

Association of Antibiotics, Airway Microbiome, and Inflammation in Infants with Cystic Fibrosis

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Abstract

Rationale: The underlying defect in the cystic fibrosis (CF) airway leads to defective mucociliary clearance and impaired bacterial killing, resulting in endobronchial infection and inflammation that contributes to progressive lung disease. Little is known about the respiratory microbiota in the early CF airway and its relationship to inflammation.

Objectives: To examine the bacterial microbiota and inflammatory profiles in bronchoalveolar lavage fluid and oropharyngeal secretions in infants with CF.

Methods: Infants with CF from U.S. and Australian centers were enrolled in a prospective, observational study examining the bacterial microbiota and inflammatory profiles of the respiratory tract. Bacterial diversity and density (load) were measured. Lavage samples were analyzed for inflammatory markers (interleukin 8, unbound neutrophil elastase, and absolute neutrophil count) in the epithelial lining fluid.

Results: Thirty-two infants (mean age, 4.7 months) underwent bronchoalveolar lavage and oropharyngeal sampling. Shannon diversity strongly correlated between upper and lower airway samples from a given subject, although community compositions differed. Microbial diversity was lower in younger subjects and in those receiving daily antistaphylococcal antibiotic prophylaxis. In lavage samples, reduced diversity correlated with lower interleukin 8 concentration and absolute neutrophil count.

Conclusions: In infants with CF, reduced bacterial diversity in the upper and lower airways was strongly associated with the use of prophylactic antibiotics and younger age at the time of sampling; less diversity in the lower airway correlated with lower inflammation on bronchoalveolar lavage. Our findings suggest modification of the respiratory microbiome in infants with CF may influence airway inflammation.

Keywords: cystic fibrosis; respiratory infection; bacterial infection; pediatric lung disease inflammation; microbiota

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Cystic fibrosis (CF) lung disease begins during infancy. Bacterial infection in CF airways induces an intense neutrophilic response (1) that contributes to airway injury (2). The cycle of infection, neutrophilic inflammation, oxidative stress, and overwhelmed antiprotease defenses leads to irreversible airway destruction. In infants and young children with CF, lower airway infection and inflammation have been linked to poorer respiratory outcomes, including expiratory airflow limitation (3) and bronchiectasis (4–6).

Historically, interventions have focused on specific organisms associated with progressive disease, such as *Pseudomonas aeruginosa* (7, 8). However, it has become increasingly clear that the lungs of patients with CF have greater microbial diversity than previously recognized. In older children and adults, sputum and bronchoalveolar lavage (BAL) microbiomes in CF are relatively constant, particularly during periods of clinical stability (9–11), and lower bacterial diversity has been associated with more advanced CF lung disease (12–15). It is unknown whether reduced bacterial diversity in the adult airway contributes to lung disease or occurs secondary to disease progression or its treatment.

The airway microbiome changes during infancy (16, 17). In a recent retrospective study, we examined the evolution of lower airway microbiota over time, analyzing serial BAL samples collected more than 2 decades ago (18). The microbiota in the CF lower airway appeared dynamic in these retrospective samples, and microbial diversity decreased in serial BAL samples in association with increased patient age. Little is known about the evolution of the bacterial microbiota in infancy or its relationship to early lung disease and airway inflammation in children with CF.

Bacterial colonization in infancy has the potential to change the physiology and native immunity of CF airways. The early microbiota of the CF airway represents a potentially modifiable factor that could affect mucosal inflammation and disease progression (19–21). In this manuscript, we describe the bacterial and inflammatory profiles in BAL fluid and oropharyngeal secretions collected prospectively from infants with CF seen at four U.S. and Australian centers, building on our previous work. We hypothesized that reduced microbial diversity would be associated with increased antibiotic exposure, particularly

use of prophylactic antibiotics (amoxicillin-clavulanate), and that differences in lung microbiota would be linked to differences in inflammation in the lower airways.

