Mild CF Lung Disease is Associated with Bacterial Community Stability

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Abstract

Microbial communities in the airways of persons with CF (pwCF) are variable, may include genera that are not typically associated with CF, and their composition can be difficult to correlate with long-term disease outcomes. Leveraging two large datasets characterizing sputum communities of 167 pwCF and associated metadata, we identify five bacterial community types. These communities explain 24% of the variability in lung function in this cohort, far more than single factors like Simpson diversity, which explains only 4%. Subjects with *Pseudomonas*-dominated communities tended to be older and have reduced percent predicted FEV₁ (ppFEV₁) than subjects with *Streptococcus*-dominated communities, consistent with previous findings. To assess the predictive power of these five communities in a longitudinal setting, we used random forests to classify 346 additional samples from 24 subjects observed 8 years on average in a

range of clinical states. Subjects with mild disease were more likely to be observed at baseline, that is, not in the context of a pulmonary exacerbation, and community structure in these subjects was more self-similar over time, as measured by Bray-Curtis distance. Interestingly, we found that subjects with mild disease were more likely to remain in a mixed *Pseudomonas* community, providing some support for the climax-attack model of the CF airway. In contrast, patients with worse outcomes were more likely to show shifts among community types. Our results suggest that bacterial community instability may be a risk factor for lung function decline and indicates the need to better understand factors that drive shifts in community composition.

Introduction

Cystic fibrosis (CF) is a genetic disease affecting about 30,000 people in the United States and 70,000 world-wide (1). It has long been recognized that airway infection by *Pseudomonas aeruginosa* predicts morbidity and mortality in young children with CF (2). Direct sampling of the CF airway demonstrates that a small set of established CF pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae* and species from genera *Burkholderia*, *Stenotrophomonas*, and *Achromobacter* dominate early infections in children with CF (3). The prevalence of *Pseudomonas* infection in the CF community increases with age (4), and it has been reported that few pathogens other than *Pseudomonas* are detectable in end stage disease (5). Not surprisingly, the prevailing thought was once that CF lung disease might be caused by a single pathogen (6-7), although not necessarily the same pathogen in all people (8). Consequently, treatments were directed at specific pathogens such *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae* (9). Evidence from the last 15 years

demonstrates that genera outside the established set of CF pathogens, such as *Streptococcus*, and anaerobes like *Prevotella* and *Veillonella* also play important roles in lung disease for persons with CF (pwCF) (10-12).

CF lung disease is now considered a polymicrobial phenomenon (13) that can be described using ecological concepts like species diversity. Diversity has been associated with improved lung function (14-17) but increased diversity may also signal the onset of exacerbation (18).

Longitudinal analysis, in which a pwCF is followed over time, generally reveals that the CF airway microbiome as relatively stable (18-21) and changes modestly during cycles of antibiotic treatment and pulmonary exacerbations, acute phenomena characterized by a rapid decline in overall health, accompanied by decreased lung function (19, 22-24) during exacerbations pwCF experience rapid decline in overall health frequently accompanied by permanently decreased lung function (19). More frequent exacerbations, and failure to recover fully from these exacerbations, are thought to differentiate people with moderate or severe disease from those with mild CF lung disease (26, 27). Intravenous antibiotic treatment in a hospital setting is recommended during a pulmonary exacerbation (20) and recovery usually occurs over a period of weeks (21). Complete recovery of lung function to levels prior to exacerbation is more common in younger pwCF (22) and those with fewer exacerbations. Recovery may be mediated by response to antibiotics like tobramycin, which is at least partly predictable from polymicrobial signatures (23). It is possible that some exacerbations are triggered by increasing relative abundance of anaerobes in the two weeks leading up to an exacerbation (24) or by specific species of *Streptococcus*, including *anginosus* group streptococci (AGS), also known as

the *milleri* group streptococci (25-27) . Collectively, recent findings paint a complex picture of how exacerbations occur in CF.

One model of how dynamic changes in the CF airway community might be associated with changes in patient health suggests that the CF airway microbiome is normally a stable climax community (28) dominated by a classic pathogen such as *Pseudomonas*, *Achromobacter*, *Staphylococcus*. From time to time, the intrusion of facultative or obligate anaerobes, via an undescribed mechanism, leads to a fermentative “attack community” that lowers pH and favors further expansion of anaerobes (28). This population shift would increase bacterial species diversity, consistent with the increased diversity during exacerbation that has been previously observed (18). Interestingly, this is one instance where increased diversity is associated with a worse clinical outcome, rather than better outcomes as is typically reported (14, 17, 29, 30, 31). Furthermore, this climax-attack model is also consistent with the concept of stable core and dynamic satellite taxa (16) because these satellite taxa are largely the same genera that are thought to constitute the attack community, and it has been shown that satellite genera are more dynamic across disease states (32).

