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Bronchiolitis obliterans syndrome susceptibility and the pulmonary microbiome

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BACKGROUND: Lung transplantation outcomes remain complicated by bronchiolitis obliterans syndrome (BOS), a major cause of mortality and retransplantation for patients. A variety of factors linking inflammation and BOS have emerged, meriting further exploration of the microbiome as a source of inflammation. In this analysis, we determined features of the pulmonary microbiome associated with BOS susceptibility.

METHODS: Bronchoalveolar lavage (BAL) samples were collected from 25 patients during standard of care bronchoscopies before BOS onset. Microbial DNA was isolated from BAL fluid and prepared for metagenomics shotgun sequencing. Patient microbiomes were phenotyped using *k*-means clustering and compared to determine effects on BOS-free survival.

RESULTS: Clustering identified 3 microbiome phenotypes: Actinobacteria dominant (AD), mixed, and Proteobacteria dominant. AD microbiomes, distinguished by enrichment with Gram-positive organisms, conferred reduced odds and risks for patients to develop acute rejection and BOS compared with non-AD microbiomes. These findings were independent of treatment models. Microbiome findings were correlated with BAL cell counts and polymorphonuclear cell percentages.

CONCLUSIONS: In some populations, features of the microbiome may be used to assess BOS susceptibility. Namely, a Gram-positive enriched pulmonary microbiome may predict resilience to BOS.
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Outcomes for lung transplantation patients notably lag behind other solid organ transplants, with median survivals averaging approximately 5.5 years. The development of bronchiolitis obliterans syndrome (BOS) persists as the major cause of mortality and retransplantation for patients who survive beyond the first year.[1] BOS is characterized by alloimmune, autoimmune, and inflammatory reactivity leading to fibroproliferative infiltrates in the bronchiolar tree, resulting in end-stage obstruction and parenchymal damage.[2] BOS develops in greater than 50% of lung allograft recipients within 5 years after transplant and in greater than 75% within 10 years.[1]

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Inflammation has been linked to pathogenesis of BOS and other rejection phenotypes,[3,4] but inflammation can be influenced by a variety of factors. Acute rejection, human leukocyte antigen mismatching, and the development of autoimmunity to collagen are direct sources of immune reactivity linked to BOS development. External mediators of inflammation are less well understood. For example, the lung's exposure to the environment can increase susceptibility to infection, which is further complicated by immunosuppression.[5] Cytomegalovirus, *Aspergillus*, and *Pseudomonas* infections are also associated with increased incidence of BOS,[6–8] but contributions from the pulmonary microbiome remain poorly defined.

Host–microbe interactions are capable of modulating immune responses, inducing resilience or exacerbation of certain inflammatory and fibrotic processes.[9,10] The lung, previously presumed sterile, exhibits a microbiome.[11] Pulmonary dysbiosis has been associated with another fibroproliferative pulmonary disorder, idiopathic pulmonary fibrosis.[12] Consistent with this observation, notable changes occur in the microbiome after lung transplantation,[13,14] suggesting that a relationship with BOS merits further exploration.

Recent advances in the area of microbiome research have enabled improved diagnostic and analytic techniques related to the microbiome, which we used in our study.[15] These advances also represent new avenues to explore biomarkers and pathophysiology in BOS. Standard of care surveillance bronchoscopies provide material to investigate the effects of allograft colonization within months. In this study, we analyzed whether exposure to elements of the pulmonary microbiome contribute to BOS development in lung transplant patients. Using metagenomic shotgun sequencing of bronchoalveolar lavage (BAL) samples, we identified a pulmonary microbiome phenotype associated with resilience to BOS.

Methods

Identification of study participants and sample acquisition

Lung transplant recipients at the University of California, Los Angeles were enrolled in an observational registry study that included collection of additional BAL fluid for research purposes at the time of standard of care bronchoscopies. This study was approved by the University of California, Los Angeles Institutional Review Board (IRB# 2515-0085). All recipients were provided written informed consent to participate in the study. This study included standardized medical record abstraction, including demographic, transplantation, and outcome-related variables. From this registry, 360 patients contributed 1,639 samples to a biorepository. From this cohort, we established a case-control study to distinguish features between patients with early-onset BOS (within the first 3 years after transplant) and BOS-free patients. BOS was defined using International Society of Heart and Lung Transplantation criteria.[16]

The study excluded patients without a BOS diagnosis who died before 3 years of follow-up. Because bacterial infections have downstream implications on the microbiome and vary in origin, patients with positive bacterial cultures were also excluded. From these criteria, 73 BOS and 85 BOS-free patients were identified (Table 1). For study of the microbiome, 25 recipients were randomly selected, of which 10 developed early-onset BOS and 15 remained BOS-free. Because BOS was the outcome of interest and exclusion criteria included death within 3 years, death was not considered a relevant risk in the study design. Patients were monitored until BOS development, censor, or study end date (May 2013).

Bronchoscopies were performed as standard of care, and eligible patients were required to have had at least 1 bronchoscopy. Up to 3 longitudinal samples per patient were obtained, and 53 samples were acquired from 25 patients. Sample processing and microbiome analysis were performed after patient selection was limited to those patients.