Methods

Subjects

Infants with CF at 3 months of age were enrolled in a prospective, observational, multicenter study at Riley Children's Health (Indianapolis, Indiana), St. Louis Children's Hospital (St. Louis, Missouri), Princess Margaret Hospital for Children (Perth, Australia), and the Royal Children's Hospital (Melbourne, Australia). All subjects were identified through newborn screening, with diagnosis of CF defined as at least one clinical feature of CF plus a documented sweat chloride concentration greater than 60 mEq/L by quantitative pilocarpine iontophoresis and/or compatible genotype with two identified mutant *CFTR* alleles. Families were approached consecutively. Clinical information collected included sex, *CFTR* genotype, clinical presentation, tobacco smoke exposure, previous bacterial infection or infections, medications, weight, height, and vital signs. Families were contacted weekly to inquire about respiratory symptoms and antibiotic use (treatment and prophylactic). BAL was performed at 6 (\pm 2) months of age, using a standard protocol at all sites. Interim history and chart review was performed before bronchoscopy, including hospitalizations, culture data, and antibiotic use. Human subjects committees at all sites approved this study, informed parental consent was obtained for all subjects, and a National Institutes of Health-approved Observational Safety Monitoring Board reviewed and approved the protocol before study initiation.

Specimens

BAL fluid and oropharyngeal swabs were collected on the same day. A total of four 1 ml/kg aliquots of sterile normal saline were instilled into subsegmental bronchi of right middle lobe and lingula. Routine bacterial culture was performed on BAL fluid according to clinical procedures at each site. Lavage fluid and oropharyngeal swabs were processed according to study protocols (*see the online supplement*) and sent to Washington University for inflammatory marker and microbiome analyses.

Measurement of Inflammatory Markers

Interleukin 8 (IL-8) concentrations and functional activity of unopposed neutrophil elastase were measured in BAL supernatant (22). Values were corrected for dilution by measuring urea concentrations in BAL supernatant and serum (*online supplement*) (23). Lavage cell counts and absolute neutrophil counts (ANC) were determined at each site, using established techniques (24).

Nucleic Acid Extraction

Total nucleic acid was extracted from BAL cell pellets and oropharyngeal specimens, using the NucliSENS easyMAG instrument (bioMérieux, Inc, Marcy l'Etoile, France; *online supplement*) (18).

16S rRNA Gene Quantitative Polymerase Chain Reaction

The BactQuant assay (25) was used to determine the number of copies of 16S rRNA gene per milliliter of each sample as an approximation of bacterial load (*online supplement*) (18). Samples that did not have detectable bacterial concentrations were assigned a value of 10 copies per microliter for analysis of bacterial load, which was the minimum detected using a cloned 16S rRNA gene in a standard curve (*online supplement*).

16S Amplicon Sequencing

Sequencing was performed on those samples with bacterial loads above the detection limit of the BactQuant assay. The V1–V3 region of the 16S rRNA gene was amplified, sequenced on the Roche 454 GS FLX Titanium instrument (Roche/454 Life Sciences, Branford, CT), and processed according to methods published by the Human Microbiome Project (26) (*online supplement*). 16S sequences were classified using the Ribosomal Database Project classifier (27), version 10.2, training set 10. Sequence data were deposited into GenBank under BioProject PRJNA311065 (18).

Sequencing Controls

As a sample control, sterile saline was washed through bronchoscopes before sample collection in a subset of subjects to ensure bacterial signals in BAL samples were distinct from background, residual bacterial DNA from the equipment (*online supplement*). Each control was run as a separate sample, and consistently had undetectable 16S signal by quantitative

polymerase chain reaction (qPCR) assay (Figure E1A). BAL samples collected from participants directly after control washes and using the same bronchoscope had low detectable levels of bacteria by qPCR (Figure E1A). For sequencing, the entire volume of the 16S amplification reaction from each of the controls was included in the pool of samples. Sequence reads generated from controls were lower in number than those from patient samples, and patient samples had distinct bacterial taxa and community structures compared with controls. 16S qPCR and sequencing were also performed on buffers used for sample processing and PCR-negative controls. These negative controls had undetectable levels of bacteria in the 16S qPCR and low numbers of sequence reads.

