Lung microbiome composition and bronchial epithelial gene expression in patients with COPD versus healthy individuals: a bacterial 16S rRNA gene sequencing and host transcriptomic analysis

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Summary

Background Chronic obstructive pulmonary disease (COPD) is associated with airway inflammation and bacterial dysbiosis. The relationship between the airway microbiome and bronchial gene expression in COPD is poorly understood. We aimed to identify differences in the airway microbiome from bronchial brushings in patients with COPD and healthy individuals and to investigate whether any distinguishing bacteria are related to bronchial gene expression.

Methods For this 16S rRNA gene sequencing and host transcriptomic analysis, individuals aged 45–75 years with mild-to-moderate COPD either receiving or not receiving inhaled corticosteroids and healthy individuals in the same age group were recruited as part of the Emphysema versus Airways Disease (EvA) consortium from nine centres in the UK, Germany, Italy, Poland, and Hungary. Individuals underwent clinical characterisation, spirometry, CT scans, and bronchoscopy. From bronchoscopic bronchial brush samples, we obtained the microbial profiles using 16S rRNA gene sequencing and gene expression using the RNA-Seq technique. We analysed bacterial genera relative abundance and the associations between genus abundance and clinical characteristics or between genus abundance and host lung transcriptional signals in patients with COPD versus healthy individuals, and in patients with COPD with versus without inhaled corticosteroids treatment.

Findings Between February, 2009, and March, 2012, we obtained brush samples from 574 individuals. We used 546 of 574 samples for analysis, including 207 from healthy individuals and 339 from patients with COPD (192 with inhaled corticosteroids and 147 without). The bacterial genera that most strongly distinguished patients with COPD from healthy individuals were *Prevotella* (median relative abundance 33·5%, IQR 14·5–49·4, in patients with COPD *vs* 47·7%, 31·1–60·7, in healthy individuals; p<0·0001), *Streptococcus* (8·6%, 3·8–15·8, *vs* 5·3%, 3·0–10·1; p<0·0001), and *Moraxella* (0·05%, 0·02–0·14, *vs* 0·02%, 0–0·07; p<0·0001). *Prevotella* abundance was inversely related to COPD severity in terms of symptoms and positively related to lung function and exercise capacity. 446 samples had assessable RNA-seq data, 257 from patients with COPD (136 with inhaled corticosteroids and 121 without) and 189 from healthy individuals. No significant associations were observed between lung transcriptional signals from bronchial brushings and abundance of bacterial genera in patients with COPD without inhaled corticosteroids treatment and in healthy individuals. In patients with COPD treated with inhaled corticosteroids, *Prevotella* abundance was positively associated with expression of epithelial genes involved in tight junction promotion and *Moraxella* abundance was associated with expression of the IL-17 and TNF inflammatory pathways.

Interpretation With increasing severity of COPD, the airway microbiome is associated with decreased abundance of *Prevotella* and increased abundance of *Moraxella* in concert with downregulation of genes promoting epithelial defence and upregulation of pro-inflammatory genes associated with inhaled corticosteroids use. Our work provides further insight in understanding the relationship between microbiome alteration and host inflammatory response, which might lead to novel therapeutic strategies for COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by persistent airflow obstruction and airway inflammation,[1] typically associated with airway bacterial dysbiosis.[2–5] Although associations of airway host transcriptome, protein biomarkers, and inflammatory

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Research in context

Evidence before this study

We searched PubMed for studies on chronic obstructive pulmonary disease (COPD) published from database inception to July 1, 2020, with the search terms "COPD" AND "microbiome" AND "transcriptome", with no language restrictions. We found two articles with these search criteria. The first study was a single centre trial done in 2016, that analysed the sputum of eight Taiwanese Han men with moderate or severe COPD. The second study was a longitudinal, single centre trial done in 2019, that used 101 sputum samples from 16 healthy individuals and 43 patients with COPD to investigate host–microbiome interactions. The study found that the genus *Haemophilus* was associated with host responses in stable disease and during exacerbation states, whereas *Moraxella* was associated with host factors during COPD exacerbations. We found no studies about the relationship between airway microbiome dysbiosis and gene expression of bronchial brush-derived cells in COPD.

Added value of this study

To our knowledge, this was the first and largest (comprising 339 patients with COPD and 207 healthy individuals) multicentre study to date that investigated the host–microbiome interaction from the same bronchial brush samples in patients with mild-to-moderate COPD. Our study showed a distinct microbial profile in patients with COPD compared with that of healthy individuals. We found that bacteria from the Bacteroidetes, Firmicutes, and Proteobacteria phyla were the major contributors for discriminating between COPD and the healthy lung microbiome, with *Prevotella*, *Streptococcus*, and *Moraxella* being the key genera. Microbiome dysbiosis in COPD was associated with downregulation of epithelial genes involved in the repair of epithelium (associated with increased *Moraxella* abundance) and upregulation of inflammatory pathway responses (associated with reduced *Prevotella* abundance).