DNA isolation, library construction, and sequencing

Microbial content was isolated from BAL fluid aliquots by centrifugation at 22,000 rpm. The resulting pellet was pre-treated with deoxyribonuclease, followed by lysis using lysozyme and proteinase K. DNA was isolated using the QIAamp MinElute Virus Spin Kit (QIAGEN). DNA concentrations were measured using the Qubit 2.0 fluorometer (Invitrogen). For the purposes of this study, metagenomics shotgun sequencing was used due to enhanced specificity and sensitivity for taxonomic identification.[17] Sequencing libraries were constructed and indexed from isolated DNA content using the Nextera XT kit (Illumina). Samples were

Table 1 Cohort Characteristics[a]

Characteristic	Eligible (n = 158)	Included (n = 25)	Not included (n = 133)
Age, mean (SD), years	57.6 (10.5)	58.8 (8.7)	57.4 (10.9)
Sex, No. (%)			
Male	89 (56)	14 (56)	75 (56)
Female	69 (44)	11 (44)	58 (44)
Pre-transplant disease, No. (%)			
Restrictive lung disease	91 (58)	16 (64)	75 (56)
COPD	49 (31)	7 (28)	42 (32)
CF/bronchiectasis	7 (4)	0	7 (5)
Pulmonary hypertension	7 (4)	1 (4)	6 (5)
Other	4 (3)	1 (4)	3 (2)
BOS, No. (%)	73 (46)	10 (40)	63 (47)

BOS, bronchiolitis obliterans syndrome; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease.

[a]A description of patients meeting inclusion and exclusion criteria (see Methods) from the lung transplant registry at the University of California at Los Angeles.

sequenced on the Illumina MiSeq platform using a V3-600 kit (Illumina).

Taxonomic profiling

The metagenomic sequencing reads that were produced were processed with a custom pipeline hosted on the University of Illinois at Chicago supercomputer "Extreme." First, the sequences were quality controlled by filtering out all low-quality reads (<25 on Phred quality score), short reads (<100 base pairs), or any human reads. High-quality microbial short reads were assembled into contigs using MetaVelvet.[18] For each sample, taxonomic profiles were constructed using Weighted Voting Taxonomic Identification (WEVOTE).[19] Because WEVOTE is an ensemble classifier, we used Kraken,[20] CLARK,[21] and Basic Local Alignment Search Tool[22] as base classifiers for WEVOTE.

Ordination and clustering

Statistical analyses were performed using R 3.3.0 software (The R Foundation for Statistical Computing).[23] Sequencing results, taxonomic annotations, and metadata were collated using the *phyloseq* package.[24] To determine sample similarity, principle coordinates analysis (PCoA) was performed on all 53 samples using Bray-Curtis dissimilarity measures from the *vegan* package.[25] The *k*-means method was used for clustering and defining microbiome phenotypes based on the first two axes defined from the PCoA. The amount of clusters was determined using the gap statistic method from the *cluster* package.

Survival analysis

Relationships between microbiome phenotypes and rejection outcomes were measured using odds and risk ratios calculated by the *epitools* package. Acute rejection scores were available at the time of sampling for each sample. Kaplan-Meier survival analysis was performed using the *survival* and *survminer* packages to determine potential BOS-free survival advantages in a given microbiome phenotype. Baseline phenotypes for this analysis were microbiome phenotypes at the first sampling after transplant. Cox regression models were constructed to distinguish whether the microbiome or other covariates, such as immunosuppression and prophylaxis, influenced outcomes. For Cox models, significance was determined using the Fisher exact test, and findings were reported as hazard ratios.

Enrichment testing and network analysis

Generalized linear models were constructed using the *DESeq2* package for differential abundance testing on all 53 samples.[26] Models included cluster phenotypes and sampling time frame to

Table 2 Patient Characteristics[a]

Patient	Time from transplant (days)	Follow-up (days)	Microbiome cluster	Sex	Age (years)	Pre-transplant diagnosis	(+) *Aspergillus*	Acute rejection	BOS
1	(1) 106	1,591	(1) AD	M	70	RLD	TP1	None	Free
2	(1) 85, (2) 372	2,058	(1) PD, (2) AD	M	57	RLD	None	(1) B1	Free
4	(1) 36, (2) 117, (3) 389	1,957	(1) AD, (2) M, (3) AD	F	61	COPD	TP2	(3) A1	Free
5	(1) 28, (2) 373	759	(1) AD, (2) AD	M	53	RLD	None	None	Free
6	(1) 137, (2) 375	3,087	(1) M, (2) AD	M	64	RLD	TP1	(2) A1 B1	Free
7	(1) 189, (2) 377	2,834	(1) AD, (2) AD	F	60	COPD	None	None	Free
11	(1) 36, (2) 100, (3) 274	2,927	(1) AD, (2) AD, (3) AD	F	63	RLD	None	None	Free
14	(1) 202	1,825	(1) AD	F	34	LAM	TP1	(1) A2	Free
15	(1) 36	1,203	(1) AD	M	66	COPD	None	None	Free
16	(1) 371	2,785	(1) AD	M	61	COPD	None	None	Free
19	(1) 32, (2) 95, (3) 364	2,291	(1) AD, (2) AD, (3) AD	F	58	RLD	None	None	Free
21	(1) 37, (2) 87	2,129	(1) M, (2) AD	M	69	RLD	TP2	None	Free
22	(1) 180	788	AD	F	65	COPD	None	None	Free
24	(1) 33, (2) 187	1,866	(1) AD, (2) AD	M	46	RLD	None	(1) A2 B1	Free
25	(1) 53, (2) 82	1,324	(1) AD, (2) AD	M	69	RLD	TP2	(1) A2 B1	Free
3	(1) 51, (2) 72	817	(1) PD, (2) M	F	56	COPD	TP2	(1) A2	BOS
8	(1) 74, (2) 686	739	(1) M, (2) M	F	59	COPD	TP1	(1) B1, (2) A1 B1	BOS
9	(1) 61, (2) 90, (3) 572	791	(1) M, (2) M, (3) AD	F	62	RLD	TP2	(1) A1, (3) B1	BOS
10	(1) 85, (2) 469	720	(1) M, (2) M	F	57	COPD	TP1	(1) B1, (2) A1 B1	BOS
12	(1) 33, (2) 91, (3) 410	510	(1) AD, (2) AD, (3) AD	M	62	RLD	TP2	None	BOS
13	(1) 33, (2) 173, (3) 565	630	(1) M, (2) AD, (3) AD	M	60	PH	None	(1) A2, (3) A1 B1	BOS
17	(1) 35, (2) 90, (3) 370	943	(1) PD, (2) PD, (3) AD	M	65	RLD	TP2	None	BOS
18	(1) 177	1,081	(1) AD	F	47	RLD	None	None	BOS
20	(1) 33, (2) 193, (3) 368	535	(1) AD, (2) M, (3) AD	M	60	RLD	None	None	BOS
23	(1) 31, (2) 184, (3) 311	715	(1) M, (2) M, (3) M	M	43	RLD	None	(1) A1, (2) A1	BOS