Statistical Analyses

Subject demographics were compared using rank-sum testing or Fisher's exact test, as appropriate. The R library *vegan* was used for statistical analysis of alpha diversity. Sequence data from each sample were adjusted (rarefied) to the number of sequences found in the sample with the fewest reads before calculating richness and plotting the data. Lavage and oropharyngeal sample data sets were treated separately, with BAL rarefied to 1,631 sequences and the oropharyngeal sample rarefied to 10,503 sequences. *LabDSV* (28) was used to calculate beta diversity and carry out principle coordinates analysis, and *rgl* (29) was used for three-dimensional plotting. The statistical significance of diversity and inflammatory marker measurements between defined groups was assessed using rank-sum testing with continuity correction. Spearman correlations were calculated between measures. Differences in bacteria between groups by antibiotic regimens were identified using *MetaStats* (30); only taxa that accounted for $\geq 0.1\%$ of the total classified reads were included. Associations between alpha diversity and other factors were examined using linear regression with backward stepwise regression and partial F testing (*Stata*; *StatCorp*, TX). Variables used in the initial regression model were chosen based on significance in bivariate analyses.

Results

Subjects

Complete data were collected on 32 infants (16 Melbourne, 11 Indianapolis, 4 St. Louis,

Table 1. Subject demographics by continent of enrollment

	Australia (N = 17)	United States (N = 15)
Male	8 (47%)	3 (20%)
Caucasian/White	16 (94%)	15 (100%)
F508del homozygous	9 (53%)	8 (53%)
Age at bronchoscopy, mon	3.5 (± 0.9)*	6 (± 1.6)
Weight at bronchoscopy, kg	6.3 (± 1)	7 (± 1.3)
Lavage sequencing performed	16 (94%)	11 (73%)
Exclusively breast fed	6 (35%)	4 (27%)
Smoke exposure	2 (12%)	0
Prophylactic antibiotics	16 (94%)*	0
Treatment antibiotics	3 (18%)*	11 (73%)
No antibiotics	1 (6%)	4 (27%)

Subject demographics and characteristics by continent of enrollment, shown for all subjects (n = 32), presented as number and percentage or mean and standard deviation. Australian subjects were significantly younger than U.S. subjects at the time of bronchoscopy. All but one of the Australian subjects received prophylactic antibiotics, whereas no U.S. subjects were treated with antistaphylococcal prophylaxis ($P < 0.01$). U.S. subjects were more likely to receive treatment courses of antibiotics. * $P \leq 0.01$ for difference between Australian and U.S. subjects.

1 Perth) who underwent bronchoscopy at a mean age of 4.7 (± 1.8) months. Subject demographics by continent are described in Table 1. Australian subjects were younger than U.S. subjects at the time of bronchoscopy. All but one of the Australian subjects were treated with antistaphylococcal prophylactic antibiotics; no U.S. subjects received prophylaxis. U.S. subjects were more likely to receive treatment courses of antibiotics than Australians. Six subjects (five U.S., one Australian) had received antibiotics [intravenous (1), oral (4), or inhaled (1)] during the 14 days before bronchoscopy, including three who were still receiving treatment at the time of the procedure. Although most subjects were at their clinical baseline at the time of sampling, six children had increased respiratory

symptoms at the time of bronchoscopy (three U.S., three Australian) (table E1).

Microbial Communities

A range of bacterial loads was seen, particularly on BAL, even when corrected for volume of BAL fluid recovered and dilution (23). All oropharyngeal samples (n = 32) had detectable bacterial loads and underwent nucleotide sequencing for microbiome analysis; 22 of the BAL samples had bacterial loads above the level of detection and were sequenced. Analyses involving BAL bacterial diversity were restricted to these 22 subjects; all other analyses included all 32 subjects. There were no significant demographic differences between subjects with or without detectable bacterial loads on BAL.

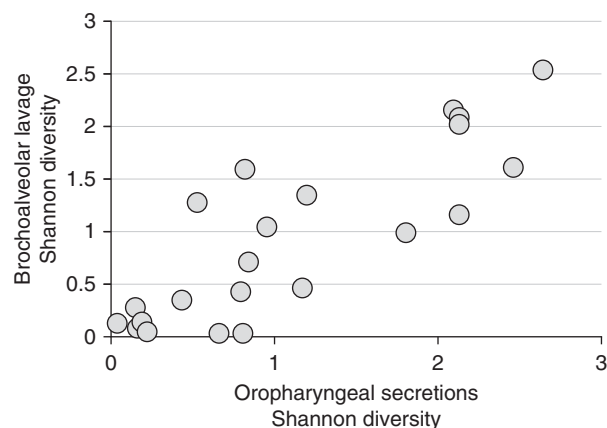


Figure 1. Correlation of Shannon diversity from lavage and oropharyngeal samples from individual subjects. Spearman correlation = 0.8; $P < 0.0001$.

