Evaluating Sinus Microbiology by Transplant Status in Persons With Cystic Fibrosis: A Matched Cohort Study

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Abstract

Objective. Sinus disease is prevalent in persons with cystic fibrosis (PwCF) and may be a reservoir of airway infection in postlung transplant (pTx) patients. The microbial composition of cystic fibrosis sinuses and its associations with chronic rhinosinusitis (CRS) is relatively unexplored. We aimed to examine the sinus and lower airway microbiome and their associations with CRS in PwCF and pTxPwCF.

Study Design. Prospective single-centre study.

Setting. A total of 31 sex and age (±2 years) matched PwCF and pTxPwCF.

Methods. Demographic and clinical data along with sinus swabs and sputum were collected. CRS was assessed using Sinonasal Outcome Test-22 (SNOT-22) (patient reported outcome) and Lund-McKay (computed tomography sinus) scores. Samples underwent MiSeq Illumina sequencing of the universal 16S ribosomal RNA gene.

Results. A total of 31 PwCF (15 pTxPwCF) were included. Aggregate airways microbiome composition was dominated by *Pseudomonas* (46%), *Haemophilus* (14%), *Staphylococcus* (11%), *Streptococcus* (10%), and *Fusobacterium* (6%). α-diversity was significantly lower in post-Tx samples across both sputum and sinus samples ($P = .005$). β-diversity was significantly different between sputum ($P = .004$), but not sinus ($P = .75$) samples by transplant status. While there was a trend in higher β-diversity associated with lower SNOT-22 score at time of first visit, this did not reach significance ($P = .05$).

Conclusion. Sinus and airway microbiomes differed in PwCF and pTxPwCF, but the prevalent organisms remained consistent. Elucidating the relationship of the microbiome with clinical status to better understand when to intervene accordingly is needed to optimize sinus disease management in PwCF.

The upper airways are continuously exposed to inhaled microbes from the environment. In persons with cystic fibrosis (PwCF) the sinonasal cavity is considered as the first site colonized by pathogenic microbes with potential to seed the lower respiratory tract. The "unified airway hypothesis" has been used to describe the link between the upper and lower CF airways whereby treatment of upper respiratory tract symptoms may improve lower respiratory tract and vice versa.[1] Chronic rhinosinusitis (CRS), an inflammatory disease of the nasal and paranasal sinus mucosa, is a defining feature in PwCF with prevalence reaching nearly 100%.[2-4] CRS has been correlated with a negative impact on quality of life across in children and adults with cystic fibrosis (CF).[5] Moreover, the adverse impact of CRS on disease progression has been demonstrated through their associations with pulmonary exacerbation (PEx) and associated sequelae.[6,7]

Culture-independent methods studying the microbiome have demonstrated that the sinuses mirror the lower respiratory tract with polymicrobial communities that progressively become dominated by classical CF pathogens with advancing disease.[8-12] PwCF who have

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undergone lung transplantation are a unique subgroup in whom the airway microbiome is even less understood. Concordance between the pre- and posttransplant sputum microbiome of PwCF suggests the sinuses are a persistent nidus of airways infection.[13] Microbiome studies in posttransplant PwCF suggest that these communities are distinct with reduced diversity compared to other chronic lung diseases.[14] Moreover, aberrant microbial flora (ie, presence and/or re-establishment of *Pseudomonas aeruginosa*) may contribute to graft injury posttransplant, including development of the bronchiolitis obliterans syndrome (BOS).[15-18] The sinonasal tract is of particular importance in this population given the established microbial reservoir prior to transplant that may be further confounded with immunosuppression.

With increasing recognition that sinuses in CF are linked to respiratory health, elucidating the role of the sinus microbial milieu is essential. The goal of our study was to identify the microbial communities in the sputum and paranasal sinuses in PwCF and postlung transplant persons with cystic fibrosis (pTxPwCF) and characterize association(s) with CRS during periods of stability and illness. We hypothesized that there will be similar microbial populations in the upper and lower airways with differing diversity in the pre- and posttransplant settings. Further, microbial communities will associate with sinus disease parameters.