AD, Actinobacteria dominant; BOS, bronchiolitis obliterans syndrome; COPD, chronic obstructive pulmonary disease; F, female; LAM, lymphangioleiomyomatosis; M (sex), male;
M, mixed; PD, Proteobacteria dominant; PH, pulmonary hypertension; RLD, restrictive lung disease; TP, time point.

[a]Lung transplant recipients ($n = 25$) underwent bronchoscopy, and bronchoalveolar lavage samples were obtained. As clinically available, longitudinal samples were obtained. Clinical features at time of sampling are provided for each patient and are denoted by time point numbers (1, 2, 3) for respective patients. Only positive acute rejection (both A and B scores) are shown. Patients were monitored until BOS development or censor.

account for time-dependent associations, and the Wald method was used to determine significance. Findings were reported as β (enrichment) values, and were used to identify organisms significantly enriched in given microbiome phenotypes. To determine effects of community structures in samples, co-occurrence matrices were constructed using the *spieceasi* package[27] and visualized using Cytoscape 3.5.1 software (Cytoscape Consortium).[28] Enriched taxa were modeled against corresponding cytology data as clinically available using linear regression on GraphPad Prism 4.03 software (GraphPad Software).

Results

Patient characteristics

Patients were recruited and monitored for at least 2 years after transplant. Mean follow-up time was 2 years for early-onset BOS and 5.4 years for BOS-free patients. At least 1, and up to 3 BAL samples were obtained per subject (53 total samples). BOS development was analyzed as the primary outcome for this analysis, and other clinical (Table 2) and therapeutic (Table 3) features were used as secondary covariates. Our multivariate analysis showed no clinical covariates were significantly correlated with BOS development (Supplementary Table S1, available online at www.jhltonline.org).

Ordination and clustering identify distinct microbiome profiles

Our taxonomic analysis identified 2,726 unique microbial species in the BAL samples. Taxa were agglomerated at the

Table 3 Treatment Regimens[a]

Patient	Induction	Maintenance	Prophylactics	Other	BOS
1	Basiliximab	Tacrolimus, mycophenolate, prednisone	Valganciclovir		Free
2	ATG + PLEX	Tacrolimus, mycophenolate, prednisone	Bactrim, acyclovir		Free
4	ATG	Tacrolimus, mycophenolate (TP1, TP2), prednisone	Bactrim, valganciclovir, voriconazole		Free
5	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, itraconazole (TP1), voriconazole (TP2)		Free
6	ATG	Tacrolimus, mycophenolate, prednisone	None		Free
7	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir		Free
11	Basiliximab	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir		Free
14	ATG	Tacrolimus, sirolimus, prednisone	Bactrim, valganciclovir		Free
15	Basiliximab	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, CytoGam,[b] voriconazole, isoniazid		Free
16	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, acyclovir, voriconazole		Free
19	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim		Free
21	Basiliximab	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir		Free
22	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, acyclovir, fluconazole		Free
24	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir		Free
25	Basiliximab	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir		Free
3	ATG	Tacrolimus, mycophenolate, prednisone	ganciclovir, itraconazole	(TP2) Meropenem, ciprofloxacin	BOS
8	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, ganciclovir, itraconazole	(TP2) cefepime, tobramycin	BOS
9	Basiliximab	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, itraconazole (TP1, TP2)	(TP2) azithromycin, (TP3) minocycline	BOS
10	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, voriconazole (TP1)		BOS
12	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, posaconazole (TP3)		BOS
13	ATG	Tacrolimus, mycophenolate (TP1, TP3), sirolimus (TP2), prednisone	Bactrim, valganciclovir, voriconazole (TP1), fluconazole (TP2, TP3)		BOS
17	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir (TP1), ganciclovir (TP2), itraconazole (TP2)	(TP2) colistin	BOS
18	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, voriconazole		BOS
20	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir, isoniazid, voriconazole (TP2, TP3)		BOS
23	ATG	Tacrolimus, mycophenolate, prednisone	Bactrim, valganciclovir		BOS

ATG, Anti-thymocyte globulin; BOS, bronchiolitis obliterans syndrome; PLEX: plasma exchange; TP, time point.