Implications of all the available evidence

This study supports a pathogenic role for lower airway microbiome dysbiosis in COPD and presents new evidence of the consequent effect on the host bronchial epithelial inflammatory and repair responses. Our findings suggest altered host epithelial repair mechanisms in association with microbial dysbiosis. This study offers a framework for future investigations, which might lead to the discovery of novel therapies targeting microbial dysbiosis or epithelial repair for COPD.

cells[3,6,7] with specific bacterial pathogens have been investigated, a systems biology approach examining both microbiome and host transcriptome profiles has not been used.

The airway microbiome is affected by corticosteroid therapy. Oral corticosteroids are associated with a reduction in microbial diversity and an increased Proteobacteria-to-Firmicutes ratio;[3,8] however, the effects of inhaled corticosteroids on the lung microbiome of patients with COPD are poorly understood.

To date, most studies of airway dysbiosis have used sputum samples, which might be affected by upper airway contamination, to investigate the airway microbiome in patients with COPD, whereas studies based on bronchoscopy, which minimises upper airway contamination, have generally involved small numbers of individuals.

We hypothesised that the airway microbiome from bronchial brush samples would be different between patients with mild-to-moderate COPD and healthy controls. We also hypothesised a difference in airway microbiome between patients with COPD with and without inhaled corticosteroids treatment, and that specific bacterial 16S rRNA gene signals would associate with specific host cell gene expression. To test these hypotheses, we used 16S rRNA gene sequencing and RNA-Seq to obtain microbiome and host transcriptome profiles from patients with COPD with and without inhaled corticosteroids treatment and from healthy controls.

See Online for appendix

Methods

Study design and participants

For this bacterial gene sequencing and host transcriptomic analysis, adults aged 45–75 years who were patients with COPD or healthy controls were recruited as part of the Emphysema versus Airways Disease (EvA) Consortium from nine clinical centres in five European countries (Leicester, Manchester, and Coventry [UK]; Munich, Marburg, and Freiburg [Germany]; Ferrara [Italy]; Warsaw [Poland]; and Budapest [Hungary]).[9] A diagnosis of COPD was based on a post-bronchodilator ratio of forced expiratory volume in 1 s (FEV_1) to forced vital capacity (FVC) lower than 70%. Patients were excluded if they had very severe COPD (FEV_1 % of predicted <30% predicted or FEV_1 <1 L), had bronchodilator reversibility greater than 400 mL, had smoked within the preceding 12 months, or had a primary diagnosis of asthma, bronchiectasis, or any other relevant respiratory or other comorbid diseases such as symptomatic coronary artery disease, arrhythmias, uncontrolled hypertension, or severe liver and kidney diseases. The same exclusion criteria were applied for individuals in the healthy control group to match their criteria with those of patients with COPD. All participants provided written informed consent and local ethics approvals were obtained for this study (appendix p 3).[9] Additional information on methods, study participants, DNA and RNA sequencing, reagent controls, and biostatistical analyses is provided in the appendix (pp 3–5).

Procedures

All participants had clinical characterisation, including lung function testing before and after bronchodilatation, 6-min walk distance, quantification of dyspnoea with the modified Medical Research Council (MRC) scale, thoracic CT, venous blood sampling, and sputum induction. The BODE index was determined for all participants by use of body-mass index, airflow obstruction, dyspnoea, and 6-min walk distance values.

Participants underwent video-assisted bronchoscopy, with bronchial brushings done in the right lung with sheathed brushes with a diameter of 5 mm at bristle level (#BC-202D 5010; Olympus, Hamburg, Germany). We used the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) to extract RNA and DNA from bronchial brush samples, following the manufacturer's protocol.

We used the Hiseq 2000 sequecing system (Illumina, San Diego, CA, USA) with 100 bp paired-end reads for the RNA-Seq. Samples with low sequencing throughput (<10 million reads) were removed from the analysis. Additional details of the RNA sequencing protocol are described in the appendix (p 4).