A variety of bacterial communities was detected in both BAL and oropharyngeal samples (Figures E1 and E2). Of 195 distinct taxa identified from the 22 subjects with both oropharyngeal and BAL sequencing data, 102 (52%) were identified in both oropharyngeal and BAL samples; 53 (27%) were identified only in oropharyngeal samples, and 40 (21%) in BAL only. Relative abundance was higher for taxa identified in both oropharyngeal and BAL samples compared with oropharynx or BAL only ($P < 0.01$).

Several genera previously reported as possible reagent contaminants (31) were

found in some samples, including *Bradyrhizobium* and *Rhizobium* (Figures E2 and E3). *Bradyrhizobium* was also detected in several negative controls; *Rhizobium* was not. All potential contaminant taxa were present at exceptionally low levels, whereas the dominant signals defining bacterial communities were from genera consistent with the human respiratory tract.

Agreement between Lavage and Oropharyngeal Sample Diversity

Lavage and oropharyngeal communities had similar diversity. Principle coordinates

analysis from subjects with sequence data from both sample types showed clustering of BAL and oropharyngeal communities. Importantly, both sample types clustered distinctly from negative control samples (Figure E1C). Alpha diversity (Shannon index) showed strong intrasubject correlation between oropharyngeal and lavage samples (Figure 1). Intrasubject agreement between oropharyngeal and lavage sample bacterial loads was low ($R = 0.02$).

When considering specific taxa found in the BAL and oropharyngeal communities (Figure E3), *Streptococcus spp.* largely