The primary aim of this study was to improve our understanding of the baseline community types, including 1) identifying discrete CF airway community types, 2) assessing whether community types are associated with different health outcomes, 3) describing how community types in pwCF change over time, and 4) whether community stability is associated with disease severity. This analysis included a variety of machine learning techniques applied to both cross-sectional and longitudinal data sets. Collectively, our results are consistent with a recent report

that bacterial communities in stable patients have less variation in community structure over time (33), and our results support the climax-attack model of CF airway disease (28). In addition, we identify a five-community model that accounts for 24% of the variability in lung function, far more than any single factors explored previously.

Methods

Data processing. Publicly available 16S rRNA gene amplicon library sequence reads and associated metadata from recent studies by Carmody et al. (PRJNA423040) (18) and Cuthbertson et al. (PRJEB30646) (17) were downloaded from Bioproject (<https://www.ncbi.nlm.nih.gov/bioproject/>). Reads in fastq format were mapped to unique bacterial genera using QIME2 (34).

Cross-sectional data set: We assembled a cross-sectional data set by combining baseline data (i.e., not exacerbating / receiving treatment for exacerbation) set from 167 unique pwCF, 84 from the Cuthbertson et al. study (17) and 83 from the Carmody study (18). In those instances where more than one baseline sample was available from a given subject, the first sample taken was used, together with metadata (e.g. ppFEV₁). The two data sets were similar, but not identical in terms of age and lung function. The 84 subjects included in the cross-sectional data set from the Carmody et al. (17) were slightly younger, with a mean age of 25.6 ± 10.0 SD compared to a mean age of 30.27 ± 10.9 SD. OTU level mapping and metadata for the cross-sectional two data sets (17, 18) is provided in Supplemental Table 1

(<https://doi.org/10.6084/m9.figshare.14261084.v1>) and Supplemental Table 2

(<https://doi.org/10.6084/m9.figshare.14261219.v1>)

Longitudinal data set: The longitudinal data set was a subset of samples from the Carmody et al. (18) study, specifically, 346 16S rRNA gene amplicon datasets from 24 subjects that were observed at least 10 times. Age at time of sampling ranged from 11 to 54, with median of 27 and an interquartile range of 8. Lung function (ppFEV₁) of subjects in the longitudinal data set ranged from 9 to 123%, with a median of 50 and an interquartile range of 36. Longitudinal subjects are more fully described in Table 1. OTU level mapping and metadata for the longitudinal data set (18) is provided in Supplemental Table 3 (<https://doi.org/10.6084/m9.figshare.14261432.v1>).

Statistical analyses. Data analysis was performed in R 4.0.1 (<https://www.R-project.org/>). Bray-Curtis dissimilarity was calculated using ecodist (35). Simpson diversity was calculated with vegan 2.5-7 (36). Clustering was performed using kmeans in base R and clusters were visualized and gap analysis was performed using factoextra 1.0.7 (37). Random forests were generated using randomForest 4.6-14 (38). Discrete Time Markov chains were determined using the markovchain package 0.8.5-4 (39).

Results

Five Clusters to Explain Two Large Cross-sectional Data Sets

As the emphasis in CF airway microbiome investigation has shifted toward a polymicrobial interpretation (13), data analysis has increasingly focused on multivariate comparisons. We therefore began our analysis to identify polymicrobial community clusters by calculating Bray-Curtis dissimilarity (40) between genus level taxonomic assignments in bacterial 16S rRNA gene amplicon sequencing data from 167 sputum samples from people with CF. Bray-Curtis similarity (40) is a common measure used to summarize the similarity between microbial communities, and places a larger weight on species that are commonly observed, partly mitigating the problem of random sampling errors associated with rare species. Samples for this analysis were chosen as a cross sectional subset of two recently published studies (17, 18), and included clinically stable subjects aged 8 to 69 from 14 CF centers in the US and Europe creating a snapshot that should include many of the community types present in pwCF. We calculated the Bray-Curtis similarity across 167 cross-sectional samples from these two independent studies: a cross-sectional study from Cuthbertson et al. colleagues (17), from which 83 samples were analyzed, and a longitudinal study by Carmody et al. (18), from which we selected a single representative sample from each patient for this analysis (84 samples).

