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Supplementary appendix

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Online Data Supplement for "Lung microbiota predict chronic rejection in a prospective cohort study of healthy lung transplant recipients"

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Experimental Materials and Methods

Study Participants

Per institutional protocol, all lung transplant recipients receive immunosuppression with prednisone, calcineurin inhibition and an antiproliferative immunosuppressive medication. Basiliximab is used for induction immunosuppression in cases with peri-operative renal insufficiency. The choice of calcineurin inhibitor (cyclosporine vs. tacrolimus) is based on patient tolerance and the dose is titrated to therapeutic serum trough levels per protocol. Antiproliferative medication management (azathioprine vs. mycophenolate) was determined by patient tolerance and biopsy results (patients with acute rejection were preferred to receive mycophenolate), with decisions to reduce the dose, hold, and resume the medication based on pre-specified clinical criteria (e.g. ongoing treatment for an infection, post-transplant lymphoproliferative disorder, lymphopenia; Appendix Table 5). All patients received routine *Pneumocystis jiroveci* prophylaxis and antiviral prophylaxis per protocol. Antifungal prophylaxis was provided to patients with pre-transplant *Aspergillus* colonization or who had otherwise increased risk for invasive fungal disease.

Outcome & Predictor Variables

After lung transplantation, patients' baseline FEV_1 values were calculated as the average of the two highest values after transplant taken at least 21 days apart.[1] The diagnosis of CLAD was established after 3 months of sustained, definitive decline in FEV_1 to ≤80% of baseline in the absence of another explanation for pulmonary function decline (e.g. airway stenosis, persistent pleural effusion or pulmonary edema, surgical factors, myopathy/neuropathy, weight gain, etc.).[1] The date of CLAD onset was defined as the first day on which a patient had definitive decline in FEV_1. The date and cause of death were determined by medical record review. For our primary endpoint, we elected to evaluate a composite of development of CLAD or death at 500 days after the one-year surveillance bronchoscopy. This timepoint was selected because it met our criteria of, first, allowing sufficient time for patients to develop CLAD and, second, because we had complete follow-up data on all patients and thus avoided the potential bias associated with censored data.

Demographic predictor variables were obtained upon enrollment in the prospective cohort trial and included age, sex, pre-lung transplant diagnosis, and bilateral vs. single transplant. Clinical predictor variables were obtained via medical record review and included FEV_1 in litres, calcineurin inhibitor regimen, antiproliferative immunosuppression regimen, primary graft dysfunction (PGD) immediately after transplant, average cumulative rejection scores, donor-specific antibodies (DSA) presence, BAL cell count, culture, BAL culture results, BAL respiratory virus PCR results, any history of CMV pneumonitis, any history of community-acquired respiratory viral infections, and recent antibiotic use. FEV_1 was obtained per-protocol at all clinic visits according to American Thoracic Society/European Respiratory Society guidelines.[2] The FEV_1 value at the nearest available time prior to bronchoscopy was analyzed. Calcineurin inhibitor regimen and antiproliferative immunosuppression on the date of the bronchoscopy were assessed via documentation in the medical record and University of Michigan Organ Transplant Center Information System. PGD was assessed at the earliest timepoint available after transplantation, consistent with prior work.[3] PGD was graded as grade 0 if chest imaging was not consistent with pulmonary edema. For patients whose imaging was consistent with pulmonary edema, PGD severity was determined on the basis of PaO_2/FiO_2 ratio (if PaO_2 was unavailable, the SpO_2/FiO_2 ratio was used) as described in the ISHLT Consensus Report: grade 1 for PaO_2/FiO_2 >300 (SpO_2/FiO_2 >315), grade 2 for PaO_2/FiO_2 200-300 (SpO_2/FiO_2 315-235), and grade 3 for PaO_2/FiO_2 <200 (SpO_2/FiO_2 <235).[4] Cumulative rejection scores were calculated by adding the ordinal values of each biopsy specimen's A or B score divided by the total number of biopsies performed.[5] DSA were evaluated via Luminex bead assay and analyzed as positive or negative per institutional protocol (mean fluorescence intensity ≥3000 interpreted as positive). BAL cell type percentage, bacterial culture, and respiratory virus PCR were assessed and reported per the clinical laboratory protocol. BAL bacterial culture was analyzed as negative (no bacterial growth on culture), oral flora (positive culture that was deemed not clinically significant and, therefore, not speciated per microbiology laboratory protocol), and positive culture (any positive culture for which a bacterial species was identified). History of CMV pneumonitis was determined by CMV inclusions seen on histopathology from transbronchial biopsy or a positive CMV culture from BAL in the presence of lower respiratory tract symptoms. History of community-acquired respiratory viral culture was defined as positive respiratory viral PCR for: influenza A, influenza B, respiratory syncytial virus (RSV), parainfluenza virus, human metapneumovirus, adenovirus, coronavirus, or human rhinoenterovirus in the presence of lower respiratory tract symptoms or radiographic infiltrates.[6] Antibiotic usage was analyzed as receipt of a systemic or inhaled antibiotic, other than *Pneumocystis* prophylaxis, in the 30 days preceding the bronchoscopy (Appendix Table 6).

Specimen processing
BAL fluid was filtered through sterile gauze to remove noncellular material and/or mucous. Cells were separated via centrifugation (1000 g for 4 min) and the cell-free supernatant was frozen at −80 °C for subsequent assays. Cell-free supernatants were subsequently centrifuged (22,500g for 30 min), and the resulting pellet was used for DNA isolation. Acellular BAL pellets resuspended in 360µl ATL buffer (Qiagen DNeasy Blood & Tissue kit). Sterile laboratory water and AE buffer used in DNA isolation were collected and analyzed as potential sources of contamination, as were extraction controls (empty isolation tubes) and blank sequencing wells.

Bacterial DNA isolation
Genomic DNA was extracted from BAL pellets (Qiagen DNeasy Blood & Tissue kit, Qiagen, Hilden, Germany) using a modified protocol previously demonstrated to isolate bacterial DNA.[7] Sterile laboratory water and AE buffer used in DNA isolation were collected and analyzed as potential sources of contamination. Specimens were processed in a randomized order to minimize the risk of false pattern formation due to reagent contamination.[8]

16S rRNA gene sequencing
The V4 region of the 16S rRNA gene was amplified using published primers[9] and the dual-indexing sequencing strategy developed by the laboratory of Patrick D. Schloss.[10] Sequencing was performed using the Illumina MiSeq platform (San Diego, CA), using a MiSeq Reagent Kit V2 (500 cycles), according to the manufacturer's instructions with modifications found in the Schloss standard operating procedure.[11] Accuprime High Fidelity Taq was used in place of Accuprime Pfx SuperMix. Primary PCR cycling conditions were 95°C for two minutes, followed by 20 cycles of touchdown PCR (95°C 20 seconds, 60°C 20 seconds and decreasing 0.3 degrees each cycle, 72°C 5 minutes), then 20 cycles of standard PCR (95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 5 minutes), and finished with 72°C for 10 minutes.

Bacterial DNA quantification
Bacterial DNA was quantified using a QX200 Droplet Digital PCR System (BioRad, Hercules, CA). Primers and cycling conditions were performed according to a previously published protocol.[12] Specifically, primers were 5'- GCAGGCCTAACACATGCAAGTC-3' (63F) and 5'- CTGCTGCCTCCCGTAGGAGT-3' (355R). The cycling protocol was 1 cycle at 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, 1 cycle at 4°C for 5 minutes, and 1 cycle at 90°C for 5 minutes all at a ramp rate of 2°C/second. The BioRad C1000 Touch Thermal Cycler was used for PCR cycling. Droplets were quantified using the Bio-Rad Quantisoft software. No-template control specimens were used and were run alongside BAL specimens.

Statistical analysis
The sequence data from BAL fluid and control specimens were processed and analyzed using the software *mothur* v.1.38.0 according to the standard operating procedure for MiSeq sequence data.[13] A shared community file and a phylotyped (genus-level grouping) file were generated using operational taxonomic units (OTU) binned at 97% identity generated using the dist.seqs, cluster, make.shared and classify.otu commands in *mothur*, as previously described.[14,15]

The OTU numbers referenced in the manuscript were assigned arbitrarily during the binning process. Classification of OTU was carried out using the *mothur* implementation of the Ribosomal Database Project (RDP) Classifier and the RDP taxonomy training set 14 (Trainset14_032015.rdp), available on the *mothur* website. Sequences are available via the NCBI Sequence Read Archive (accession number PRJNA615630). OTU, taxonomy, and metadata tables are available at https://github.com/combspulmonarydata/HealthyLungTx.

Microbial ecology analysis was performed using the *vegan* package and *mvabund* in R version 3.6.1(R Foundation for Statistical Computing, Vienna, Austria).[16,17] All OTU were included in diversity analysis. For relative abundance and ordination analysis, samples were normalized to the percent of total reads and we restricted analysis to OTU that were present at greater than 1% of the sample population, as previously described.[15] We performed ordinations using a principal component analysis on Hellinger-transformed normalized OTU tables generated using Euclidean distances; we presented these graphically as a biplot.[18] We determined significance in community composition comparisons using both the *adonis* function of *vegan*,[16] which performs permutational multivariate analysis of variance (PERMANOVA) using 10,000 permutations, and *mvabund*,[17] a model-based approach to analysis of multivariate abundance data.

Adequacy of sequencing and exclusion of specimens
Bacterial community analysis (using 16S rRNA gene amplicon sequencing) was performed on all specimens. We obtained 23,571 ± 12,914 16S rRNA gene copies per specimen. Three specimens had inadequate amplification (<1,000 16S rRNA gene copies) and were excluded from sequencing analysis (though included in ddPCR analysis). No bacterial taxa were excluded from analysis.

Supplemental Results
The microbiota of one-year surveillance BAL specimens are distinct from those of background sequencing controls
Cell-free BAL specimens are commonly used in lung microbiome studies.[19-21] However, similar to all low-biomass microbiome studies, these samples are vulnerable to contamination from bacterial DNA present in regents used for DNA extraction and library

preparation.[22] Therefore, our first priority was to confirm that we could detect a bacterial signal in the cell-free BAL specimens samples that was distinct from the no-template specimens (n=26), AE buffer specimens (n=8), sterile water used in DNA extraction (n=8), and extraction control specimens (n=4) that were included as negative controls.

First, we used droplet digital PCR to quantify bacterial DNA. The bacterial DNA burden in the BAL samples had a range of 1,007—15,708,014 copies/mL, with a median of 3563 copies/mL (IQR 2377-8316). In comparison, the bacterial DNA burden in our negative control samples had a range of 222—1,386 copies/mL, with a median of 776 copies/mL (IQR 678-934). Overall, we found a significantly higher burden in BAL specimens vs· no-template control specimens ($p < 0{\cdot}0001$, Appendix Figure 1A).

Next, we used 16S rRNA gene sequencing to demonstrate that there was a difference in bacterial community composition between BAL specimens and negative controls ($p<0{\cdot}0001$). Principle components analysis showed distinct clustering of BAL specimens from the various negative control specimens, although some overlap was observed (Appendix Figure 1B). Likewise, rank abundance analysis showed obvious differences in the relative abundance of taxa in the negative controls and BAL specimens (Appendix Figure 1C). We observed significant differences in the relative abundance of the following bacterial families: Comamonadaceae, Flavobacteriaceae, Prevotellaceae (all 0.001), Verrucomicrobiaceae (0.002), and Streptococcaceae (0.005).

Evaluating the association of microbiome characteristics with demographic and clinical variables
We performed multiple exploratory analyses to evaluate whether any of the available demographic or clinical variables were associated with bacterial burden, community diversity, community richness, or overall community composition.

Previous research has demonstrated that primary graft dysfunction,[3] acute cellular rejection,[5] lymphocytic bronchiolitis,[24] presence of DSA,[25,26] CMV pneumonitis,[27,28] and community-acquired respiratory viral infections[6] represent risk factors for subsequent development of CLAD. Therefore, we first investigated if known risk factors for CLAD were correlated with bacterial burden or any other microbiome characteristic. We found no detectable association between microbiome characteristics and PGD grade assessed immediately after transplant (Appendix Figure 13), cumulative Grade A rejection score (Appendix Figure 14), cumulative Grade B rejection score (Appendix Figure 15), DSA presence (Appendix Figure 16), any history of community-acquired respiratory viral infections (Appendix Figure 21), or any history of CMV pneumonitis (Appendix Figure 22). We thus concluded that the lung microbiome characteristics are not correlated with known clinical risk factors for CLAD.

Next we asked if there were associations between microbiome characteristics and immunosuppression regimen and recent antibiotic exposure. As our study was not designed or powered to evaluate more remote exposures, we chose to limit our analyses to medications received at or near the time of one-year surveillance bronchoscopy. There was no association between microbiome characteristics and calcineurin inhibitor regimen (Appendix Figure 11), antiproliferative immunosuppression regimen (Appendix Figure 12), antibiotic use—other than *Pneumocystis jiroveci* prophylaxis—in the 30 days prior to bronchoscopy (Appendix Figure 23), and receiving azithromycin for CLAD prevention (Appendix Figure 24). Although the use of induction immunosuppression at our center was rare, we did find that induction immunosuppression with basiliximab was associated with having a lower bacterial burden (Appendix Figure 10A); but had no significant effect on community diversity, community richness, or overall community composition (Appendix Figure 10B-10D). We thus concluded that immunosuppression regimen and having any recent antibiotic exposure at the time of one-year surveillance bronchoscopy are not associated with difference in microbiome characteristics.

We then investigated whether other clinical variables— e.g. lung function, BAL cellularity, current BAL bacterial culture results, or having a history of prior positive respiratory bacterial cultures—have any association with microbiome characteristics. Perhaps unsurprisingly, we found that having a BAL bacterial culture with either oral flora or a speciated pathogen was associated with having a higher bacterial burden (Appendix Figure 19A) and with differences in overall community composition (Appendix Figure 19D) relative to having a BAL bacterial culture with no growth. BAL culture results were not correlated with either community diversity (Appendix Figure 19B) or community richness (Appendix Figure 19C). Additionally, we observed a difference in overall community composition between samples with BAL neutrophil ≥15% vs. those with BAL neutrophil <15% (Appendix Figure 17D). Of note, this dichotomization was chosen based on a clinical cutoff to facilitate visualization; when BAL neutrophil percent was analyzed a continuous variable, there was no significant association with community composition. There was no association between BAL neutrophil percentage and bacterial burden, community diversity, and community richness. There was no association between patients' one-year post-transplant FEV_1 values (Appendix Figure 9), BAL lymphocyte percentage (Appendix Figure 18), and number of prior positive respiratory cultures (Appendix Figure 20) and any microbiome characteristics.

Last, among the demographic variables, patient age was inversely correlated with bacterial burden (Spearman's $\rho = -0{\cdot}201$, $p = 0{\cdot}017$, Appendix Figure 5A). Community diversity, community richness, and overall community composition was not associated with patients' age (Appendix Figure 5B-5D). There was no association between any microbiome characteristics and patients' sex (Appendix Figure 6), pre-transplant diagnosis (Appendix Figure 7), double vs. single lung transplant (Appendix Figure 8).

Increased lung bacterial burden predicts shorter CLAD-free survival over 500 days of follow-up, more rapid CLAD development over 500 days of follow-up, and shorter CLAD-free survival over 2000 days of follow-up

We used a univariate Cox regression models to evaluate the association of each of the variables included in Table 1 on the development of CLAD or death. These analyses are presented in Table 2. Next, to account for potential confounding, we used a multivariable Cox regression model to more rigorously evaluate the association between bacterial burden and CLAD-free survival. In this model we included: the available known risk factors for CLAD (pre-transplant diagnosis,[23] primary graft dysfunction,[3] acute cellular rejection,[5] lymphocytic bronchiolitis,[24] presence of DSA,[25,26] CMV pneumonitis,[27,28] and community-acquired respiratory viral infections[6]), variables that were associated with our primary outcome in univariate analyses (antiproliferative immunosuppression regimen, Table 1), and variables associated with bacterial burden (age [Appendix Figure 5], induction immunosuppression [Appendix Figure 10], and concurrent BAL bacterial culture results [Appendix Figure 19]). We observed collinearity between age and pre-transplant diagnosis; thus, to minimize overfitting, age was not included in the final model. As shown in Table 2, increasing lung bacterial burden was an independent predictor of developing CLAD or death, whether analyzed as a continuous variable (HR 2·49 per $\log_{10}$ increase, 95% CI 1·38 – 4·48; p=0·0024) or as tertiles (Middle vs. Lowest bacterial burden HR 4·94, 95% CI 1·25-19·21, p=0·022; Highest vs. Lowest bacterial burden HR 10·56, 95% CI 2·53-44·08, p=0·0012). We thus concluded that lung bacterial burden is an independent predictor of CLAD-free survival.

To evaluate the robustness of this finding, we then performed a sensitivity analysis to evaluate the effect of lung bacterial burden on the development of CLAD alone. For this analysis, the five patients who died prior having established the diagnosis of CLAD via repeated pulmonary function testing as described in the 2019 ISHLT CLAD consensus document[29] were considered to not have developed the outcome of interest. In univariate survival analyses (Appendix Figure 3) patients with the highest bacterial burden has increased risk of developing CLAD compared to those with the lowest bacterial burden (p=0·0068). In multivariate analysis, lung bacterial burden remained a predictor of CLAD when analyzed as a continuous variable (HR for each 10-fold increase in bacterial burden 2·05, 95% CI 1·10-3·94, p=0·030), and categorically as tertiles (Lowest vs. Middle, HR 4·71, 95% CI 1·10-20·06, p=0·036, Lowest vs. Highest, HR 9·26, 95% CI 2·04-42·07, p=0·0039; Appendix Table 3).

Finally, we evaluated the effect of lung bacterial burden on CLAD-free survival at more distant time points. When analyzed as a continuous variable, bacterial burden remained a significant predictor of developing CLAD or death through 2000 days of follow-up (HR 1·55 per $\log_{10}$ increase, 95% CI 1·04 – 2·30, p=0·030; Appendix Table 4). The Kaplan Meier survival curves for patients stratified into bacterial burden tertiles are presented in Appendix Figure 4. In univariate survival analyses, bacterial burden tertile is a significant predictor of developing CLAD or death at all the time points between 300 and 800 days of follow-up. In the multivariable survival analysis, bacterial burden tertile remains a significant predictor through 1000 days of follow-up. We thus concluded that the association of increased lung bacterial burden with shorter CLAD-free survival is robust to alternative analytic approaches.

Evaluating for potential confounding and interactions between the lung microbiome, CLAD-free survival, and relevant predictor variables

As with all cohort studies, our study is limited by the potential of confounding or interaction between our primary outcome and predictor variables. In order to robustly interrogate our key finding that bacterial burden is associated with shorter CLAD free survival, we performed *post hoc* analyses evaluating the potential interactions between bacterial burden, relevant predictor variables, and our composite outcome.