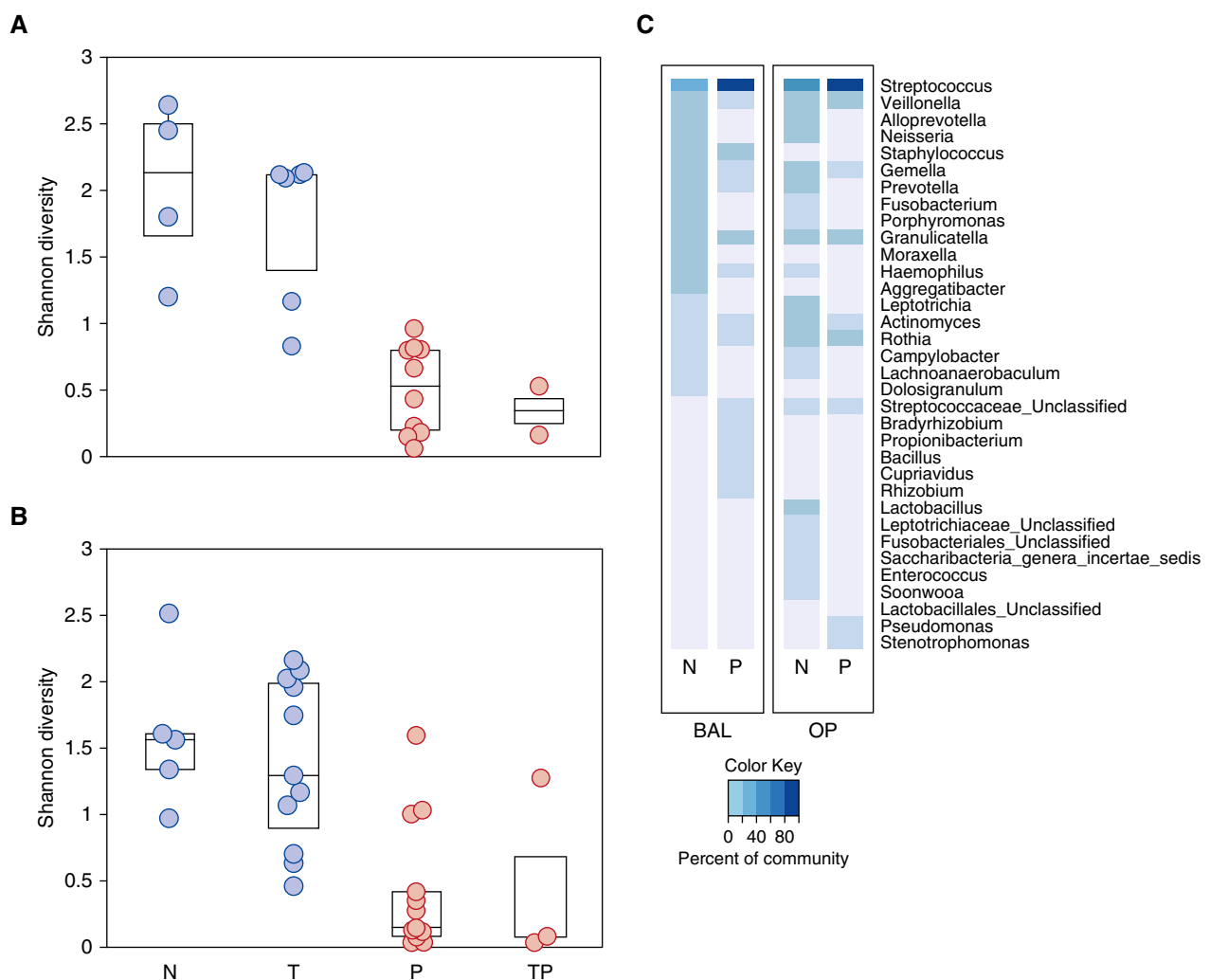


Figure 2. Shannon diversity differs with exposure to daily prophylactic antibiotics in lavage fluid and oropharyngeal swabs. Subjects are divided by antibiotic exposure, with subjects who received prophylactic antibiotics designated in *red* and those who did not in *blue*. Shannon diversity in (A) lavage and (B) oropharyngeal samples of subjects who received no antibiotics (labeled *N*), only prophylactic antibiotics (labeled *P*), only intermittent antibiotics for treatment of illness or eradication (labeled *T*), and those who received both daily prophylactic antibiotics and courses of antibiotics for treatment of illness or eradication (labeled *TP*). Significant differences were seen between *N* and *P* ($P < 0.01$), and *N* and *TP* ($P < 0.01$). (C) Composite bacterial communities in lavage (BAL) and oropharyngeal (OP) samples for all subjects segregated by exposure to daily prophylactic antibiotics (*N* and *P* represent no prophylaxis and prophylaxis, respectively), with each row representing a distinct bacterial taxon and relative abundance reflected by color intensity.

dominated both sample types. Intrasubject agreement between oropharyngeal and BAL samples for individual taxa varied widely, but agreement was stronger for the 10 taxa most abundant in the entire study population for either BAL or oropharyngeal sampling, which made up 88% and 92% of the total BAL and oropharyngeal sequences, respectively. Frequently, the major components of the BAL samples consisted of the same genera found in the oropharynx (Table E2).

Comparisons between Conventional Bacterial Culture and Sequencing of Lavage Samples

Using standard clinical laboratory microbial culture techniques, 9 of 32 BAL samples (28%) reported no growth, 13 yielded only normal oropharyngeal flora, and 10 produced oropharyngeal flora with growth additional bacterial species, including methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Moraxella catarrhalis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Morganella morganii*. Nine of these 10 samples had adequate bacterial load for sequencing, with identification of all but two taxa (*Pseudomonas* and *Klebsiella*) at the genus level, though some had few reads. Conventional culture of the 10 BAL samples with bacterial load below the level of qPCR yielded a specific pathogen (methicillin-sensitive *Staphylococcus aureus*) in a single subject; six had no significant growth, and three were reported as normal oropharyngeal flora.

Association of Bacterial Load and Diversity, Inflammation, and Antibiotic Prophylaxis

Using bivariate analysis, subjects receiving daily amoxicillin-clavulanate therapy as antistaphylococcal prophylaxis had slightly lower bacterial loads in oropharyngeal, but not BAL samples than those who did not receive prophylaxis (Figure E4). Use of antibiotic prophylaxis, however, was strongly associated with lower Shannon diversity in both BAL and oropharyngeal samples (Figure 2). There was not a significant difference in diversity between subjects with intermittent exposure to antibiotics (e.g., for treatment of exacerbations or nonrespiratory infections) and subjects who never

received antibiotic therapy, nor was there a difference in diversity in subjects who received only prophylactic antibiotics compared with those who received both prophylaxis and intermittent treatment courses (Figure 2). Other measures of alpha diversity yielded similar results, including measures of richness, Simpson diversity index, and Pielou evenness (Figure E5). Lower diversity of oropharyngeal and BAL samples also correlated with younger age and Australian subjects.

