recurrent issue when analyzing BALF samples (24), and we controlled for sequencing contaminants as described in Schneeberger and colleagues (25).

Statistical Analysis

Bray-Curtis dissimilarity indices and non-metric multidimensional scaling ordination plots were generated using the Vegan R package (26). Random Forest analysis was conducted using the randomForest package (27). Generalized estimating equations were done with the geepack R package (28). Taxonomic differences between community state types (CSTs) were identified using the LEfSe pipeline (29).

Results

Cohort Features

Baseline patient characteristics of the GERD cohort were comparable between patients with GERD ($n = 24$) and no-GERD control subjects ($n = 51$) (Table 1), and inclusion/exclusion criteria are reported in Figure E1. Transbronchial biopsy pathology results (A-grades and B-grades), BALF culture results, CLAD diagnoses, and follow-up times are reported for each patient in Figure E2. Baseline characteristics of Nissen cohort patients are reported in Table 2.

Comparison of Bacterial Community Composition in BALF Between Lung Transplant Recipients With and Without GERD

We first assessed bacterial community composition by 16S rRNA gene sequencing (Figure 1). Using a Bray-Curtis dissimilarity matrix, we identified three compositional extremes of community composition among all collected samples, which we labeled CST 1–3 (Figure 1A). We adopted the term "community state types" from microbial ecology terminology and descriptions of the female genital tract microbiota (30) to describe a set of samples clustering together on the basis of their microbial composition. GERD cases and no-GERD control subjects had distinguishable community composition distributions (permutational multivariate analysis of variance [PERMANOVA] $P < 0.001$) (Figure 1B), but GERD status explained only a small amount of compositional variance ($R^2 = 0.013$). When CST was assigned as a categorical variable, the proportion of GERD cases with CST1 was greater than that of no-GERD control subjects, whereas the proportion of CST2 was less ($P = .014$; odds ratio [OR], 2.4; 95% confidence interval [CI], 1.2–4.4 for CST1). A similar proportion of patients in both groups presented CST3 ($P = 0.12$; OR, 1.7; 95% CI, 0.9–3.2 for CST1) (Figure 1C). To assess the effect of primary transplant indication on microbial composition, we performed a PERMANOVA analysis (Figure E3A) and compared α diversity indices (Figure E3B) between transplant indications at 3 months after transplant. There were no significant differences at the community level when comparing the lung microbiota of patients with different transplant indications.

Bacterial density and within-sample (α) diversity differed by CST (Figure 2), as expected because CSTs were defined on the basis of the composition. CST1 was characterized by the high relative abundance of oropharyngeal taxa, including *Prevotella* and *Veillonella*. The genera *Streptococcus* and *Tanerella* were significantly enriched in CST2, while CST3 was characterized by an enrichment of genera with commonly pathogenic species *Pseudomonas* and *Staphylococcus* (Figure 2A). The BALF CSTs were distinguished most strongly by the

Table 1. Baseline Patient Characteristics of the Main Cohort

Characteristic	GERD ($n = 24$)	No GERD ($n = 51$)	*P* Value
Recipient age at transplant, yr	—	—	0.23
Mean ± SD	54 ± 14	57 ± 12	—
Range	19–70	22–75	—
Male, *n* (%)	14 (58)	29 (57)	1.00
Native lung disease, *n* (%)	—	—	0.98
Chronic obstructive pulmonary disease	4 (17)	10 (20)	—
Cystic fibrosis	2 (8)	6 (12)	—
Pulmonary fibrosis	12 (50)	24 (47)	—
Other	6 (25)	11 (22)	—
Transplant type, *n* (%)	—	—	1.00
Single lung	3 (13)	7 (14)	—
Double lung	24 (87)	44 (86)	—
Donor (D) recipient (R) CMV status, *n* (%)	—	—	0.85
D^-/R^-	3 (13)	11 (22)	—
D^-/R^+	7 (29)	14 (27)	—
D^+/R^-	6 (25)	12 (24)	—
D^+/R^+	8 (33)	14 (27)	—
BALF samples available by time point, *n*	—	—	—
3 mo	23	51	—
6 mo	20	43	—
9 mo	20	49	—
12 mo	19	45	—
24-h pH impedance testing, median *n* (IQR)	—	—	—
Total reflux episodes	66 (57–71)	8 (5–15)	<0.001
Proximal reflux episodes	38 (26–54)	5 (2–8)	<0.001
On PPI at the time of GERD testing, *n* (%)	23 (96)	46 (90)	0.66

Definition of abbreviations: BALF = BAL fluid; CMV = cytomegalovirus; GERD = gastroesophageal reflux disease; PPI = proton pump inhibitors.

genera *Prevotella*, *Veillonella*, *Streptococcus*, *Pseudomonas*, and *Staphylococcus* with mean decreases of classification accuracy of 0.102, 0.041, 0.025, 0.02, and 0.018 in a Random Forest model that omitted these taxa, respectively (model classification accuracy = 82% and Cohen's Kappa = 73.9%). A comparison of the composition by GERD status recapitulated the differences observed between CSTs (Figure E4). Indeed, taxa enriched in GERD-associated CST1 were also mostly found to be enriched in patients with GERD.

The median bacterial density (16S rRNA gene copies/ml BALF) was approximately 10-fold higher in CST1 than in CST2 or 3, as was the absolute abundance of the CST1-associated genus *Prevotella* as measured by 16S rRNA gene and *Prevotella*-specific qPCR, respectively (Figure 2B). Although taxonomic richness was highest in CST1, composite (Shannon) diversity was lower than CST2 and 3 because of the high relative abundance of *Prevotella*. CST2 had the greatest evenness/lowest tendency toward dominated communities, while CST3 was characterized by high variability in density, diversity, and taxonomic dominance, with many samples highly dominated by a single taxon (Figure 2C). The most abundant genera in the highly dominated communities in CST3 were *Staphylococcus* and *Pseudomonas*. Thus, CST1 seems to represent a high bacterial density state dominated by oropharyngeal taxa, CST2 a low bacterial density state, and CST3 a variable density state commonly characterized by dominance with pathogenic taxa. Although the proportion of samples in each CST differed by GERD status, compositional differences between samples with and without GERD within CSTs were not observed.