[a]Immunosuppression induction, immunosuppressive therapy, and prophylactic regimens are provided for each patient. Time point–specific changes to treatment regimens are denoted by the TP number for each patient.

[b]CSL Behring.

phylum level to analyze sample composition (Figure 1A), and PCoA was performed at the species level. Stratification of the results based on BOS development showed no significant distinction in sample microbiomes. Further analysis was performed on all sample microbiomes using *k*-means clustering. Three clusters were identified along the first 2 principle coordinate axes, accounting for a combined 45.9% of the variance across samples (Figure 1B). Actinobacteria dominant (AD), mixed, and Proteobacteria dominant clusters were defined based on different phylum composition. Notably, the AD cluster had minimal variance along axis 1, suggesting a well-defined phenotype that was confirmed using the analysis of similarities technique[29] (Figure 1C). Analysis of similarities also determined that there was significant dissimilarity between the clusters ($R = 0.728$, $p = 0.001$). Prophylactic anti-fungal, anti-viral, and anti-biotic usage was not significantly associated with cluster designation (not shown). During the course of the study, 47% of patients with more than 1 sample maintained the same cluster phenotype across all of their samples (Figure 1D).

Pulmonary microbiome clusters implicated in susceptibility to allograft rejection

Given the ability of microbial communities to modulate inflammation,[10] we assessed the relationship of the pulmonary microbiome with allograft rejection. Acute rejection scores (A, airway; B, vascular) were obtained at the time of each bronchoscopy. Although no patients suffered high-grade rejection (A or B grade ≥3), a link was detected between acute rejection and our microbiome phenotypes. Specifically, patients with an AD microbiome had an odds ratio of 0.24 and risk ratio of 0.55, indicating reduced risk of developing airway or vascular acute rejection compared with other clusters (Fisher exact $p < 0.05$). Previous reports show that acute rejection is one of the strongest indicators of susceptibility to chronic rejection,[30,31] and we determined odds and risk ratios linking the microbiome with BOS. To standardize the effect that microbiome exposure had on BOS, samples were divided based on time from transplantation. For samples collected within 3 months after transplant, AD microbiomes

