Sputum microbiome α-diversity is a key feature of the COPD frequent exacerbator phenotype

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Low sputum microbiome α-diversity and decreased compositional stability (increased β-diversity) are associated with the COPD frequent exacerbator phenotype. Low sputum microbiome α-diversity is also associated with lung inflammation. https://bit.ly/3SEA8M5

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Abstract

Background The lung microbiome is an inflammatory stimulus whose role in COPD pathogenesis is incompletely understood. We hypothesised that the frequent exacerbator phenotype is associated with decreased α-diversity and increased lung inflammation. Our objective was to assess correlations between the frequent exacerbator phenotype, the microbiome and inflammation longitudinally during exacerbation-free periods.

Methods We conducted a case–control longitudinal observational study of the frequent exacerbator phenotype and characteristics of the airway microbiome. 81 subjects (41 frequent and 40 infrequent exacerbators) provided nasal, oral and sputum microbiome samples at two visits over 2–4 months. Exacerbation phenotype, relevant clinical factors and sputum cytokine values were associated with microbiome findings.

Results The frequent exacerbator phenotype was associated with lower sputum microbiome α-diversity (p=0.0031). This decrease in α-diversity among frequent exacerbators was enhanced when the sputum bacterial culture was positive (p<0.001). Older age was associated with decreased sputum microbiome α-diversity (p=0.0030). Between-visit β-diversity was increased among frequent exacerbators and those who experienced a COPD exacerbation between visits (p=0.025 and p=0.014, respectively). Sputum cytokine values did not differ based on exacerbation phenotype or other clinical characteristics. Interleukin (IL)-17A was negatively associated with α-diversity, while IL-6 and IL-8 were positively associated with α-diversity (p=0.012, p=0.012 and p=0.0496, respectively). IL-22, IL-17A and IL-5 levels were positively associated with *Moraxella* abundance (p=0.027, p=0.0014 and p=0.0020, respectively).

Conclusions Even during exacerbation-free intervals, the COPD frequent exacerbator phenotype is associated with decreased sputum microbiome α-diversity and increased β-diversity. Decreased sputum microbiome α-diversity and *Moraxella* abundance are associated with lung inflammation.

Introduction

COPD is a leading cause of death; however, the mechanisms driving its progression remain incompletely understood. One recently recognised mechanism is inflammation triggered by the lung microbiome. Cycles of recurrent lung infection, inflammation and antibiotic use may disrupt the microbiome with downstream consequences for lung function [1]. Even in the absence of clinical infection, COPD patients with pathogenic bacteria in their airways have higher levels of sputum and systemic inflammatory markers and increased pulmonary symptoms [2, 3].

The COPD frequent exacerbator phenotype identifies a subset of patients at high risk of recurrent COPD exacerbation. Frequent exacerbators suffer increased morbidity and mortality compared to those who

experience exacerbations less often [4–7]. Approximately half of COPD exacerbations are attributed to bacterial infection, and pathogenic bacteria such as *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* are often identified in the lung microbiome of COPD patients even during periods of stable lung disease [3].

Inflammatory markers are increased in the sputum of frequent exacerbators [8–10] and COPD patients colonised with potentially pathogenic bacteria [9, 11–15]. The COPD lung microbiome provides an inflammatory stimulus, even in the absence of overt lung infection. In particular, interleukin (IL)-17A, IL-8, IL-6, IL-1β, IL-22, IL-5 and leukotriene B4 (LTB4) levels in sputum have been associated with various components of the COPD lung microbiome during exacerbation-free intervals [2, 8, 11–19]. It remains unclear which particular components of the microbiome (bacterial biomass, α-diversity, the particular taxa present, *etc*.) are most closely associated with sputum inflammation or the frequent exacerbator phenotype.

We and others have shown that the sputum microbiome of frequent exacerbators has lower α-diversity compared to nonfrequent exacerbators [18, 20–26]. However, few of these studies evaluate the COPD lung microbiome solely during periods of clinical stability (when findings are less influenced by exacerbation treatments), longitudinally and including an analysis of concurrent lung inflammation. We undertook the present case–control longitudinal observational study of COPD exacerbation phenotype, the upper airway and sputum microbiome and lung inflammation to address these gaps.

Methods

Study design and recruitment

We conducted a case–control longitudinal observational study of exacerbation phenotype and characteristics of the upper airway and sputum microbiome. All participants were recruited from a single site and were aged ⩾40 years with COPD. Frequent exacerbators had at least one severe exacerbation (an exacerbation requiring hospital admission or emergency department visit) in the past 12 months, in accordance with descriptions found in Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [27]. Infrequent exacerbators must have had no exacerbations in the prior 24 months. Recruitment and all visits/samples were deferred until participants had recovered for ⩾1 month from the most recent exacerbation. The protocol was approved by the Minneapolis VA IRB (#4541-B). Additional details on recruitment and other methods can be found in the supplementary material. Sequence reads from 22 participants have been published previously (National Center for Biotechnology Information (NCBI) Sequence Read Archive Accession PRJNA543785) [20]; however, all data analysed here were re-sequenced for this analysis (NCBI Sequence Read Archive Accession PRJNA944199).

Study procedures

At visit 1, participants provided their medical history, underwent spirometry, completed the St George's Respiratory Questionnaire (SGRQ), and provided oral wash, nasal swab and induced sputum samples. All participants returned for a second study visit ∼2 months following visit 1, where they provided information on interim COPD exacerbations, repeated the SGRQ, and provided oral, nasal and sputum samples. Visit 2 was deferred (for up to 4 months following visit 1) if the participant reported any COPD exacerbations or antibiotic use in the 1 month prior to the visit. Exacerbation phenotype was determined at visit 1 and was not revised based on exacerbations observed during the study.