Longitudinal comparisons in the first year after transplant. We next assessed whether bacterial density, α diversity, CST membership, and longitudinal stability differed between GERD cases and noGERD control subjects. Using a generalized estimating equation model, we assessed stability in bacterial density over time and observed that patients with GERD had variability in microbial density and patients without GERD were stable (coefficient of correlation for patients with GERD: $\rho = 0.165$; $P = 0.332$; and for patients without GERD: $\rho = 0.153$; $P = 0.01$; a positive correlation coefficient (ρ) and a $P < 0.05$ indicate stability over time) (Figure 3A). Shannon diversity index (SDI) and Berger-Parker (BP) dominance were consistent over the first year in patients without GERD ($\text{mean}_{\text{SDI[no-GERD]}} = 2.35$, 95% CI, 2.26–2.45; $\text{mean}_{\text{BP[no-GERD]}} = 0.33$; 95% CI, 0.31–0.36). In patients with GERD, both composite metrics indicated higher dominance and decreased Shannon diversity when compared with control subjects without GERD at 3 months (MW; $\text{mean}_{\text{SDI[GERD]}} = 1.98$; $\text{mean}_{\text{BP[GERD]}} = 0.42$; $P = 0.007$ and 0.025, respectively) but not at later time points ($\text{mean}_{\text{SDI[GERD]}}$ (6–12 mo) = 2.32; 95% CI, 2.18–2.48; $\text{mean}_{\text{BP[GERD]}}$ (6–12 mo) = 0.33; 95% CI, 0.29–0.36) (Figure 3B), indicating that recovery of microbial diversity is delayed in patients with GERD.

Compositional variability over time was greatest in both groups at early sampling intervals (3–6 mo) and stabilized over time (Figure 3C). While transitions between CSTs were common in both groups, CST1 appeared to be more stable in patients with than without GERD (Figure 3D). Transitions to CST1 were associated with significant increases in absolute total bacterial abundance (Figure 3E) and absolute

Table 2. Characteristics of Patients Undergoing Nissen Fundoplication to Treat Gastroesophageal Reflux Disease

Characteristic	Nissen cohort ($N = 10$)
Recipient age at transplant, yr	
Mean ± SD	47 ± 14
Range	18–65
Male, *n* (%)	2 (20)
Native lung disease, *n* (%)	
Chronic obstructive pulmonary disease	3 (30)
Cystic fibrosis	3 (30)
Pulmonary fibrosis	0 (0)
Other	4 (40)
Days from transplant to Nissen, mean ± SD	70 ± 29

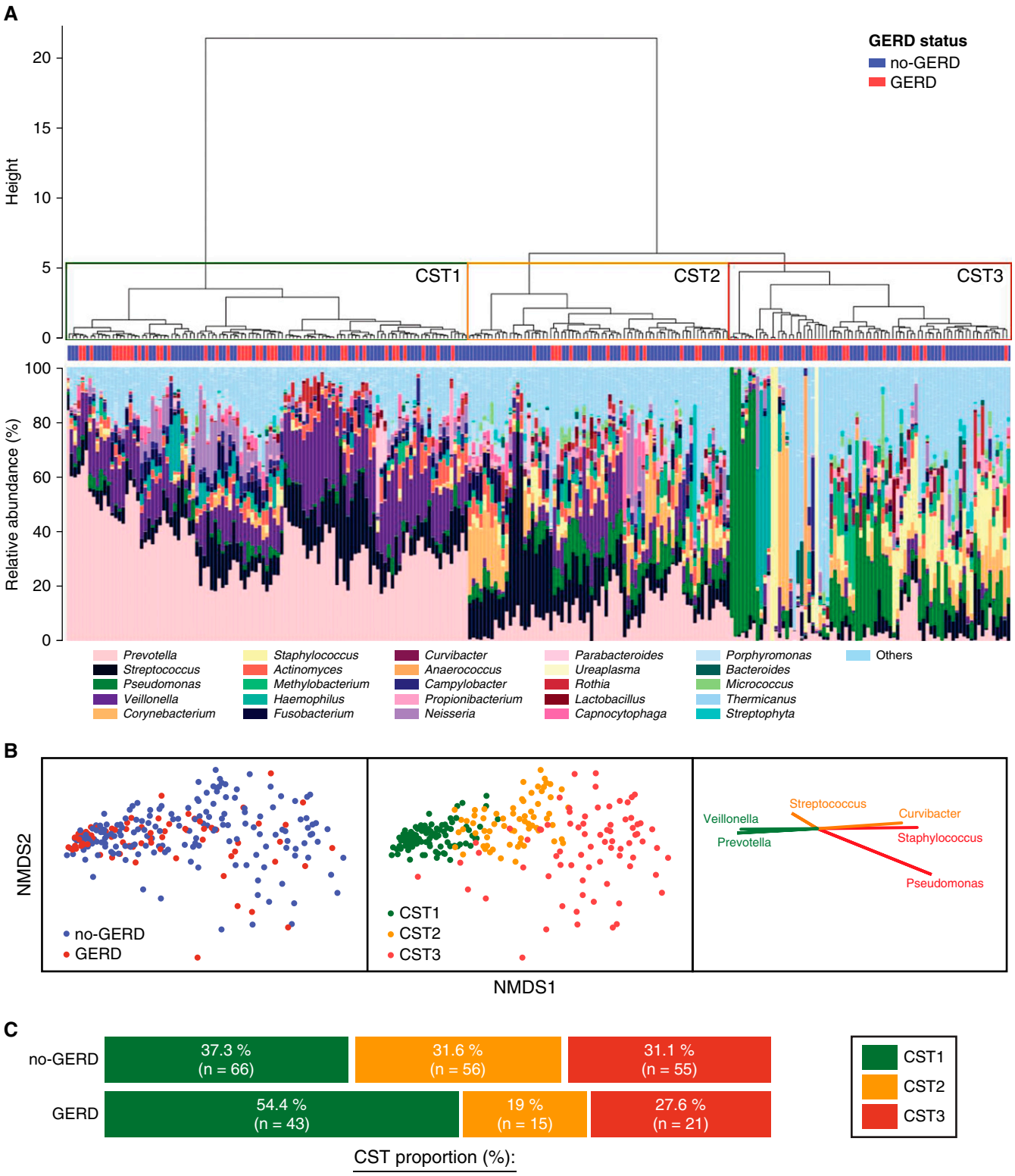

Figure 1. Composition of the lung microbiota in patients after transplant with and without gastroesophageal reflux disease (GERD) analyzed by 16S ribosomal RNA gene sequencing. (*A*) Classification of BAL fluid samples on the basis of Bray-Curtis dissimilarity measure. (*B*) Non-metric multidimensional scaling (NMDS) plots on the basis of Bray-Curtis dissimilarity measure comparing spatial ordination of patients with GERD versus without GERD, community state type (CST) 1–3 samples, and showing genera contributing most to this classification system. (*C*) Proportion of CSTs by GERD status.