Materials and Methods

Study Design, Participants, Sinus Sampling, and Clinical Evaluation

We performed a prospective, matched cohort study of 31 adult PwCF and pTxPwCF. They were matched for sex and age (±2 years) with CF and symptomatic CRS between July 2015 and December 2016. Sample size calculations were completed on the basis of 80% power to detect differences in microbial richness in the pre- and posttransplant cohorts (difference of ~10 operational taxonomic units [OTUs], $\alpha = .05$) which required a total sample size of 30 patients (15 PwCF, 15 pTxPwCF). Participants with a confirmed diagnosis of CF, age ≥ 18 years and cystic fibrosis transmembrane conductance regulator (CFTR) class I to III mutations were enrolled. Persons with percent predicted forced expiratory volume in 1 second (ppFEV$_1$) <30% at study initiation and sinus surgery either in the preceding 12 weeks or planned during the study period were excluded. During routine quarterly clinic visits and unscheduled visits during periods of exacerbation, at least 1 sinus swab and/or expectorated sputum (to represent the lower airways) as paired samples were collected as able. Sinonasal samples were collected via nasal swabs (directed at the middle meatus). Investigators who obtained the nasal swabs (R.S., L.F.) received training from an otolaryngologist (K.S.). A minimum of 2 samples per enrolled subject were required to be included in the analysis. Demographic and clinical data (age, sex, pancreatic status, CF-genotype, ppFEV$_1$) and concomitant medications were collected through chart review. This study was approved by the University of Calgary's Conjoint Health Research Ethics Board (REB15-1994).

Participants were asked to complete a validated sinus symptom score at each visit. The Sinonasal Outcome Test (SNOT-22) is a widely used and validated CRS-specific symptom quality of life questionnaire.[19] SNOT-22 score ≥ 21 has been identified as a useful threshold for concomitant CRS in PwCF and for a minimally important clinical difference previously. For those who had undergone computed tomography of the sinuses for clinical purposes, we used the validated Lund-Mackay (LM) score to grade the radiologic severity of sinonasal disease.[20,21]

DNA Extraction and 16S Ribosomal RNA (rRNA) Gene Amplicon Sequencing

DNA extraction of sputum and sinus samples were done using previously established methods.[22,23] Briefly, frozen sputum or sinus samples were thawed, homogenized through a 1-mL Tuberculin Slip Tip syringe (BD) with a needle and 300 μL of sample was mechanically lysed by bead beating. Samples were enzymatically processed using 50 μL lysozyme (100 mg/mL), 50 μL mutanolysin (10 U/μL), 25 μL sodium dodecyl sulfate (25%), 25 μL proteinase K, and 62.5 μL NaCl (5 M), and centrifuged at 13,500*g* for 5 minutes. DNA was extracted using phenol chloroform and purified using Zymo Clean and Concentrator-25 columns (Cedarlane Laboratories). 100 ng of DNA was used for further analysis. The V3 region of the 16S ribosomal DNA gene was amplified and sequenced using Illumina MiSeq.[24] Reagent blanks are run for each set of DNA extractions, and samples are excluded if these controls are positive for polymerase chain reaction products and DNA extractions repeated as necessary.

Microbial Community Analysis

Data analysis and visualization were done with R v.4.1.2 (R Core Team 2021) using the open-access phyloseq, ggplot2 and vegan packages. To account for differences in sampling depth, all samples were rarefied to the lowest sample read count and the relative abundance of each taxon was calculated (number of sequences for specific taxa divided by total number of sequences). For microbiome data, OTU tables were created and used to calculate indices of richness and diversity (Shannon Diversity Index, SDI). Principal component analysis was completed to evaluate β-diversity. To calculate bacterial evenness within samples, the Shannon Evenness Index was performed by dividing the SDI by the total number of unique species (as measured by OTUs). Values range from 0 to 1, where 1 indicates complete evenness. Paired comparisons of subject groups

were done by t tests or semiparametric methods based on spread of data. Multivariate parametric calculations (with Dirichlet-Multinomial distribution) were conducted on the microbiome data by permutational multivariate analysis of variance (PERMANOVA) using established methods.[22,23] Categorical variables are presented as numbers and frequencies. Continuous variables are presented as mean ± standard deviation or median (interquartile range [IQR]), as appropriate. Microbial measures were correlated with patient demographics, microbiologic, and clinical parameters and sinus symptom and radiographic scores.