Antiproliferation immunosuppression regimen at the time of surveillance bronchoscopy differed among patients who did and did not develop the composite outcome (Table 1), and the use of mycophenolate was associated with longer CLAD-free survival in our multivariate model (Table 2). Therefore, we investigated whether immunosuppression regimen was a potential confounding variable, and whether there was an interaction between bacterial burden and immunosuppression on CLAD-free survival. First, we more carefully investigated the fifteen patients were not receiving an antiproliferative agent for immunosuppression at the time of one-year surveillance bronchoscopy. The clinical contexts for holding immunosuppression are summarized in Appendix Table 5. Briefly, eight patients of these were receiving treatment for CMV viremia, one patient was being treated for a chronic bacterial infection, five had leukopenia or other laboratory abnormalities, one had history of PTLD, and one had immunosuppression empirically held for one day at a local hospital. Next, we excluded the possibility that immunosuppression regimen at the time of surveillance bronchoscopy was a confounder, as it was not associated with bacterial burden (Appendix Figure 12A). This analysis was unchanged if the patient who only had immunosuppression held for one day was analyzed as having received immunosuppression. Finally, we did not observe any interaction between bacterial burden and immunosuppression (Appendix Table 7). We thus concluded that antiproliferative immunosuppression is not associated with microbiome characteristics, and that the predictive significance of bacterial burden on CLAD-free survival is not attributable to variation in immunosuppression regimens.

While not statistically significant, patients with a pre-transplant diagnoses categorized as “Other” or cystic fibrosis tended to develop our composite outcome at lower rates than patients with COPD or IPF (Table 1). Thus, we further investigated whether pre-transplant diagnosis represented a potential confounder or modifier or the association between bacterial burden and CLAD-free survival. We did not find sufficient evidence to suggest confounding, as pre-transplant diagnosis is not associated with bacterial burden (Appendix Figure 7A). In models evaluating the interaction of pre-transplant diagnosis and bacterial burden we did not find evidence of an

interaction; importantly, as only three patients with cystic fibrosis and two patients who had a pre-transplant diagnosis categorized as "Other" developed our primary outcome, there was very limited power to detect possible interactions.
Finally, although pre-transplant diagnosis was not associated with either our primary outcome or bacterial burden, we did note that the age of patients with cystic fibrosis (mean 32.2 ± 8.9) and a pre-transplant diagnosis of "Other" (mean 50.4 ± 11.9) was lower than those with COPD (mean 59.5 ± 5.5) and ILD (mean 57.5 ± 7.7; all pairwise comparisons $p<0.05$ except COPD vs. ILD and ILD vs. other). Although there was an inverse correlation between age and bacterial burden (Appendix Figure 5A), age was not associated with development of our primary outcome, minimizing the possibility that age is a confounder. Furthermore, we did not find evidence of an interaction between age and bacterial burden in our survival models (Appendix Table 8). We thus concluded that there was no detectable evidence that the association of increased bacterial burden with shorter CLAD-free survival was not confounded or modified by pre-transplant diagnosis or age.

16S-identified evidence of P. aeruginosa is not predictive of CLAD or death
Because the family *Pseudomonadaceae* is comprised of many species with varying clinical significance[30], we wanted to determine if we could use 16S sequencing to identify *P. aeruginosa* among the OTU from this family. We have previously demonstrated it is possible to approximate species-level resolution among the genus *Pseudomonas* from 16S sequencing by comparing the 97% homologous representative nucleotide sequences for each OTU to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).[31] Using this approach, we found 78 speciated bacterial strains in the NCBI database that shared 100% coverage and homology with OTU 0006; of these, 76 were identified as *P. aeruginosa*. None of the representative sequences from the other five *Pseudomonas* OTU had 100% overlap with any strains identified as *P. aeruginosa* in the NCBI database. We thus concluded that OTU 0006 represented *P. aeruginosa*. Sixteen (11·9%) of the samples in our cohort had detectable OTU 0006: *P. aeruginosa* (relative abundance range 1·4%-98·6%, mean 30·9% ± 37·8%). Six of these patients had detectable *P. aeruginosa* on concurrent BAL bacterial culture, and an additional four had prior BAL cultures with *P. aeruginosa*. One patient had a positive *P. aeruginosa* culture and no evidence of OTU 0006 on 16S sequencing. There was no association between the developing CLAD or death and OTU 0006: *P. aeruginosa* when analyzed as present vs. absent (p=0·68) or by increasing relative abundance (p=0·14). We thus concluded that the association between increased bacterial DNA burden and risk of CLAD development is not attributable to the presence or relative abundance of *Pseudomonas* spp.

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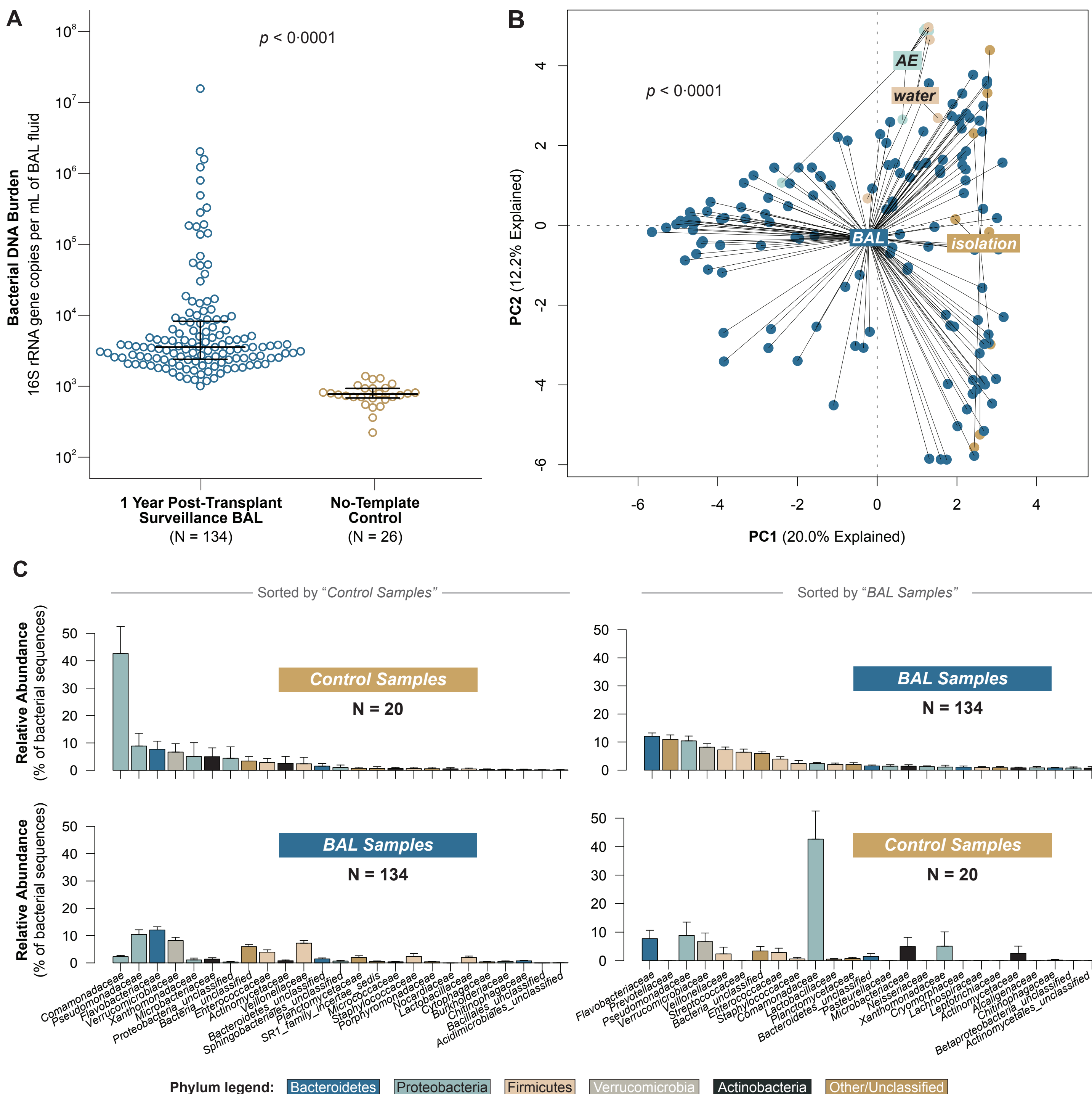
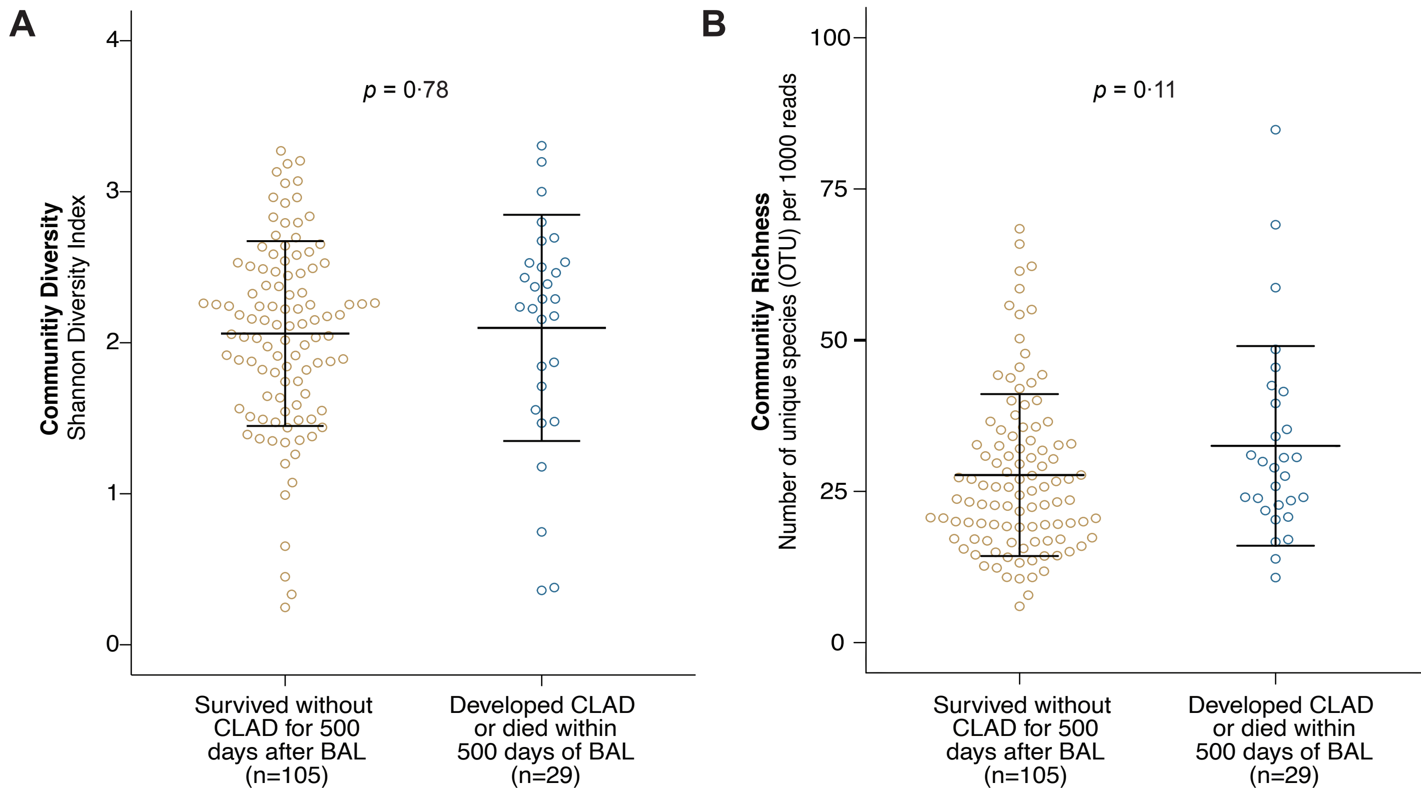
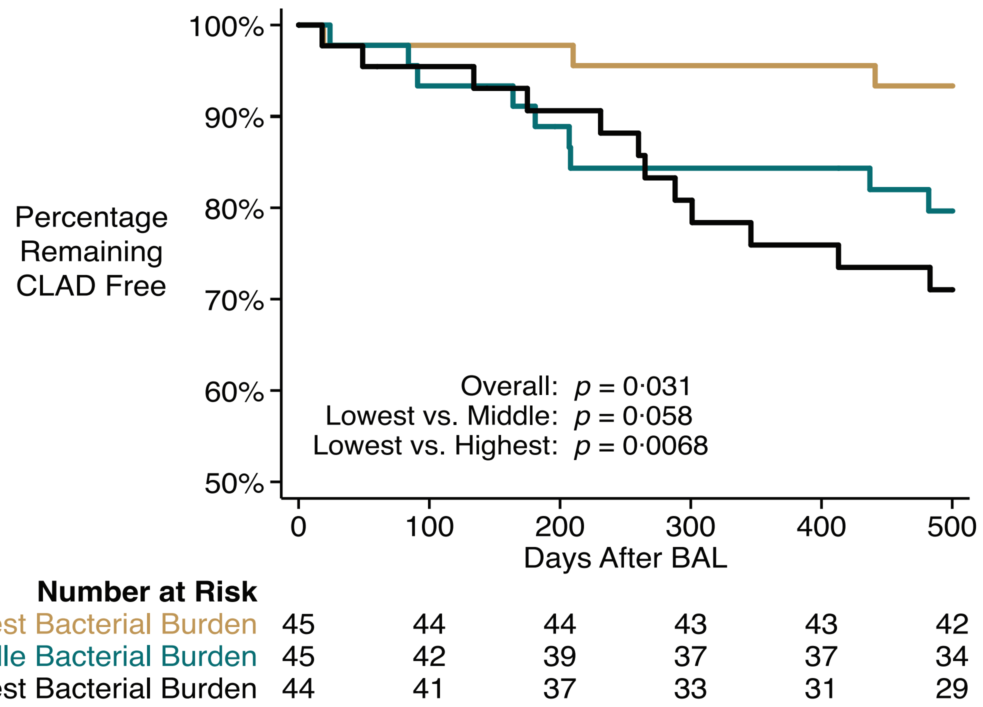


Appendix Figure 1: Evidence of distinct microbial signal in cell-free BAL specimens.
Bacterial DNA was quantified and identified in 1-year post-transplant surveillance bronchalveolar lavage (BAL) fluid using droplet digital PCR (A) and 16S RNA gene amplicon sequencing (B, C), respectively. The bacterial burden in BAL specimens was significantly higher than in no template controls. (A) Likewise, the community composition of the BAL specimens differed significantly compared to the negative control specimens. (B) Rank abundance comparison (C) of prominent taxa for the negative controls and BAL specimens. For each comparison, the 20 most abundant taxa from the reference group are displayed in decreasing order of relative abundance (mean ± SD). Asterisks indicate taxa as significantly distinct across groups. Hypotheses testing performed using Wilcoxon rank-sum test (A) PERMANOVA (B), and mvabund (C). (A) Lines indicate median and interquartile range.

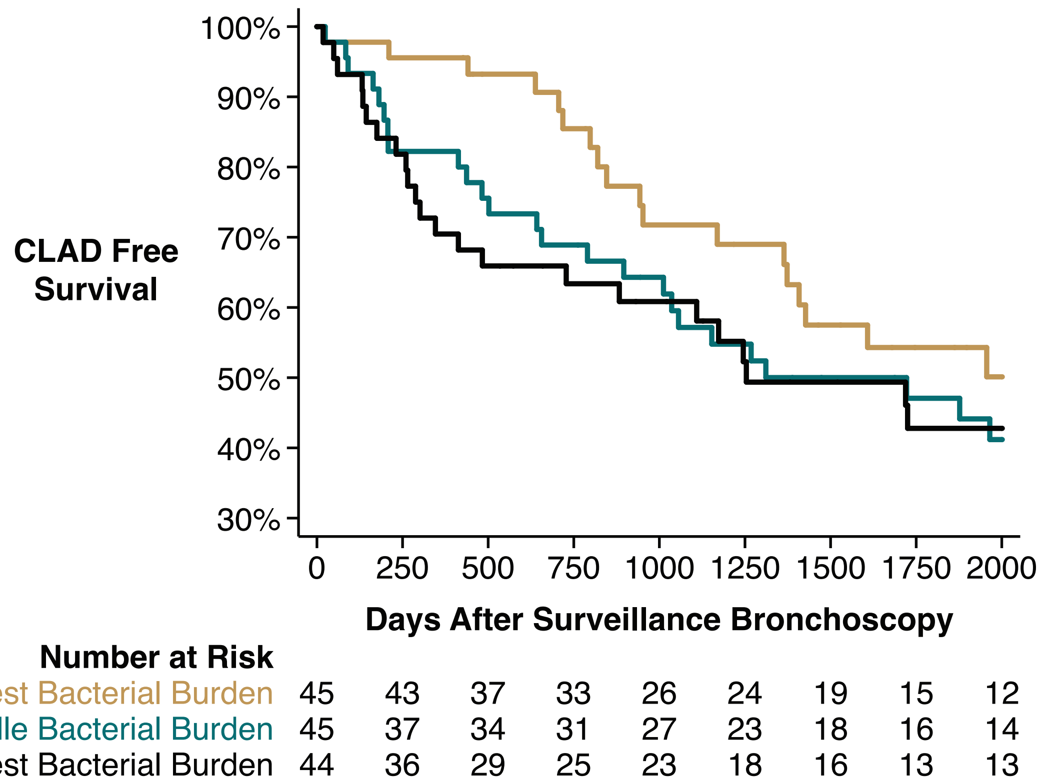


Appendix Figure 2**: Microbial diversity or richness in surveillance BAL samples are not associated with development of CLAD or death within 500 days.**