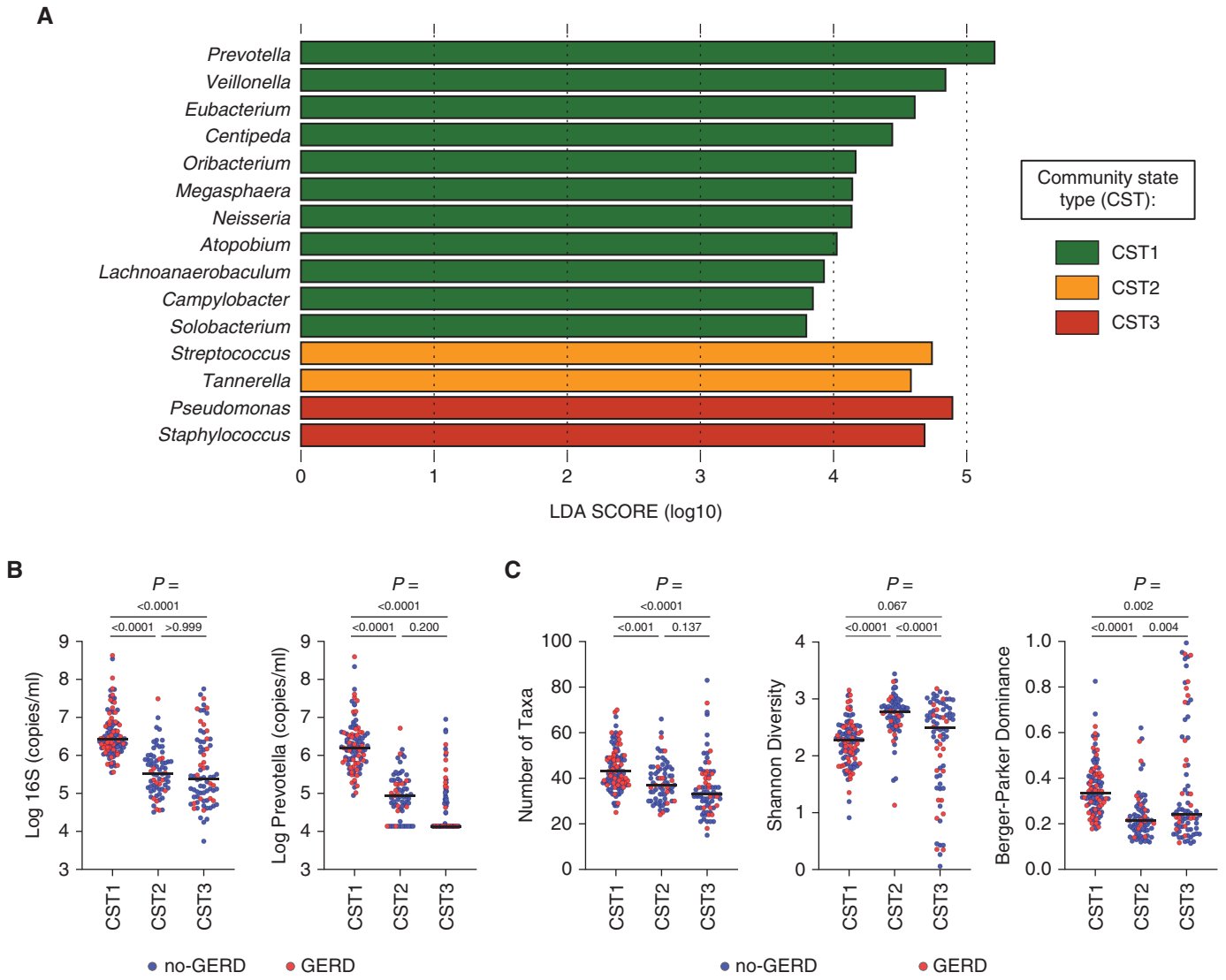

Figure 2. Microbial features of community state types in BALF samples of lung transplant recipients. (*A*) Genera enriched in each community state type (CST), identified using the LefSe (linear discriminant analysis [LDA] effect size [28]) pipeline. A higher LDA score indicates a higher difference between groups. (*B*) Comparison of total bacterial density on the basis of 16S ribosomal RNA gene copies/ml BALF and *Prevotella*-specific quantitative polymerase chain reaction by CST. (*C*) Comparison of α diversity indices by CST. Group comparison was tested using Kruskal-Wallis tests with *post hoc* Dunn tests. BALF = BAL fluid; GERD = gastroesophageal reflux disease.

abundance of the CST1-associated genus *Prevotella* (Figure 3F). Conversely, transitions from CST1 were associated with significant decreases in absolute total bacterial and *Prevotella* abundance. Transitions between CST2 and 3 were not associated with changes in absolute bacterial abundance or absolute abundance of *Prevotella*. Instability in bacterial abundance in patients with GERD during the first year is thus driven by transitions to and from CST1.

Association with bile acids. As previously reported (1), 3 months after transplant, TCA concentrations were significantly higher in individuals with GERD compared without GERD (Mann-Whitney; $P = 0.023$), whereas there were no differences in GCA or CA concentrations. At 3 months, CSTs were not significantly associated with bile acid concentrations (Figure E5). Concentrations of TCA were negatively correlated with *Actinomyces*, *Propionibacterium*, and *Porphyromonas* (Figure E6).

GERD and CST as Predictors of Inflammation, ALAD, CLAD, and Death

We have previously reported elevated inflammatory cytokine concentrations in patients with GERD, although no differences in rates of ALAD or CLAD on the basis of GERD status (1). We sought to assess whether the addition of microbial community composition and density (CST and 16s rRNA gene copy density) affected associations between allograft inflammation, ALAD, and CLAD.

Inflammation. 16S density was strongly associated with individual proinflammatory cytokine concentrations independently of GERD status (Figure 4A). Inflammation was defined as having at least two out of four proinflammatory cytokines (IL-1α, IL-1β, IL-6, and IL-8) in the 75th percentile on the basis of a similar previously published