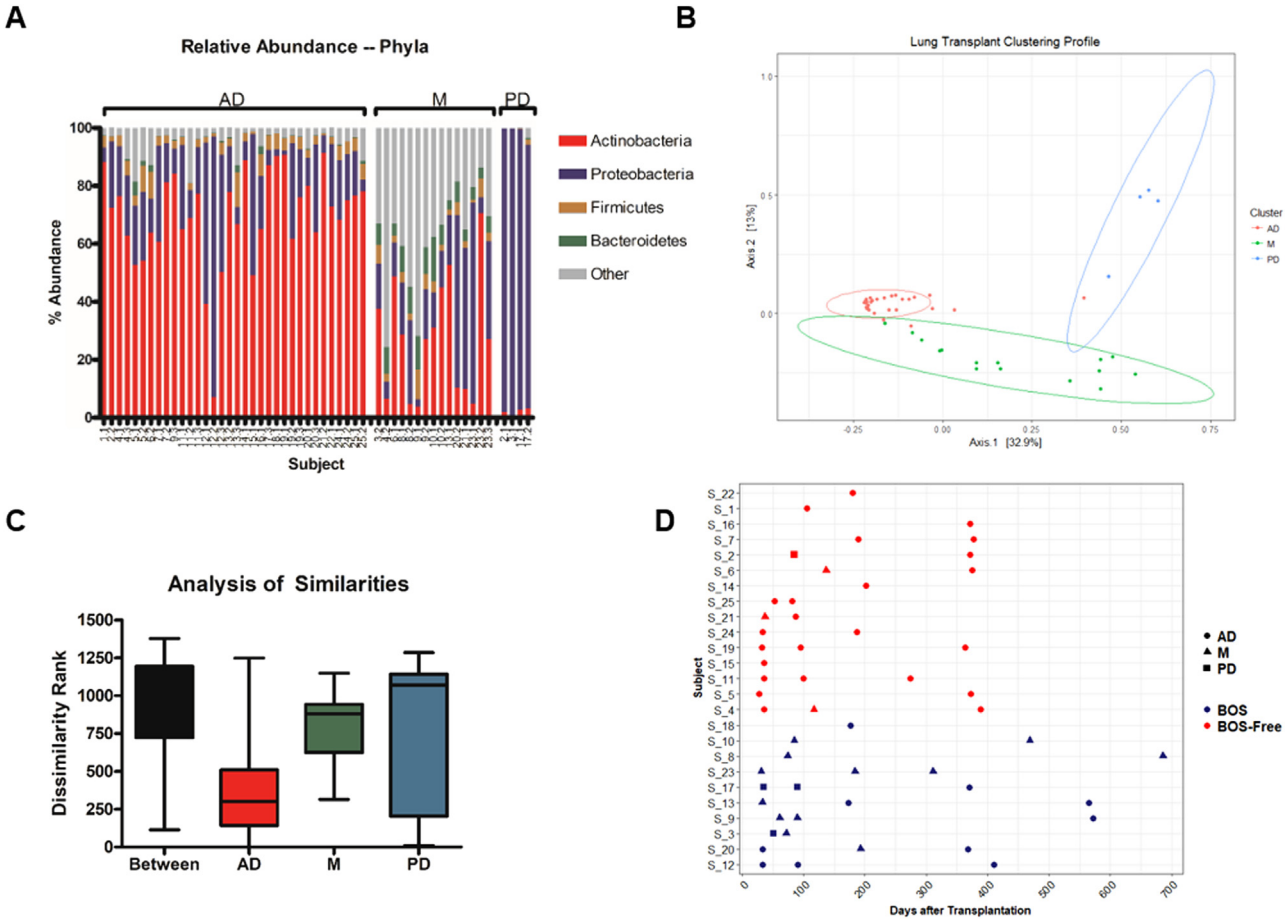


Figure 1 Clustering identifies 3 distinct microbial communities. (A) Fifty-three bronchoalveolar lavage samples from 25 subjects were clustered using the *k*-means method and characterized by dominance at the phylum level: Actinobacteria dominant (AD), mixed (M), and Proteobacteria dominant (PD) clusters. (B) Principle coordinates analysis was performed using Bray-Curtis distances. The 3 clusters are identified and their 95% confidence intervals are shown. (C) Analysis of similarities showed significant dissimilarity between groups. Minimum, maximum, median, and interquartile range of dissimilarity indices are indicated. (D) Subject microbiome clusters over time are plotted. Color represents final outcomes for each patient; shapes signify microbiome clusters at each sampling time point. BOS, bronchiolitis obliterans syndrome.

conferred significantly reduced odds and risk for BOS development, 0.057 and 0.23, respectively (Fisher exact $p < 0.01$). Odds and risk for BOS were not significantly affected by microbiome phenotype in samples collected beyond this initial surveillance period. Kaplan-Meier curves (Figure 2A) revealed significantly increased BOS-free survival for AD microbiomes ($n = 15$) compared with non-AD microbiomes at baseline ($n = 10$). These findings showed that the AD phenotype is associated with reduced BOS susceptibility, particularly within the first 3 months after transplant.

To determine whether prophylactic or immunosuppressive treatment regimens were correlated with enhanced BOS-free survival in the AD cluster, Cox regression analysis was performed. Similar to the Kaplan-Meier model, the multivariate Cox model detected a significantly reduced hazard for individuals with an AD microbiome, independent of any covariate features (Figure 2B). No covariates were independently associated with increased or decreased risk of BOS development. Together, these data suggest that AD microbiomes were correlated with improved outcomes in transplant patients and associated with a reduced risk of rejection.

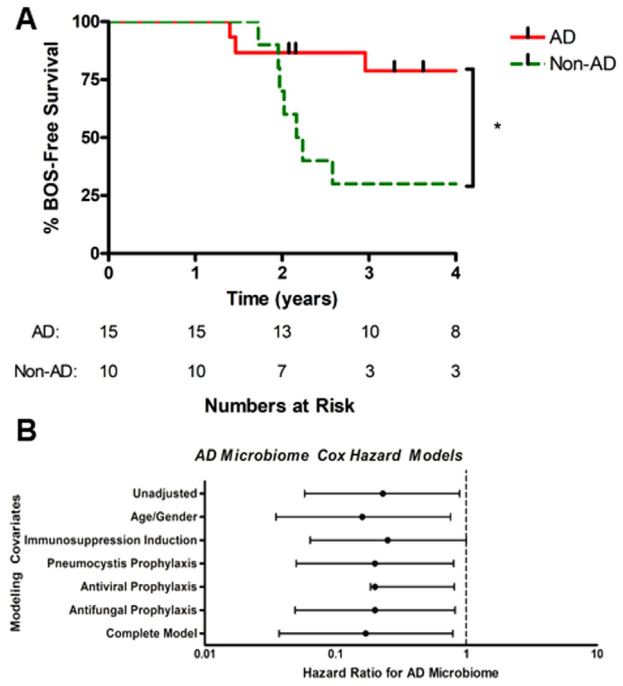


Figure 2 Microbiome clustering associates with bronchiolitis obliterans syndrome (BOS) development. Outcomes related to BOS development were tracked for each patient until BOS development or censor. (A) Kaplan-Meier analysis was used to determine the relationship between pulmonary microbiome clusters and BOS susceptibility. Actinobacteria dominant (AD) cluster patients had significantly increased BOS-free survival compared with non-AD clusters (log-rank test). Censored patients are denoted by vertical marks on the associated survival curve. Numbers at risk are shown at each year. (B) Cox hazard regression analysis was performed to identify whether clinical characteristics or treatment regimens influenced BOS-free survival in AD microbiome patients. The unadjusted model represents the hazard ratio and 95% confidence intervals of the AD microbiome independent of other covariates. The complete model factors in all immunosuppressive and prophylactic treatments for each patient. Patients with AD microbiomes had significantly reduced hazard regardless of modeling covariates. *$p < 0.05$.

Increased diversity and enrichment of key taxa associate with reduced incidence of rejection

To establish which features of the AD microbiome were associated with decreased susceptibility to allograft rejection, we analyzed community properties and the species and genera of the lower respiratory tract microbiome. The α-diversity indices showed significantly increased richness (Fisher) in the AD cluster compared with mixed and Proteobacteria dominant clusters (Figure 3A and B) to identify contributory organisms. Linear models were developed to identify clinical features that differentiate metagenomic findings between samples. Permutational multivariate analysis of variance[32] showed clustering was the strongest differentiator ($p < 0.001$). No clinical covariates significantly differentiated features of sample microbiomes. Accordingly, AD clustering was modeled against phylum and genus level counts.