We next used k-means to cluster samples using the Bray-Curtis similarity matrix we generated above. The k-means machine learning algorithm requires the user to identify how many clusters (k) are to be identified in the data, then uses an iterative approach to assign samples to each cluster to minimize distances between samples in the same cluster and maximize distances between samples in different clusters. Such machine learning approaches are commonly applied to microbiome studies (41). For example, unsupervised techniques like hierarchical clustering (42) have been used to identify core taxa in the CF airway (16) and gut (43) and to demonstrate

that samples from the same person tend to be similar to each other, even across disease and treatment (44). K-means analysis has also been used to identify groups of patients with similar patterns in inflammation, microbiota, or clinical factors (45). What makes k-means unsupervised is that similarity between clusters is based only on observed data, in this case, counts of bacterial genera. Unsupervised learning does not take sample labels (e.g., “severe disease” or “exacerbation”) into consideration. Therefore, when unsupervised clustering places samples labeled “severe disease” together, it is likely that a non-random process (e.g., the result of a biological difference) caused the samples to cluster.

As described above, the k-means algorithm requires the user to identify how many clusters (k) are to be identified in the data. Since we had no prior information to justify a particular choice of k, we used another machine learning approach, the gap statistic (46), to estimate the number of distinct clusters in our cross-sectional data. The gap statistic has recently been used to evaluate the optimal number of clusters in CF airway microbiome data (29). The gap statistic identifies the smallest value of k that minimizes distances between samples in the same cluster and maximize distances between samples. Gap analysis suggested the existence of five distinct community types in our cross-sectional data set of the 167 baseline samples analyzed here. We found that subjects belonging to different communities also differed significantly in lung function (see below); this finding suggests that clusters are based on biological differences (47) and that community types may be clinically relevant. The optimal number of clusters (five) chosen by the gap statistic algorithm is shown by the vertical dashed line in Figure 1A. The basis of this choice is driven by the break in the data after k=5.

188 A principal coordinate analysis that captures >70% of the variance of these data (Figure 1B)
 189 shows how the clusters separate into 5 largely non-overlapping groups. It is important to note
 190 that this analysis raises the possibility of alternative, potentially equally valid grouping of the
 191 samples and/or potential overlap among these communities.

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195 Figure 1A: Gap statistic for different numbers of k clusters, ranging from 2 to 10. Larger values
 196 indicate greater separation between clusters. An optimal number of clusters based on gap statistic
 197 is shown by a dashed vertical line.

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202 Figure 1B: Principal coordinate analysis of 5 clusters, based on Bray-Curtis dissimilarity matrix.

203 The legend showing the color code of each cluster is on the right. Together, the first two

204 principal coordinates capture >70% of the variance in these data. Each dot represents one of 167

205 samples in the cross-sectional analysis described in Methods.

206

207 It is reassuring that choosing 5 clusters also optimizes the ability of communities to explain

208 patient differences in lung function. Figure 1C shows the value of adjusted r^2 from linear models

209 of ppFEV₁, a measure of lung health, as a function of cluster membership. Five clusters explain

210 about 24% (adjusted $r^2 = 0.24$) of patient differences; using six clusters reduces r^2 . In summary,

211 two independent lines of evidence suggest that 5 clusters may be optimal for summarizing these

212 data.

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215 Figure 1C: Adjusted r squared from linear models of lung function (ppFEV1) using membership
 216 in a given cluster as the independent variable. Models were run for different numbers of k
 217 clusters, ranging from 2 to 10.

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219 *Clusters Membership Predicts Lung Function Better than Pseudomonas Relative Abundance,*
 220 *Age or Community Diversity*

221

222 Despite a reframing of the conversation surrounding the CF airway microbiome away from
 223 single pathogens toward ecological descriptions involving species diversity (14, 17-18, 48, 49),
 224 the relationship between *Pseudomonas* and lung function is still frequently cited (14, 23, 49, 50).
 225 We used linear models to assess the level of association between lung function and commonly
 226 identified explanatory variables such as the relative abundance of *Pseudomonas* as well as
 227 Simpson Diversity and age. As shown in Figure 2, single variables like relative abundance of

228 *Pseudomonas*, Simpson diversity and age explain less than 11% of patient variability in lung
 229 function (adjusted $r^2 < 0.11$). In other words, over 89% of variability in lung function is
 230 explained by factors other than *Pseudomonas*, Simpson diversity or age. The explanatory power
 231 of these models improves with increasing complexity, and a combined model that incorporates
 232 Simpson diversity, age and the relative abundance of *Pseudomonas* or other classical CF
 233 pathogens in a single model still explains less than 12% of patient variability in lung function. In
 234 contrast, membership in one of the 5 communities identified above explains 24% of patient
 235 variability in lung function. In other words, knowing which cluster a person's airway
 236 microbiome belongs to provides a better estimate of their lung function than knowing the relative
 237 abundance of *Pseudomonas* in their airway microbiome (Figure 2).