Sample processing, 16S rRNA gene quantification and MiSeq sequencing

All samples and negative controls were extracted using the MO BIO PowerSoil DNA Isolation Kit (QIAGEN, Germantown, MD, USA). Extracted DNA underwent 16S rRNA gene quantification using droplet digital PCR (ddPCR; supplementary table E1) and 16S rRNA gene V4 MiSeq sequencing. 16S rRNA V4 sequences were processed as described in the supplementary material.

Sputum culture results

The clinical microbiology laboratory performed Gram stain and aerobic culture on all sputum samples. Any organism identified in culture was considered a pathogen.

Cytokine analyses

Sputum samples were submitted to the University of Minnesota Cytokine Reference Laboratory for determination of LTB4, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-8, IL-6, IL-1β, IL-17A, IL-22, IL-5 and tumour necrosis factor (TNF)-α (R&D Systems, Minneapolis, MN, USA).

Statistical analyses

Data presented are from visit 1 only (V1) or both visits (BV). Linear regression, including random-effects censored regression models, linear mixed models (LMM) and generalised estimating equations (GEE), were used to test for associations between variables. Analyses of α-diversity metrics were adjusted for age, forced expiratory volume in 1 s % predicted (FEV_1pp), body mass index (BMI), current tobacco use and current alcohol use. β-diversity was assessed *via* the Bray–Curtis dissimilarity metric and illustrated *via* principal coordinates analysis (PCoA). Univariate PERMANOVA analyses of β-diversity were performed for each anatomical site. Permutation tests were used to determine if within-subject visit 1–visit 2 similarity differed by permuting levels of categorical clinical factors. After eliminating genera present in <10% of samples, tests of association between taxa and clinical characteristics used Holm's procedure to control the family-wise error rate across all taxa at 5%. GM-CSF and TNF-α were not analysed and IL-22 results were dichotomised (detected *versus* not detected), as too few samples contained quantifiable results. Random-effects censored regression models were used to test for associations between clinical or microbiome characteristics and cytokine levels (modelled as the response variable). All analyses were conducted in R version 3.6.0.

Results

Cohort

81 subjects consisting of 40 infrequent exacerbators and 41 frequent exacerbators provided data and samples during two visits over 2–4 months (supplementary table E2). Subjects were balanced with respect to age, gender, race, inhaled corticosteroid (ICS) use and dental care habits (table 1). Consistent with the Department of Veterans Affairs patient population, most subjects were male. Frequent exacerbator phenotype was associated with lower BMI (median 27.9 $kg \cdot m^{-2}$ *versus* 30.2 $kg \cdot m^{-2}$, p=0.022), lower FEV_1pp (44.0% pred *versus* 52.5% pred, p<0.001) and a higher number of COPD exacerbations in the past 12 months (median two *versus* 0, mean 2.39 *versus* 0). Frequent exacerbators were less likely than infrequent exacerbators to be current tobacco users (19.5% frequent exacerbators *versus* 42.5% infrequent exacerbators, p=0.032). Six subjects (five frequent exacerbators, one infrequent exacerbator) reported a COPD exacerbation at visit 2 and six subjects (four frequent exacerbators, two infrequent exacerbators) reported antibiotic use at visit 2. Relationships between microbiome measures at each site and clinical factors (exacerbation phenotype, age, FEV_1pp, ICS use, pack-years of tobacco use, current tobacco use, toothbrushing frequency, SGRQ score, pathogen detection in sputum samples, and experiencing a COPD

TABLE 1 Subject baseline characteristics

	Infrequent exacerbator	Frequent exacerbator	Overall	p-value[#]
Subjects	40	41	81	
Male	40 (100)	40 (97.6)	80 (98.8)	1.00
Age years, median (IQR)	69 (5)	69 (8)	69 (7)	0.632
Caucasian white	37 (92.5)	40 (97.6)	77 (95.1)	0.359
BMI $kg \cdot m^{-2}$, median (IQR)	30.2 (7.79)	27.93 (9.26)	29.57 (8.46)	0.022
COPD severity				
Moderate	24 (60)	14 (34.1)	38 (46.9)	
Severe	14 (35)	18 (43.9)	32 (39.5)	
Very severe	2 (5)	9 (22)	11 (13.6)	
FEV_1 % predicted, median (IQR)	52.5 (19)	44 (17)	48 (19)	<0.001
COPD exacerbations in the past 12 months, median (IQR)	0 (0)	2 (1)	1 (2)	<0.001
COPD hospitalisations in the past 12 months, median (IQR)	0 (0)	0 (1)	0 (0)	<0.001
Inhaled corticosteroids (yes)	11 (27.5)	14 (34.1)	25 (30.9)	0.632
Smoking pack-years, median (IQR)	41 (23.12)	50 (23)	50 (24.5)	0.059
Current tobacco use (yes)	17 (42.5)	8 (19.5)	25 (30.9)	0.032
Current alcohol use (yes)	24 (60)	32 (78)	56 (69.1)	0.096
Brush teeth at least once daily[¶]	30 (75)	30 (75)[¶]	60 (75)[¶]	1.00
SGRQ score, median (IQR)	43.62 (10.49)	52.4 (20.6)	47.29 (17.84)	0.074

Data are presented as n or n (%), unless otherwise stated. IQR: interquartile range; BMI: body mass index; FEV_1: forced expiratory volume in 1 s; SGRQ: St George's Respiratory Questionnaire. [#]: a two-sample t-test was conducted for all continuous variables and a Fisher exact test for all categorical variables; [¶]: one subject did not provide frequency of brushing teeth.

exacerbation or use of an antibiotic between study visits) were examined; unless mentioned, these analyses did not reveal an association.