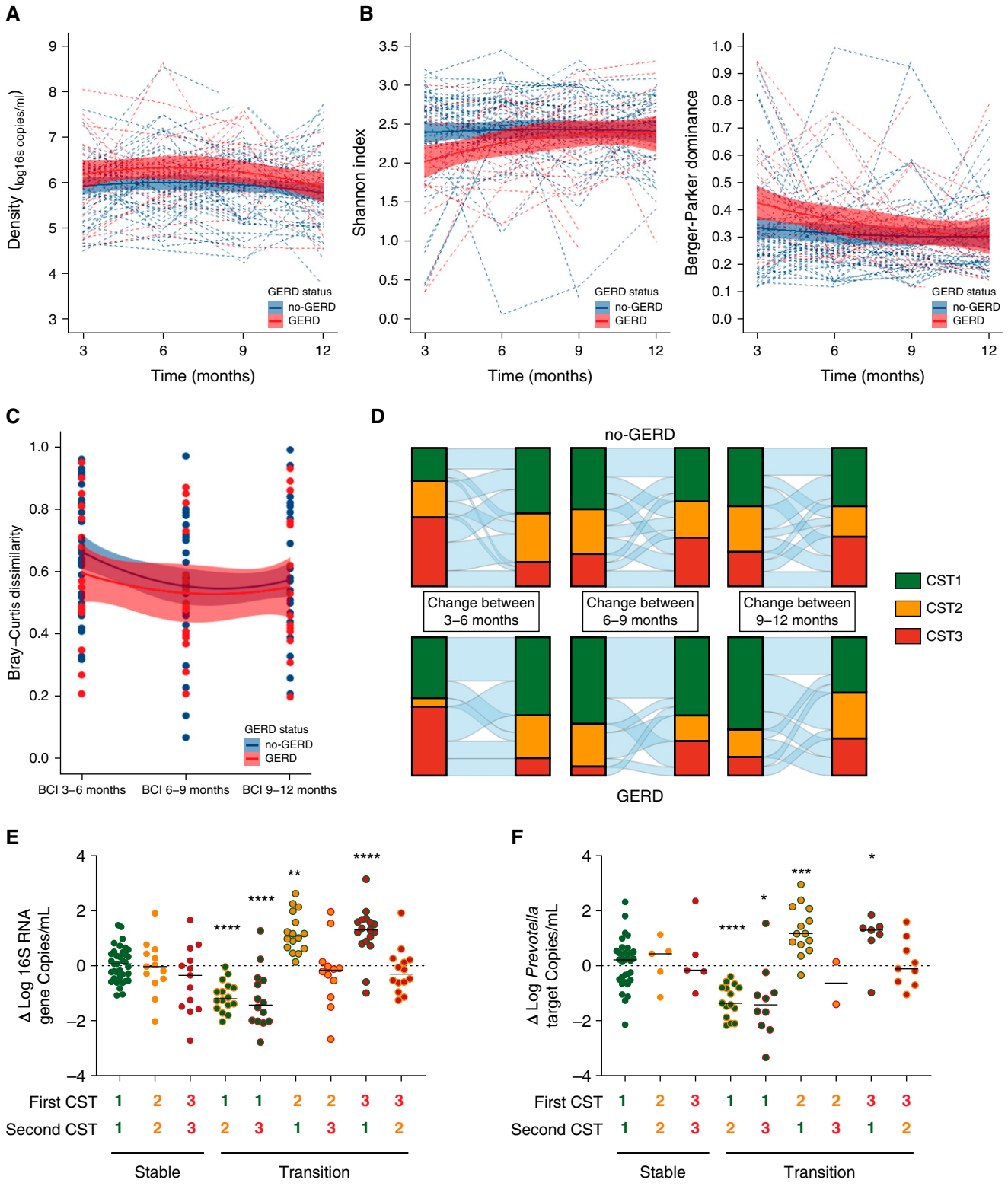

Figure 3. Analysis of the longitudinal features of the lung microbiota 1 year after transplantation by gastroesophageal reflux disease (GERD) status. (*A*) Comparison of total bacterial density variation by GERD status. (*B*) Comparison of α diversity indices changes over time by GERD status. (*C*) Patient-specific variation was measured using the Bray-Curtis dissimilarity measure between consecutive samples by GERD status. (*D*) Transitions of community state types (CSTs) between 3 and 6, 6 and 9, and 9 and 12 months after transplant, by GERD status. (*E*) Changes in total bacterial density associated with a transition to the different CSTs. (*F*) Changes in *Prevotella* density associated with CST transitions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. BCI = Bray-Curtis Index.

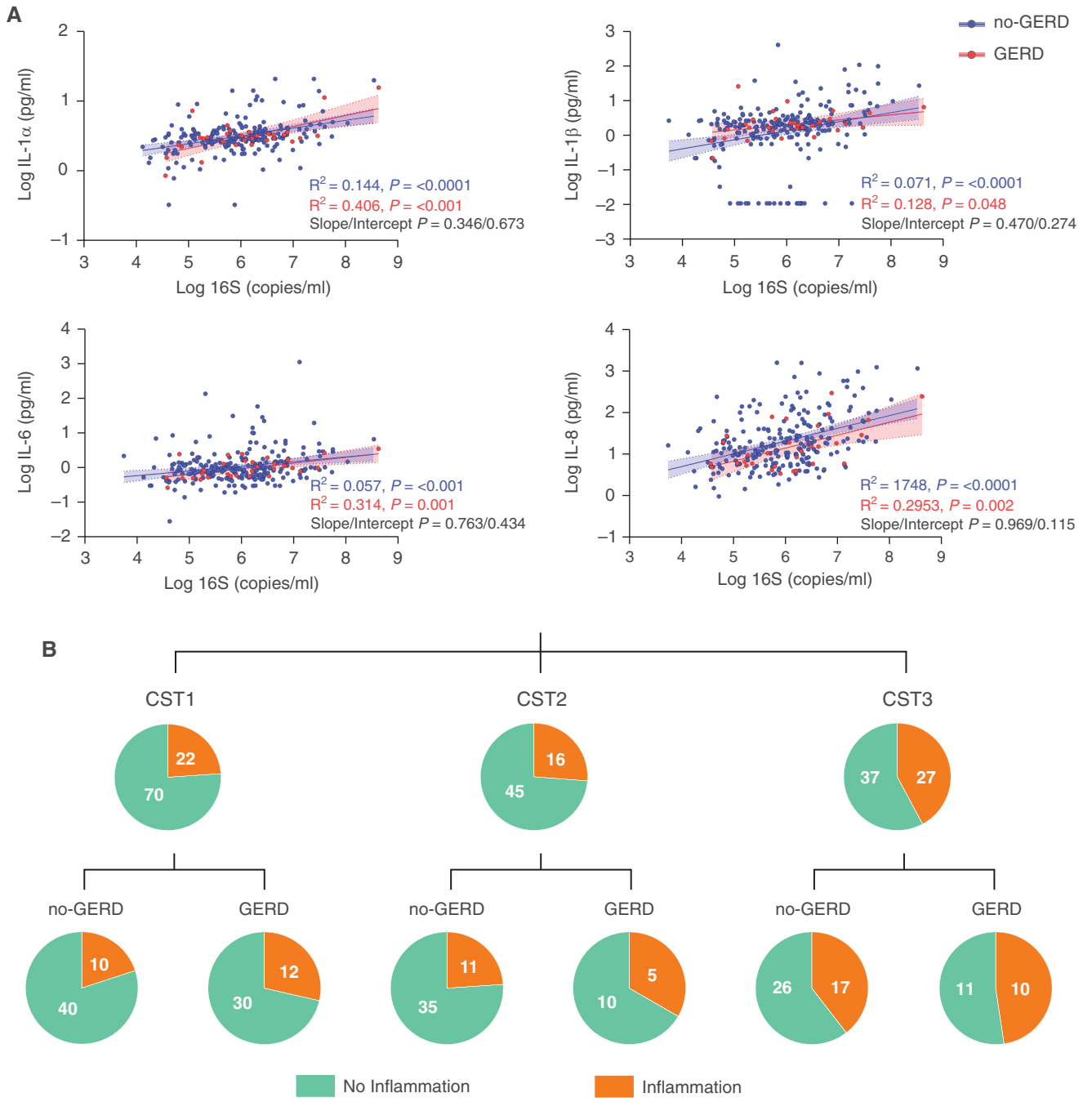

Figure 4. Associations between gastroesophageal reflux disease (GERD) status, microbial composition and density, and inflammation in BAL fluid of lung transplant recipients. (*A*) Linear regression analysis of total bacterial density and individual proinflammatory cytokines. (*B*) Proportion of samples with inflammation (defined by two or more cytokines in the top 75th percentile) by community state type (CST) and further stratified by GERD status.