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 239 Figure 2: The ability of linear models to predict ppFEV₁, as estimated by adjusted R squared.
 240 Individual factors or combinations (denoted by “+”) of covariates shown on y axis: Simpson
 241 Diversity only, Age only, *Pseudomonas* only, the combination of Simpson and Age, or Simpson
 242 and Age and the relative abundance of other classic CF pathogens, compared to membership in
 243 one of the 5 clusters.

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Top 10 Bacterial Genera Across Clusters

We identified the top 10 genera with the highest mean relative abundance across the 167 cross-sectional samples and averaged their relative abundance in each of the 5 clusters, as shown in Figure 3A. Choosing the top 10 genera for visualization still includes the vast majority of the reads (mean 90%) that mapped to a genus. The first cluster (Str.D), which contained 46 samples was, on average, dominated by *Streptococcus* (brick red). Clusters 2, 4 and 5 had large amounts of *Pseudomonas* (olive), but were distinguished by the differences in abundance and/or type of minor genera. Cluster 2 (Pa.D) was dominated by *Pseudomonas*. While Cluster 4 (Pa.M1) and Cluster 5 (Pa.M2) were similar, they clustered separately, perhaps because Cluster 5 had more *Achromobacter* and *Burkholderia* than cluster 4 ($P < .001$ asymptotic Wilcoxon-Mann-Whitney test), and Cluster 4 had more *Streptococcus* ($P < .001$, asymptotic Wilcoxon-Mann-Whitney test). Cluster 3 (Oth.D) was dominated by a genus other than *Streptococcus* or *Pseudomonas* and could be described as having an even mix of genera based on averages.

Figure 3A: Mean abundances of top 10 genera in each of the 5 clusters. Mnemonic abbreviations: Str.D = *Streptococcus* dominated, Pa.D = *Pseudomonas* dominated, Oth.D = Other dominated, Pa.M1 = *Pseudomonas* mixed community #1, Pa.M2 = *Pseudomonas* mixed community #2. The number of samples that belonged to each cluster appears beneath each cluster mnemonic.

We then mapped each of these clusters back to the two cross-sectional datasets, as shown in Table 2. The Carmody data set (18) was from a single CF center in the US, and the Cuthbertson data set (17) included subjects from Europe and the US, spanning 13 different CF centers. Some clusters, but not others, were enriched in data from particular studies perhaps as a consequence of biases in sample collection/processing, differences in subject age, and/or systematic differences in patient care. Of note, the two *P. aeruginosa* mixed communities, Pa.M1 and Pa.M2, largely clustered as a function of the dataset from which they were derived. Interestingly, the Carmody (18) data set had no patients in the *P. aeruginosa* dominant cluster (Cluster 2, Pa.D), while the Cuthbertson (17) samples, collected over 13 centers had ~1/3 of its sample map to this community cluster. In contrast, for the communities assigned to the Carmody dataset (18), more than half were described as *Streptococcus*-dominated, while the remaining samples, while containing *P. aeruginosa*, were more evenly mixed (Pa.M1) or dominated by other pathogens (Oth.D).

Cluster Number	Mnemonic	Carmody	Cuthbertson
1	Str.D	45	1
2	Pa.D	0	27
3	Oth.D	19	18
4	Pa.M1	19	8
5	Pa.M2	1	29

Table 2: Number of subjects in cross sectional data set mapping to various clusters shown in Figure 2, for the Carmody (18) and Cuthbertson (17) data sets.

Interestingly, averages do not capture the specifics of a group perfectly, as shown in Figure 3B. Individual samples that mapped to cluster 3 (Oth.D; Other dominated), which when averaged appears to have an even mix of genera, tended to be dominated by individual genera, including *Stenotrophomonas* which was not among the top 10 most abundant genera across all 167 samples. We therefore complemented our analysis of the 10 most abundant genera (on average) with an analysis of the 10 most dominant genera, that is, the top 10 genera achieving the highest relative abundance in any of the 167 samples.

Figure 3B: Abundances of top 10 genera relative abundance of cluster 3 (Oth.D) in each of the 37 samples belonging to this group.

Haemophilus, *Stenotrophomonas*, *Burkholderia*, *Prevotella* and *Achromobacter* achieved high levels of dominance (relative abundance greater than 85%) in samples in cluster 3, as shown in Figure 3C, but not in other clusters. *Streptococcus* achieved dominance in cluster 1, but not elsewhere. *Pseudomonas* achieved high levels in clusters 2, 4 and 5.

Figure 3C: Maximum abundance of 10 genera achieving the highest relative abundance in any of the 167 samples, as a function of cluster.