Results

Cohort Demographics

A total of 31 participants (16 PwCF and 15 pTxPwCF) matched by sex and age were included in the study. Baseline demographics were comparable between cohorts (**Table 1**). Among transplant recipients, the median time from lung transplantation to first study visit was 5.2 years (IQR, 3.3-7.8). Twenty-nine (94%) subjects completed all 3 visits with the remainder completing 1 or 2 visits. Four visits from 4 separate subjects were associated with PEx, all from those in the PwCF cohort. In total there were 47 sputum (40 prelung transplant and 7 pTx) and 90 sinus (46 PwCF and 44 pTxPwCF) samples.

Microbial Community Structure

DNA extraction was performed on a total of 137 samples (89 PwCF and 48 pTxPwCF) with 16S rRNA amplicon sequencing obtained from 107 samples (70 PwCF and 37 pTxPwCF samples). Samples excluded failed to amplify through 16S rRNA sequencing due to presumably low microbial burden. A total of 1,279,611 reads were generated across sample types, with a median read of 9085 (IQR, 1191-17,487). Community composition analysis identified *Pseudomonas* (44.6%), *Haemophilus* (14.6%), and *Staphylococcus* (12%), *Streptococcus* (10.7%), *Fusobacterium* (4.8%), *Neisseria* (3.1%), *Prevotella* (2.8%), and *Gemella* (1.9%). While there was relative community structuring, the dominant OTU did not differ significantly in either sinus or sputum samples between the 2 patient cohorts. Finally, given the small sample size of those with PEx, no specific associations in microbial community structure during exacerbation were observed as compared to baseline.

Microbial Diversity Composition at First Visit

α-diversity (SDI) and β-diversity measures were calculated using the first (baseline) visit to evaluate differences between the 2 groups. Sinus microbial diversity as measured by SDI had a median value of 0.53 (IQR, 0.025-1.13) with a median evenness was 0.22 (IQR, 0.07-0.36). Sinus diversity was significantly greater in the pTxPwCF (n = 14, SDI: 0.71 [IQR, 0.21-1.13]) group as compared to the PwCF group (n = 10, SDI: 0.18 [IQR, 0.025-0.68]) ($P = .04$) (**Figure 1A**). In those with sputum

Table 1. Baseline Characteristics of the Study Population

	PwCF	pTxPwCF
N (%)	16 (51.2)	15 (48.8)
Male (%)	7 (43.8)	6 (56.2)
Age, y[a]	36.7 (8.3)	38.1 (10.3)
ppFEV$_1$[b]	52.6 (13.7)	83.4 (17.3)
BMI (kg/mg^2)	21.4 (2.5)	23.6 (2.9)
CF-related diabetes (%)	6 (37.5)	8 (53.3)
CF liver disease (%)	4 (25.0)	5 (33.3)
Pancreatic Insufficiency (%)	14 (87.5)	14 (93.3)
History of sinus surgery	12 (75.0)	12 (80.0)
CF genotype		
F508del/F508del (%)	10 (62.5)	13 (86.7)
F508del/other (%)	4 (25.0)	2 (13.3)
Other/other (%)	2 (12.5)	0
Prescribed chronic antimicrobial therapy at time of study initiation		
Inhaled tobramycin (TIP or TIS) (%)	12 (75.0)	0
Inhaled aztreonam (%)	10 (62.5)	0
Oral azithromycin (%)	8 (50.0)	14 (93.3)
Other (%)	1 (8.3)	0
Prescribed chronic therapy at time of study initiation		
Inhaled corticosteroid (%)	13 (81.3)	2 (13.3)
Inhaled DNase (%)	12 (75.0)	0
Inhaled hypertonic saline (%)	9 (56.3)	0
Oral steroids (%)	0	15 (100.0)
Oxygen (%)	3 (18.8)	0
CFTR modulator (%)	0	0
Topical inhaled corticosteroid (%)	12 (75.0)	13 (86.7)
Sinus rinse agents (%)	15 (93.8)	14 (93.3)
Sputum culture in the prior year		
Pseudomonas aeruginosa (%)	12 (75.0)	5 (33.3)
Burkholderia cepacia complex (%)	0	0
Stenotrophomonas maltophilia (%)	1 (8.3)	0
Staphylococcus aureus (%)	2 (12.5)	3 (20.0)
Achromobacter spp. (%)	1 (8.3)	0
Sputum culture in the following year		
Pseudomonas aeruginosa (%)	12 (75.0)	4 (26.7)
Burkholderia cepacia complex (%)	0	0
Stenotrophomonas maltophilia (%)	1 (8.3)	0
Staphylococcus aureus (%)	4 (25.0)	5 (33.3)
Achromobacter spp. (%)	0	0