Several taxa had significant differential enrichment. The top 5 abundant phyla and 10 top abundant genera were selected based on median abundance, and their enrichment values for each cluster were plotted with confidence intervals (Figure 3C and D). Of these taxa, the Gram-positive phyla Firmicutes and Actinobacteria, including genera *Propionibacterium*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus*, were significantly enriched in only AD microbiomes. Proteobacteria, namely the Alphaproteobacteria *Sphingomonas*, were also significantly enriched in the AD cluster. Gram-negative organisms, including the Bacteroidetes phylum, namely *Flavobacterium*, and the Gammaproteobacteria *Pseudomonas* were decreased in the AD phenotype. Alphatorqueviruses, which includes torque teno virus, and *Mycoplasma*, causative agents of atypical pneumonia, were also decreased. Significantly enriched species for each cluster are provided in Supplementary Table S2 (online). These findings suggest a model where enrichment with the identified Gram-positive organisms, as well as Alphaproteobacteria, are predictive of rejection-free survival and may contribute to host resilience against BOS development. Bacteroidetes, *Pseudomonas*, *Mycoplasma*, and Alphatorquevirus enrichment favors non-AD phenotypes and increased BOS susceptibility.

To elucidate how the composition of the pulmonary microbiome may contribute to BOS, network analysis was performed to determine clustering coefficients and centrality measures from co-occurrence matrices (interactions were filtered for significant interactions between taxa; $p < 0.01$). Networks for AD and non-AD microbiomes were plotted using Cytoscape, and minimal overlap between networks was observed (Figure 3E). Two distinct communities were identified in the AD cluster, with significant interactions among the core Gram-positive genera defining one of these communities. Interactions in non-AD clusters were less extensive and centered on *Pseudomonas aeruginosa* as a hub organism. Network statistics confirmed these findings.

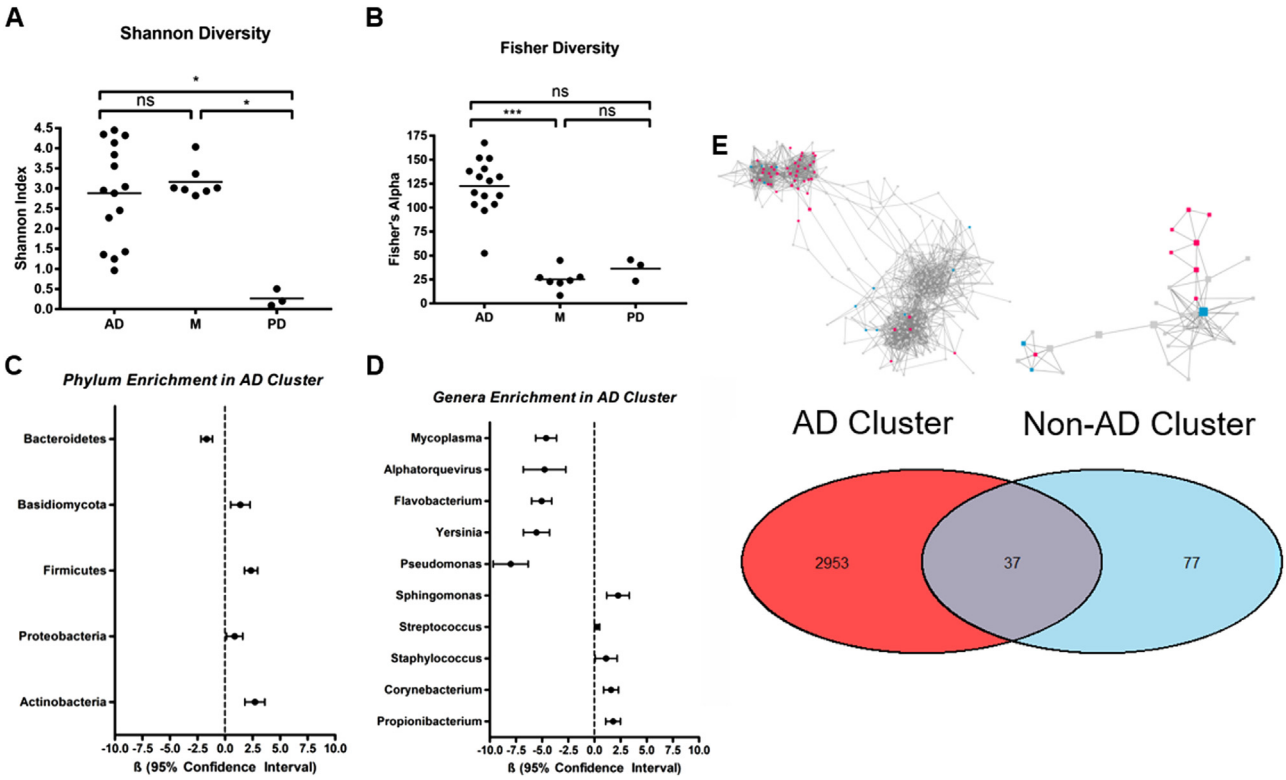


Figure 3 Diversity, enrichment models, and network data reveal key features of Actinobacteria dominant (AD) microbiomes. (A) Shannon diversity and (B) Fisher's α-diversity indices from bronchoalveolar lavage samples are shown. Samples were grouped based on their AD, mixed (M), and Proteobacteria dominant (PD) microbiome clustering. Samples counts were normalized and modeled against AD microbiomes and sampling time using the DESeq2 package in R (Bioconductor). Significance was determined using the Wald test. (C) The top 5 abundant phyla and (D) the top 5 abundant positively and negatively enriched genera are shown with their enrichment (β) values. (E) Covariance and correlation indices were calculated using the SparCC function from the SpiecEasi package on R. Co-occurrence networks for AD cluster (left) and non-AD cluster (right) were plotted using Cytoscape (Cytoscape Consortium). Previously discussed genera identified are highlighted in red (enriched in AD microbiomes) and blue (decreased in AD microbiomes). Network interactions were compiled and showed minimal overlap between AD and non-AD cluster microbiomes. $*p < 0.05$; $***p < 0.001$.