Clusters Dominated by Streptococcus and Pseudomonas Differ in Lung Function and Age

It has previously been observed that species from the *Streptococcus* genus are part of the core CF airway microbiome (16, 31, 50), that the prevalence of *Streptococcus* spp. is sometimes associated with less severe lung function loss (12, 14, 31, 51), but that some genera of *Streptococcus*, including anginosus group streptococci (AGS; also known as the milleri group streptococci) may facilitate exacerbation (25-27). Our cross-sectional analysis highlights the positive aspect of patients carrying the community dominated by *Streptococcus*; subjects whose airway microbiomes were characterized as *Streptococcus*-dominated tended to have better lung function, as shown in Figure 4A, and be younger, as shown in Figure 4B.

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318 Figure 4A: Association of communities with lung function. Subject-Lung Function profiles of 5
319 distinct communities. Box and whisker plot comparing ppFEV₁ between *Streptococcus*-
320 dominated (Str.D), *Pseudomonas*-dominated (Pa.D), communities dominated by other genera
321 (Oth.D), *Pseudomonas* mixed community #1 (Pa.M1), and *Pseudomonas* mixed community #2
322 (Pa.M2), as shown in Figure 3A. Communities sharing a common letter are not significantly
323 different; those with no letter in common differ significantly (Tukey Honest significant
324 difference $P < .05$). For example, subjects with Str.D communities had higher ppFEV₁ than those
325 with any other community type.

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Figure 4B: Association of communities with age. Subject-age profiles of 5 distinct communities. Box and whisker plot comparing age between *Streptococcus*-dominated (Str.D), *Pseudomonas*-dominated (Pa.D), communities dominated by other genera (Oth.D), *Pseudomonas* mixed community #1 (Pa.M1), and *Pseudomonas* mixed community #2 (Pa.M2), as shown in Figure 3A. Communities sharing a common letter are not significantly different; those with no letter in common differ significantly (Tukey Honest significant difference $P < .05$). For example, subjects with Str.D communities were younger than those with Pa.D communities or Pa.M2 communities but did not differ significantly in age from subjects with Oth.D or Pa.M1 communities.

Communities Observed in Subjects with Mild versus Moderate/Severe Disease are Similar

Findings in the cross-sectional data (Figure 4A, B) are consistent with previous reports showing that communities dominated by *Pseudomonas* tend to be associated with older subjects and reduced lung function (36, 39, 44). Given that young, healthier pwCF tend to have sputum communities dominated by *Streptococcus* led to the hypothesis that people with such

Streptococcus-dominated communities ultimately transition to communities dominated by *Pseudomonas* as they age. To probe this idea, we used a longitudinal data set to assess how communities changed in the 24 subjects detailed in Table 1, for whom there are 10 or more longitudinal samples collected for times periods ranging from ~6-10 years (average of ~8 years).

For this analysis we used supervised machine learning to track community type changes in individuals with CF through time. Briefly, we used random forests (52) to identify features that reliably classify a sample as belonging to one of the five previously identified communities. This classifier enabled us to identify the community type of 16S rRNA gene amplicon datasets from 24 subjects that were observed across many years (18). This longitudinal data set included 16S rRNA gene amplicon dataset measurements made in different clinical states (baseline, exacerbation, treatment and recovery) and includes clinical metadata such as whether a subject had been diagnosed as having mild or more severe disease. This approach mapped the 16S rRNA gene amplicon datasets to the four communities shown in Figure 3, specifically, Str.D., Pa.D, Pa.M1 and Oth.D. No samples in the Carmody (18) longitudinal data mapped to Pa.M2, consistent with the description of Pa.M2 cluster being specific Cuthbertson (17) dataset (see Table 2).

Roughly two or three community types were required to describe any given subject over time, recapitulating previous reports that the CF airway microbiome is fairly stable over time (15, 18, 19, 38, 45, 46), but may shift in structure around the time of exacerbation or in response to antibiotic therapy (23, 45). We did not observe large differences in community types between subjects with mild disease compared to moderate/severe disease either as a function of subject

(Figure 5A) or disease state (Figure 5B). For example, subject 117 was observed 15 times to show a *Streptococcus* dominated community (Str.D) and 5 times to show the *Pseudomonas* mixed community number 1 (Pa.M1).

We do note some associations. For example, domination by a pathogen other than *Pseudomonas* (Oth.D) was associated with decreased probability of being observed in the baseline state (p-value = 0.004, odds ratio 0.43, Fisher’s exact test). Furthermore, despite similarities, subjects with mild disease did differ from those with moderate/severe disease in specific ways. Subjects with mild disease were more likely to be observed having a community classified as Pa.M1 (p-value = 0.02, odds ratio = 1.69, Fisher’s exact test) and more likely to be observed in the baseline state (p-value = 3.1e-06, odds ratio = 2.8, Fisher’s exact test) than people with moderate/severe disease.