When q -value cutoffs (<0.1) were applied, there was no association between presence of specific taxa and prophylactic antibiotic usage, except *Fusobacterium*, which was present at very low abundances and appeared more common in infants

who did not receive prophylactic antibiotics ($q = 0.03$).

Lower lavage ANC was associated with both lower Shannon diversity on BAL and use of prophylactic antibiotics. Lower lavage IL-8 was found in BAL samples that had lower Shannon diversity, but not antibiotic prophylaxis (Figure 3). There was no correlation between bacterial load and BAL inflammatory measures (Figure E6). Three subjects had detectable free neutrophil elastase in BAL fluid; none were found in the prophylactic antibiotic cohort ($P < 0.01$). Presence of neutrophil elastase was significantly associated with respiratory symptoms at bronchoscopy ($P = 0.006$), but ANC and IL-8 concentrations were not.

Subject characteristics were next compared between prophylaxis and no

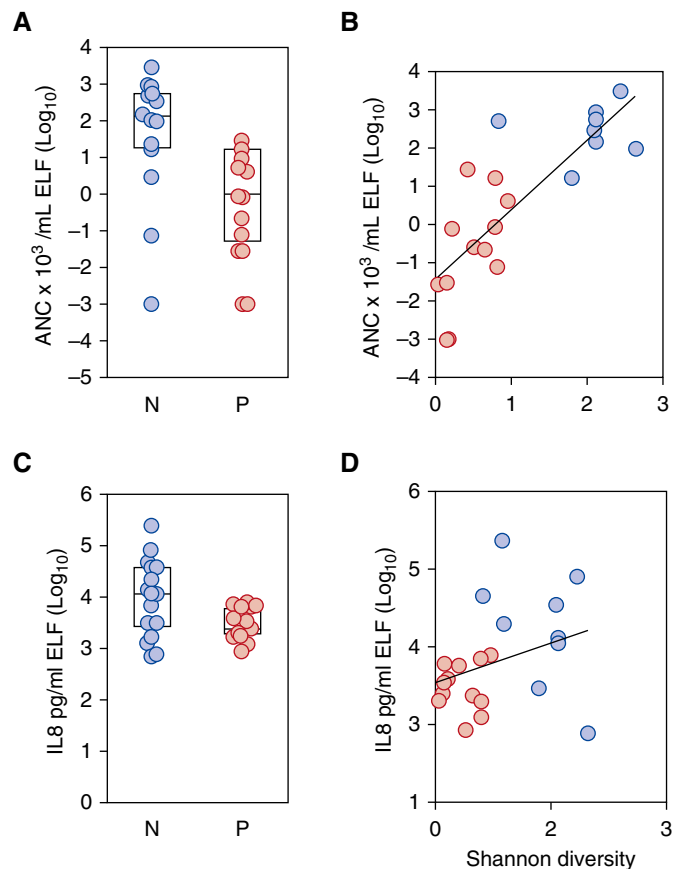


Figure 3. Association of inflammatory markers with prophylactic antibiotic use and bacterial diversity. In all panels, subjects receiving antibiotic prophylaxis (P) are shown in red circles, whereas those not receiving prophylaxis (N) are in blue. (A) Significantly lower absolute neutrophil counts (ANC) were found in the group treated with prophylactic antibiotics ($P < 0.01$). (B) Correlation between absolute neutrophil count and Shannon diversity in lavage samples ($R = 0.82$; $P < 0.01$). (C) There was no difference in interleukin 8 (IL-8) levels between the two groups ($P = 0.07$). (D) Correlation between IL-8 lavage concentration and Shannon diversity in lavage samples ($R = 0.44$; $P = 0.04$). ELF = epithelial lining fluid.

prophylaxis groups. There were no differences in sex or *CFTR* genotype between the two groups. Subjects in the prophylaxis group were all Australian, were younger at the time of sampling, and were less likely to have received a treatment course of antibiotics (Table 2).