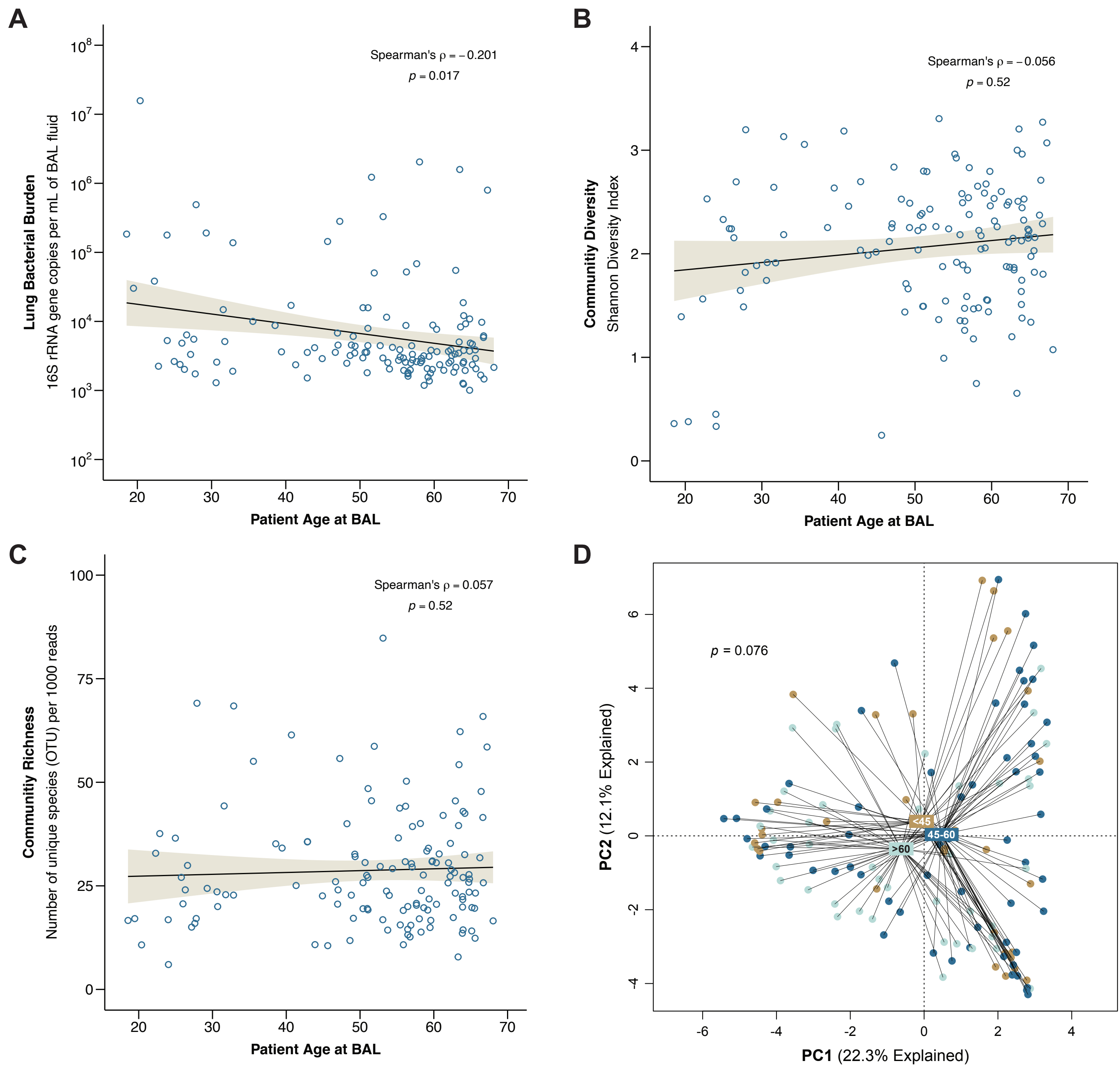
The distribution of A) community diversity (measured by the Shannon diversity index) and B) richness (number of unique taxa, i.e. operational taxonomic units [OTU], per 1000 reads) in one-year surveillance bronchoalveolar lavage (BAL) did not differ among patients who developed chronic lung allograft dysfunction (CLAD) or death within 500 days and CLAD free survivors. Hypothesis testing performed using two sample t tests. Lines indicate mean +/- standard deviation.



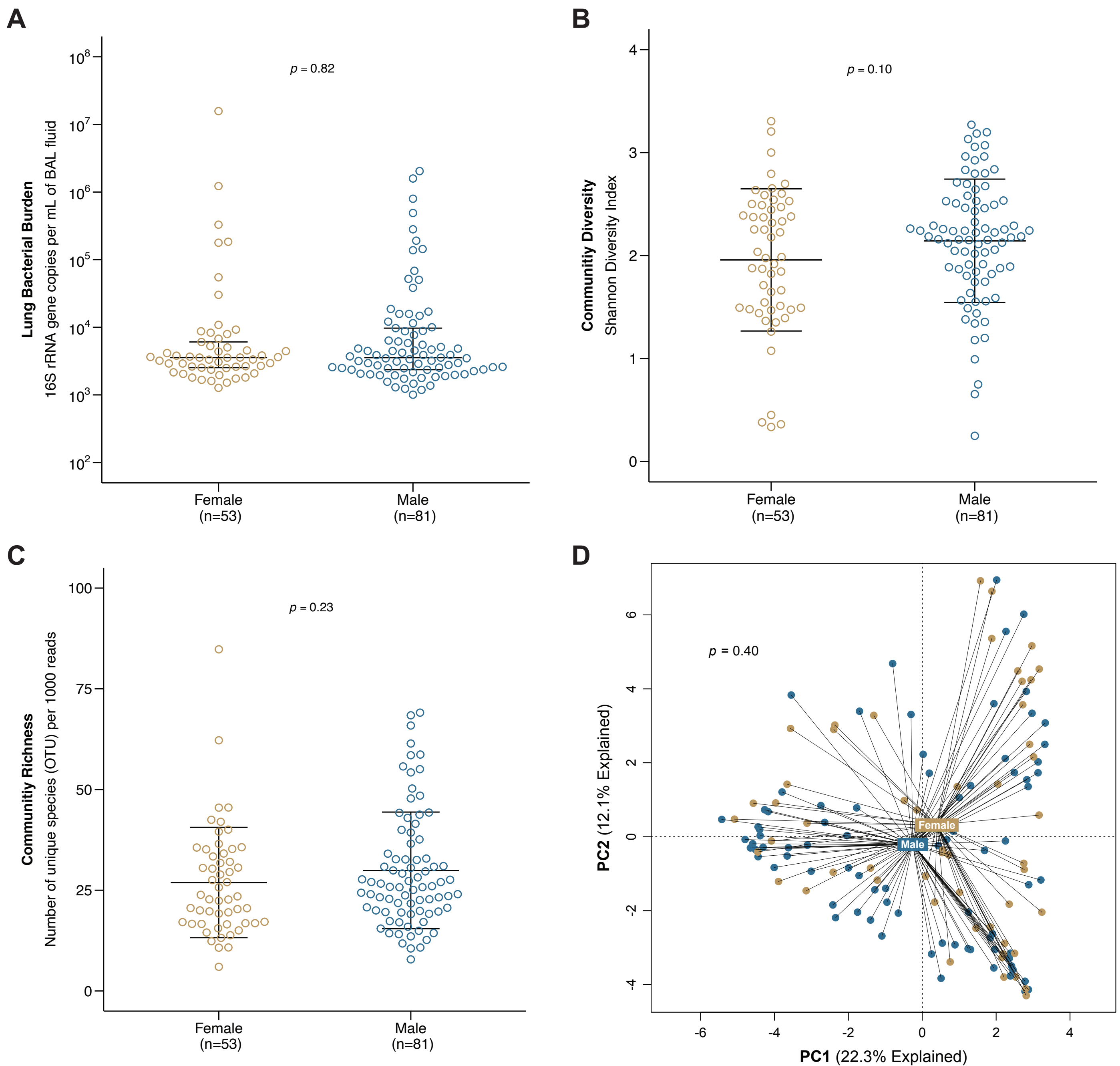
***Appendix Figure 3*: Increased lung bacterial burden predicts development of chronic lung allograft dysfunction.** We quantified bacterial DNA in asymtpomatic one-year surveillance BAL specimens using droplet digital PCR. Patients were then divided into terticles of lowest, middle, and highest bacterial burden as described elsewhere. Kaplan-Meier curves illustrating the time to developing CLAD are presented. Hypothesis testing performed using univariate Cox proportional hazards model.



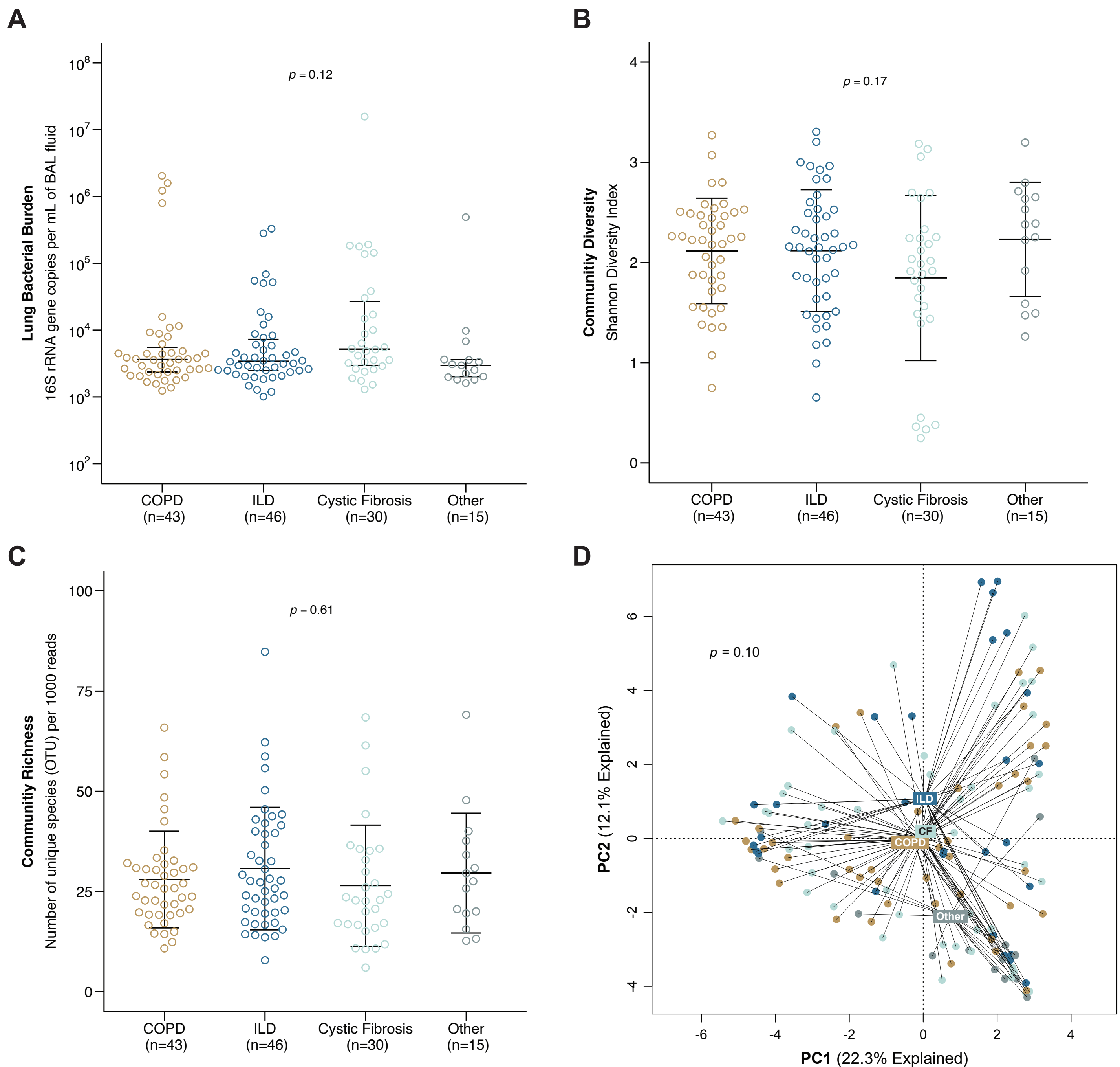
***Appendix Figure 4*: Lung bacterial burden and development of chronic rejection or death over 2000 days of follow-up.** We quantified bacterial DNA in asymtpomatic one-year surveillance BAL specimens using droplet digital PCR. Patients were then divided into terticles of lowest, middle, and highest bacterial burden as described elsewhere. Kaplan-Meier curves illustrating the time to development of CLAD or death are presented. Lung bacterial burden tertiles are associated with an increased risk of developing CLAD or death at all time points between 300 and 800 days of follow-up in univavriate Cox proportional hazards models.



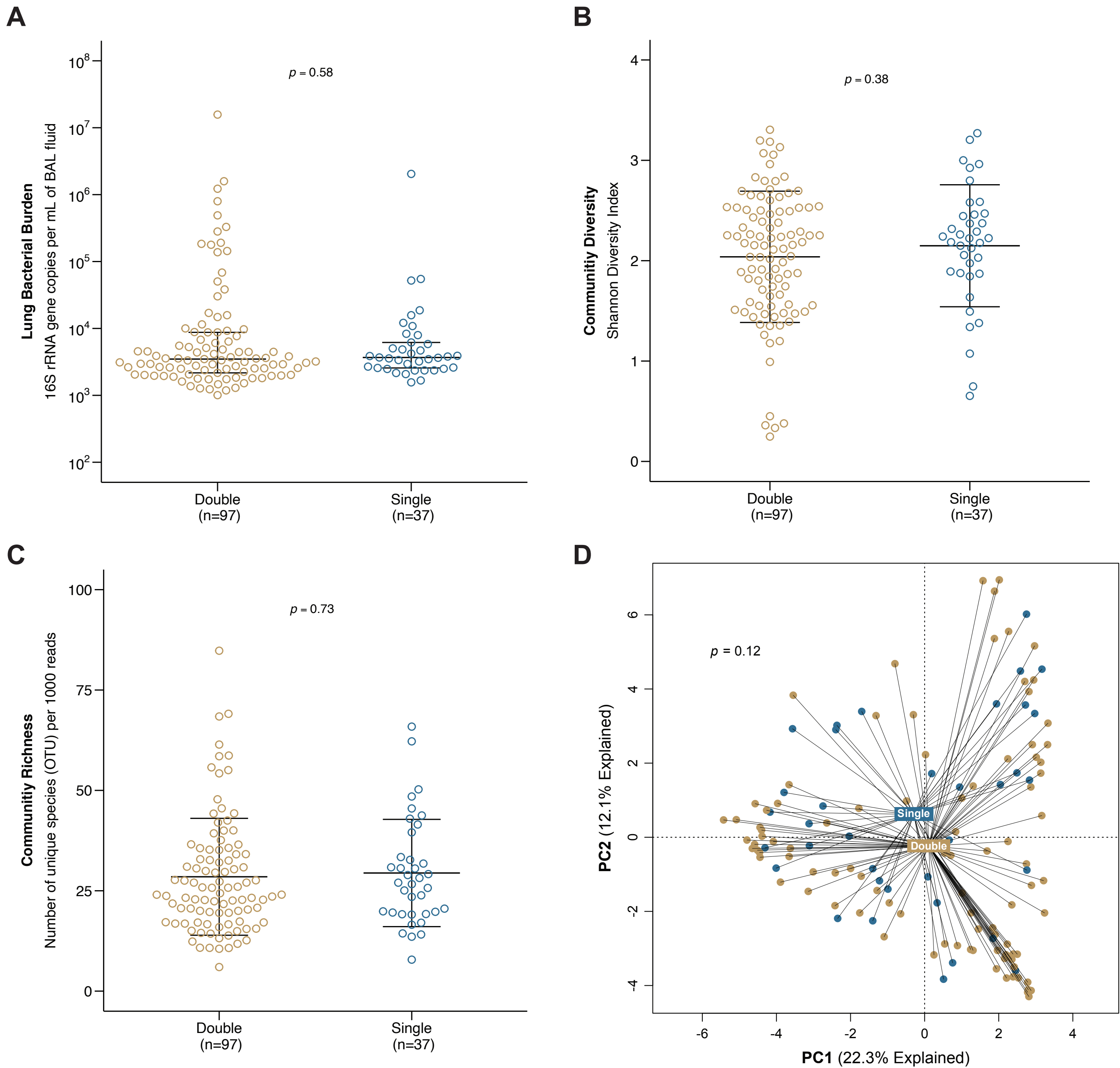
Appendix Figure 5: **Comparing respiratory microbiome characteristics on the basis of patient age.**
We compared the bacterial burden, community diversity, and community richness on the basis of patient age at the time of BAL. There was an inverse association between age and bacterial burden (A), but age was not correlated with community diversity (B), or community richness (C). To visualize differences in community composition, patients were grouped into those younger than 45 (N=32), between 45 and 60 (N=60), and older than 60 (N=42). There was no difference in community composition between patients when grouped by age (D). Hypothesis testing performed using Spearman's correlation (A-C) and *adonis* (D). Lines indicate best-fit linear regression, shaded areas indicate standard error (95% confidence level) (A-C).