Figure 5A: Frequency of observing various community types in subjects designated as having mild or moderate/severe disease. Subjects with mild disease (N=14, left) are compared to subjects with moderate/severe disease (N=10, right). “Subject identifier” corresponds to “isolate”

in Table 1, a field provided in the Carmody (18) data set. Community types identified in these longitudinal samples were deemed similar to those in Figure 3A by random forest classifier.

Figure 5B: Frequency of observing various clinical states for the 14 subjects with mild disease aggressiveness (left) and 10 subjects with moderate/severe disease (right) as shown in Table 1.

Clinical states are defined as (B = baseline, E = exacerbation, T = treatment, R = Recovery).

Community types identified in these longitudinal samples were deemed similar to those in Figure 3A by random forest classifier.

Interestingly, we note that people with mild disease have bacterial communities that are more stable over time based on being observed in a specific community category (Figure 5). We tested this association using a continuous variable by calculating Bray-Curtis distances for all pairwise comparison of samples taken from any given subject over time. As shown in Figure 6A,

communities from subjects with mild disease were less dissimilar to each other than communities from subjects with moderate/severe disease, suggesting that variation in community composition is associated with worse disease outcomes.

Figure 6A: Median within-subject pairwise distances (Bray-Curtis dissimilarity) for N=14 subjects with mild disease aggressiveness (left) and N=10 subjects with moderate/severe disease (right) as shown in Table 1. Subjects with mild disease had samples that were significantly more similar to each other (“*” signifies $P < .05$, Wilcoxon rank sum exact test).

As a second approach to assess the association between community stability and clinical outcome, a Markov chain analysis was performed to track subjects and their associated community types across time. Markov models simplify dynamic systems by representing them as a set of finite states that are connected to each other enabling transitions between the states. With enough data, the geometry of these systems (i.e., states that are connected to each other, enabling

a transition) can be determined. In addition, the probability of transitioning between any two connected states can be estimated. Markov modeling of this system suggests that this self-similarity arises at least partly because subjects with mild disease are repeatedly observed with a Pa.M1 community type (Figure 6B). Specifically, given that a subject was characterized as having mild disease and was last observed with community type Pa.M1, they had a 73% probability of being observed in Pa.M1 again. In contrast, the geometry associated with moderate/severe disease was more complex, and subjects were less likely to remain in the Pa.M1 state (60%) and also found more often in the Pa.D community (Figure 6C). Overall, repeated observation of Pa.M1 was significantly more likely in subjects with mild disease compared to moderate/severe disease (red arrow in 6B: “*” indicates $P = .03$, Fisher’s exact test, odds ratio 1.78). Together, these data indicate that patients with stable communities tend to have better airway function.

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431 Figure 6B: Markov chain diagram for community transitions in subjects with mild disease (see
 432 Table 1). Each node in the diagram (orange circles) represents the state of being observed with a
 433 specific bacterial community. Edges leading from each node represent possible transitions,

434 including the possibility of observing the same community again (loop). Numbers on arrows are
435 estimates of the probability of taking a specific path.

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439 Figure 6C: Markov chain diagram for community transitions in subjects with moderate/severe
440 disease (see Table 1). Each node in the diagram (orange circles) represents the state of being
441 observed with a specific bacterial community. Edges leading from each node represent possible

transitions, including the possibility of observing the same community again (loop). Numbers on arrows are estimates of the probability of taking a specific path.

DISCUSSION

We present two major findings in this report. First, the previously reported climax-attack model postulates that an attack community composed of genera like *Streptococcus*, *Veillonella* and *Porphyromonas*, perturbs a climax community dominated by *Pseudomonas* and *Staphylococcus* (28), resulting in a metabolic shift toward fermentation and decreasing pH. These conditions are more favorable to fermentative bacteria like *Prevotella*, *Veillonella*, *Streptococcus*, *Rothia* and *Granulicatella* (28). Our observation that mild disease is associated with community stability, and that stability is associated with a *Pseudomonas*-dominated community are therefore consistent with the climax-attack model of CF.

Second, we have shown that a relatively small number of CF microbiome types explain patient differences in lung function, and that they explain these differences much better than single variables like the relative abundance of *Pseudomonas*, age or Simpson diversity. This finding is important because developing interventions to treat the community as the pathogenic unit will require a reasonable number of experimentally tractable model communities. Without such models, we would be faced by a dizzying array of patient differences. Fortunately, the airway community in a pwCF over multiple samplings generally looks more like “self” than any other patient when using relative abundance of bacteria as a metric of what defines a community increasing the likelihood of identifying an in vitro model that might help design more effective

treatment regimens for a pwCF that has a similar community composition. Our findings raise the question of what combinations of abundant microbes (*Pseudomonas*, *Streptococcus*, *Staphylococcus*) and less abundant community members (i.e., anaerobic, fermentative organisms) really matter? Does it matter if a patient has 90% *Pseudomonas* versus 80% or 70%? Intriguingly, *Pseudomonas*, often listed as the quintessential CF pathogen, is highly abundant in long lived people with CF, an aspect of a “survivor phenotype” (53). Alternatively, does it matter if the dominant microbe in a community is *Streptococcus* rather than *Pseudomonas*, or if the minor players are *Prevotella* versus *Veillonella* versus *Gemella*? Although unsupervised machine learning cannot answer these questions with precision, we have demonstrated that as few as five communities may represent a useful starting point as pwCF with a particular community type experience significantly similar lung function as measured by ppFEV₁.