Abbreviations: BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ppFEV$_1$, percent predicted forced expiratory volume in 1 second; pTx, postlung transplant; PwCF, persons with cystic fibrosis; TIP, tobramycin inhalational powder; TIS, tobramycin inhalational solution.
[a]Means (standard deviation) unless otherwise stated.
[b]Lung function and BMI are at baseline (ie, initiation of study).

samples (16 pretransplant; 3 posttransplant), there were no differences in diversity by transplant status ($P = .08$). When β-diversity was assessed by sample type, sputum (**Figure 1B**; $P = .04$) but not sinus (**Figure 1C**),
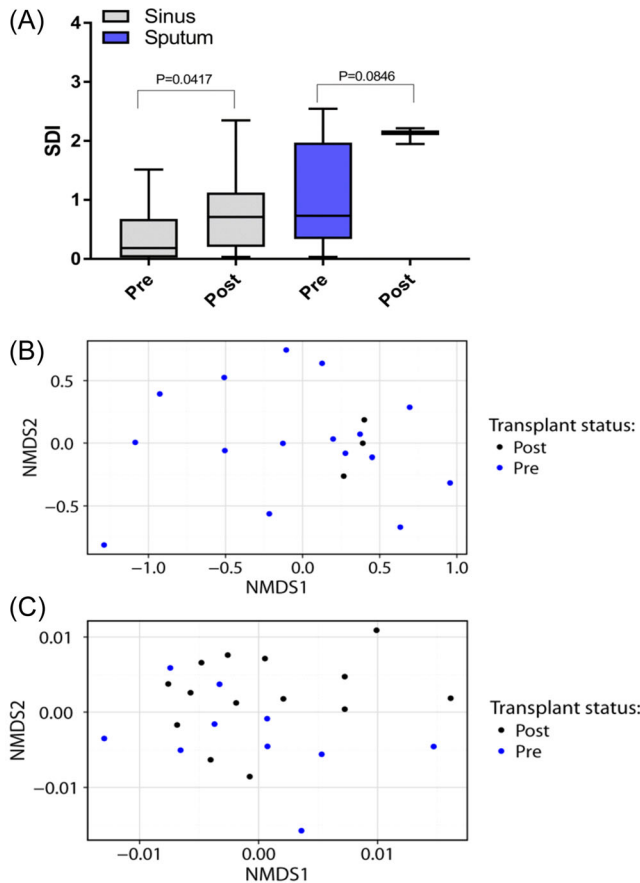
Figure 1. (A) α-diversity comparisons by transplant and sample type. NMDS plots based on Bray-Curtis distances were used to compare between groups by sputum ($P = .042$, PERMANOVA) (B) and sinus samples (C). NMDS, nonmetric multidimensional scaling; PERMANOVA, permutational multivariate analysis of variance; SDI, Shannon Diversity Index.

significantly clustered based on transplant status at time of first visit.

Microbial Composition and CRS Symptom Scores

Using an established SNOT-22 score of 21 as a cut-off (based on a prior study in CF),[19] we observed no significant differences in microbial composition ($P = .15$) using first visit data. A sensitivity analysis using a SNOT-22 cut-off of 26 (median of the data) also was not significantly associated with differences in microbial composition ($P = .07$). Similar results were observed when α-diversity (SDI) of subjects were compared at first visit using only sinus samples, using SNOT-22 score cut-off of 21 or 26 (**Figure 2A**). However, when all visit data were included (with exclusion of exacerbation time points), there was a significant difference in α-diversity based on a SNOT-22 cut-off score of 21 (**Figure 2B**).