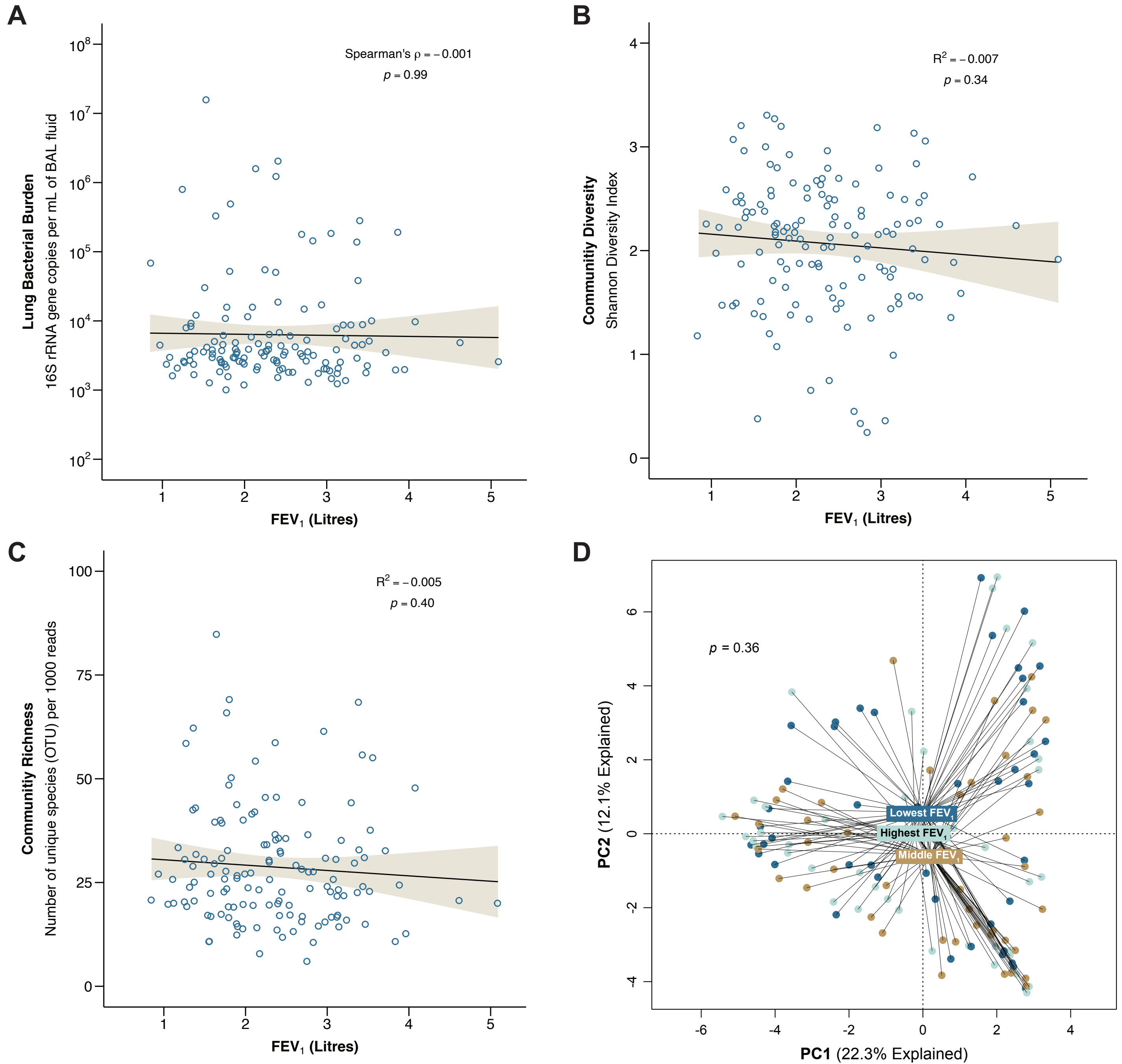
Appendix Figure 6: **Comparing respiratory microbiome characteristics on the basis of sex.**
We compared the bacterial burden, community diversity, community richness, and overall community composition between male (N=81) and female (N=53) patients undergoing one-year surveillance bronchoscopy. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or community composition (D) on the basis of sex. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C) and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



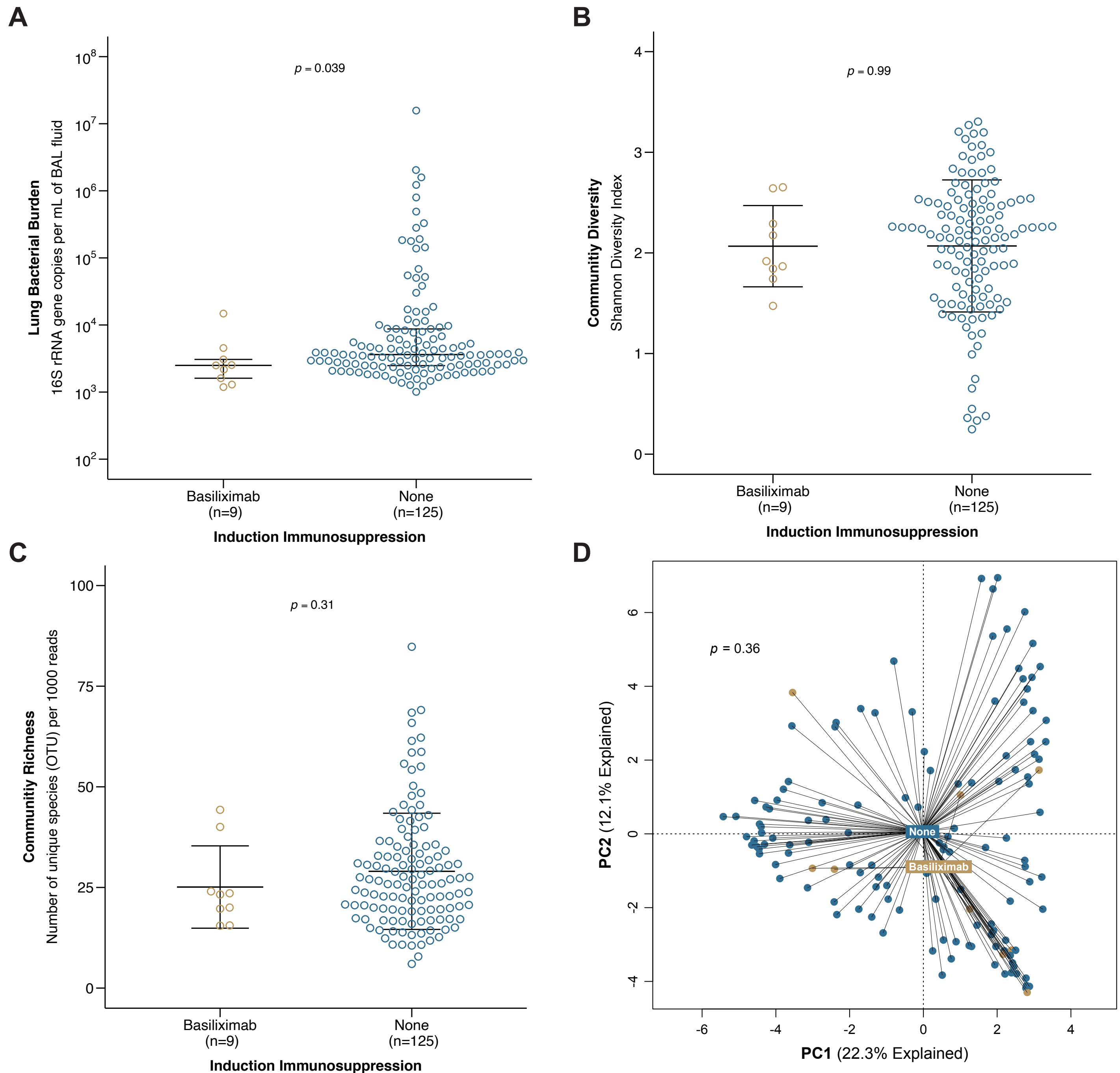
Appendix Figure 7: **Comparing respiratory microbiome characteristics on the basis of pre-transplant diagnosis.** We compared the bacterial burden, community diversity, community richness, and overall community composition between patients who had a pre-transplant diagnosis of COPD/Emphysema (N=43), interstitial lung disease (N=46), cystic fibrosis (N=30), and other indications (N=15) who were undergoing one-year surveillance bronchoscopy. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or community composition (D) on the basis of pre-transplant diagnosis. Hypothesis testing performed using Kruskal-Wallis test (A), one-way ANOVA (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



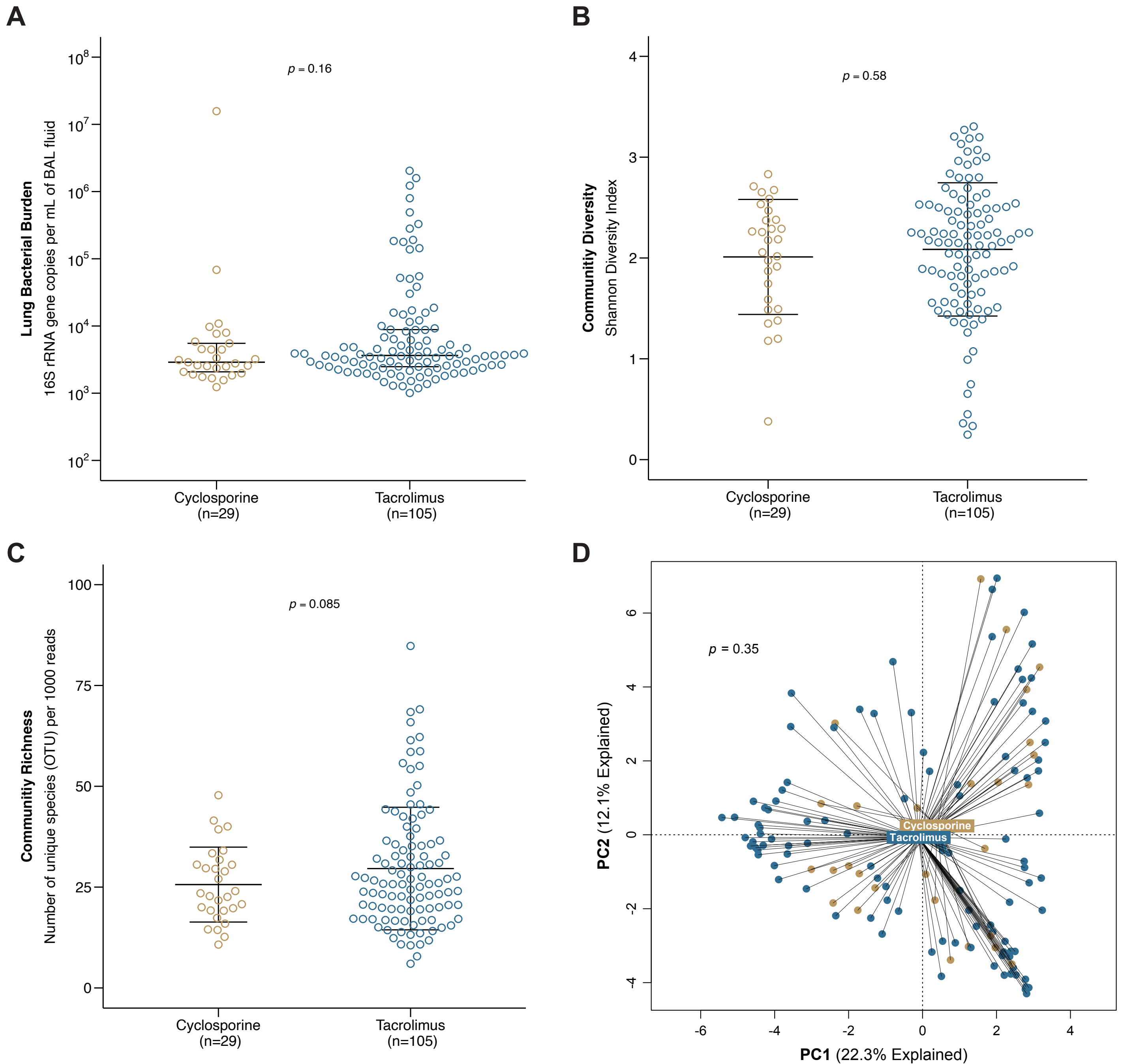
***Appendix Figure 8:* Comparing respiratory microbiome characteristics on the basis of double vs. single lung transplant.** We compared the bacterial burden, community diversity, community richness, and overall community composition between patients who recieved a double (N=97) vs. single (N=37) lung transplant recipients undergoing one-year surveillance bronchoscopy. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or community composition (D) on the basis of double vs. single lung transplant. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C) and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



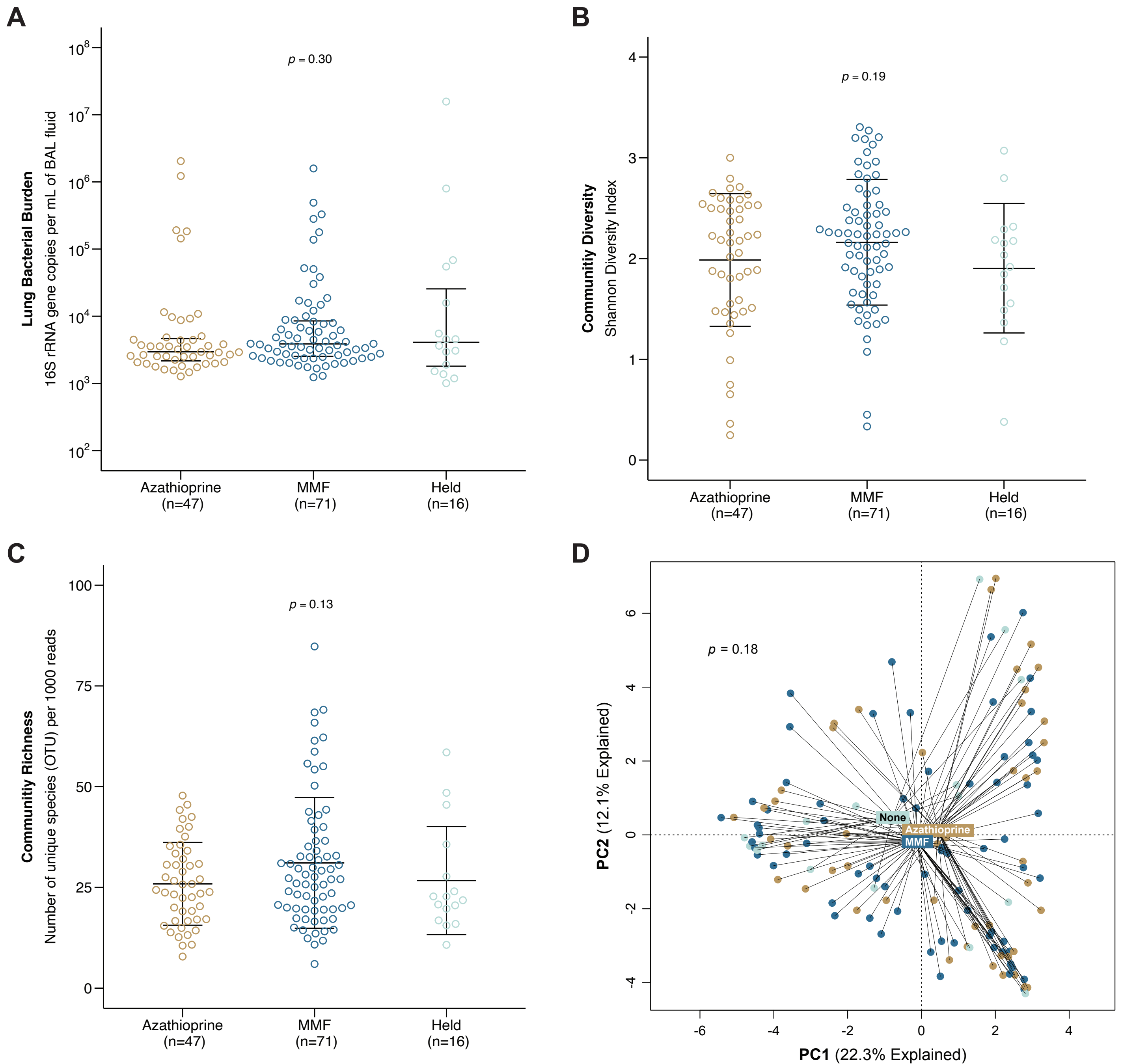
***Appendix Figure 9:* Comparing respiratory microbiome characteristics on the basis of FEV_1 values.**
We compared the bacterial burden, community diversity, and community richness on the basis of patient's FEV_1 values in litres at the time of BAL. There was no association between FEV_1 values and bacterial burden (A), community diversity (B), or community richness (C). These analyses were unchanged when FEV_1 was analyzed as % predicted vs. litres. To visualize differences in community composition, patients were divided into tertiles of lowest (N=45), middle (N=45) and highest (N=44) FEV_1 values. There was no difference in community composition between patients when grouped by FEV_1 values (D). Hypothesis testing performed using Spearman's correlation (A), linear regression (B,C) and *adonis* (D). Lines indicate best-fit linear regression, shaded areas indicate standard error (95% confidence level (A-C).



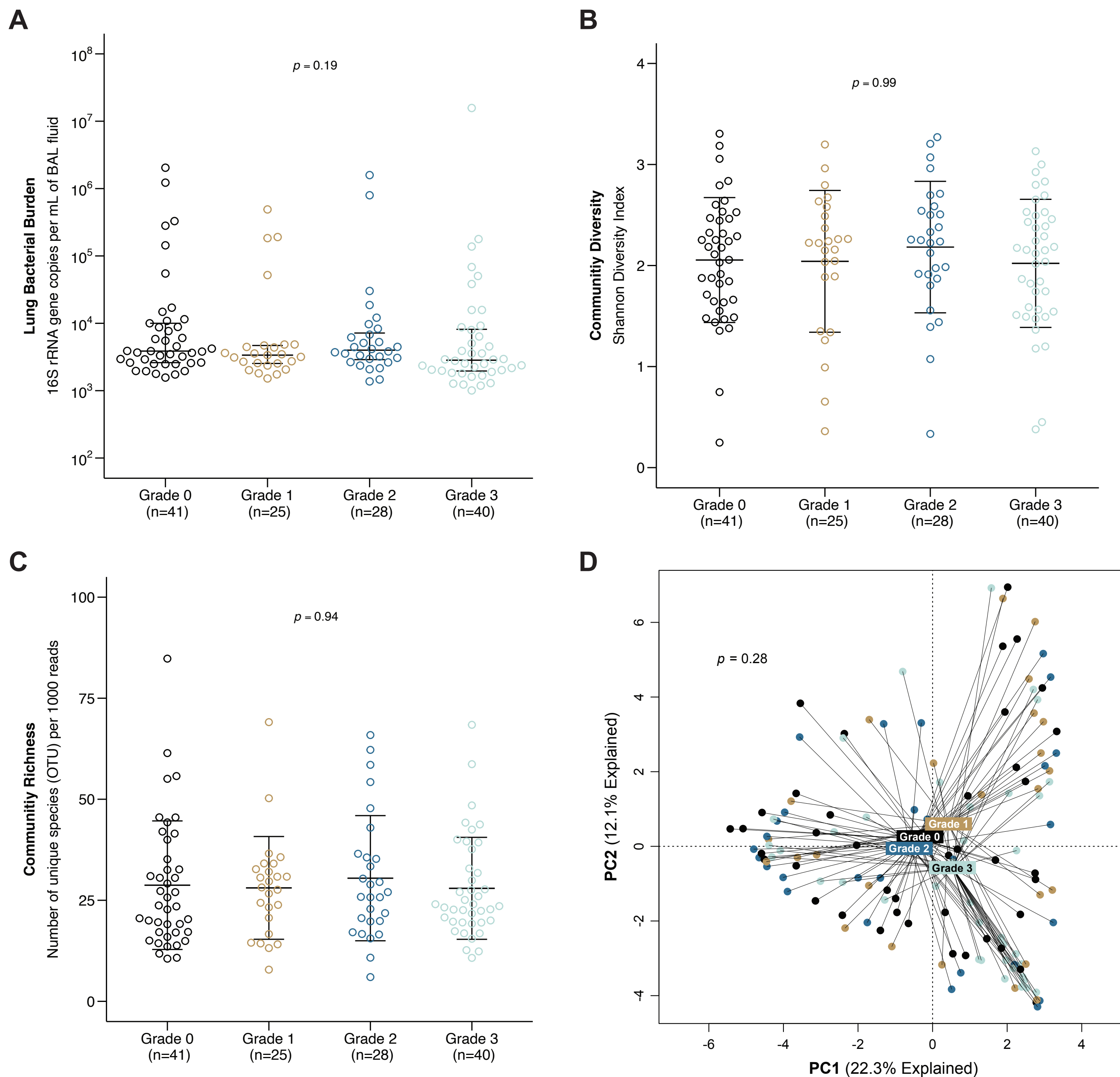
Appendix Figure 10: **Comparing respiratory microbiome characteristics on the basis of induction immunosuppression.** We compared the bacterial burden, community diversity, community richness, and overall community composition between patients who recieved basiliximab for induction immunosuppression (N=9) vs. those who did not (N=125). Patients who recieved basiliximab for induction had lower bacterial burden than those who did not recieve induction immunosuppression(A). Community diversity (B), community richness (C), and overall community composition (D) did not vary between patients who did and did not recieve induction immunosuppression. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C) and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



Appendix Figure 11: **Comparing respiratory microbiome characteristics on the basis of calcineurin inhibitition regimen.** We compared the bacterial burden, community diversity, community richness, and overall community composition between patients who were recieving cyclosoprine (N=29) or tacrolimus (N=105) at the time of one-year surveillance bronchoscopy. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or community composition (D) on the basis of pre-transplant diagnosis. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C) and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).