The association between bacterial diversity and subject characteristics, antibiotic use, and inflammation was examined using linear regression analyses, performed separately for oropharyngeal and BAL samples. Variables in the initial model were chosen based on significance in bivariate analyses, and included bacterial diversity and load, prophylactic antibiotic use, treatment antibiotic use, age at bronchoscopy, continent, BAL inflammatory markers (ANC and IL-8), symptoms at time of bronchoscopy, and volume of fluid collected (BAL analysis only). Treatment antibiotic use was modeled as a categorical variable (treatment antibiotics within 14 days of bronchoscopy, >14 days from bronchoscopy, or never). Only use of prophylactic antibiotics and younger age at bronchoscopy showed significant associations with lower bacterial diversity in both upper and lower respiratory tract samples. Bacterial load showed a slight association with diversity in oropharyngeal samples, but was not associated with diversity in BAL samples (Table 3).

Discussion

As part of a novel, international collaboration comprehensively examining

the earliest origins of CF lung disease, we have identified associations between bacterial diversity of the respiratory tract microbiome and antibiotic exposure in young infants with CF. Lower bacterial diversity correlated with prophylactic antibiotic use and younger age at sampling. We also found a strong intrasubject correlation between respiratory microbial diversity in BAL and oropharyngeal samples, particularly for the presence of more abundant taxa, although exact community structures often differed.

Our understanding of the complex polymicrobial community in the lower airways of patients with CF has begun to alter our thinking on the role of colonizing and infecting bacteria in CF airways. Culture-independent methods for quantitative profiling of bacterial populations have opened a new era in investigation (32), demonstrating that respiratory tract microbial profiles can change with age (20) and antibiotic use (33), and evolve in young children (16). The upper and lower respiratory tract samples we have collected from infants across four centers in two continents provides a unique opportunity to understand the diversity and complexity of the airway microbiota in infants with CF, and has yielded insights into factors that may influence airway bacterial communities in early life.

We recently reported an association between lower diversity in BAL samples and increasing age in a historical cohort of young Australian children with CF sampled before routine use of antistaphylococcal

prophylaxis (18). We applied similar processing and sequencing approaches to examine airway bacterial diversity in infants with CF in the era of routine prophylaxis. Our findings suggest that administration of prophylactic antibiotics decrease bacterial diversity of the CF airway in infancy. Interestingly, prophylactic antibiotic use was not associated with lower bacterial load in BAL samples (although all bacterial loads detected were relatively low). Intermittent administration of treatment courses of antibiotics was not associated with differences in microbial diversity of the lower airways, though our small sample size and variability of timing of antibiotics in relation to sampling limited our ability to fully explore this relationship.

We found that bacterial diversity in oropharyngeal secretions strongly correlated with lower airway samples, which is in agreement with previous studies (32). The most abundant taxa found were represented in both samples, but the exact community compositions differed. Strong intrasubject correlation between oropharyngeal and lavage sample diversity, combined with similar findings of the association of age and prophylactic antibiotic use with diversity on oropharyngeal and lavage sampling, strengthens our findings, as each type of sampling has different limitations. Further, these observations could influence sampling modality choice in future studies investigating airway diversity in infants, as oropharyngeal secretions are more easily obtainable than BAL fluid and may represent a more consistent means of

Table 2. Subject characteristics by administration of prophylactic antibiotics

	All Subjects (N = 32)		Subjects with Lavage Sequencing Data (N = 22)	
	No Prophylaxis (N = 16)	Antibiotic Prophylaxis (N = 16)	No Prophylaxis (N = 10)	Antibiotic Prophylaxis (N = 12)
Male	3 (18%)	8 (50%)	3 (30%)	5 (42%)
Caucasian/White	16 (100%)	15 (94%)	10 (100%)	12 (100%)
F508del homozygous	9 (56%)	8 (50%)	7 (70%)	7 (58%)
U.S. subject*	15 (94%)	0	9 (90%)	0
Age at bronchoscopy, mo*	5.8 (±1.7)	3.5 (±0.9)	5.8 (±1.8)	3.5 (±1)
Weight at bronchoscopy, kg	6.9 (±1.3)	6.3 (±0.9)	7.2 (±1.2)	6.2 (±0.9)
Exclusively breast-fed	4 (25%)	6 (37%)	3 (30%)	3 (25%)
Smoke exposure	0	2 (12%)	0	2 (17%)
Treatment antibiotics*	11 (69%)	3 (18%)	6 (60%)	2 (17%)
No antibiotics	5 (31%)		4 (40%)	

Subject characteristics by administration of prophylactic antibiotics (amoxicillin-clavulanic acid) for all subjects and the subgroup with lavage sequencing data. No significant differences were seen between the groups with and without lavage sequencing data.