There are several critical factors and caveats to consider in light of the interpretations we make here. First, although a gap statistic suggests five communities may be optimal for the cross-sectional data set used here, one should avoid construing “five” as the number of distinct populations in the CF community. Children younger than eight were not included in this study, and it is possible that broad sampling of young pwCF would have enabled us to identify additional clusters. In the same vein, although cluster 3 (Oth.D) contains subjects that were dominated by genera other than *Pseudomonas*, it is understood that broader sampling of this group would create additional clusters for each genus in cluster 3. Nonetheless, some of the findings here mirror the conclusions from previous studies with smaller data sets (12, 15, 44). Second, this study includes both cross-sectional and longitudinal observations of airway microbiomes and associated clinical parameters, enabling us to identify associations between

488 them. However, this type of study cannot prove causation. Indeed, our interpretation of the data
489 here should rightly be viewed as hypotheses to be tested either via additional clinical studies or
490 in the laboratory. Third, and related to the previous point, the most useful aspect of this work is
491 that it does set a framework for hypothesis testing, for example, can we build the communities
492 we observe here *in vitro* and assess their function (i.e., are some clusters of microbes more/less
493 cytotoxic or more/less recalcitrant to antibiotics)? Alternatively, are similar communities
494 associated with positive/negative health outcomes in other cohorts of pwCF or in other disease
495 settings? For example, a recent report by Frey et. al. (29) concludes that airway diversity is a
496 biomarker of lung disease severity that links dysbiosis to lung function decline in pwCF. They
497 observed a negative correlation between inflammatory cytokine secretion and airway community
498 diversity, suggesting that more diverse communities are for some reason more salubrious than
499 less diverse communities (a common but unexplained finding across many microbiome studies).
500 Using a slightly different unsupervised approach to identify CF airway communities (i.e.,
501 hierarchical clustering of Unifrac distances), these investigators found that high diversity maps to
502 an oropharyngeal-like community typically found in younger people. The authors carefully avoid
503 making the claim that bacterial diversity reduces inflammation, although others have reported
504 this association (50) or found a positive association between higher lung function and increased
505 bacterial diversity (14, 17). The diverse, oropharyngeal-like community identified by Frey is
506 characterized by anaerobes like *Prevotella* and *Veillonella* and aerobic commensals including
507 *Neisseria* and *Streptococci*, making it very similar to the *Streptococcus* dominated community
508 (Str.SD) identified in this report (Figure 3); our Str.D community was also associated with higher
509 lung function (Figure 4A) and relative youth (Figure 4B) in pwCF. Thus, orthogonal approaches

appear to converge on similar conclusions as to community composition associated with less severe health outcomes.

The findings here and reported by Frey et. al. (29) and others (14, 50, 51) raise the question of whether remodeling CF airway communities to make them more diverse and more similar to the oropharyngeal communities found in younger people with higher lung function, if possible, might improve outcomes in CF. There are several reasons to question the wisdom of attempting to turn back the hands of time in older subjects and enhance the abundance of oropharyngeal flora in their airways. First, as described above, it is possible that the introduction of diversity into an established community (for example, one dominated by *Pseudomonas*) would destabilize the community and lead to enhanced inflammation, as suggested by the climax-attack model of CF (54). Second, as demonstrated by Muhlebach (55), inflammation increases when pediatric pwCF are first colonized by oropharyngeal flora; although this finding should not be surprising, it is worth pointing out that the best airway community is probably no community at all. Third, even in adults, increased diversity is not uniformly benign. Studies by Raghuvanshi et al. (24) show a linear increase in the microbiome alpha-diversity and in the log ratio of anaerobes to pathogens two weeks before a pulmonary exacerbation, confirming similar observations by Carmody et al. (18) showing that bacterial community diversity and the relative abundance of anaerobes increased during exacerbations, i.e., before antibiotic treatment. Others have shown that treating adults with antibiotics primarily affects taxa other than classic CF pathogens (56, 57), and that antibiotics reduce the abundance of *Streptococcus*, *Prevotella* and *Veillonella*, but not *Pseudomonas*, the ostensible target of treatment. Collectively then, the results cited support

the somewhat surprising conclusion that oropharyngeal flora are associated with worse airway disease, and that treatments work because they reduce the abundance of oropharyngeal genera.