The AD microbiomes had a greater clustering coefficient (0.358 to 0.248) and a greater network diameter (12 to 9) compared with non-AD samples. These network analyses indicate that the pulmonary milieu, not just individual taxa, contributes to BOS susceptibility.

Pulmonary cytology associates with microbiome findings

To establish links between features of the microbiome and host immune response factors, taxa abundance was correlated with corresponding cytology data from BAL. We hypothesized that AD-enriched taxa would display fewer inflammatory profiles and that AD-decreased taxa would exhibit more inflammatory properties. Using linear regression, count data from previously described organisms was modeled against bronchoalveolar cell profiles. Notably, we identified significant associations between taxa and total cell counts and polymorphonuclear cell (PMN) percentage (Figure 4). As expected, as AD-enriched *Propionibacterium* became more abundant, total BAL cell count decreased, suggesting a decreased inflammatory profile. These findings were reversed when analyzing AD-decreased taxa: *Flavobacterium*, *Mycoplasma*, and *Yersinia* were all significantly positively correlated with total BAL cell count. AD-enriched *Corynebacterium* had a significant negative correlation with PMN percentage, suggesting that members of the AD microbiome are inversely related to neutrophil presence in the lungs.

Neutrophil-associated inflammation has been linked to BOS, and neutrophilia has been purported as a biomarker for BOS.[33] Cutoffs for neutrophilia at 16% have been described.[34] Applying this cutoff to our study, we observed that normal samples had increased abundance of *Corynebacterium* compared with PMN-high samples. These findings suggest a role for the pulmonary microbiome in shaping the inflammatory cell profile of lung transplant patients, with implications for BOS susceptibility.

Discussion

Recent studies have postulated that changes in the lower respiratory microbiome contribute to allograft physiology. To define a community of organisms associated with BOS susceptibility, we used metagenomic shotgun sequencing on BAL samples from lung transplant patients. Cluster-based phenotyping identified enrichment with the genera *Propionibacterium*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, and *Sphingomonas* was significantly associated with improved outcomes. A similar lower airway microbiome