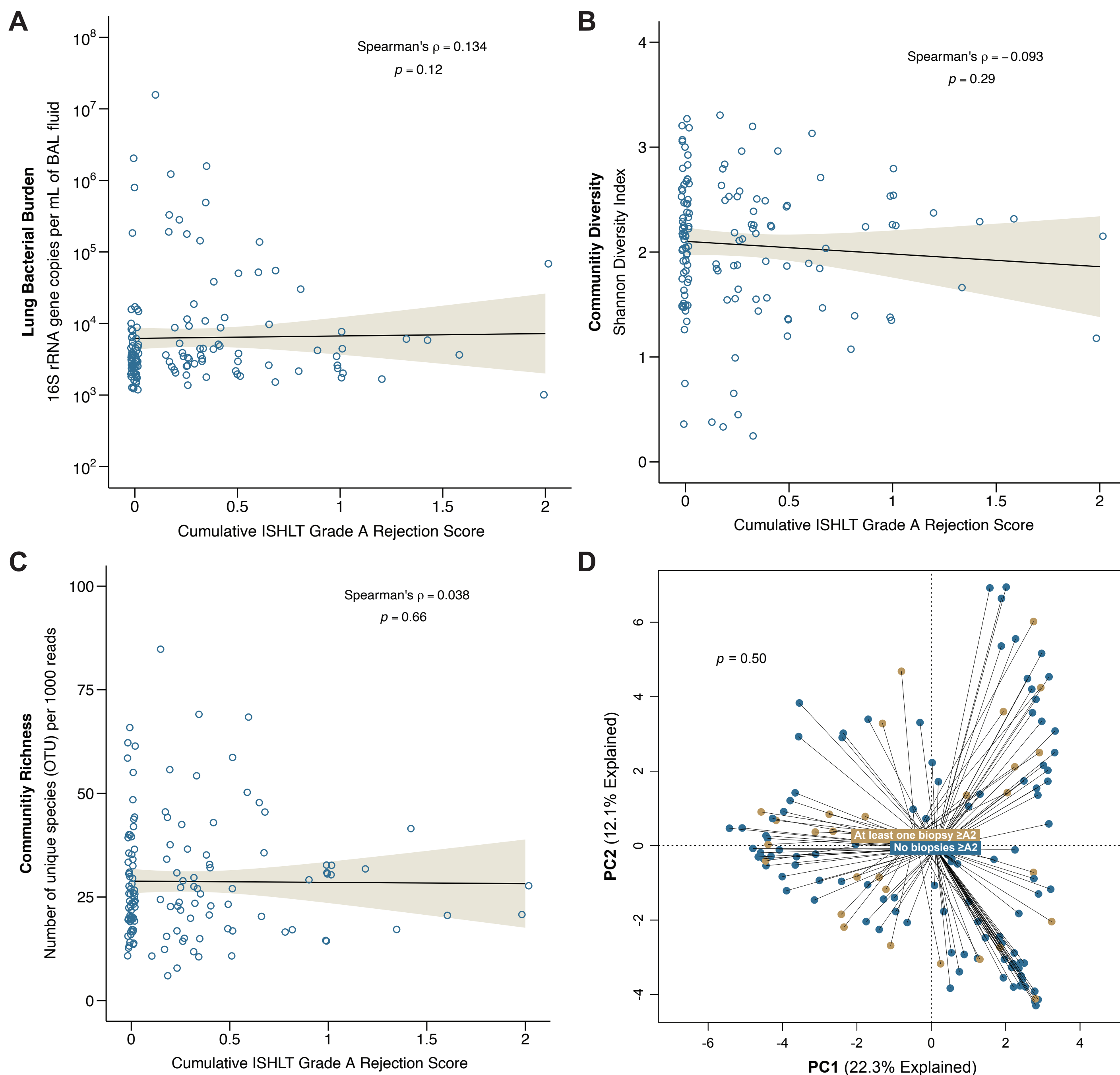


Appendix Figure 12: **Comparing respiratory microbiome characteristics on the basis of antiproliferative immunosuppression regimen.**
We compared the bacterial burden, community diversity, community richness, and overall community composition between patients receiving azathioprine (N=47), mycophenolate (N=72), and patients whose antiproliferative immunosuppression was held (N=15) at the time of one-year surveillance bronchoscopy. There was no difference in bacterial burden (A), community diversity (B), or community richness (C) on the basis of immunosuppression. Likewise, there was no difference in community composition identified on principle components analysis (D). Hypothesis testing performed using Kruskal-Wallis test (A), one-way ANOVA (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



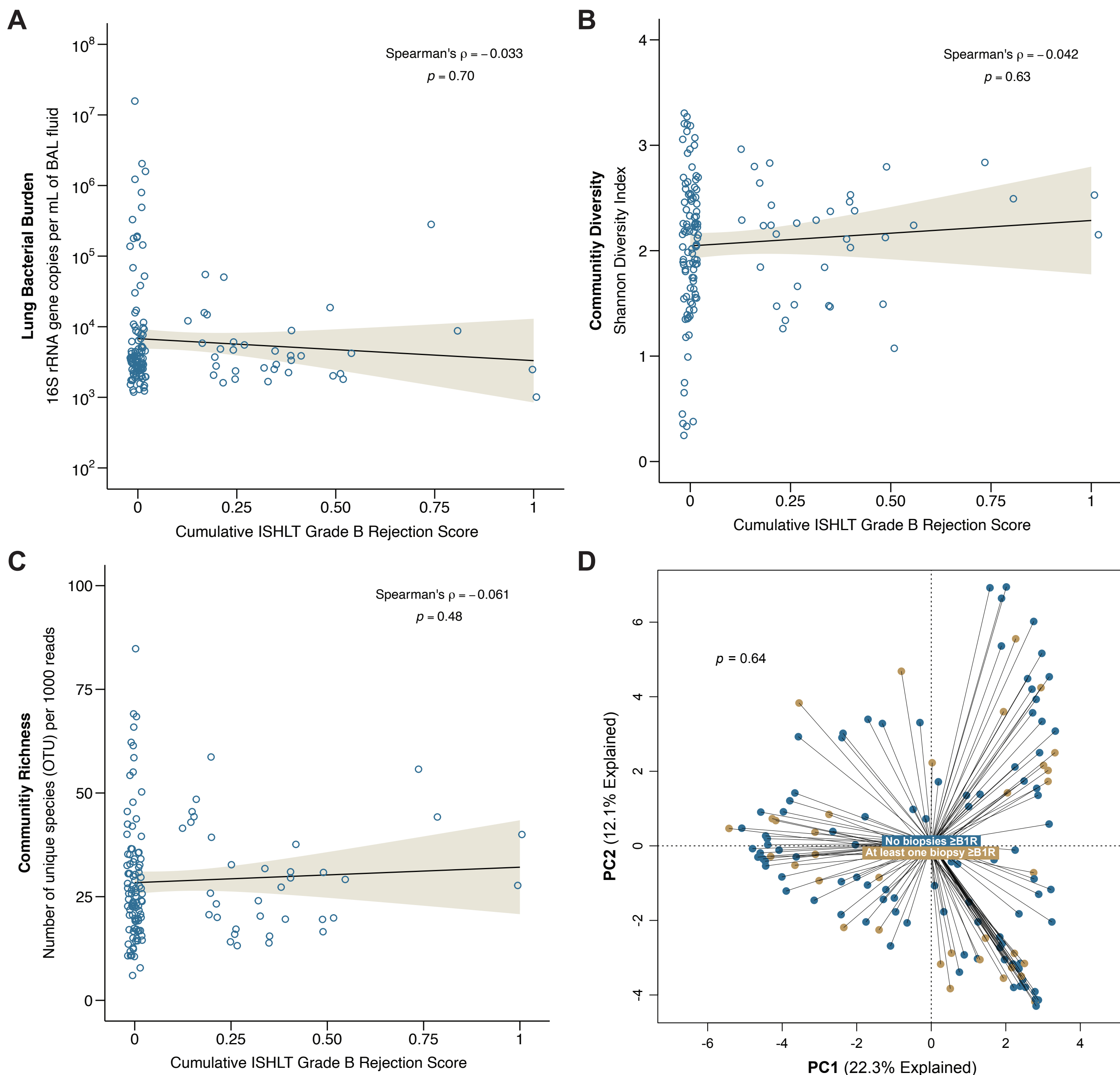
Appendix Figure 13: **Comparing respiratory microbiome characteristics on the basis of primary graft dysfunction immediately following lung transplantation.**

We compared the bacterial burden, community diversity, community richness, and overall community composition in BAL collected at one-year surveillance bronchoscopy between patients who had primary graft dysfunction (PGD) grade 0 (N=41), PGD grade 1 (N=25), PGD grade 2 (N=28), and PGD grade 3 (N=40) based evaluation immediately following lung transplantation. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or overall community composition (D) on the basis of PGD history. Hypothesis testing performed using Kruskal-Wallis test (A), one-way ANOVA (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).

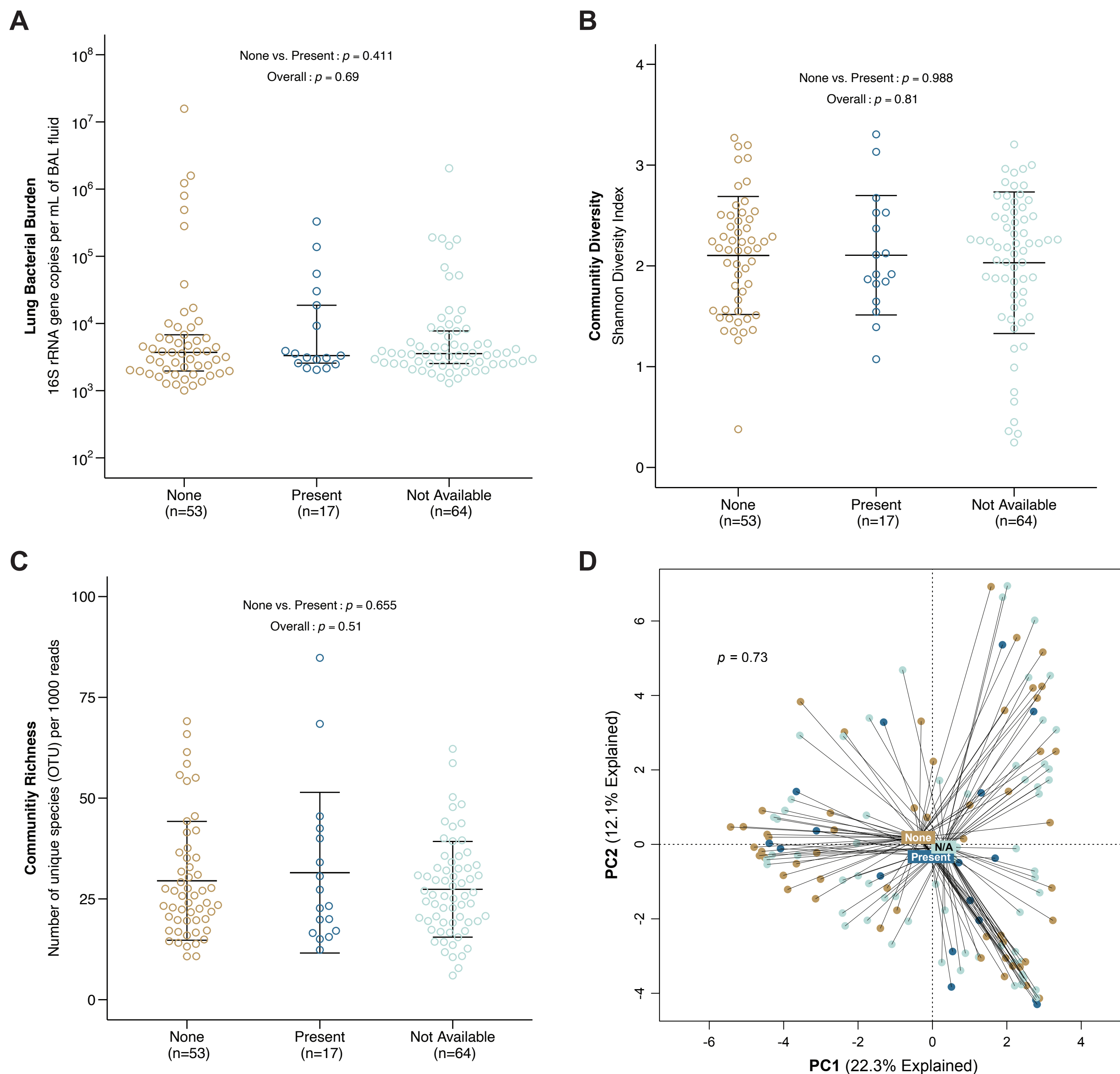


Appendix Figure 14: **Comparing respiratory microbiome characteristics on the basis of biopsy-proven acute cellular rejection episodes.**

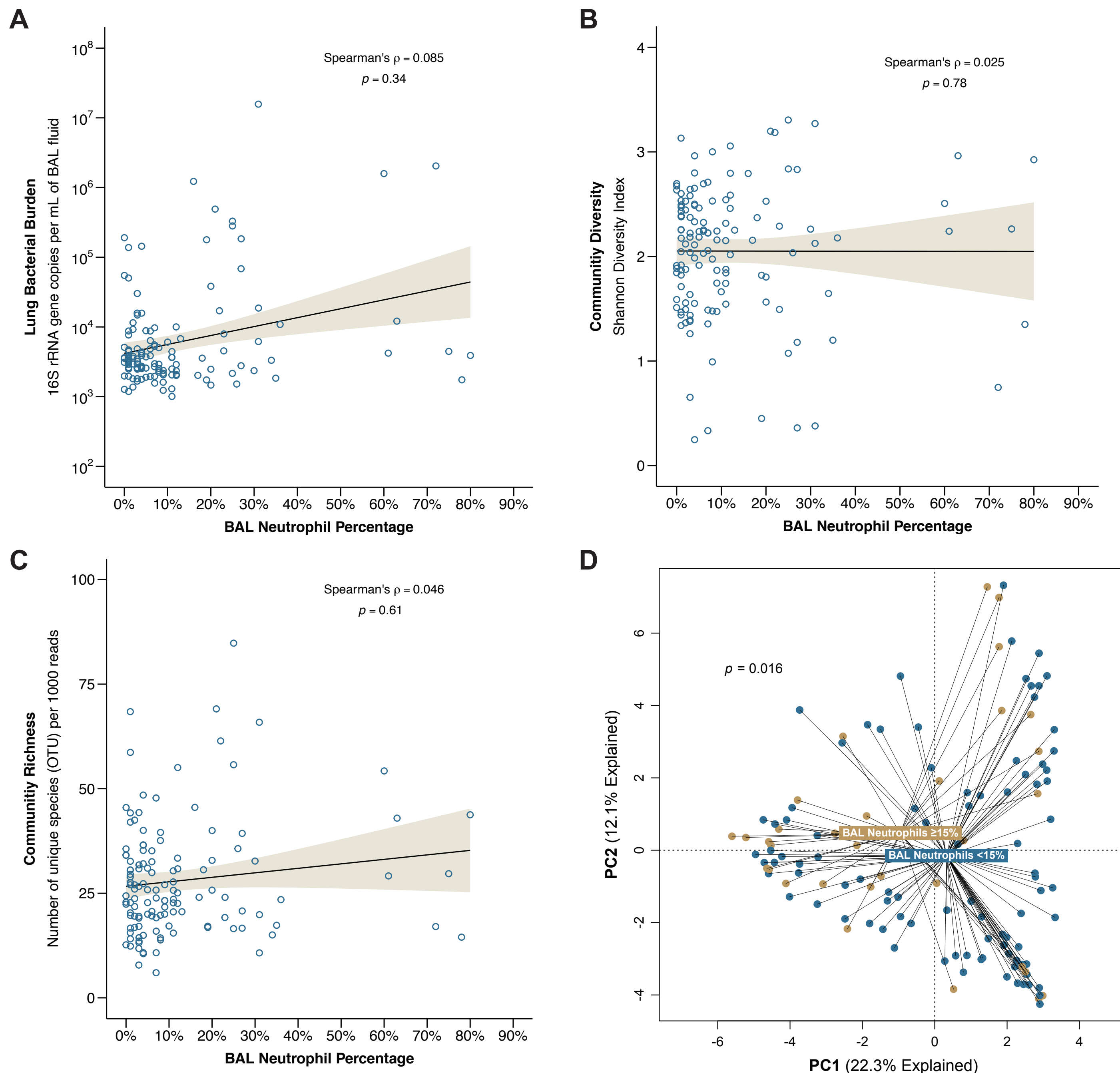
We compared the bacterial burden, community diversity, and community richness on the basis of cumulative ISHLT Grade A Rejection score (calculated by adding the ordinal values of each biopsy specimen's A score divided by the total number of biopsies performed in the first year post-transplant). There was association between cumulative ISHLT grade A rejection score and bacterial burden (A), community diversity (B), or community richness (C). To visualize differences in community composition, patients were grouped as either having no biopsies with a score of A2 or higher (N=105) or at least one biopsy with a score of A2 (N=29). There was no difference in community compositoin between patients who had had biospy-proven high-grade acute cellular rejection and those who had not (D). Hypothesis testing performed using Spearman's correlation (A-C) and *adonis* (D). Lines indicate best-fit linear regression, shaded areas indicate standard error (95% confidence level) (A-C).