In evaluating the relationships between microbial communities, SNOT-22 score and transplant status at the first visit, there were no significant differences in microbial composition based on interactions between
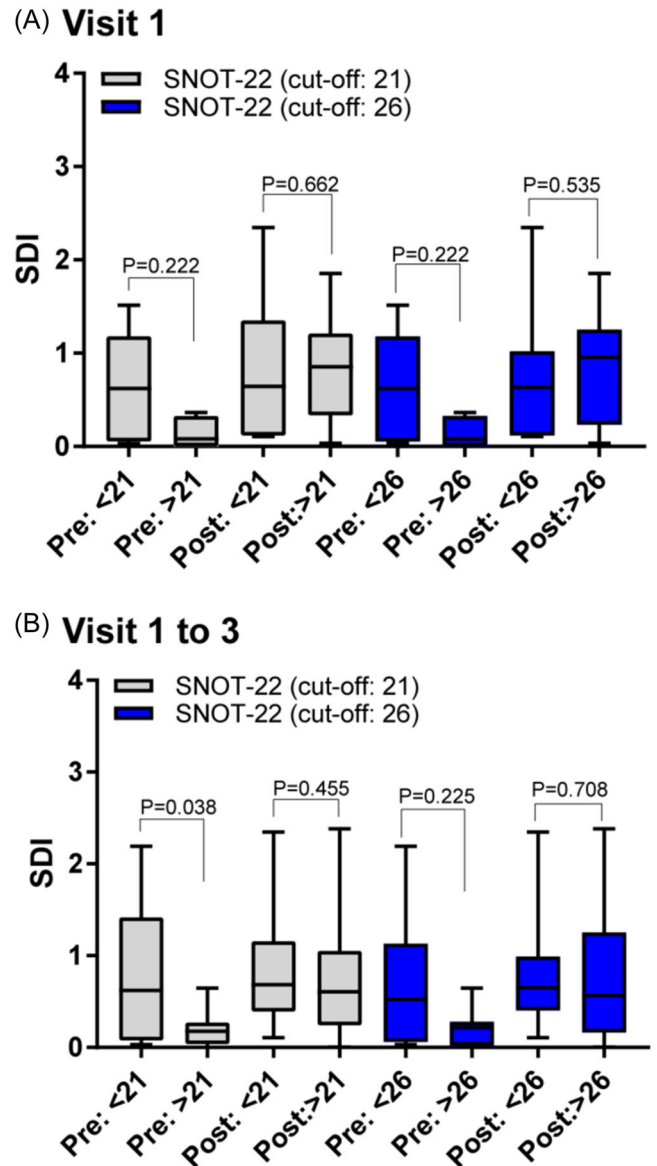

Figure 2. α-diversity comparisons from sinus samples by the SNOT-22 score at baseline (A) or all visits (B) time points in the patient groups. Wilcoxon rank-sum (Mann-Whitney) tests were performed. SDI, Shannon Diversity Index; SNOT-22, Sinonasal Outcome Test.

both variables (SNOT-22 score and lung transplant status) when the SNOT-22 cut-off scores was categorized either as 21 or 26 ($P = .15$ and .28, respectively). When all time points in the study were included, there was a statistically significant interaction between SNOT-22 scores and lung transplant status for both SNOT-22 cut off scores of 21 and 26 ($P = .01$ and .03, respectively).

To examine the association of SNOT-22 scores with the sinus microbiome at baseline (ie, visit 1) or over time (ie, any visit) in both the pre- and posttransplant groups, a PERMANOVA analysis based on Bray-Curtis distances was completed. When the association of SNOT-22 score with the microbiome was evaluated over time, the microbiome composition was significantly associated
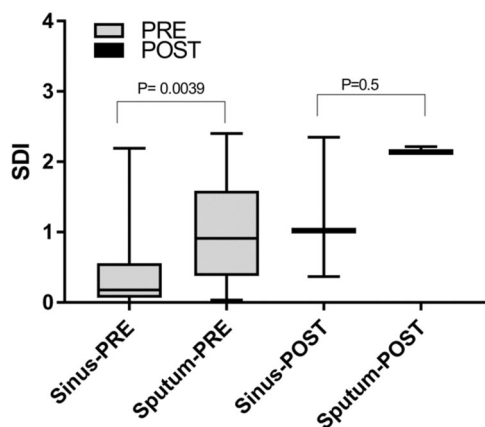
Figure 3. α-diversity comparisons as a function of lung transplant status and by sample type. Wilcoxon matched pairs signed rank test were performed. SDI, Shannon Diversity Index.

with SNOT-22 using the established cut-off of 21 in PwCF when all visit samples were included for analysis, but not in the pTxPwCF cohort. α-diversity was significantly different at a SNOT-22 cut-off score of 21 in the PwCF (**Figure 2A**), but not pTxPwCF cohort (**Figure 2B**) across all visits.

In the 18 individuals with LM score data available, there were no significant differences identified in microbial composition based on the LM score at the first visit (using a cut-off score of 15 as the median value) in either patient cohort ($P = .14$ and .64, respectively). Similarly, there were no differences α- or β-diversity based on LM score (only run in the pTxPwCF group due to small sample size).