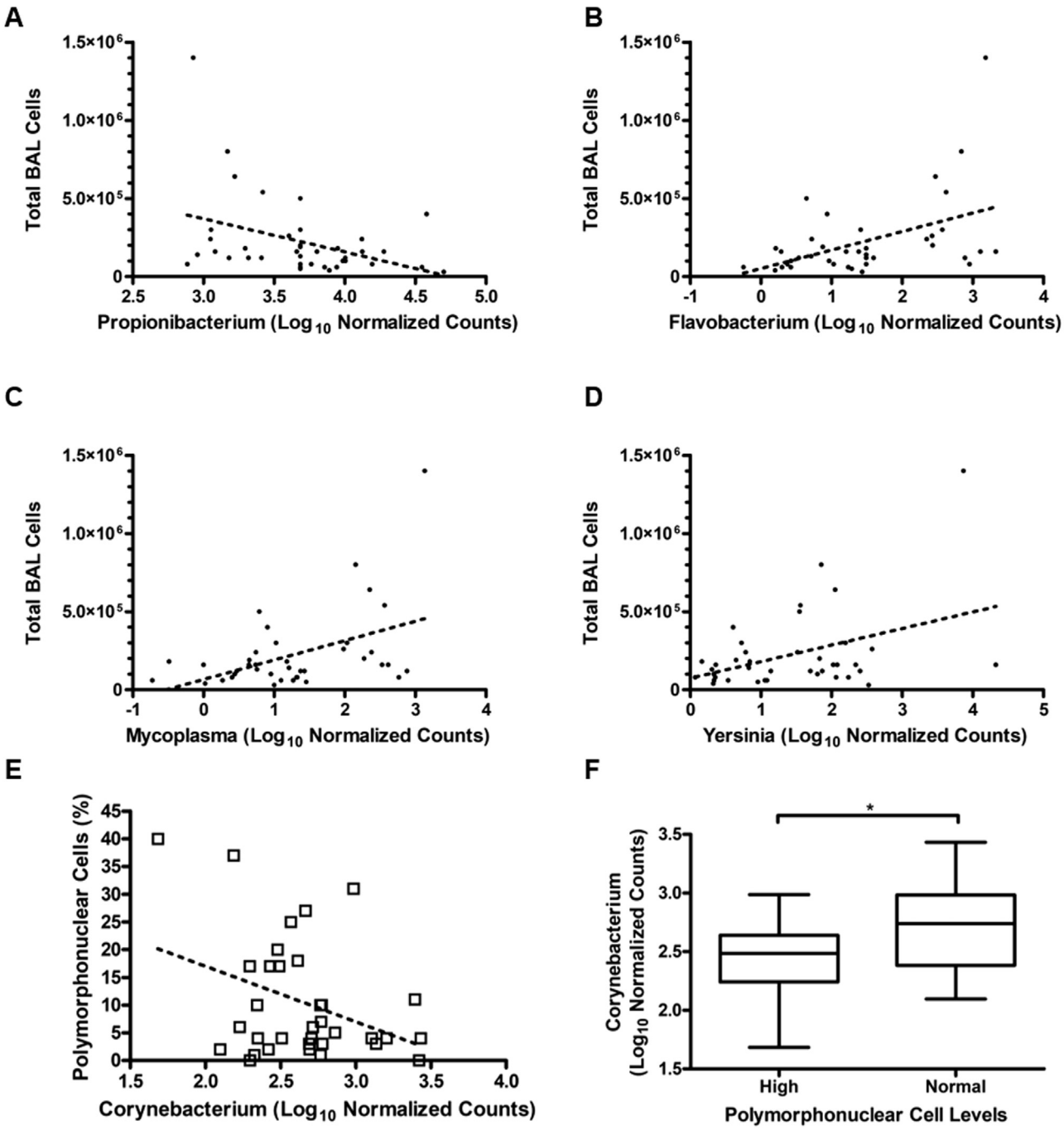


Figure 4 Metagenomic findings correlate with bronchoalveolar lavage (BAL) cytology. Associated cytology findings for each sample were compiled and correlated with corresponding metagenomics data. The relationship between taxa and cytology findings was determined with linear regression. (A) *Propionibacterium* was negatively correlated with total cell counts identified from BAL. (B) *Flavobacterium*, (C) *Mycoplasma*, and (D) *Yersinia* were positively correlated with total cell counts identified from BAL. (E) *Corynebacterium* was negatively correlated with polymorphonuclear cell percentage. (F) *Corynebacterium* was decreased in neutrophilic samples. Minimum, maximum, median, and interquartile range of normalized counts are indicated. For pairwise comparison, significance was determined using the Mann-Whitney *U* test. $*p < 0.05$.

phenotype marked by enrichment with *Propionibacterium*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus* had been previously described and was associated with reduced pulmonary inflammation.[35] *Staphylococcus* has recently been linked to destructive remodeling of lung architecture in allograft recipients.[13] Those findings did not account for community interactions between organisms, which are known to modulate bacterial behavior.[36] Whether these organisms represented reconstitution from the recipient's commensal organisms, a factor reported to contribute to BOS-free survival,[37] remains unclear.

Our study demonstrates that AD-decreased organisms include Gram-negative populations and that their enrichment leads to worse outcomes. These findings include *Flavobacterium* and *Pseudomonas*. Interestingly, AD-decreased taxa also included Alphatorquevirus, a genus that includes torque teno

virus, whose abundance has been correlated with higher levels of immunosuppression in transplant recipients.[38] *Mycoplasma*, a causative agent of atypical pneumonia, was also decreased. Conversely, the resilient AD microbiomes were significantly enriched with Gram-positive organisms. We speculate that the microbiome influences rejection outcomes through interactions triggering innate immune mechanisms. Of those mechanisms, Toll-like receptor 4 signaling and bacterial lipopolysaccharide (LPS) exposure have been implicated in BOS development.[39] Unsurprisingly, neutrophilic responses have been associated with worse outcomes in transplant patients.[33] Thus, it is possible that the resilient AD phenotype promotes graft survival by reduced Toll-like receptor 4 signaling, thereby promoting immune tolerance. Interestingly, the sole Gram-positive AD-enriched taxa *Sphingomonas* contains glycosphingolipids in place of LPS. Likewise, our analysis of BAL cell profiles suggests that the AD microbiome associates with reduced inflammatory cell recruitment to the lung. Future studies analyzing links between the microbiome and inflammatory activity of BAL cells are warranted.

The microbiome may serve both mechanistic and diagnostic purposes. Our findings are enhanced by the sampling time frame, which occurred before relative to BOS onset. Surveillance bronchoscopies occurred within the first year after transplant and were monitored for at least 2 years. Notably, our findings show that uncovering an AD phenotype in patients, particularly within 3 months after transplant, confers significantly reduced odds and risk for patients to develop BOS. Our observation that changes in the microbiome occur before BOS development indicates that microbiome profiles may serve as clinically significant biomarkers for the development of BOS. Specifically, modulating prophylactics and immunosuppression at an earlier clinical stage may improve outcomes for those susceptible to BOS.

Although we differentiated outcomes in clinical subgroups after lung transplantation, the retrospective nature of our study affected experimental design considerations. Our analyses were limited by sample size, non-standardized sampling time frames for participants, lack of environmental background, and exclusion criteria. To account for these potential confounders, differential abundance testing was applied to identify significantly enriched organisms and to limit contributions of environmental background and outlier organisms. The linear model applied to the package considered variations in sampling time of the patients, thereby negating any effect that time to surveillance bronchoscopy had on microbial enrichment. Although this study did not consider the role bacterial infections may play in BOS-related dysbiosis, such infections are already established risk factors for BOS.[40] By excluding these samples, we delineate sub-clinical exposures priming the lung for chronic rejection.

In this study, we examined whether the microbiome contributes to and may be predictive of susceptibility or resilience to BOS. We established a significant relationship between the pulmonary microbiome and BOS susceptibility. A resilient AD phenotype had significant enrichment with well-characterized Gram-positive genera and was associated with reduced inflammatory cell recruitment to the lung. Likewise, increased Alphatorquevirus and Gram-negative organisms were associated with increased inflammatory cell recruitment. These analyses suggest that modulating immunosuppression or introduction of microbiome-based therapeutics (probiotics or phages) to favor an AD phenotype may provide a novel opportunity to influence lung allograft rejection.

Disclosure statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at www.jhltonline.org.

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