α-Diversity

α-Diversity was assessed using Shannon diversity, Simpson diversity and Chao1 metrics. For simplicity, Simpson diversity findings are discussed in detail here (supplementary figure E1), as they are largely consistent with the Shannon and Chao1 diversity findings.

Nasal samples

No significant relationships between nasal sample Simpson diversity and any of the evaluated clinical factors were observed.

Oral samples

In multiple models of oral wash Simpson diversity, FEV_1pp (a model covariate) was consistently associated with increased oral wash Simpson diversity (frequent exacerbator phenotype V1 linear regression, FEV_1pp coefficient estimate (CE) 0.0008, 95% CI 0.00002 to 0.0016; p=0.027 (figure 1a); BV GEE, FEV_1pp CE 0.0007, 95% CI 0.0001 to 0.0013; p=0.023). In light of this association, we further evaluated potential relationships between frequent exacerbator phenotype, FEV_1pp and Simpson diversity. In a model of frequent exacerbator phenotype, FEV_1pp and their interaction, frequent exacerbator phenotype and the interaction of frequent exacerbator phenotype and FEV_1pp were associated with oral wash Simpson diversity (interaction BV GEE CE 0.0010, 95% CI 0.00002 to 0.0020; p=0.048). Higher SGRQ scores (indicating worse quality of life) were associated with lower oral wash Simpson diversity (V1 linear regression, CE −0.0007, 95% CI −0.0013 to −0.00011; p=0.018 (figure 1b); and BV GEE, CE −0.0005, 95% CI −0.0009 to −0.0001; p=0.020).

Sputum samples

The frequent exacerbator phenotype was associated with lower Simpson diversity in sputum at visit 1 and both visits (V1 linear regression, CE −0.077, 95% CI −0.15 to −0.0048; p=0.041 (supplementary figure

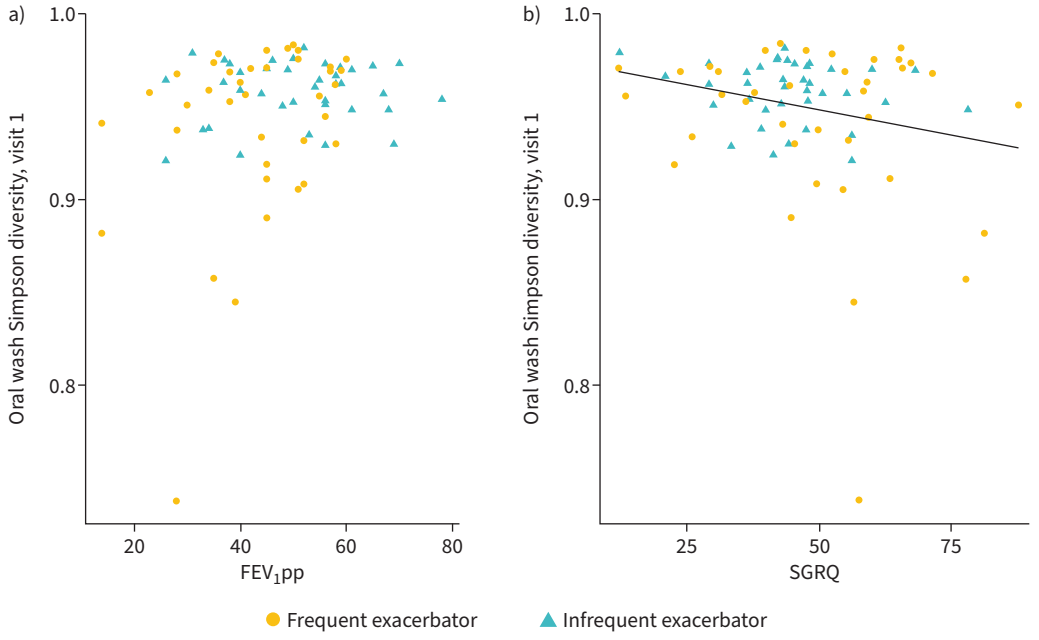

FIGURE 1 Oral wash α-diversity is associated with forced expiratory volume in 1 s % predicted (FEV_1pp) and COPD-related quality of life. a) Oral wash Simpson diversity at visit 1 is associated with FEV_1pp (a model covariate) in a model of frequent exacerbator phenotype (frequent exacerbator phenotype V1 linear regression (LR), FEV_1pp coefficient estimate (CE) 0.0008, 95% CI 0.00002 to 0.0016; p=0.027. FEV_1pp is associated with low Simpson diversity in the adjusted model. b) Oral wash Simpson diversity at visit 1 is associated with St George's Respiratory Questionnaire (SGRQ) score at visit 1 (LR, CE −0.0007, 95% CI −0.0013 to −0.00011; p=0.018). Higher SGRQ scores indicate worse COPD-related quality of life.

E2); BV GEE, CE −0.075, 95% CI −0.12 to −0.025; p=0.0031). Older age (another model covariate) was also associated with lower sputum Simpson diversity in a model of exacerbation phenotype (V1 linear regression, age CE −0.0090, 95% CI −0.015 to −0.0025; p=0.0080 (figure 2a and supplementary figure E3); BV GEE, age CE −0.0074, 95% CI −0.012 to −0.0025; p=0.0030).