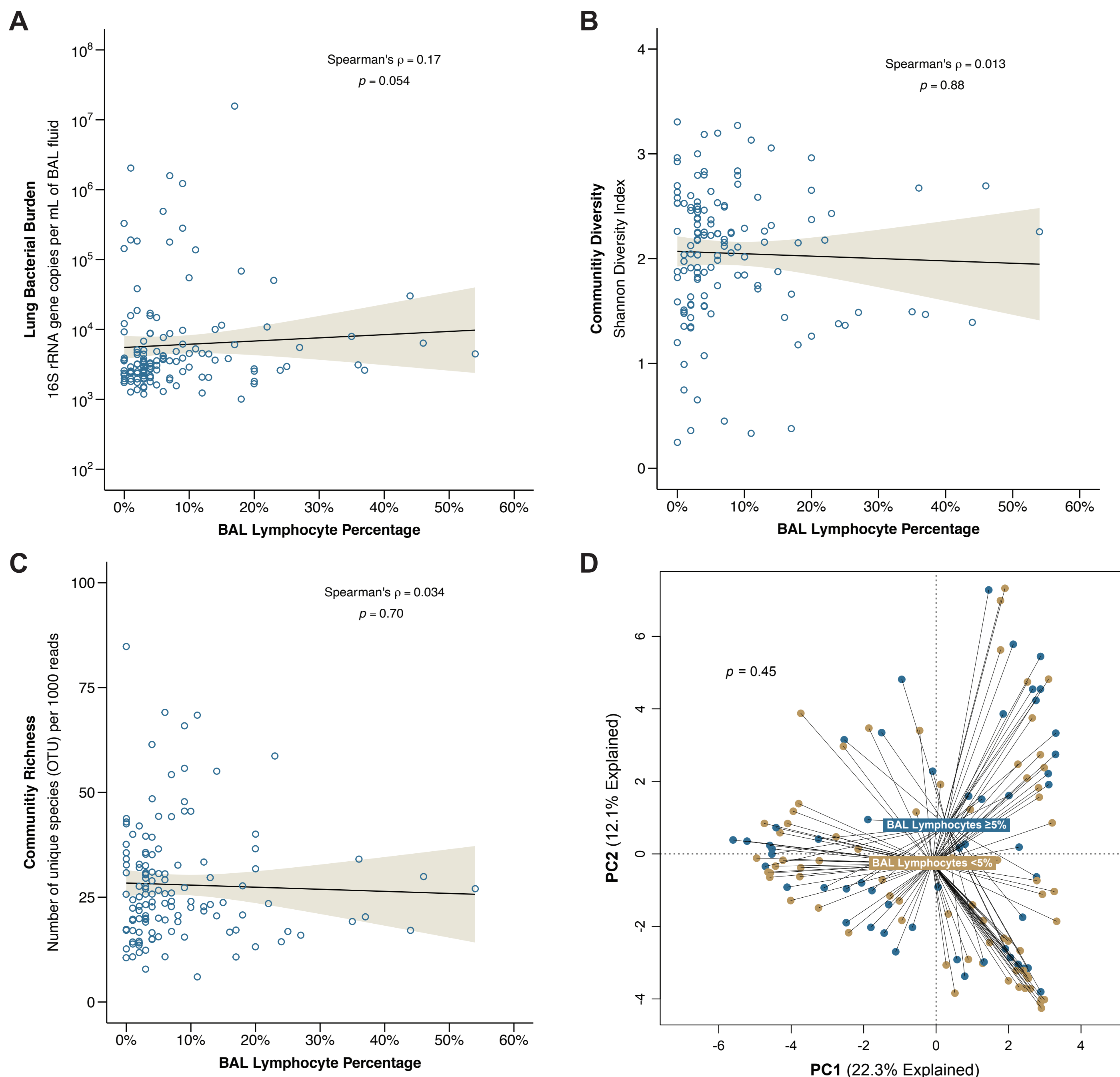
Appendix Figure 15: **Comparing respiratory microbiome characteristics on the basis of biopsy-proven lymphocytic bronchiolitis episodes.**
We compared the bacterial burden, community diversity, and community richness on the basis of cumulative ISHLT Grade B Rejection score (calculated by adding the ordinal values of each biopsy specimen's B score divided by the total number of biopsies performed in the first year post-transplant). There was no association between cumulative ISHLT grade B rejection score and bacterial burden (A), community diversity (B), or community richness (C). To visualize differences in community composition, patients were grouped as either having no biopsies with a score of B1R or higher (N=99) or at least one biopsy with a score of B1R (N=35). There was no difference in community compositoin between patients who had had any episodes of biopsy-proven lymphocytic bronchiolitis and those who had not (D). Hypothesis testing performed using Spearman's correlation (A-C) and *adonis* (D). Lines indicate best-fit linear regression, shaded areas indicate standard error (95% confidence level) (A-C).



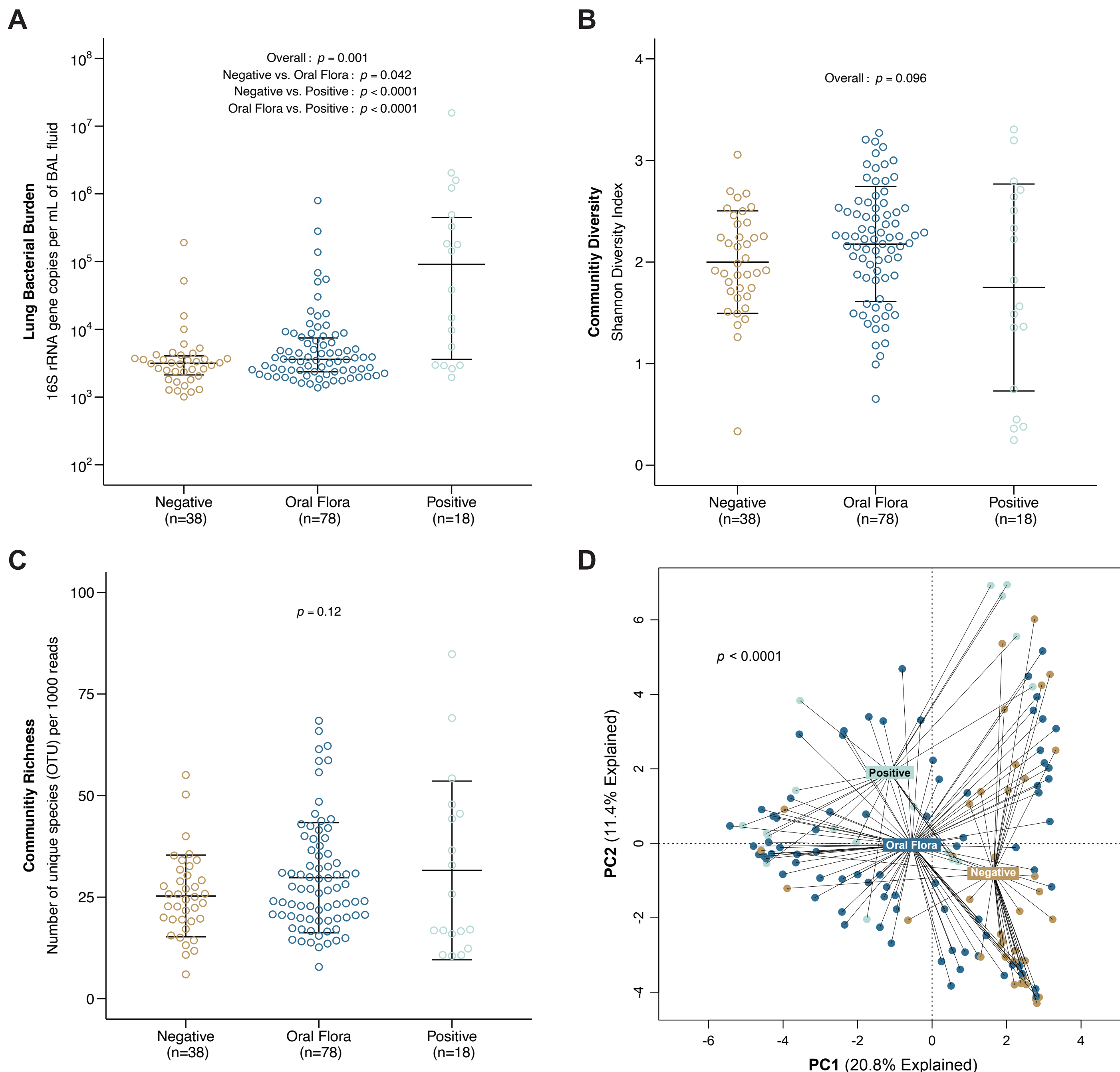
Appendix Figure 16: **Comparing respiratory microbiome characteristics on the basis of donor specific antibody presence.** We compared the bacterial burden, community diversity, community richness, and overall community composition in BAL collected at one-year surveillance bronchoscopy between patients who had evidence of donor specific antibodies (N=), no donor specific antibodies (N=) and patients for whom no donor specific antibody evaluation was avaialble (N=64). There was no difference in bacterial burden (A), community diversity (B), community richness (C), or overall community composition (D) on the basis of donor specific antibody presence. Hypothesis testing performed using Wilcoxon rank-sum test (None vs. Present) and Kruskal-Wallis test (Overall) (A), two-sample t test (None vs. Present) and one-way ANOVA (Overall) (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



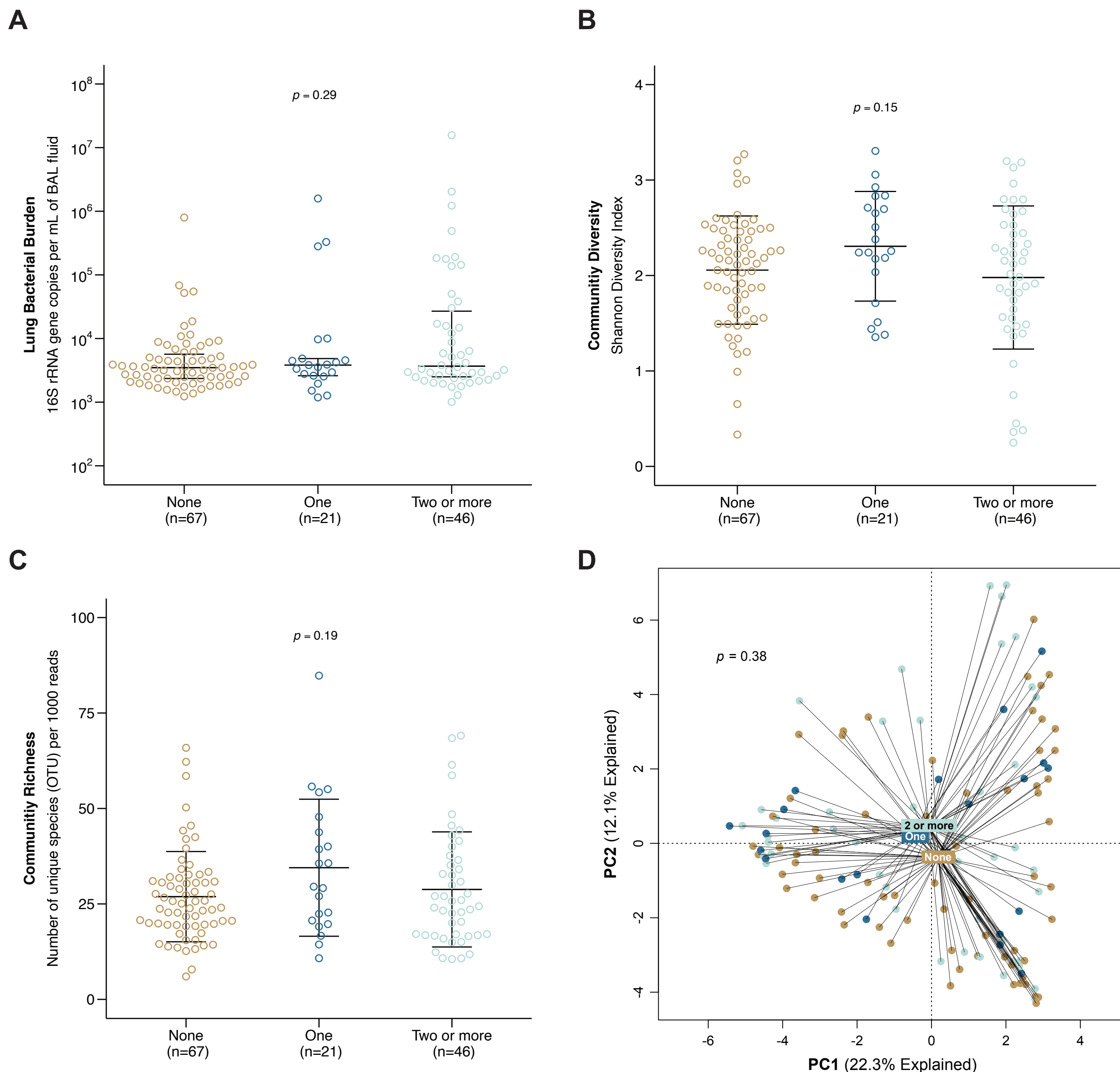
Appendix Figure 17: **Comparing respiratory microbiome characteristics on the basis of BAL neutrophil percentage.** We compared the bacterial burden, community diversity, and community richness on the basis of BAL neutrophil percentage in the one-year post-transplant surveillance bronchsocopy. There was no association between BAL neutrophil percentage and bacterial burden (A), community diversity (B), or community richness (C). To visualize differences in community composition, samples were classified as having ≥15% neutrophils (N=33) or <15% neutrophils (N=96). There was a difference in community composition between patients when categoried according to ≥15% vs. <15% BAL neutrophils (this difference was not observed when BAL neutrophil percent was analyzed as a continuous variable, p=0.123) (D). Hypothesis testing performed using Spearman's correlation (A-C) and *adonis* (D). Lines indicate best-fit linear regression, shaded areas indicate standard error (95% confidence level) (A-C).



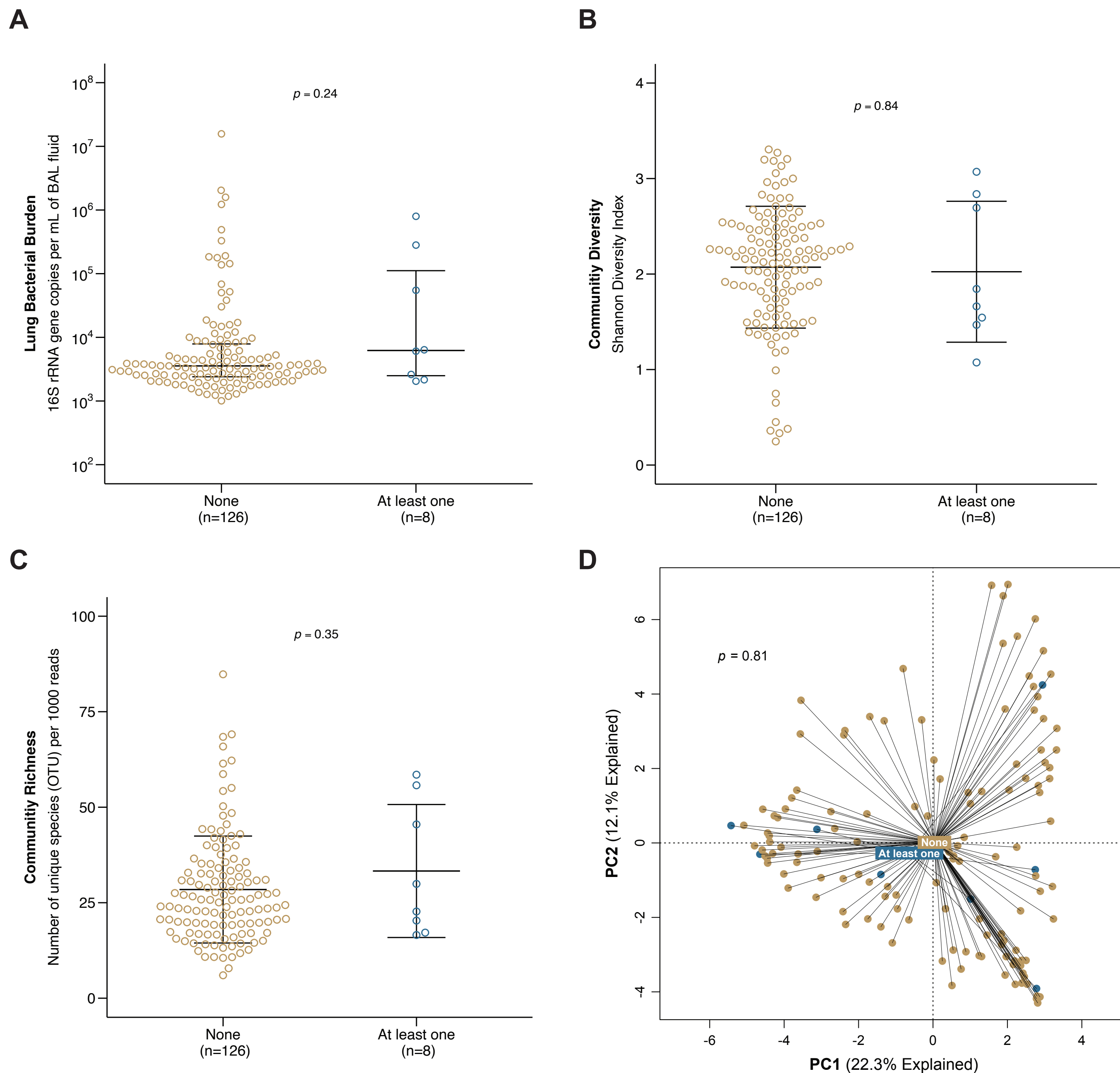
Appendix Figure 18: **Comparing respiratory microbiome characteristics on the basis of BAL lymphocyte percentage.** We compared the bacterial burden, community diversity, and community richness on the basis of BAL lymphocyte percentage in the one-year post-transplant surveillance bronchsocopy. There was no association between BAL lymphocyte percentage and bacterial burden (A), community diversity (B), or community richness (C). To visualize differences in community composition, samples were classified as having >5% lymphocytes (N=96) or ≤5% lymphocytes (N=33). There was no difference in community composition between patients with >5% vs. ≤5% BAL lymphocytes (D). Hypothesis testing performed using Spearman's correlation (A-C) and *adonis* (D). Lines indicate best-fit linear regression, shaded areas indicate standard error (95% confidence level) (A-C).



Appendix Figure 19: **Comparing respiratory microbiome characteristics on the basis of a current respiratory culture results.** We compared the bacterial burden, community diversity, community richness, and overall community composition among BAL from one-year surveillance bronchoscopy on the basis of culture results. Relative to BAL with negative cultures, BAL with cultures showing oral flora had higher bacterial burden, and BAL with cultures positive for pathogenic bacteria high higher bacterial burden than BAL with negative cultures and BAL with cultures showing oral flora (A). Community diversity (B) and community richness (C) did not vary based on BAL culture results. Overall community composition (D), however, did differ on the basis of current culture BAL culture results. Hypothesis testing performed using Kruskal-Wallis test (A), one-way ANOVA (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).