approach (31). Samples with and without inflammation showed distinct distributions of proinflammatory cytokine concentrations (Figure E7A). Inflammation was associated with higher bacterial density at 3, 6, and 12 months after transplant (Figure E7B). Despite bacterial density being significantly higher in patients with CST1, the proportion of patients with inflammation was significantly higher in patients with CST3 when compared with CST1 (Fisher's Exact OR, 2.3; 95% CI, 1.2–4.7; $P = 0.022$), with a nonsignificant difference in the CST3–inflammation relationship when compared with CST2 (OR, 2.1; 95% CI, 1.0–4.4; $P = 0.090$). There were no differences in the proportion of samples with inflammation from patients with and without GERD within CST1 (Fisher's Exact OR, 1.6; 95% CI, 0.6–4.0; $P = 0.462$), CST2 (Fisher's Exact OR, 1.6; 95% CI, 0.5–5.3;

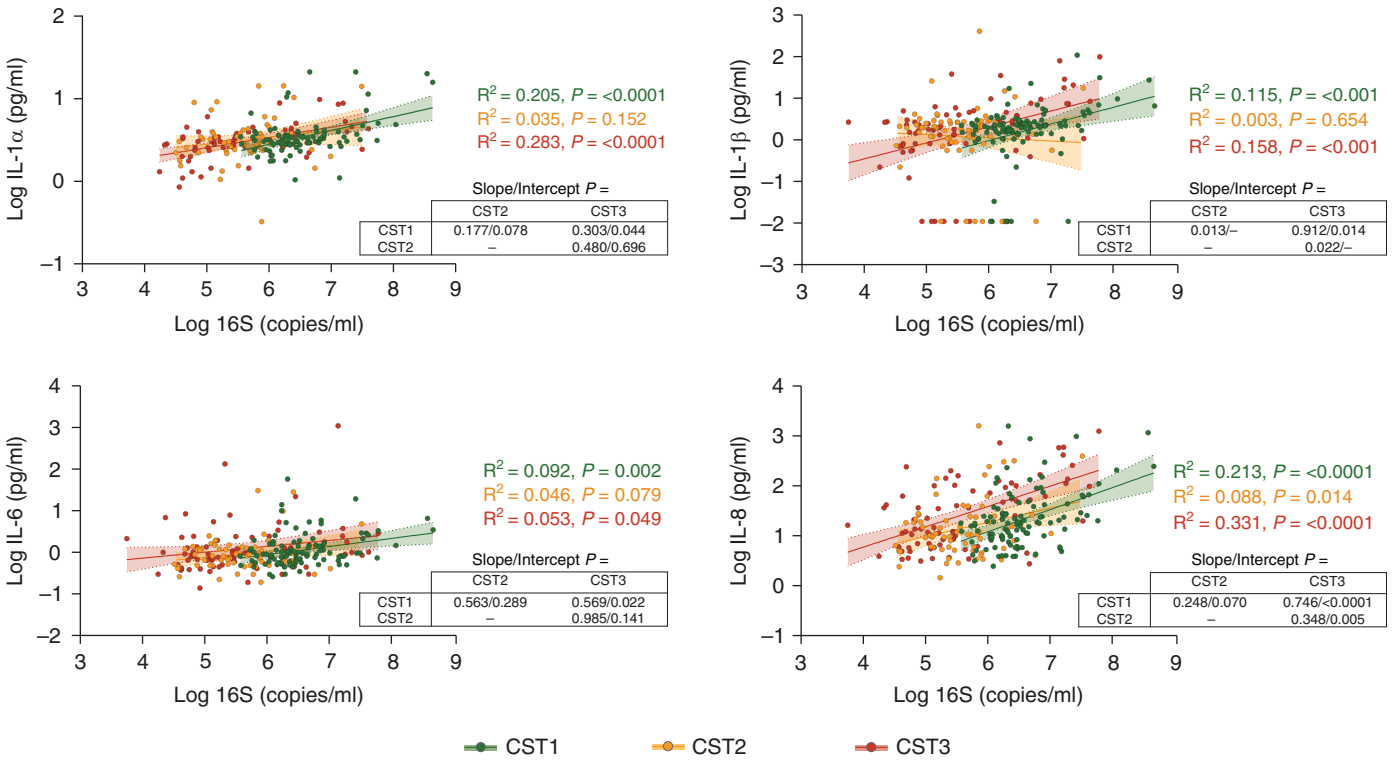

Figure 5. Linear regression analysis of total bacterial density and individual proinflammatory cytokines, stratified by community state type (CST) in lung transplant recipients (CST1 = green; CST2 = yellow; and CST3 = red).

$P = 0.510$), and CST3 (Fisher's Exact OR, 1.4; 95% CI, 0.5–3.9; $P = 0.596$) (Figure 4B). A comparison of individual proinflammatory cytokines showed no differences between CSTs, with the exception of CST2, which was associated with lower IL-8 concentrations compared with CST3 (Figure E7C). Notably, while bacterial density–inflammation correlations were independent of GERD diagnosis, they differed significantly by CST (Figure 5), and correlations between CST3 and inflammatory markers were observed starting at a lower bacterial density (measured by 16S qPCR) compared with CST1 and CST2. All four proinflammatory cytokines (IL-1α, IL-1β, IL-6, and IL-8) were higher in CST3 compared with CST1 for the same bacterial density (*P* values for differences of regression intercepts between CSTs were 0.044, 0.014, 0.022, and <0.0001, respectively). This may, in part, explain why, although GERD is associated with higher and more variable bacterial density in the first transplant year, it is not associated with increased inflammation in this dataset.

ALAD, CLAD, and death. At 3 months, IL-1α and IL-8 concentrations were significantly associated with ALAD (logistic regression OR, 2.12; 95% CI, 1.4–407.1; $P = 0.034$; OR, 4.0; 95% CI, 1.4–13.5; $P = 0.003$, respectively), but not IL-1β and IL-6. Inflammation as a binary variable (as defined above) was also significantly associated with ALAD (Fisher's Exact OR, 4.7; 95% CI, 1.0–18.8; $P = 0.050$). A diagnosis of GERD was not significantly associated with concurrent ALAD (Fisher's Exact OR, 1.4; 95% CI, 0.4–6.5; $P = 0.692$). Increased bacterial burden was also not associated with ALAD (logistic regression OR, 1.2; 95% CI, 0.5–3.2; $P = 0.587$). However, ALAD was more common in patients with CST3 than with CST1 (Fisher's Exact OR, infinity; 95% CI, 1.5–infinity; $P = 0.030$) but not different from CST2 (Fisher's Exact OR, 1.8; 95% CI, 0.3–9.3; $P = 0.694$). A Cox proportional hazards model was used to assess the association between GERD status and the number of CST3 events at different intervals with time to CLAD and death, as shown in Table 3. One patient was excluded from this analysis because of missing metadata. GERD status was not associated with CLAD. However, patients with higher cumulative numbers of CST3 events at 6, 9, and 12 months after transplant were more likely to develop CLAD compared with CST1 or CST2, even when adjusted for sex, age at transplant, cytomegalovirus serostatus mismatch, and primary disease. This relationship was also seen at 3 months after transplant when adjusted for sex and cytomegalovirus mismatch. GERD status and the number of CST3 events were not significantly associated with death.