When exacerbation phenotype, age, and their interaction were included in a model of sputum α-diversity, their interaction was significantly associated with lower Simpson diversity (V1 linear regression, interaction CE −0.013, 95% CI −0.024 to −0.0024; p=0.020 (figure 2a); BV GEE, interaction CE −0.0099, 95% CI −0.018 to −0.0017; p=0.017). This shows that the association between exacerbation phenotype and sputum Simpson diversity differs based on age.

Sputum samples from six subjects (three frequent exacerbators and three infrequent exacerbators) were positive for clinically relevant respiratory pathogens (*M. catarrhalis*, *H. influenzae*, methicillin-resistant *S. aureus* and *Klebsiella aerogenes*) at visit 1. Presence of a pathogen was associated with lower sputum Simpson diversity at visit 1 and both visits (V1 linear regression, CE −0.19, 95% CI −0.31 to −0.074; p=0.0028; and BV LMM, CE −0.20, 95% CI −0.26 to −0.14; p<0.001). When frequent exacerbator phenotype, pathogen and their interaction were included in the model, only their interaction was significant at visit 1 and both visits (V1 linear regression, CE −0.46, 95% CI −0.63 to −0.30; p<0.001 (figure 2b); BV LMM, CE −0.27, 95% CI −0.38 to −0.15; p<0.001). This shows that the association between sputum culture positivity during clinically stable periods and Simpson diversity differs based on exacerbation phenotype.

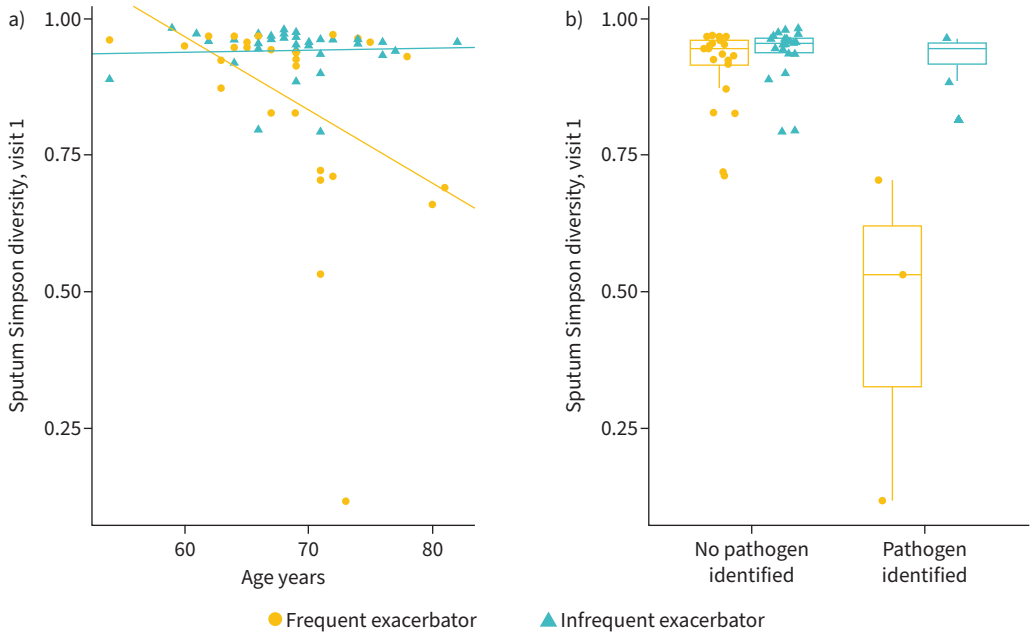

FIGURE 2 Sputum α-diversity is associated with exacerbation phenotype, age and culture results. a) Both the frequent exacerbator phenotype and older age were significantly associated with lower Simpson diversity at visit 1 (linear regression (LR), coefficient estimate (CE) −0.077, 95% CI −0.15 to −0.0048; p=0.041 and CE −0.0090, 95% CI −0.015 to −0.0025; p=0.0080, respectively). When the interaction of exacerbation phenotype and age was added to the model, only the interaction of age and exacerbation phenotype was significant (LR, CE −0.013, 95% CI −0.024 to −0.0024; p=0.020). The regression lines represent the association between phenotype, age and Simpson diversity. The significant interaction between age and phenotype indicates that older frequent exacerbators have lower sputum α-diversity than younger frequent exacerbators or older infrequent exacerbators. b) Presence of a pathogen in sputum culture was associated with lower sputum Simpson diversity at visit 1 (LR, CE −0.19, 95% CI −0.31 to −0.074; p=0.0028). When frequent exacerbator phenotype, pathogen and their interaction were included in the model, only their interaction was significant (visit 1 LR, CE −0.46, 95% CI −0.63 to −0.30; p<0.001). The association between sputum culture positivity during clinically stable periods and Simpson diversity differs based on exacerbation phenotype.

In summary, sputum α-diversity is associated with frequent exacerbator phenotype, age and pathogen detection in sputum culture during clinically stable periods. The association between frequent exacerbator phenotype and decreased sputum α-diversity is also modified by older age or the identification of a pathogen from sputum culture.

α-Diversity over time

Visit 1 values were significantly associated with visit 2 values at all sites (data not shown). When visit 1 values were included in the model, self-reported COPD exacerbation or self-reported use of an antibiotic between study visits was not associated with α-diversity at visit 2. Exacerbation phenotype, age, FEV_1pp, current tobacco use, pack-years of tobacco use and SGRQ score were not associated with a change in α-diversity between study visits.