Microbial Composition in Subjects With Paired Sinus and Sputum Samples

To examine the microbial concordance between sinuses and the lower respiratory system, we next compared subjects (24 PwCF and 3 pTxPwCF) with paired samples (ie, sputum and sinus) from the same visit. Across the pretransplant group, there was a significant difference in α-diversity between sinus and sputum samples, however, this was not observed in pTxPwCF (**Figure 3**). Similarly, β-diversity of microbiome composition between sample types were significantly different ($P = .001$) in the PwCF cohort (**Figure 4A**) but not statistically significant in the very limited sampling conducted in the pTxPwCF group despite large scoring differences ($P = .1$; **Figure 4B**).

Discussion

Ours is one of the first prospective matched cohort studies to investigate the microbial communities in PwCF paranasal sinuses in the non-transplant and transplant contexts and characterize associations with clinical disease. We demonstrated that the sinus and airway microbiome differ by transplant status in PwCF, but the prevalent organisms, as defined by most abundant OTU,
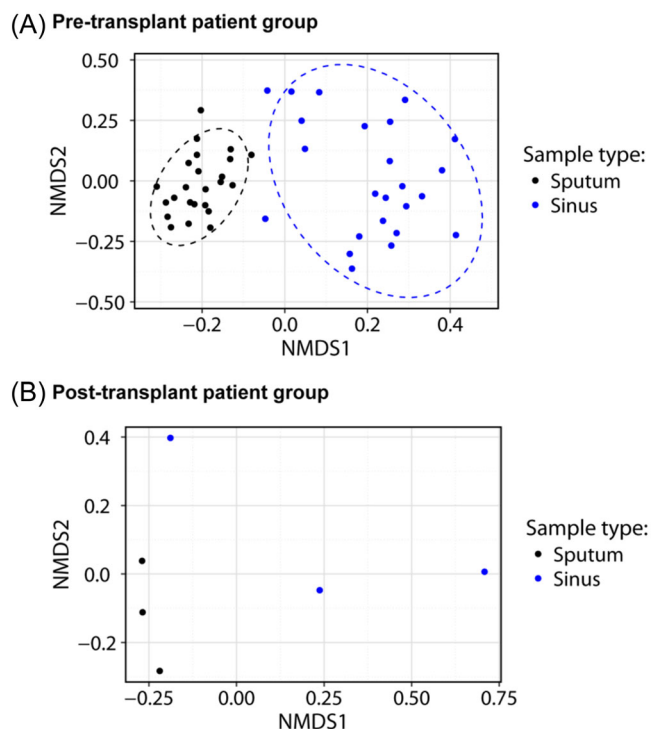

Figure 4. β-diversity analysis by NMDS plot from Bray-Curtis distance based between sputum (black) and sinus (blue) samples in the PwCF group (A) and in the pTxPwCF group (B). Ellipses denote 95% confidence intervals. NMDS, nonmetric multidimensional scaling; pTxPwCF, postlung transplant persons with cystic fibrosis; PwCF, persons with cystic fibrosis.

remained consistent. While there were differences in diversity across the sinus and airway microbiome between the 2 patient groups, the overall changes in community structure were modest across sample types. Moreover, we found the prevalent organisms, as defined by highest OTU through culture-independent analysis and traditional culture-dependant growth, remained consistent across sample types, expanding on prior observations of a potential pathogenic reservoir in transplant recipients. These findings were supposed by relative low community evenness, suggesting communities were dominated by a subset of taxa present. Finally, our study was the first to associate sinus microbiome compositional structure with sinonasal clinical outcomes by transplant status in PwCF as noted by patient reported questionnaires. Not surprisingly, those with greater microbial diversity had improved symptom scores consistent with earlier findings.[25] Finally, we did not observe a significant association with LM scores and microbiome composition and were likely limited by sample size similar to others.[26,27]