Association of Nissen Fundoplication with Inflammatory Cytokines and Bacterial Density

In a cohort of 10 patients who underwent Nissen fundoplication for GERD, we assessed bacterial load and proinflammatory cytokine concentrations in BALF supernatants before and after the procedure. Similar to our previous report (1), significant decreases in proinflammatory cytokines were observed after fundoplication (Figure 6A). While no significant changes were observed in overall bacterial loads before and after fundoplication, individuals with the greatest decreases in inflammatory cytokines also had the greatest decreases in bacterial density as measured by 16S rRNA gene qPCR and had the highest baseline densities of the CST1-associated genus *Prevotella*. This suggests that fundoplication may have direct or

Table 3. Cox Proportional Hazards Associated with Gastroesophageal Reflux Disease and Events of Community State Type 3

Outcome	Predictor	HR (95% CI) GERD Status (GERD)	*P* Value	HR (95% CI) CST3 Samples at 3 mo	*P* Value	HR (95% CI) CST3 Samples Up to 6 mo	*P* Value	HR (95% CI) CST3 Samples Up to 9 mo	*P* Value	HR (95% CI) CST3 Samples Up to 12 mo	*P* Value
CLAD		*n* = 74, EV = 21		*n* = 67, EV = 20		*n* = 72, EV = 19		*n* = 70, EV = 19		*n* = 69, EV = 18	
		0.55 (0.20–1.51)	0.24	1.99 (0.82–4.87)	0.12	2.01 (1.08–3.75)	0.02	1.77 (1.05–2.97)	0.03	1.67 (1.01–2.74)	0.04
	Adjusted for										
	Sex (M)	0.53 (0.19–1.46)	0.22	2.43 (0.96–6.13)	0.06	2.56 (1.29–5.07)	0.01	2.11 (1.21–3.68)	0.01	1.76 (1.08–2.85)	0.02
	Age at tx, yr	0.41 (0.14–1.20)	0.11	1.87 (0.76–4.61)	0.17	1.88 (0.98–3.63)	0.05	1.68 (0.97–2.91)	0.06	1.57 (0.95–2.61)	0.07
	CMV mismatch (Yes)	0.55 (0.20–1.51)	0.24	2.20 (0.87–5.53)	0.09	2.05 (1.10–3.82)	0.02	1.76 (1.05–2.97)	0.03	1.67 (1.01–2.78)	0.04
	Primary disease	0.46 (0.16–1.29)	0.14	1.89 (0.75–4.76)	0.17	1.82 (0.96–3.46)	0.06	1.56 (0.93–2.62)	0.09	1.55 (0.94–2.54)	0.08
DEATH		*n* = 74, EV = 19		*n* = 67, EV = 17		*n* = 72, EV = 17		*n* = 70, EV = 15		*n* = 69, EV = 14	
		0.37 (0.11–1.28)	0.12	1.30 (0.50–3.38)	0.59	1.35 (0.66–2.73)	0.41	1.21 (0.66–2.22)	0.54	1.26 (0.72–2.23)	0.42
	Adjusted for										
	Sex (M)	0.36 (0.11–1.26)	0.11	1.51 (0.57–4.04)	0.41	1.50 (0.71–3.19)	0.29	1.31 (0.70–2.44)	0.39	1.29 (0.75–2.26)	0.36
	Age at tx, yr	0.36 (0.10–1.29)	0.12	1.29 (0.49–3.40)	0.6	1.49 (0.69–3.22)	0.31	1.25 (0.66–2.35)	0.49	1.27 (0.71–2.26)	0.41
	CMV mismatch (Yes)	0.37 (0.11–1.29)	0.12	1.49 (0.55–4.04)	0.43	1.43 (0.72–2.86)	0.31	1.23 (0.68–2.21)	0.49	1.28 (0.74–2.21)	0.37
	Primary disease	0.36 (0.10–1.24)	0.11	1.22 (0.46–3.25)	0.7	1.51 (0.72–3.16)	0.27	1.24 (0.68–2.27)	0.48	1.24 (0.72–2.11)	0.44

Definition of abbreviations: CI = confidence interval; CLAD = chronic lung allograft dysfunction; CMV = cytomegalovirus; CST = community state type; EV = events; GERD = gastroesophageal reflux disease; HR = hazard ratio; tx = transplant.
GERD status and the cumulative number of CST3 samples between different time intervals during the first year after transplant were used to predict CLAD and death and adjusted for potential confounders.

indirect effects on the allograft microbiota that vary by baseline community composition (Figure 6B).

Discussion

This study is the first to systematically compare the allograft microbiota in lung transplant recipients with and without GERD. We believe we add the following observations to our understanding of allograft microbiota–host associations in the context of GERD:

1) The taxonomic compositional variability of BALF allograft community composition has 3 "extremes", CST 1–3, which, when applied categorically, reveal differences between bacterial density, diversity, and host–microbiome associations across samples;
2) The proportion of individuals with each CST differed between GERD and no-GERD groups, with the high-density, oropharyngeal taxa-enriched CST1 being more common in patients with GERD than no GERD;
3) GERD was associated with a more variable community composition and bacterial abundance in the first year after transplant, with more frequent transitions between lower density CST2/3 and CST1 in patients with GERD than without;
4) CST–inflammation associations exceeded any association between GERD status and inflammation in this cohort;
5) The CST most common in lung allograft recipients with GERD (CST1) was associated with lower inflammatory cytokine concentrations than the pathogen-dominated CST3 across the range of bacterial densities observed;
6) Models used to predict inflammation, ALAD, and CLAD revealed CST (particularly CST3) but not GERD-status associations;
7) In some patients, Nissen fundoplication was associated with decreased bacterial load, which correlated with decreased concentrations of proinflammatory cytokines, especially in individuals with a high prefundoplication density of the CST1-associated genus *Prevotella*.