β-Diversity

Environmental, equipment and regent control samples were distinct from subject samples (supplementary figure E4, supplementary table E3). After subsampling to include only subject samples from visit 1, PCoA revealed significant clustering by anatomic site (PERMANOVA, p=0.001 for all pairwise testing; figure 3).

PERMANOVA analyses

Clustering on PCoA based on exacerbation phenotype and other clinical factors was investigated using PERMANOVA analyses. The analyses were conducted at each anatomic site separately and using visit 1 data, unless noted below. At visit 1, nasal samples clustered based on FEV_1pp (PERMANOVA, R^2=0.029, p=0.033). Several other PERMANOVA results with p<0.10 are provided in supplementary table E4. When visit 2 data were analysed, sputum samples from participants who reported between-visit antibiotic use for any indication clustered separately from participants who did not report between-visit antibiotic use (PERMANOVA, R^2=0.029, p=0.049).

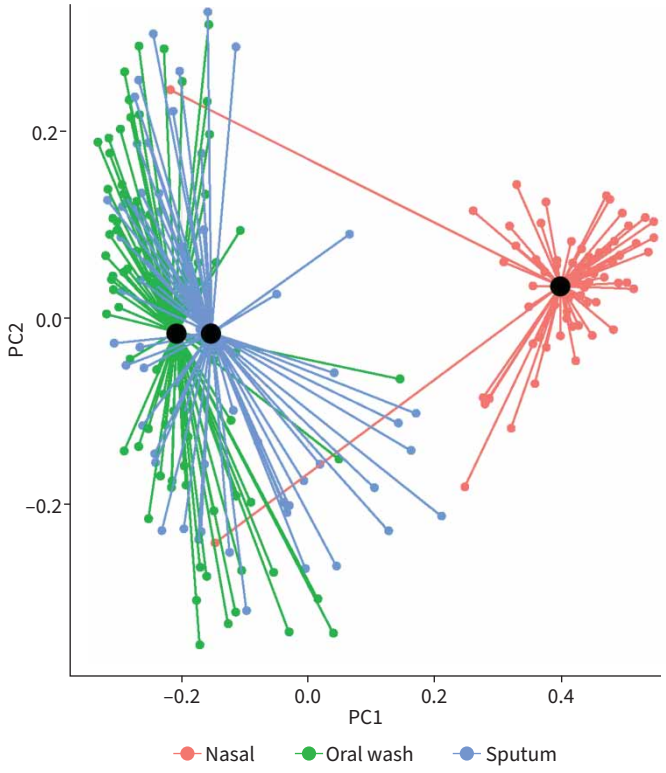

FIGURE 3 β-Diversity reveals clustering by anatomic site. Principal coordinates (PC) analysis using Bray–Curtis dissimilarity on visit 1 samples demonstrating significant clustering by anatomic site (PERMANOVA, p=0.001 for all pairwise testing). Centroids are illustrated by black dots, with lines connecting each sample to its corresponding centroid.

β-Diversity over time

Visit 1 samples were compared to corresponding visit 2 samples for all subjects and sites to assess the stability of microbiome composition over time. Among nasal samples, the frequent exacerbator phenotype and antibiotic use between study visits was associated with decreased similarity between paired samples (permutation testing (P), p=0.044 and p=0.032, respectively). There were no associations with oral wash similarity between visits. Among sputum samples, the frequent exacerbator phenotype (*versus* infrequent exacerbator phenotype) and experiencing a COPD exacerbation (*versus* no COPD exacerbation) between visits were associated with lower similarity between paired samples (P, p=0.025 and p=0.014, respectively; figure 4 and supplementary figure E5).

Bacterial taxa

We investigated the bacterial taxa present in each sample to determine potential associations with relevant clinical factors (97 tests). Many taxa were associated with clinical site, in accordance with clinical findings and the human microbiome literature. Nasal samples were enriched with *Corynebacterium*, *Staphylococcus*, *Cutibacterium* and *Moraxella* (among others) compared with oral and sputum samples (linear regression, all p<0.05 following Holm correction; supplementary table E5). Oral and sputum samples were enriched with *Veillonella*, *Rothia*, *Prevotella*, *Streptococcus* and *Haemophilus* (among others) when compared with nasal samples (linear regression, all p<0.05 following Holm correction; supplementary table E5). Across all anatomic sites, *Mannheimia* abundance was positively associated with