Previous studies have demonstrated the sinonasal cavity as an initial site of bacterial colonization, including canonical pathogens such as *P. aeruginosa*, with development of a microbial reservoir that can then seed the lungs of PwCF.[2,28-30] Moreover, in patients identified with new *P. aeruginosa* infection who then receive eradication therapy, nearly a quarter are recolonized with the same

strain as cultured from their sinuses, suggesting presence of a latent reservoir.[31] Likewise, the sputum microbiome in PwCF pretransplant has been shown to be similar to posttransplant including isolating the same strains of *P. aeruginosa*[13] suggesting the sinuses remain a persistent nidus of chronic infection. Our study expanded on these findings to demonstrate *P. aeruginosa* as the most prevalent taxa by both molecular and standard clinical culture techniques. While small in numbers, our analysis demonstrated concordance between the sputum and sinus microbiota, suggesting preservation of unique communities within individuals. These findings are novel in that our cohort evaluated those postlung transplant and address a pre-existing gap in the literature. Moreover, our findings have therapeutic implications as they suggest a potential role for interventions to attenuate future reinfection and risk of graft rejection through earlier therapeutic interventions. Vital et al performed endoscopic sinus surgery in PwCF who underwent lung transplantation with the goal to eliminate bacterial colonization of the sinuses immediately post transplantation.[32] Compared to subjects that continued to demonstrate persistent *P. aeruginosa* airway colonization, those with clearance had improved 5-year survival and a decreased incidence of BOS. Somewhat disappointingly, 65% of patients had persistent colonization of *P. aeruginosa* after transplantation despite aggressive surgery and antimicrobial therapy, highlighting the difficulty in eradication and importance of multimodal attempts in this patient population.

The successful introduction of highly-effective modulator therapy (HEMT) to a large fraction of the CF population (estimated >90% eligible in PwCF in some countries) [33] has been a pivotal milestone in the fight to control and mitigate CF. However, as airways infections persist with relatively modest restructuring a great many questions have arisen including effect(s) on the sinus microbiome and relation to lower respiratory tract infections. To address this, the upcoming PROMISE (NCT04038047), a large US multi-disciplinary prospective study assessing the broad impacts of long-term elexacaftor-tezacaftor-ivacaftor (ETI) therapy in PwCF ≥ 6 years aims to clarify some of these questions raised by evaluating both culture-dependent and independent measures of pathogen/microbiome-constituent abundance[34] in the postmodulator era.

A number of limitations must be considered. Although the samples were derived from non-transplanted and transplanted cohorts, both groups had CF and would be expected to have altered microbiota and potentially similar microbiota given that both groups had CF-related sinus disease. This was a single-center study, but we enrolled a matched cohort with a high (94%) of study completion. We had fewer sputum samples in the pTxPwCF group (due to their inability to expectorate in the same manner as PwCF) limiting our ability to conduct some of our comparative analyses. Regardless, we were able to assess microbial composition in the sinuses within and between groups over time. Although we collected clinical data including times of illness and medications, and were powered to identify differences in microbial composition, we had limited power to assess differences by subgroups. We matched for age, and sex but there is potential for other confounding. As with observational studies, associations are not synonymous with causal inferences and our findings must be validated in larger cohorts. Finally, in our study, none of the of subjects were receiving CFTR modulator therapy, limiting our ability to evaluate confounding effects on the microbiome or symptoms. Moreover, our study preceded ETI, introduced in 2019, and thus may have relative sinus microbial restructuring that remains to be explored.

In summary, in our prospective matched cohort study of PwCF and pTxPwCF, we identified that despite modest differences in microbiome composition in the sinuses and lower airways, prevalent organisms remained consistent in both groups. These findings support the notion that sinuses may act a pathogenic reservoir particularly in the post-transplant setting and have implications for optimizing therapeutic approaches. SNOT-22 scores correlated with microbiome structure and diversity and may be important as a longitudinal metric in addition to clinical and microbiologic indices in the care of PwCF. Future evaluations of the sinuses in the HEMT era will also be relevant to determine if it can serve as a surrogate for infection surveillance of the lower airways in PwCF.

Author Contributions

Ranjani Somayaji, supervision, conceptualization, funding acquisition, methodology, formal analysis, writing—original draft, writing—review and editing; **Christina S. Thornton**, project administration, formal analysis, writing—review and editing; **Nicola Acosta**, investigation, methodology, project administration, writing—review and editing; **Kristine Smith**, project administration, writing—review and editing; **Jessica Clark**, formal analysis, writing—review and editing; **Linda Fatovich**, project administration, writing—review and editing; **Mitesh V. Thakrar**, project administration, writing—review and editing; **Michael D. Parkins**, supervision, conceptualization, funding acquisition, methodology, formal analysis, writing—original draft, writing—review and editing.

Disclosures

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