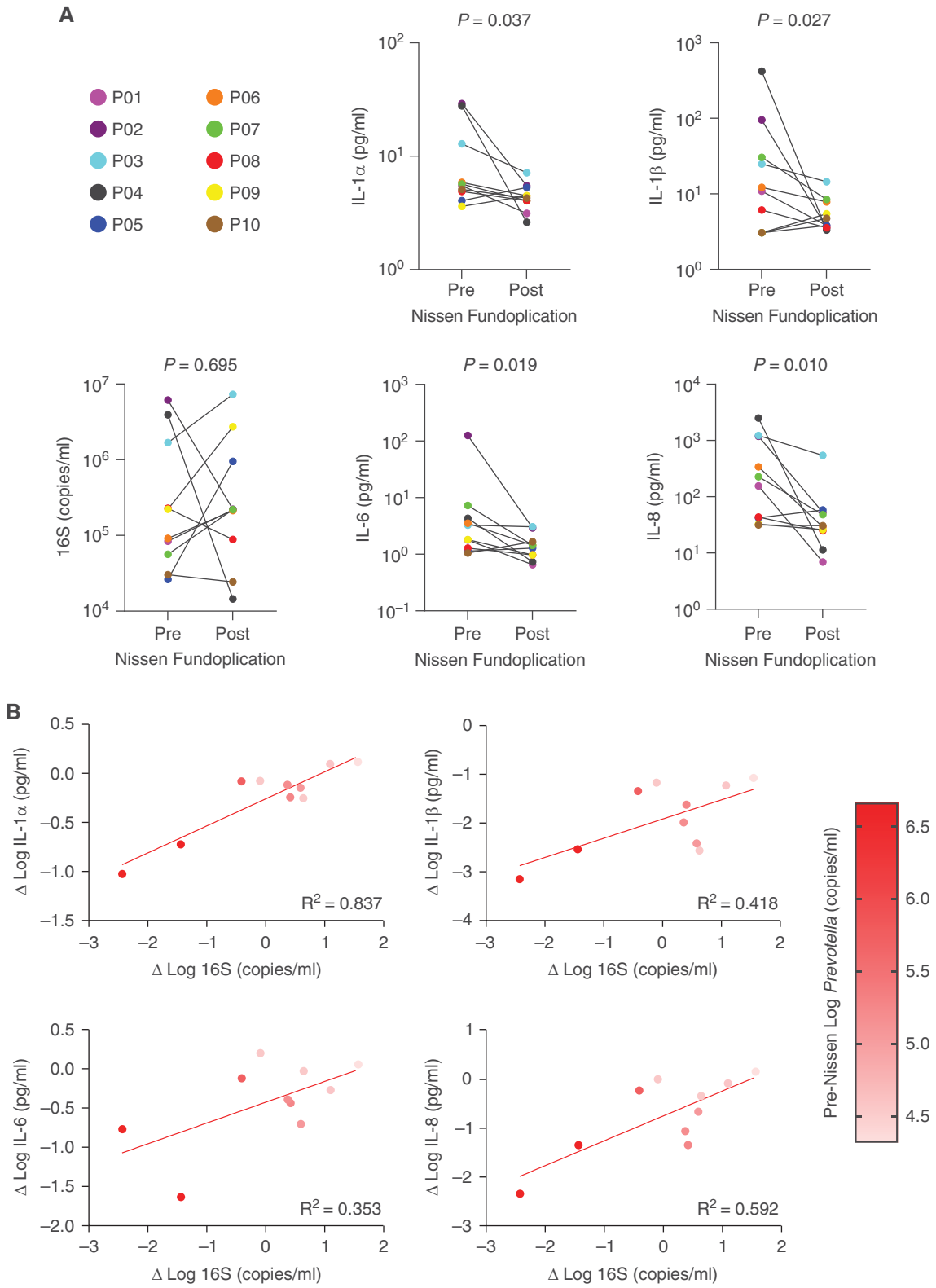

Figure 6. Comparison of BAL fluid supernatant bacterial density and cytokine concentrations before and after Nissen fundoplication in lung transplant recipients. (*A*) Changes in total bacterial density and cytokines by patient. (*B*) Regression between individual cytokines and total bacterial density, weighted by the density of the community state type 1-associated genus, *Prevotella*, before Nissen.

Our observation of discernible compositional clusters underlying the β diversity of the lung allograft confirms the results of others (5, 8). Importantly, these compositionally similar poles represent extremes of a compositional continuum defined by the presence of oropharyngeal taxa (CST1), low density (CST2), and pathogen abundance/dominance (CST3) but are not "discrete" in the same way as CSTs in the female genital tract (i.e., allograft CSTs are overlapping). We note that there are taxonomic differences in these CSTs compared with those reported by others, which may in part be because of technical factors (e.g., sequencing methods and sequence variant annotation) or differences in methods to identify and remove taxa suspected to be reagent contaminants, which is especially consequential for low-density samples in CST2 (25). Thus, we believe our findings, with respect to community composition, are likely to be generalizable to other cohorts, with the caveat that a structured comparison of categorical (e.g., CST) and continuous (nondiscrete) descriptions of the allograft microbiota in lung transplant recipients are needed.

We were unable to assess whether enrichment for CST1 among individuals with GERD was because of actual aspiration of refluxed oropharyngeal content in this observational study. However, we found that decreases in inflammatory cytokines after Nissen fundoplication were greatest in individuals with significant decreases in bacterial density and high initial *Prevotella* absolute abundance in BALF supernatant. One potential explanation is that GERD is associated with aspiration of oropharyngeal taxa, leading to inflammation. However, alternative models are also possible; for example, GERD may be associated with biases in upper gastrointestinal or oropharyngeal communities, which are then aspirated, or aspiration of nonmicrobial factors because of GERD may affect relative rates of extinction or proliferation of airway resident species. These hypotheses cannot be directly addressed in this observational study.

CST was not significantly associated with bile acid concentrations, although we cannot exclude the possibility that this association exists, and future datasets can reassess this in larger cohorts. Conjugated bile acids, like TCA and GCA, have been associated with poor clinical outcomes and bacterial infections (1, 32). This may explain the weak trends in CST1 and CST3, CSTs that contain aspiration-related microbes and pathogens.

A similar number of patients with and without GERD had CST3, suggesting that this CST arises independently of reflux. CST3 was characterized by the increased relative abundance of *Pseudomonas* and *Staphylococcus*, two taxa with known pathogenic species. Despite CST1 being enriched for GERD and having higher bacterial density, CST1 and CST2 were similarly associated with decreased rates of inflammation, CLAD, and ALAD compared with CST3. The association of pathogen communities, inflammation, and host status replicates the findings of others (5, 8). Importantly, our study highlights how the relationship between GERD and clinical outcomes may be confounded by the underlying microbial composition of the lung because the GERD-associated CST1 correlated with less inflammation, ALAD, and CLAD. Future studies assessing relationships between GERD and host status or prognosis should control for the potential confounding by underlying CST or analyze it as an interacting variable.