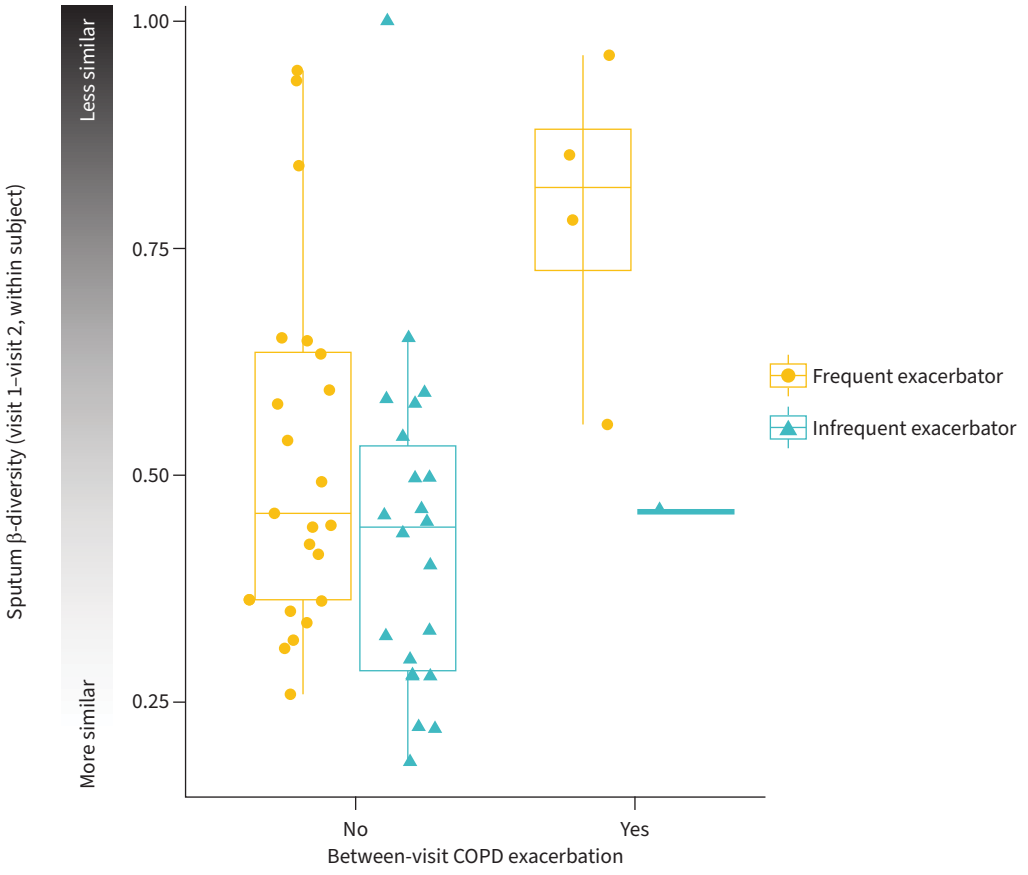

FIGURE 4 Within-subject microbiome composition stability. Self-report of a COPD exacerbation between study visits corresponded with an increase in microbiome compositional changes (increased β-diversity, or a decrease in sample similarity) compared with subjects who did not experience a COPD exacerbation between study visits. Please note that one frequent exacerbator who reported an exacerbation between study visits is not represented here, as this subject did not provide both sputum samples. The frequent exacerbator phenotype also corresponded with an increase in microbiome compositional changes (increased β-diversity, or a decrease in sample similarity) between study visits compared with the infrequent exacerbator phenotype (permutation analyses, p=0.014 and p=0.025, respectively).

age (linear regression, p=0.0014); *Mogibacterium* abundance was lower among frequent exacerbators compared to infrequent exacerbators (p=0.029); *Leuconostoc* abundance was negatively associated with FEV_1pp (p=0.020); *Bulleidia* abundance was higher among current tobacco users (p=0.013); and *Pseudomonas* abundance was positively associated with pack-years of tobacco use (p<0.0001; supplementary table E6).

Sputum cytokine analyses

Sputum sample cytokine levels were tested for an association with clinical factors and sputum microbiome characteristics. Cytokines were chosen for analysis based on prior reported associations with culture or microbiome results, and analyses of the seven evaluable cytokines are provided here. Samples from all available visits were analysed using GEE or random-effects censored regression models, as appropriate, accounting for visit.

Cytokines associated with clinical characteristics

None of the clinical factors (exacerbation phenotype, age, FEV_1pp, pack-years of tobacco exposure, current tobacco use or SGRQ score) were associated with cytokine levels in a random-effects censored regression model.

Cytokines associated with α-diversity (Simpson)

Three sputum cytokines were associated with sputum sample α-diversity on univariate analysis. IL-17A levels were negatively associated with Simpson diversity, while IL-6 and IL-8 were positively associated with Simpson diversity (random-effects censored regression model, IL-17A CE −1.3, 95% CI −2.2 to −0.50; p=0.012; IL-6 CE 6.0, 95% CI 2.2 to 9.8; p=0.012; and IL-8 CE 4.2, 95% CI 1.0 to 7.4; p=0.0496, respectively; figure 5). IL-22, IL-5, IL-1β and LTB4 were not associated with Simpson diversity.

Cytokines associated with pathogen abundance

Presence of typical COPD pathogens in sputum samples, such as *Streptococcus*, *Moraxella* or *Haemophilus*, have been associated with exacerbation phenotype, increased inflammation and decreased α-diversity. We found that IL-22, IL-17A and IL-5 levels were positively associated with *Moraxella* abundance (GEE with Holm correction, IL-22 CE 10.26, 95% CI 3.96 to 16.55; p=0.027; random-effects censored regression model with Holm correction, IL-17A CE 2.01, 95% CI 1.02 to 2.99; p=0.0014; and IL-5 CE 1.87, 95% CI 0. 93 to 2.81, p=0.002, respectively; figure 6). There were no significant associations with other cytokines or the genera *Streptococcus* or *Haemophilus*.