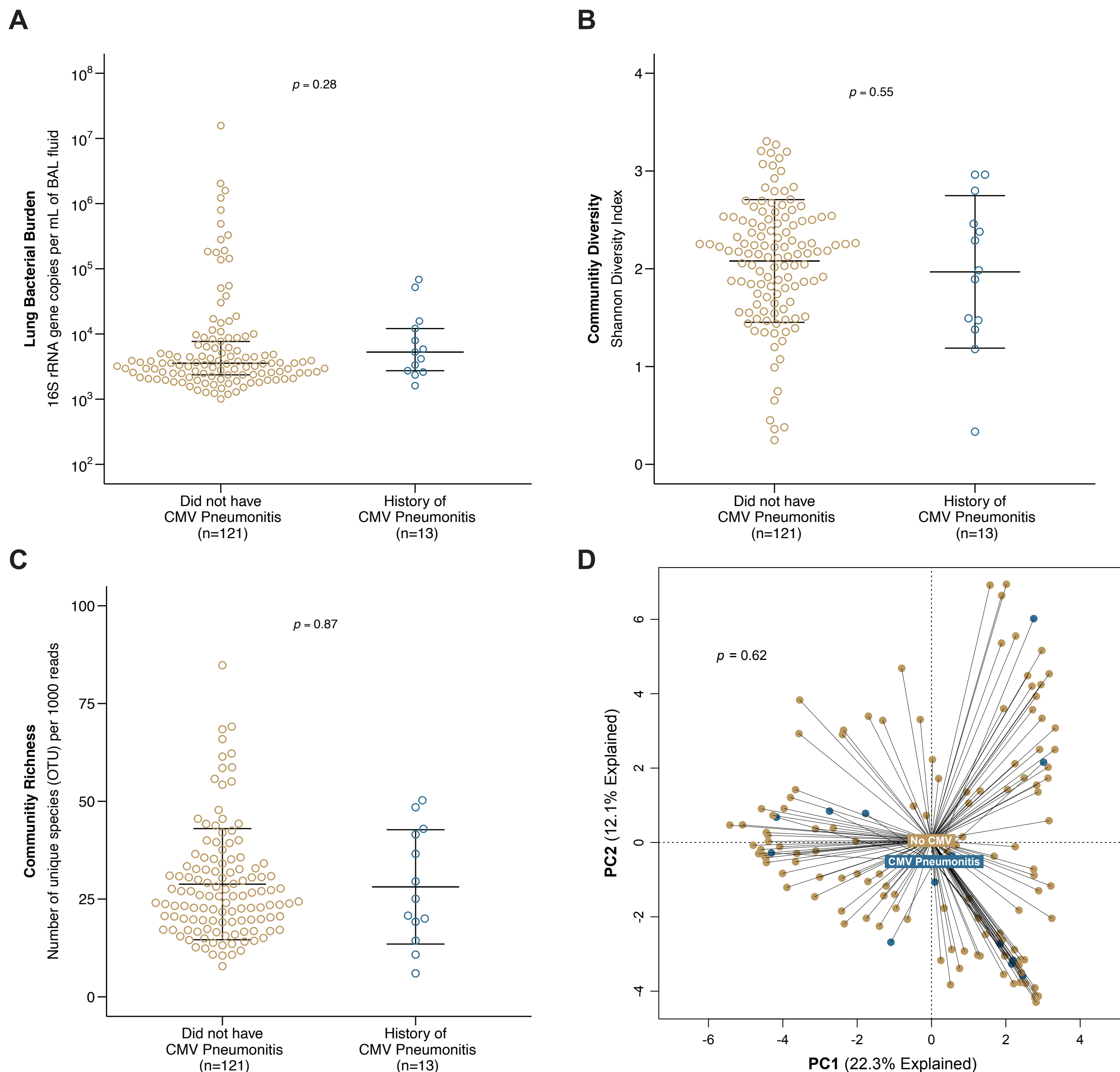


Appendix Figure 20: **Comparing respiratory microbiome characteristics on the basis of prior positive respiratory cultures.** We compared the bacterial burden, community diversity, community richness, and overall community composition in BAL collected at one-year surveillance bronchoscopy between patients who had no BAL with bacterial cultures positive for pathologic bacteria in the first year after transplant (N=89), patients who had one BAL with pathologic bacteria subsequent to transplant (N=26), and patients who had two or more BAL with cultures positive for pathologic bacteria (N=19). Of note, 18 patients had a pathologic bacteria isolated on their one-year surveillance BAL; of these, 4 were the only positive culture in the first year after transplant and 14 had at least one other BAL culture positive for pathologic bacteria. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or overall community composition (D) on the basis of a history of positive respiratory cultures. Hypothesis testing performed using Kruskal-Wallis test (A), one-way ANOVA (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).

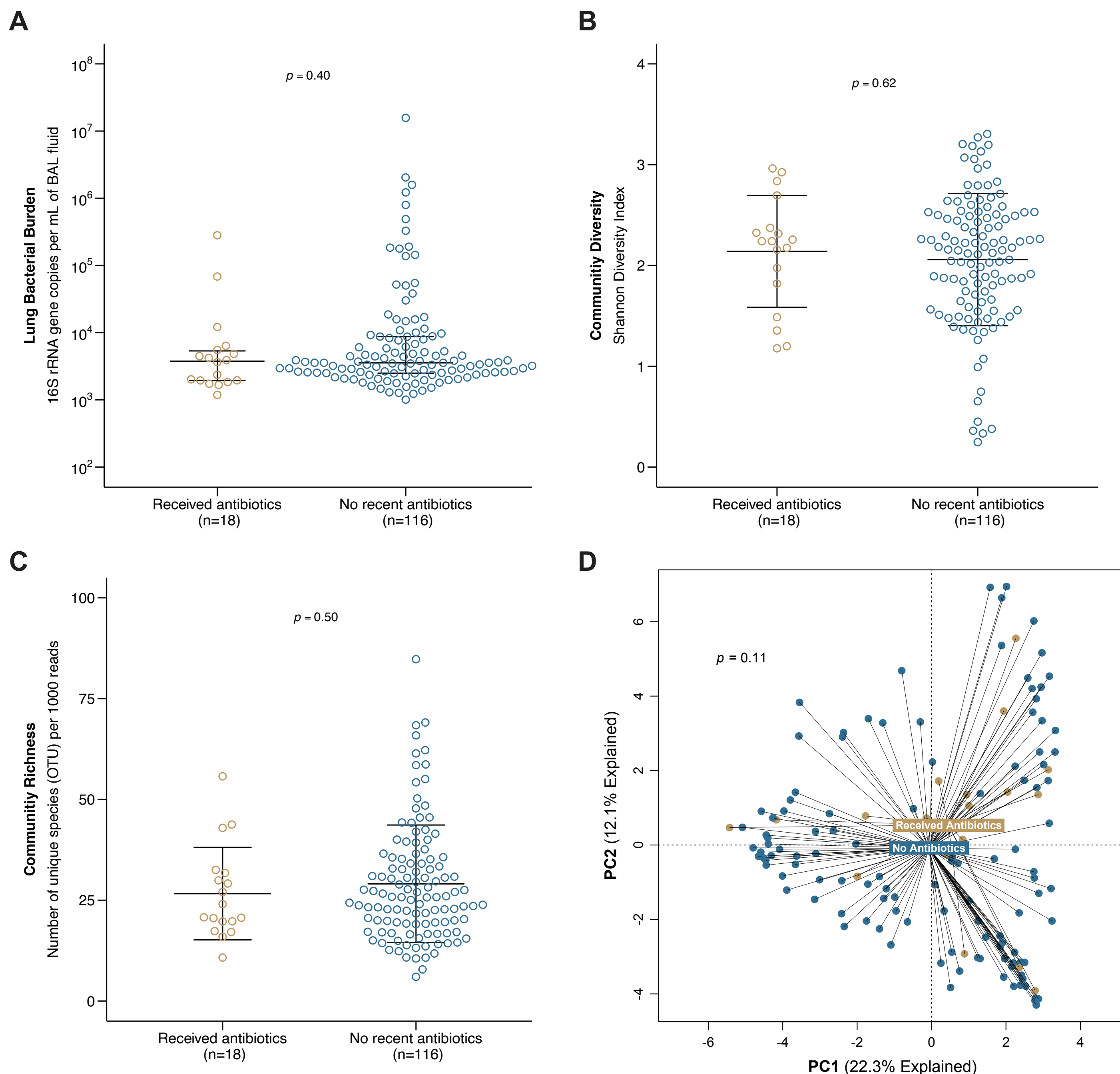


Appendix Figure 21: **Comparing respiratory microbiome characteristics on the basis of prior community-acquired respiratory viral infections.**

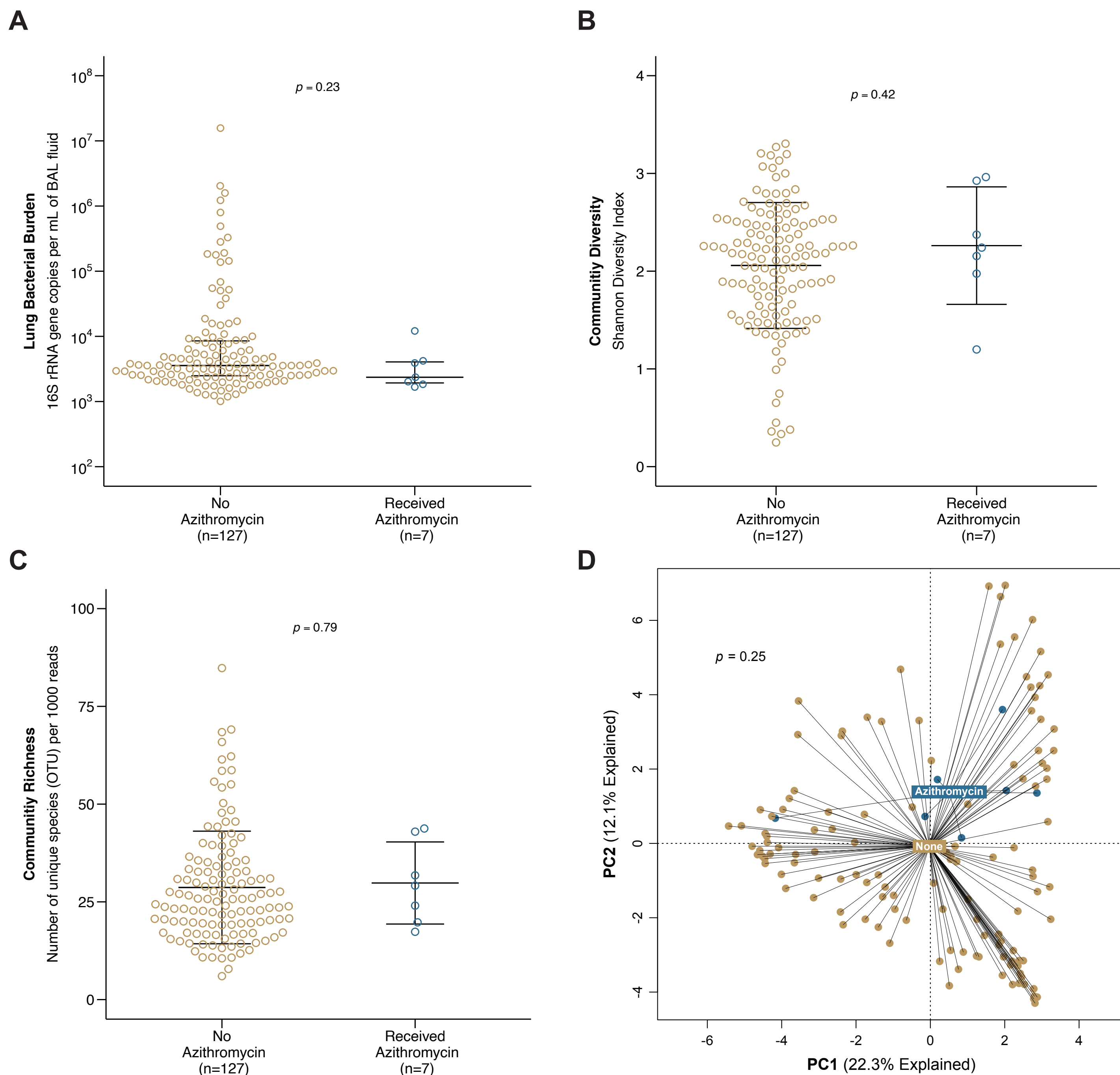
We compared the bacterial burden, community diversity, community richness, and overall community composition among BAL from one-year surveillance bronchoscopy among patients with (N=8) and without a history of having a community-acquired respiratory infection (CARV, N=128). CARV were defined as positive respiratory viral PCR and/or viral culture results in conjection with respiratory symptoms, pulmonary function decline, or radiographic infiltrate. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or overall community composition (D) between patients who did and did not have any prior CARV. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



Appendix Figure 22: **Comparing respiratory microbiome characteristics on the basis of prior CMV pneumonitis.** We compared the bacterial burden, community diversity, community richness, and overall community composition among BAL from one-year surveillance bronchoscopy among patients with (N=13) and without a history of CMV pneumonitis (N=121). CMV pneumonitis was defined by CMV inclusions seen on histopathology from transbronchial biopsy or a positive CMV culture from BAL in the presence of lower respiratory tract symptoms. There was no difference in bacterial burden (A), community diversity (B), community richness (C), or overall community composition (D) between patients who did and did not have a history of CMV pneumonitis. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C), and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



Appendix Figure 23: **Comparing respiratory microbiome characteristics on the basis of recent antibiotic exposure.**
We compared the bacterial burden, community diversity, community richness, and overall community composition between patients who had recieved antibiotics within the 30 days prior to one-year surveillance bronchoscopy (N=18) and patients who had not recieved any antibiotics, other than routine *Pneumocystis jiroveci* prophylaxis (N=116). There was no difference in bacterial burden (A), community diversity (B), community richness (C), or community composition (D) on the basis of recent antibiotic exposure. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C) and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).



Appendix Figure 24: **Comparing respiratory microbiome characteristics on the basis of receipt of azithromycin for CLAD prevention.**

We compared the bacterial burden, community diversity, community richness, and overall community composition between patients who had recieved azithromycin for CLAD prevention at the time of one-year surveillance bronchoscopy (N=7) and patients who were not (N=127). There was no difference in bacterial burden (A), community diversity (B), community richness (C), or community composition (D) on the basis of recent antibiotic exposure. Hypothesis testing performed using Wilcoxon rank-sum test (A), two-sample t test (B-C) and *adonis* (D). Lines indicate median and interquartile range (A) and mean +/- standard deviations (B-C).

Appendix Table 1: Comparison of community composition by taxonomic level on between patients who develop CLAD or death within 500 days of one-year surveillance bronchoscopy vs. CLAD-free survivors

	Analytic Method	
Taxonomic Level	*PERMANOVA p*-value*	*Multivariate GLM p-value*†
Phylum	0.14	0.057
Class	**0.036**	**0.024**
Family	**0.047**	0.091
Genus	**0.045**	0.11
OTU	0.065	0.086

Definition of abbreviations: PERMANOVA = permutational multivariate analysis of variance, GLM = generalized linear model, OTU = operational taxonomic unit.
*Computed using the *adonis* function in *vegan,*[13] requesting 10,000 permutations.
†Computed using the *mvabund* package.[14]

Appendix Table 2: Patients with evidence of *P. aeruginosa* on BAL culture or 16S rRNA gene sequencing

Patient ID	Age	Pre-Transplant Diagnosis	BAL Culture Result	Relative abundance of OTU 0006: *P. aeruginosa* (%)	Pre-Transplant Culture positive for *P. aeruginosa**	Colonized with *P. aeruginosa* post-lung transplant†
74	52	COPD/Emphysema	*P. aeruginosa*	0.0%	n/a	Not colonized
67	56	IPF	Negative	1.4%	n/a	Not colonized
23	29	Cystic Fibrosis	Negative	1.7%	n/a	Colonized
85	39	Cystic Fibrosis	Oral Flora	2.0%	Positive	Not colonized
1	59	IPF	Negative	3.0%	Negative	Colonized
130	56	COPD/Emphysema	Oral Flora	3.1%	n/a	Not colonized
94	51	COPD/Emphysema	Oral Flora	3.3%	n/a	Not colonized
77	28	Cystic Fibrosis	*P. aeruginosa*	5.3%	n/a	Colonized
51	47	Cystic Fibrosis	Oral Flora	8.4%	n/a	Colonized
56	60	COPD/Emphysema	Oral Flora	11.4%	n/a	Not colonized
32	32	Cystic Fibrosis	*P. aeruginosa*	16.5%	Positive	Colonized
116	54	COPD/Emphysema	Oral Flora	26.7%	n/a	Not colonized
14	27	Cystic Fibrosis	Negative	57.4%	Positive	Colonized
55	53	RA-associated ILD	*P. aeruginosa*	64.4%	n/a	Colonized
25	20	Cystic Fibrosis	*P. aeruginosa*	94.3%	n/a	Colonized
24	46	Cystic Fibrosis	*P. aeruginosa*	97.4%	Positive	Colonized
57	19	Cystic Fibrosis	*P. aeruginosa*	98.4%	n/a	Colonized

Definition of abbreviations: BAL = bronchoalveolar lavage, COPD = Chronic obstructive lung disease, IPF = idiopathic pulmonary fibrosis, RA = rheumatoid arthritis, ILD = interstitial lung disease, n/a = not available.

*Based on BAL or sputum samples available prior to transplant. As the University of Michigan is a lung transplant referral center, some patients with CF did not receive their pre-transplant care at the University of Michigan and, thus, did not have pre-transplant culture data available.

†Patients with multiple BAL cultures positive of P. aeruginosa, regardless of symptoms, were considered to be colonized. 16S rRNA gene sequencing was not available at other time points and not considered for colonization.

Appendix Table 3: Predictors of developing CLAD in the 500 days after one-year post-transplant surveillance BAL

Variable	**HR (95% CI)**	***p*-value***
Pre-Transplant Diagnosis		
COPD	1	Referent
ILD	1.50 (0.53 - 4.22)	0.45
Cystic Fibrosis	0.25 (0.05 - 1.31)	0.10
Other	0.93 (0.17 - 5.15)	0.93
Induction Immunosuppression		
No Induction	1	Referent
Basiliximab	0.27 (0.03 - 2.57)	0.25
Antiproliferative Immunosuppression†		
Azathioprine	1	Referent
Mycophenylate	0.37 (0.12 - 1.11)	0.075
None/Held	1.99 (0.40 - 10.03)	0.40
Primary Graft Dysfunction Immediately After Transplant		
Grade 0	1	Referent
Grade 1	0.80 (0.22 - 2.98)	0.74
Grade 2	0.35 (0.08 - 1.55)	0.17
Grade 3	1.39 (0.47 - 4.16)	0.55
Average Cumulative A Rejection Score, per 1‡	0.47 (0.12 - 1.83)	0.28
Average Cumulative B Rejection score, per 1‡	0.35 (0.03 - 4.18)	0.40
DSA Present¶	2.25 (0.66 - 7.68)	0.19
BAL Bacterial Culture Results§		
Negative	1	Referent
Oral Flora	1.16 (0.40 - 3.40)	0.78
Speciated Result	0.62 (0.10 - 4.00)	0.62
History of Community-Acquired Respiratory Viral Infection‖,**	0.58 (0.07 - 4.70)	0.61
History of CMV Pneumonitis‖,††	1.75 (0.31 - 9.73)	0.52
Lung Bacterial Burden, per $\log_{10}$ increase§§	2.05 (1.07 - 3.94)	**0.030**
Lung Bacterial Burden Tertiles§§		
Lowest Bacterial Burden Tertile	1	Referent
Middle Bacterial Burden Tertile	4.71 (1.10 - 20.06)	**0.036**
Highest Bacterial Burden Tertile	9.26 (2.04 - 42.07)	**0.0039**

Definition of abbreviations: BAL = bronchoalveolar lavage, CLAD = chronic lung allograft dysfunction, COPD = Chronic obstructive lung disease, ILD = interstitial lung disease, DSA = donor specific antibodies.