Our study has several important limitations. The primary analysis was on the basis of a case-control, retrospective, single-center cohort requiring validation. The cohort was composed of patients with strictly defined GERD or no GERD, and our findings, therefore, may not apply to individuals with intermittent or less frequent/severe reflux. Importantly, this limitation would be expected to exaggerate the differences between cases and control subjects, as our cohort represents an "extreme" GERD phenotype. In addition, we did not comprehensively assess the relationships between CST, GERD, and a wide array of soluble or other host factors in BALF and restricted our analysis to four proinflammatory cytokines, ALAD and CLAD. Associations between GERD and other soluble or host-derived factors in BALF may be present. Furthermore, while we assessed the association between microbiota and Nissen fundoplication, the study was not powered to assess the effect of specific nonsurgical treatments on the allograft microbiome, such as proton pump inhibitors (more than 90% of patients were on these medications), antibiotics, or immunosuppressants. Finally, our small Nissen fundoplication cohort was assessed without sequencing because we had only BALF supernatants available, which are compositionally different from BALF pellets or raw BALF (uncentrifuged or fractionated).

Conclusions

Increased bacterial density and taxonomic composition dominated by oropharyngeal taxa are associated with GERD. Surgical treatment of GERD showed a correlation between changes in bacterial density and concentrations of proinflammatory cytokines. Similar to what was recently published, we showed that a pathogen-dominated community independent of GERD status is associated with an increased risk of lung allograft dysfunction. Future studies should investigate the exact effect of GERD treatment and whether it promotes or protects from colonization of the lung ecological niche by CLAD-associated microbial communities. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the Centre for the Analysis of Genome Evolution and Function (CAGEF) for providing support in the sequencing experiments as well as bioinformatics support for the taxonomic profiling. The authors also thank the Toronto Lung Transplant Program Biobank team for helping with sample collection and retrieval; the Database team for data collection and organization; and clinical staff who contributed to patient phenotyping.

References

1. Zhang CYK, Ahmed M, Huszti E, Levy L, Hunter SE, Boonstra KM, *et al.*; CTOT-20 investigators. Bronchoalveolar bile acid and inflammatory markers to identify high-risk lung transplant recipients with reflux and microaspiration. *J Heart Lung Transplant* 2020;39:934–944.
2. Verleden GM, Glanville AR, Lease ED, Fisher AJ, Calabrese F, Corris PA, *et al.* Chronic lung allograft dysfunction: definition, diagnostic criteria, and approaches to treatment-A consensus report from the pulmonary council of the ISHLT. *J Heart Lung Transplant* 2019;38:493–503.
3. Razia D, Mittal S, Bremner R, Bansal S, Ravichandran R, Smith M, *et al.* Pretransplant GERD-induced immune response predisposes to CLAD. *J Heart Lung Transplant* 2021;40:S59.
4. King BJ, Iyer H, Leidi AA, Carby MR. Gastroesophageal reflux in bronchiolitis obliterans syndrome: a new perspective. *J Heart Lung Transplant* 2009;28:870–875.
5. Combs MP, Wheeler DS, Luth JE, Falkowski NR, Walker NM, Erb-Downward JR, *et al.* Lung microbiota predict chronic rejection in healthy lung transplant recipients: a prospective cohort study. *Lancet Respir Med* 2021;9:601–612.
6. Willner DL, Hugenholtz P, Yerkovich ST, Tan ME, Daly JN, Lachner N, *et al.* Reestablishment of recipient-associated microbiota in the lung allograft is linked to reduced risk of bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med* 2013;187:640–647.
7. Royer P-J, Olivera-Botello G, Koutsokera A, Aubert J-D, Bernasconi E, Tissot A, *et al.*; SysCLAD consortium. Chronic lung allograft dysfunction: a systematic review of mechanisms. *Transplantation* 2016;100:1803–1814.
8. Mouraux S, Bernasconi E, Pattaroni C, Koutsokera A, Aubert J-D, Claustre J, *et al.*; SysCLAD Consortium. Airway microbiota signals anabolic and catabolic remodeling in the transplanted lung. *J Allergy Clin Immunol* 2018; 141:718–729.e7.
9. Dickson RP, Erb-Downward JR, Huffnagle GB. Towards an ecology of the lung: new conceptual models of pulmonary microbiology and pneumonia pathogenesis. *Lancet Respir Med* 2014;2:238–246.
10. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Beck JM, Huffnagle GB, *et al.* Spatial variation in the healthy human lung microbiome and the adapted island model of lung biogeography. *Ann Am Thorac Soc* 2015;12:821–830.
11. Dickson RP, Erb-Downward JR, Huffnagle GB. Homeostasis and its disruption in the lung microbiome. *Am J Physiol Lung Cell Mol Physiol* 2015;309:L1047–L1055.
12. Tinckam KJ, Keshavjee S, Chaparro C, Barth D, Azad S, Binnie M, *et al.* Survival in sensitized lung transplant recipients with perioperative desensitization. *Am J Transplant* 2015;15:417–426.
13. Gray AL, Mulvihill MS, Hartwig MG. Lung transplantation at Duke. *J Thorac Dis* 2016;8:E185–E196.
14. Levy L, Huszti E, Tikkanen J, Ghany R, Klement W, Ahmed M, *et al.* The impact of first untreated subclinical minimal acute rejection on risk for chronic lung allograft dysfunction or death after lung transplantation. *Am J Transplant* 2020;20:241–249.
15. Davis WA, Finlen Copeland CA, Todd JL, Snyder LD, Martissa JA, Palmer SM. Spirometrically significant acute rejection increases the risk for BOS and death after lung transplantation. *Am J Transplant* 2012;12: 745–752.
16. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology (Reading)* 2002;148: 257–266.
17. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 2012;6:1621–1624.
18. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–2461.
19. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;10:996–998.
20. Edgar RC. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. bioRxiv; 2016: 081257.
21. Edgar R. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. bioRxiv; 2016: 074161.
22. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73:5261–5267.
23. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335–336.
24. Carney SM, Clemente JC, Cox MJ, Dickson RP, Huang YJ, Kitsios GD, *et al.* Methods in lung microbiome research. *Am J Respir Cell Mol Biol* 2020;62:283–299.
25. Schneeberger PHH, Prescod J, Levy L, Hwang D, Martinu T, Coburn B. Microbiota analysis optimization for human bronchoalveolar lavage fluid. *Microbiome* 2019;7:141.
26. Dixon P. VEGAN, a package of R functions for community ecology. *J Veg Sci* 2003;14:927–930.
27. Breiman L. Bagging predictors. *Mach Learn* 1996;24:123–140.
28. Højsgaard S, Halekoh U, Yan J. The R package geepack for generalized estimating equations. *J Stat Softw* 2005;15:1–11.
29. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
30. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, *et al.* The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2014;2:1–19.
31. Masson L, Passmore J-AS, Liebenberg LJ, Werner L, Baxter C, Arnold KB, *et al.* Genital inflammation and the risk of HIV acquisition in women. *Clin Infect Dis* 2015;61:260–269.
32. Urso A, Leiva-Juárez MM, Briganti DF, Aramini B, Benvenuto L, Costa J, *et al.* Aspiration of conjugated bile acids predicts adverse lung transplant outcomes and correlates with airway lipid and cytokine dysregulation. *J Heart Lung Transplant* 2021;40:998–1008.