Finally, as mentioned above, we present just one approach for analyzing the extant microbial community data associated with CF airway infections, but an approach that has both statistical support and practical utility. That is, it is likely that only controlled experiments using model communities in laboratory or animals can unravel the mechanistic role played by oropharyngeal-like flora in an established adult microbiome (14, 25, 50, 58) and to understand the functional consequences of community composition. That is, we have identified 5 communities comprised of 10 genera, which presents an experimentally tractable numbers of communities and microbes. As another example, Crabbe et al. (59) recently reported using a nine member community for in vitro studies. Going forward, this and other studies can help inform the nature of laboratory model communities used to probe the hypotheses we and others have developed by analyzing clinical datasets.

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Table 1. Characteristics of 24 subjects with at least 10 longitudinal samples.

isolate	Aggressiveness	N	Period (yr)	Median Age	Median FEV1	Clinical States	FEV1/yr
75	Moderate/Severe	16	8.99	14.9	32.5	R,E,T,T,E,R,B,B,R,T,E,B,B,R,B,R	-2.23
139	Moderate/Severe	15	6.64	15.1	34	B,B,B,T,R,T,B,B,B,R,B,T,T,R	-5.17
94	Moderate/Severe	13	9.16	17.2	39	T,T,E,E,T,B,E,B,B,B,R,R	1.56
229	Moderate/Severe	11	7.27	19.2	34	R,R,R,B,B,T,R,B,T,B,B	-4.50
52	Moderate/Severe	18	9.74	23.0	43.5	T,E,R,R,T,R,R,B,R,R,B,E,E,B,B,E,T,R,R	-1.84
179	Moderate/Severe	24	8.66	23.8	41	B,E,T,T,B,E,T,R,B,B,E,B,R,R,E,R,T,B,T,E,R,B,E,E	-3.08
159	Mild	11	7.97	24.3	82	B,B,B,E,B,B,B,B,B,R	-1.20
87	Mild	17	7.54	25.0	82	T,T,R,B,E,B,B,B,B,R,T,R,B,R,B,B	1.84
160	Mild	13	8.01	25.2	73	B,T,B,B,R,B,R,B,R,B,T,E,B	0.79
142	Moderate/Severe	20	10.14	25.4	67	B,T,T,T,T,E,B,R,T,T,E,E,R,B,T,E,B,R,T,E	-1.16
141	Mild	12	8.91	26.3	85.5	R,E,R,B,B,B,R,T,B,R,B,T	-0.20
124	Moderate/Severe	18	10.24	26.3	54.5	B,B,T,B,T,B,E,T,B,E,B,E,B,E,T,B,E,B,B	-1.00
147	Moderate/Severe	18	8.17	27.2	36.5	B,R,E,E,B,B,B,B,T,R,T,T,T,B,B,R,B	-3.73
209	Moderate/Severe	16	7.87	28.0	37.5	T,B,T,B,E,T,R,E,R,R,R,T,R,R,B,R	-6.97
53	Mild	10	5.35	28.6	69.5	E,R,T,E,B,E,B,E,B,NA,NA	1.55
182	Mild	14	5.69	29.0	83.5	T,B,R,E,E,B,B,B,B,B,E,E,E	-2.28
118	Mild	10	7.33	30.3	58.5	E,T,B,R,B,B,B,T,R,E	-1.33
244	Mild	10	6.42	30.8	74.5	E,R,B,B,R,B,B,B,E,B	-2.23
222	Mild	10	7.47	33.5	97	B,R,B,T,B,T,B,B,B,NA	-0.21
136	Mild	15	7.6	36.8	57	B,R,B,E,B,B,T,R,B,R,E,E,B,T,B	-2.99
119	Mild	14	7.72	37.1	36	E,B,T,B,B,B,B,T,B,B,B,R,B,B	-3.42
195	Mild	11	8.24	41.4	54	E,B,E,E,B,E,B,T,R,B,R	0.45
50	Mild	10	6.27	43.5	70.5	B,E,R,B,E,B,B,B,B,R	-2.27
117	Mild	20	9.72	49.6	51	E,E,B,R,B,E,B,E,T,T,B,T,B,E,B,E,T,T,B,B	-0.31

^aDisease aggressiveness for each subject was based on publicly available metadata.

^bN represents the number of sputum microbiomes available for each subject, observed over the period, given in years.

^cClinical states observed: B = baseline, E = exacerbation, T = treatment, R = Recovery.

^dLinear regression estimates of annual change in FEV1 percent predicted for each subject shown at far right. The table is sorted by increasing median age.

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