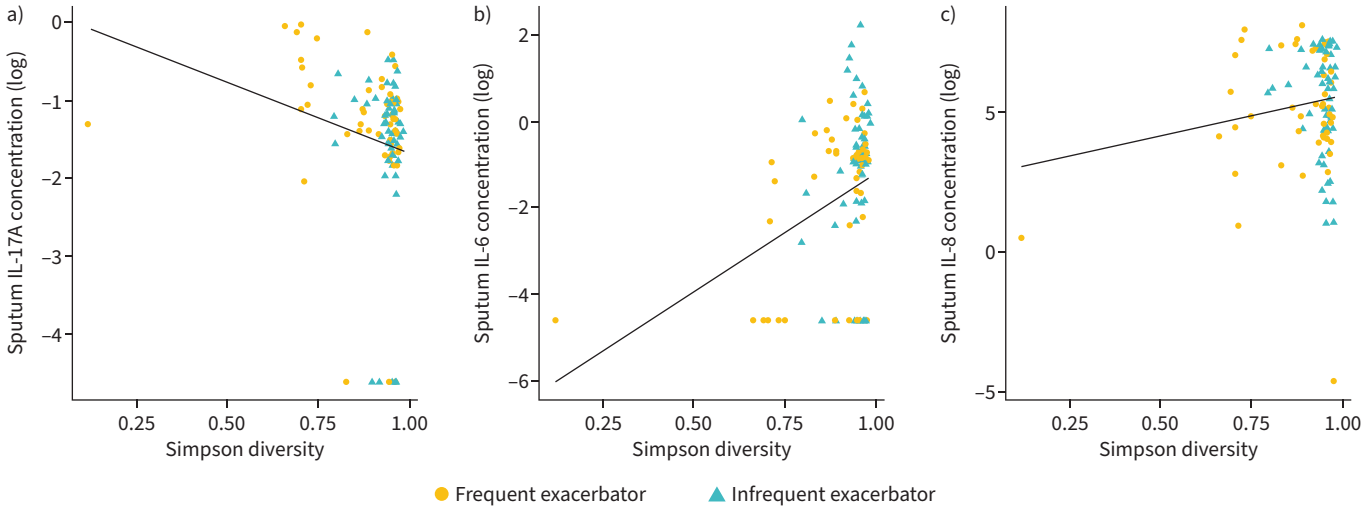

FIGURE 5 Multiple sputum cytokines are associated with α-diversity. Increased sputum concentrations of interkeukin (IL)-17A were associated with decreased Simpson diversity in the sputum, while increased sputum concentrations of IL-6 and IL-8 were associated with increased Simpson diversity (random effects censored regression model, IL-17A coefficient estimate (CE) −1.3, 95% CI −2.2 to −0.50; p=0.012; IL-6 CE 6.0, 95% CI 2.2 to 9.8; p=0.012; and IL-8 CE 4.2, 95% CI 1.0 to 7.4; p=0.0496, respectively).

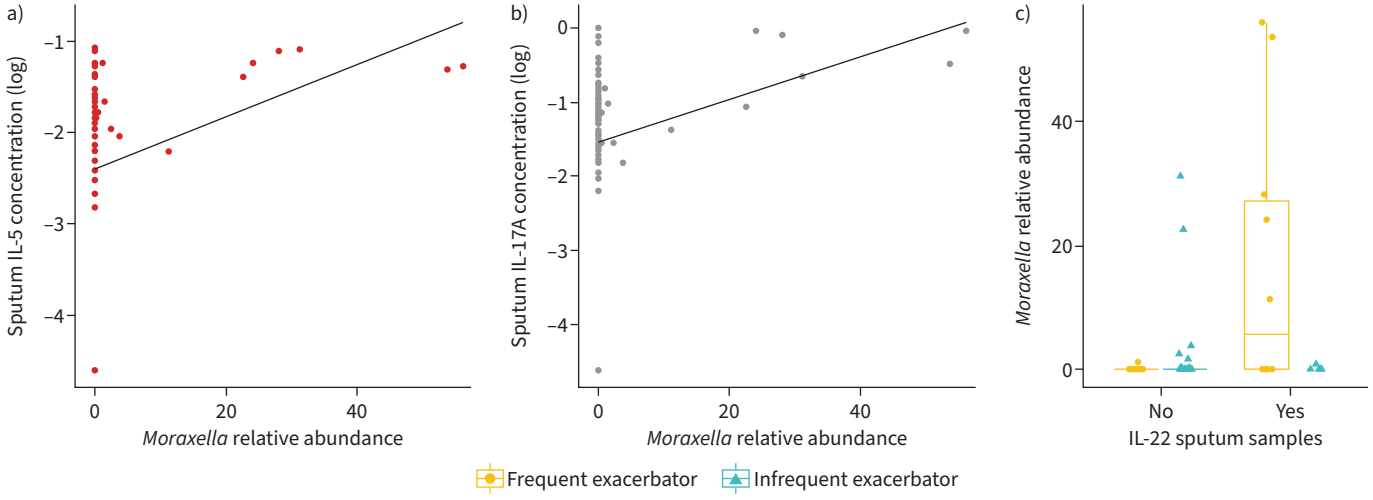

FIGURE 6 Sputum cytokines are associated with *Moraxella* abundance. Increased sputum concentrations of interleukin (IL)-22, IL-17A and IL-5 were correlated with increased abundance of *Moraxella* in sputum samples (generalised estimating equations with Holm correction, IL-22 coefficient estimate (CE) 10.26, 95% CI 3.96 to 16.55; p=0.027; random-effects censored regression model with Holm correction, IL-17A CE 2.01, 95% CI 1.02 to 2.99; p=0.0014; and IL-5 CE 1.87, 95% CI 0.93 to 2.81; p=0.002, respectively).

Discussion

Our case–control longitudinal observational study of the upper airway and sputum microbiome during periods of clinical stability identified low sputum microbiome α-diversity as a key feature of the COPD frequent exacerbator phenotype. In turn, low sputum microbiome α-diversity was associated with airway bacterial colonisation and lung inflammation, two other characteristics consistent with the increased morbidity and mortality associated with the frequent exacerbator phenotype. Additionally, the frequent exacerbator phenotype was associated with decreased microbiome compositional stability (increased β-diversity) on longitudinal sputum sampling. These findings suggest that low α-diversity and an unstable sputum microbiome are key features of the frequent exacerbator phenotype.