* $P < 0.05$ comparing no prophylaxis and prophylaxis groups both among all subjects and only those with lavage sequencing data.

Table 3. Linear regression results: association of diversity with age and antibiotic prophylaxis

Variable	β Coefficient	95% Confidence Interval		P Value
For OP diversity				
Prophylactic antibiotics	-0.521	-0.979	-0.064	0.03
Age (months) at bronchoscopy	0.222	0.093	0.352	<0.01
Bacterial load, copies per ml	2.52E-10	1.76E-12	5.02E-10	0.049
For BAL diversity				
Prophylactic antibiotics	-0.865	-1.264	-0.466	<0.01
Age at bronchoscopy, mo	0.229	0.114	0.343	<0.01

Beta coefficients from linear regression analysis for association of bacterial diversity in bronchoalveolar lavage (BAL) and oropharyngeal (OP) samples (analyzed separately) with use of prophylactic antibiotics and age (in months) at time of bronchoscopy. Lower diversity was associated with younger age and prophylactic antibiotics use. Bacterial load was associated with diversity in the model in oropharyngeal but not lavage samples. Other variables that were not significantly associated with bacterial diversity (and thus removed from the model by backward stepwise regression) included continent, interleukin-8 concentration, absolute neutrophil count, and volume pelleted (for lavage analysis only).

surveying bacterial diversity of the respiratory tract in studies of children with CF.

This study has several limitations. As it is an observational study, participants were not randomly assigned to receive prophylactic antibiotics, and thus this intervention was strongly tied to geography. Several subjects had respiratory symptoms and/or were treated with antibiotics at the time of bronchoscopy, which could influence diversity and inflammatory measures. Australian subjects were, as a whole, studied at a younger age than their American counterparts. Differences seen in microbial diversity could also be influenced by differences in the local environment, age, or both. However, historic samples in children younger than 1 year of age from the Royal Children's Hospital, collected before initiation of antistaphylococcal prophylaxis, had markedly higher diversity indices than our current samples from the same institution (18), suggesting geography is not the sole contributor to differences noted within our study populations.

Compliance with antibiotic regimens was not monitored; thus, it is possible some subjects in the prophylaxis group did not receive antibiotics as prescribed. The timing of treatment courses of antibiotics and sampling was not uniform, and could have an effect on observed associations. Small sample size also limited our ability to explore associations between specific taxa in BAL fluid and lung inflammation. Although low total BAL bacterial loads may have limited our ability to identify associations of load and inflammation, the microbial communities and bacterial densities observed were significantly different between subject samples and methodological controls, making it unlikely that our findings were spurious, related to background signal, or contamination. We were unable to speciate bacteria identified, and thus cannot comment on pathogenicity of specific organisms.

Australian subjects had lower volumes of BAL samples available for analysis, related to yields from small infants, which could have had an effect on the detection of

taxa present at low levels. Despite these limitations, BAL volume was not associated with bacterial load or with bacterial diversity in the final regression model. The strong correlation between oropharyngeal and BAL diversity, as well as the association of both oropharyngeal and BAL diversity with prophylactic antibiotic use, suggests differences in diversity are not simply related to differences in lavage sample volume or technique. We believe the uniqueness of our sample set of prospectively collected lavage samples from infants and the counterarguments outlined here more than balance the limitations of this study. In summary, we have shown in a cohort of infants with CF that microbial diversity of the upper and lower respiratory tract is associated with administration of prophylactic antibiotics, and reduced diversity in the lower airways is associated with less inflammation in the lower airways. We continue to follow these infants, which will allow us to determine whether these findings persist. By recognizing the earliest origins of CF lung disease, we expect to better understand pathogenesis and identify therapeutic targets or interventions that could alter its trajectory (19). ■

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

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