*Calculated using Cox proportional hazards regression models.

†As determined at the time of one-year post-transplant surveillance bronchoscopy only. The immunosuppression regimen over the 500 days of follow up, including decisions to stop or start immunosuppression, transition within class, and add other agents was at the discretion of the transplant physician.

‡Calculated by adding the ordinal values of each biopsy specimen's A or B score divided by the total number of biopsies performed in the first year post-transplant.

¶Defined as a mean fluorescence intensity of ≥3000 via single antigen bead testing of the patient's serum prior to or at the time of 1-year post-transplant surveillance BAL. Sixty-four patients had no DSA information available and were analyzed as unique category (not reported).

§Identified in the 1 year surveillance BAL.

‖At any point from transplant to 1-year surveillance bronchoscopy.

**Defined as any positive RPAN in the presence of respiratory symptoms, transient decline in spirometry, or radiographic infiltrate.

††Defined as any CMV detected on transbronchial biopsy, CMV culture from BAL, or CMV shell antigen from BAL.

‡‡Excluding routine pneumocystis carinii prophylaxis.

§§Unique analyses were performed for Bacterial burden as a continuous variable and tertiles.

Appendix Table 4: Predictors of developing CLAD or death in the 2000 days after one-year post-transplant surveillance BAL

Variable	HR (95% CI)	*p*-value[*]
Pre-Transplant Diagnosis		
COPD	1	Referent
ILD	0.65 (0.34 - 1.25)	0.19
Cystic Fibrosis	0.42 (0.20 - 0.92)	**0.030**
Other	0.89 (0.34 - 2.31)	0.81
Induction Immunosuppression		
No Induction	1	Referent
Basiliximab	0.52 (0.13 - 2.04)	0.35
Antiproliferative Immunosuppression[†]		
Azathioprine	1	Referent
Mycophenylate	1.02 (0.54 - 1.94)	0.94
None/Held	2.95 (1.21 - 7.19)	**0.018**
Primary Graft Dysfunction Immediately After Transplant		
Grade 0	1	Referent
Grade 1	0.92 (0.42 - 2.02)	0.84
Grade 2	0.63 (0.29 - 1.35)	0.24
Grade 3	0.93 (0.47 - 1.85)	0.84
Average Cumulative A Rejection Score, per 1[‡]	1.11 (0.57 - 2.17)	0.75
Average Cumulative B Rejection score, per 1[‡]	0.53 (0.11 - 2.64)	0.44
DSA Present[¶]	1.25 (0.53 - 2.95)	0.61
BAL Bacterial Culture Results[§]		
Negative	1	Referent
Oral Flora	0.90 (0.50 - 1.63)	0.73
Speciated Result	0.77 (0.31 - 1.94)	0.58
History of Community-Acquired Respiratory Viral Infection[ǀ,**]	0.77 (0.22 - 2.62)	0.67
History of CMV Pneumonitis[ǀ,††]	1.80 (0.77 - 4.18)	0.17
Lung Bacterial Burden, per log_{10} increase[§§]	1.55 (1.04 - 2.30)	**0.030**
Lung Bacterial Burden Tertiles[§§]		
Lowest Bacterial Burden Tertile	1	Referent
Middle Bacterial Burden Tertile	1.52 (0.79 - 2.93)	0.21
Highest Bacterial Burden Tertile	1.86 (0.90 - 3.85)	0.093

Definition of abbreviations: BAL = bronchoalveolar lavage, CLAD = chronic lung allograft dysfunction, COPD = Chronic obstructive lung disease, ILD = interstitial lung disease, DSA = donor specific antibodies.

[*]Calculated using Cox proportional hazards regression models.

[†]As determined at the time of one-year post-transplant surveillance bronchoscopy only. The immunosuppression regimen over the 500 days of follow up, including decisions to stop or start immunosuppression, transition within class, and add other agents was at the discretion of the transplant physician.

[‡]Calculated by adding the ordinal values of each biopsy specimen's A or B score divided by the total number of biopsies performed in the first year post-transplant.

[¶]Defined as a mean fluorescence intensity of ≥3000 via single antigen bead testing of the patient's serum prior to or at the time of 1-year post-transplant surveillance BAL. Sixty-four patients had no DSA information available and were analyzed as unique category (not reported).

[§]Identified in the 1 year surveillance BAL.

[ǀ]At any point from transplant to 1-year surveillance bronchoscopy.

[**]Defined as any positive RPAN in the presence of respiratory symptoms, transient decline in spirometry, or radiographic infiltrate.

[††]Defined as any CMV detected on transbronchial biopsy, CMV culture from BAL, or CMV shell antigen from BAL.

[‡‡]Excluding routine pneumocystis carinii prophylaxis.

[§§]Unique analyses were performed for Bacterial burden as a continuous variable and tertiles.

Appendix Table 5: Detailed clinical information for patients who were not receiving an antiproliferative immunosuppression medication at the time of one-year post-transplant surveillance bronchoscopy.

ID	Sex	Age	Pre-Transplant Diagnosis	Duration Immunosuppression Held at BAL	Previous Antiproliferative Immunosuppressive Medication	Total 16S copies/mL	Shannon Diversity Index	Number of unique OTU per 1000 reads	Clinical Vignette
20	Female	60	COPD/ Emphysema	1 day	MMF	3641	2.32	20.5	Seen at a local hospital for malaise and poor oral intake and was empirically treated with antibiotics. She was subsequently transferred to the University of Michigan where she was found to be at her baseline, with the working diagnosis for her initial presentation attributed to dehydration. The surveillance bronchoscopy was done as an inpatient for convenience's sake; immediately thereafter the patient was promptly discharged with no antibiotics and her immunosuppression was resumed.
98	Male	58	ILD	3 weeks	Azathioprine	1186	2.17	19.7	Admitted for cellulitis three weeks prior bronchoscopy, completed a two-week course of antibiotics. Immunosuppression was not restarted upon completion of antibiotics due to mild elevation in liver function tests; shortly after the bronchoscopy these had resolved, and immunosuppression was resumed.
18	Female	48	COPD/ Emphysema	1 month	Azathioprine	4551	1.71	22.7	CMV viremia diagnosed one month prior to surveillance BAL, initiated on valganciclovir. Shortly after starting treatment, she returned to her baseline. Remained on antiviral treatment at the time of her surveillance bronchoscopy.
34	Female	55	Other	1 month	Azathioprine	3067	1.92	15.6	WBC was <2.5, per protocol immunosuppression had been reduced and then stopped.
122	Female	53	ILD	2 months	MMF	2951	1.36	16.8	CMV pneumonitis two months prior, was treated with valganciclovir for three weeks with improvement in CT imaging. WBC had decreased to <2.5 while on antiviral treatment and immunosuppression remained on hold at time of surveillance bronchoscopy pending improvement in leukopenia.
76	Female	20	Cystic Fibrosis	3 months	MMF	15708014	0.38	10.7	Patient diagnosed with CMV colitis/viremia and was started on valganciclovir and immunosuppression was held. GI symptoms had resolved shortly after treatment; no respiratory issues at diagnosis or time of bronchoscopy.
51	Female	42	Cystic Fibrosis	3 months	MMF	1518	2.04	35.7	Patient had persistent low-grade CMV viremia requiring treatment with valganciclovir and CMV immunoglobulin, her immunosuppression was held per protocol while on these treatments.
59	Female	62	ILD	3 months	MMF	54850	1.84	45.5	WBC was <2.5, per protocol immunosuppression had been reduced and then stopped.
134	Male	64	ILD	3 months	MMF	1007	2.15	27.7	CMV pneumonitis three months prior, was treated with valganciclovir and immunosuppression was held. Symptomatically improved in the interim and was back to baseline lung function. Immunosuppression remained on hold while awaiting results of surveillance bronchoscopy.
110	Male	59	COPD/ Emphysema	4 months	MMF	1372	1.56	21.8	CMV viremia four months prior, started on valganciclovir and immunosuppression was held. Viremia persistent at time of bronchoscopy but patient was at baseline respiratory status.

106	Male	47	ILD	5 months	MMF	4536	2.29	24.0	CMV viremia diagnosed five months prior, started on valganciclovir and immunosuppression was held. Viremia had resolved as of bronchoscopy, immunosuppression remained on hold pending results of bronchoscopy.
47	Male	57	ILD	5 months	None	68416	1.18	20.8	Patient was found to have a *Mycobacterium chelonae* infection of his pleural space after his transplant. To manage this infection his immunosuppression was held, and he was on treatment with long-term antibiotics.
88	Male	67	COPD/ Emphysema	5 months	MMF	796254	3.07	58.5	Previously had CMV viremia which had resolved after treatment of valganciclovir; immunosuppression was not resumed due to persistent WBC <2.5. Otherwise asymptomatic with stable/improving lung function.
78	Male	27	Cystic Fibrosis	6 months	MMF	5520	1.49	15.9	CMV viremia diagnosed six months prior to surveillance bronchoscopy, started on valganciclovir and immunosuppression was held. Viremia had resolved as of bronchoscopy, with plan to resume immunosuppression pending bronchoscopy results.
67	Male	32	Cystic Fibrosis	9 months	MMF	1899	2.18	22.8	Patient had PTLD shortly after transplant for which immunosuppression had been held. One month prior to surveillance BAL, PET scan showed complete resolution of PTLD.

Definition of abbreviations: BAL = bronchoalveolar lavage, COPD = Chronic obstructive lung disease, CMV = cytomegalovirus, CT = computed tomography, ID = sample identifier, ILD = interstitial lung disease, MMF = mycophenolate mofetil, OTU = operational taxonomic unit, PET = positron emission tomography, PTLD = post-transplant lymphoproliferative disorder, WBC = white blood cell count.

Appendix Table 6: Detailed clinical information for patients who received antibiotics within 30 days of one-year post-transplant surveillance bronchoscopy.

ID	Sex	Age	Pre-Transplant Diagnosis	Days prior to BAL that antibiotics were discontinued	Antibiotics Received	Total 16S copies/mL	Shannon Diversity Index	Number of unique OTU per 1000 reads	BAL Culture Results	Clinical Vignette
81	Male	26	ILD	0	Azithromycin	2020	2.15	24	Oral flora	Was taking azithromycin three times weekly for CLAD prevention at time of bronchoscopy.
37	Male	26	Cystic Fibrosis	0	Azithromycin	6369	2.69	29.9	Oral flora	Prior bronchoscopy with *Mycobacterium chelonae abscesses*, for which the patients was started on chronic suppressive antibiotics. Most recent CT scan had shown no signs of active infection and BAL AFB culture was negative.
133	Male	27	Cystic Fibrosis	0	Inhaled Colistin	1737	1.82	17.1	Oral flora	The patient had multiple BAL cultures were positive for *P. aeruginosa* after transplant, prompting initiation of inhaled antibiotics every other month for suppressive therapy (currently using at time of bronchoscopy). Since starting this regimen eight months prior to surveillance bronchoscopy, subsequent BAL cultures had not shown persistent *P. aeruginosa* and the patient was asymptomatic from a respiratory perspective.
68	Male	54	ILD	0	Azithromycin; Inhaled Tobramycin	4208	2.24	29.1	Negative	Prior BAL results showed persistent positive cultures for *P. aeruginosa*, prompting initiation of inhaled antibiotics for suppression. The patient was not symptomatic for multiple visits prior to surveillance bronchoscopy. In addition to the inhaled antibiotics, he was taking azithromycin three times weekly for CLAD prevention.
15	Male	55	ILD	0	Azithromycin	3898	2.92	43.8	Oral flora	Was taking azithromycin three times weekly for CLAD prevention at time of bronchoscopy.
47	Male	57	ILD	0	Moxifloxacin; Azithromycin	68416	1.18	20.8	Oral flora	Patient was found to have a *Mycobacterium chelonae* infection of his pleural space after transplant and was treated with long-term antibiotics. At the time of surveillance bronchoscopy, he had no acute issues and the antibiotics were discontinued after negative BAL cultures.
65	Male	62	ILD	0	Azithromycin	1839	1.2	17.3	Oral flora	Was taking azithromycin three times weekly for CLAD prevention at time of bronchoscopy.
31	Male	63	ILD	0	Azithromycin	12111	2.96	43	Oral flora	Was taking azithromycin three times weekly for CLAD prevention at time of bronchoscopy.
107	Female	65	COPD/ Emphysema	0	Amoxicillin	2358	1.97	19.8	Oral flora	Had upper respiratory infection symptoms and was empirically treated with amoxicillin 10 days prior to bronchoscopy without improvement. At the time of bronchoscopy, the patient denied cough or shortness of breath; her PFT values were at her baseline and chest imaging was clear.
103	Female	66	COPD/ Emphysema	0	Azithromycin	1670	2.37	31.8	Negative	Was taking azithromycin three times weekly for CLAD prevention at time of bronchoscopy.
109	Male	47	ILD	1	Azithromycin	281163	2.84	55.7	Oral flora	Developed upper respiratory infection symptoms the week prior to surveillance bronchoscopy and was given an empiric course of antibiotics.

20	Female	60	COPD/ Emphysema	1	Vancomycin; Ceftriaxone; Levofloxacin	3641	2.32	20.5	Oral flora	Seen at a local hospital for malaise and poor oral intake and was empirically treated with antibiotics. She was subsequently transferred to the University of Michigan where she was found to be at her baseline, with the working diagnosis for her initial presentation attributed to dehydration. The surveillance bronchoscopy was done as an inpatient for convenience's sake; immediately thereafter the patient was promptly discharged with no antibiotics and her immunosuppression was resumed.
98	Male	58	ILD	6	Piperacillin-Tazobactam	1186	2.17	19.7	Negative	Admitted for cellulitis three weeks prior bronchoscopy, was empirically treated for a two-week course. Patient reported no respiratory symptoms during or after treatment.
78	Male	27	Cystic Fibrosis	17	Ciprofloxacin	5520	1.49	15.9	*P. aeruginosa*	Five weeks prior to surveillance bronchoscopy he underwent sinus surgery for persistent sinusitis, he was treated with two weeks of ciprofloxacin for prophylaxis post-operatively.
82	Male	55	COPD/ Emphysema	18	Levofloxacin	1954	1.35	10.8	*E. coli*	Received a 10-day course of antibiotics for upper respiratory infection symptoms; symptomatically improved by end of course.
85	Male	64	ILD	20	Levofloxacin	1947	2.32	32.5	Oral flora	Diagnosed with *H. influenzae* pneumonia one month prior, completed two-week course of antibiotics; was back to his baseline after treatment.
32	Male	51	COPD/ Emphysema	27	Levofloxacin; Amoxicillin-Clavulanate	4467	2.26	27	Oral flora	Had a productive cough and CT scan with consolidation 5 weeks prior to bronchoscopy, completed empiric two-week course of antibiotics. At clinic visit one week prior to surveillance bronchoscopy the patient's cough had improved and the CT scan showed resolution of the infiltrate.
80	Male	26	Cystic Fibrosis	29	Doxycycline	4842	2.24	20.7	Oral flora	Had a sinus infection six weeks prior to bronchoscopy, for which he was empirically treated with a two-week course of antibiotics.

Definition of abbreviations: AFB = acid fast bacteria, BAL = bronchoalveolar lavage, CLAD = chronic lung allograft dysfunction, COPD = Chronic obstructive lung disease, CT = computed tomography, ID = sample identifier, ILD = interstitial lung disease, OTU = operational taxonomic unit, PFT = pulmonary function testing.

Appendix Table 7: Evaluating the independent effects and interaction of immunosuppression regimen at the time of surveillance bronchoscopy and bacterial burden on developing CLAD or death in the 500 days after one-year post-transplant surveillance BAL

*Model 1: Bacterial Burden as continuous variable**		
Variable	**HR (95% CI)**	***p*-value**
Antiproliferative Immunosuppression[†]		
Azathioprine	1	Referent
Mycophenylate	0.13 (0.00 - 6.88)	0.32
None/Held	1.16 (0.03 - 42.05)	0.94
Lung Bacterial Burden, per $\log_{10}$ increase[§§]	1.64 (0.89 - 3.02)	0.11
Interaction: Antiproliferative Immunosuppression x Bacterial Burden		
Mycophenylate x Bacteria Burden	1.31 (0.51 - 3.33)	0.58
No Immunosuppression x Bacterial Burden	1.10 (0.48 - 2.51)	0.83
*Model 2: Bacterial Burden as tertiles**		
Variable	**HR (95% CI)**	***p*-value**
Antiproliferative Immunosuppression[†]		
Azathioprine	1	Referent
Mycophenylate	0.92 (0.06 - 14.77)	0.96
None/Held	3.77 (0.24 - 60.30)	0.35
Lung Bacterial Burden Tertiles		
Lowest Bacterial Burden Tertile	1	Referent
Middle Bacterial Burden Tertile	10.27 (1.28 - 82.13)	**0.028**
Highest Bacterial Burden Tertile	9.43 (1.05 - 84.40)	**0.045**
Interaction: Antiproliferative Immunosuppression x Bacterial Burden		
Mycophenylate x Middle Bacterial Burden Tertile	0.09 (0.00 - 2.92)	0.18
Mycophenylate x Highest Bacterial Burden Tertile	0.59 (0.03 - 12.26)	0.73
No Immunosuppression x Middle Bacterial Burden Tertile	0.28 (0.01 - 6.81)	0.44
No Immunosuppression x Highest Bacterial Burden Tertile	0.66 (0.03 - 14.72)	0.80

Definition of abbreviations: BAL = bronchoalveolar lavage, CLAD = chronic lung allograft dysfunction.
*Calculated using Cox proportional hazards regression models.

Appendix Table 8: Evaluating the independent effects and interaction of patient age and bacterial burden on developing CLAD or death in the 500 days after one-year post-transplant surveillance BAL

*Model 1: Bacterial Burden as continuous variable**		
Variable	**HR (95% CI)**	***p*-value**
Age, per 1 year increase	1.09 (0.98 - 1.22)	0.11
Lung Bacterial Burden, per log_{10} increase	4.83 (1.49 - 15.68)	**0.0088**
Interaction: Age x Bacterial Burden	0.98 (0.96 - 1.00)	0.12
*Model 2: Bacterial Burden as tertiles**		
Variable	**HR (95% CI)**	***p*-value**
Age, per 1 year increase	0.97 (0.90 - 1.04)	0.42
Lung Bacterial Burden Tertiles		
Lowest Bacterial Burden Tertile	1	Referent
Middle Bacterial Burden Tertile	0.11 (0.00 - 35.66)	0.46
Highest Bacterial Burden Tertile	0.74 (0.01 - 44.90)	0.89
Interaction: Age x Bacterial Burden		
Age x Middle Bacterial Burden Tertile	1.07 (0.96 - 1.19)	0.22
Age x Highest Bacterial Burden Tertile	1.04 (0.96 - 1.13)	0.32

Definition of abbreviations: BAL = bronchoalveolar lavage, CLAD = chronic lung allograft dysfunction.
*Calculated using Cox proportional hazards regression models.

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