In addition to our findings on the frequent exacerbator phenotype, we also detected associations between the sputum, oral or nasal microbiome and age and COPD-related symptom severity. Also, among the small number of subjects who experienced an exacerbation between study visits, we observed compositional changes (increased β-diversity) in the microbiome following the occurrence of a COPD exacerbation. Our study determined that the frequent exacerbator phenotype is associated with low sputum microbiome α-diversity. Low α-diversity among frequent exacerbators has been found by many, but not all, investigators, possibly related to differences in how exacerbation frequency was analysed [20, 22–26, 28, 29]. In the case of the sputum microbiome, this homogeneity was enhanced among older participants and among participants with airway bacterial colonisation. This phenotype also correlated with increased *Mogibacterium*, an oral taxon associated with oral inflammation and stable COPD [30, 31].

In this study and others, older age was associated with decreased α-diversity among sputum samples [23, 28]. Here, we also determined that older age is associated with a further decrease in α-diversity among frequent exacerbators. Although ageing is associated with an increased likelihood of COPD diagnosis, as well as increased susceptibility to lung infections, declines in lung function, and additional courses of antibiotics, the associations between ageing and the microbiome are relatively understudied. The ageing COPD lung microbiome and the gut–lung axis may exist at a juncture between declining lung function, immunosenescence, nutritional changes and increased antibiotic exposure [32]. Our work suggests that age itself may influence the COPD lung microbiome, possibly *via* mechanisms independent of typical factors (such as COPD exacerbations) that are known to influence the lung microbiome.

Our analysis of associations between the microbiome and tobacco use encompassed both pack-years of tobacco use as well as current (*versus* former) tobacco use status. Consistent with many of the previous studies [33–35], which identified more tobacco-associated microbiome changes of the upper *versus* lower airway, we identified that greater pack-years of tobacco exposure were associated with increased *Pseudomonas* abundance, while current (*versus* former) tobacco use was associated with increased

Bulleidia abundance, primarily in the oropharynx. *Bulleidia* has previously been associated with tobacco use and lung cancer [36, 37].

The present study also evaluated the longitudinal stability of the upper and lower airway microbiome. Although microbiome findings were generally similar on repeated sampling (low β-diversity), we identified several scenarios in which β-diversity was increased. The sputum microbiome of frequent exacerbators exhibited decreased similarity (increased β-diversity) compared with infrequent exacerbators. Subjects who experienced a COPD exacerbation between study visits also exhibited decreased compositional similarity *versus* those who did not report interim exacerbations.

We also found significant differences in sputum cytokine levels which associated with sputum microbiome α-diversity, but not related to exacerbation phenotype, COPD severity, age, tobacco use or COPD-related quality of life. Low sputum α-diversity was associated with increased concentrations of sputum IL-17A and decreased concentrations of IL-6 and IL-8. *Moraxella* abundance in the sputum microbiome was also associated with increased concentrations of IL-22, IL-17A and IL-5. IL-17A, IL-22 and IL-6 are key mediators of a T-helper (Th)17 response, often at mucosal sites [38]. IL-8 is a neutrophil chemoattractant. IL-5 is involved in Th2 responses and eosinophil recruitment. In prior COPD studies, elevated sputum IL-6 and IL-8 levels have been associated with tobacco use, lower FEV_1pp, frequent exacerbations and acute exacerbation (*versus* clinical stability) [8, 39–42]. Our finding of a positive association between IL-6 and IL-8 levels and α-diversity is somewhat unexpected, but it is possible that the acute rise in IL-6 and IL-8 observed during exacerbations is not reflected in our samples, which were collected during exacerbation-free intervals. Furthermore, few investigators have assessed the sputum microbiome (specifically low α-diversity) in relation to inflammatory cytokines.

Our manuscript has several strengths. We used well-defined frequent exacerbator and infrequent exacerbator phenotypes consistent with GOLD guidelines [27], allowing us to address associations between the microbiome and exacerbation phenotype. We deferred all study visits for 1 month following a COPD exacerbation or antibiotic use for any reason, in order to focus on the microbiome during periods of clinical stability. This approach minimises the influence of recent antibiotic or systemic steroid use on our microbiome findings, to the extent possible in an observational study of COPD. Lastly, our longitudinal approach allowed us to assess the stability of microbiome composition in relationship to exacerbation phenotype and recent COPD exacerbations. Our use of sputum inflammatory cytokines, in the context of the microbiome findings, identified clinical correlates of our microbiome findings.

Despite these strengths, our study had several relative weaknesses. We are unable to assess the influence of sex on the microbiome, as our single-centre study was conducted at a Veterans Affairs hospital with a limited female population. Sputum samples may be contaminated by saliva during expectoration and therefore may not reflect only the lower airway microbiome. Despite this potential limitation, we note that most of our key microbiome findings were identified only in the sputum microbiome and not identified in the oral microbiome. This suggests that sputum analysis can identify microbiome associations unique to the lower airways, despite potential upper airway contamination.

In conclusion, we found that frequent exacerbators exhibit lower sputum microbiome α-diversity, which is enhanced by older age or bacterial colonisation of the airways. Sputum microbiome α-diversity is a significant correlate of lung inflammation. The sputum microbiome composition of frequent exacerbators changes more over time when compared to the compositional stability of infrequent exacerbators.

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