After using the Qiagen kit, we measured the total bacterial burden using quantitative PCR by targeting the 16s rRNA gene, as previously described.[10] We have previously shown this extraction kit to achieve less efficient extraction from bacteria than bacteria-specific DNA extraction kits, but that all major phyla are detected.[11] For microbiomic analyses, we amplified the V4 and V5 hypervariable regions of the 16S rRNA gene with PCR, and we did 2×300 bp pair-ended DNA sequencing of amplified DNA fragments on the Illumina MiSeq platform. Samples were randomised to reduce the batch effect and, altogether, seven MiSeq runs were done to sequence all the samples. Two negative and two positive controls (additional details in appendix p 4) were included in each of the sequencing runs, and additional measures were taken to minimise potential contamination (ie, evaluating the potential effect of bacterial burden and using protected bronchial brushes to minimise upper airway contamination; appendix p 4). After trimming the sequence reads and removing the adaptors with Trimmomatic (version 0.36), we used Quantitative Insights into Microbial Ecology pipeline (version 1.9.1) to process the sequences. Pair-ended sequences were joined and potential host sequences and chimeras were removed. We selected a rarefaction depth of 10 000 reads per sample for 16S rRNA sequencing on the basis of a jack-knifed principal coordinate re-sampling analysis. The sequence reads were subject to a close reference operational taxonomic unit picking (97% identity cutoff), in which reads were clustered against the Greengenes reference database (version 13.8) and their taxonomic identities were assigned by the RDP classifier (version 2.12) using naive Bayes classification. Additional details on the microbiome analysis are described in the appendix (p 4).

Statistical analysis

We did comparisons between patients with COPD and healthy individuals, as well as between patients with COPD with and without inhaled corticosteroids treatment, using two-sided Wilcoxon-Mann-Whitney tests for continuous variables (ie, relative abundance of bacterial phyla and genera) and χ^2 tests for categorical variables (ie, clinical characteristics such as modified MRC dyspnoea scale and BODE index). We did a permutation multivariate analysis of variance based on both unweighted and weighted UniFrac distance to compare β diversity between patients with COPD and healthy individuals or between patients with COPD receiving and those not receiving inhaled corticosteroids treatment. We used a generalised linear mixed model to identify the bacterial taxa and α diversity indices that were significantly different between patients with COPD and healthy individuals or between patients with COPD receiving and those not receiving inhaled corticosteroids treatment. We used the same method to assess the association of clinical characteristics such as lung function, symptoms, and physical function with microbiome profile. Several factors were adjusted for different confounders including age, sex, clinical centre, batch effect, pack-year history, and Global Initiative for Chronic Obstructive Lung Disease (GOLD) grades.[1] We used clinical centre and batch effect as random factors. We did Cliff's δ effect size test to assess clinically relevant results. Linear discriminant analysis effect size analysis was done to rank the discriminating taxonomic groups between healthy individuals and patients with COPD and, separately, between patients with COPD receiving and those not receiving inhaled corticosteroids treatment, on the basis of the linear discriminant analysis score. We used the compositionality corrected by renormalisation and permutation method, based on an n-dimensional checkerboard score, to construct interaction networks of all detected bacterial genera in the lung microbiome. We analysed differential gene expression and did a linear mixed model analysis of gene expression with the presented bacterial genera in the network analysis with a significant association with combined patients with COPD and controls as continuous variables, using limma and lme4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were done using STRING (version 11.0). We used false discovery rate (FDR) to adjust the p value of all of our analyses according to the Benjamini and Hochberg method.[12] We considered adjusted p values lower than 0·05 statistically significant. Additional details on the statistical analysis are described in the appendix (p 5). All analyses were done in R, version 3.5.1.

Role of the funding source

The study funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

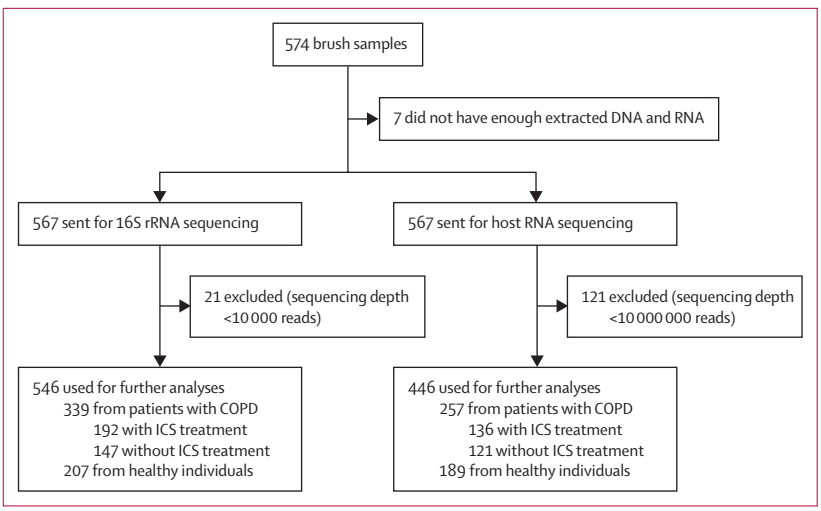


Figure 1: **Flow diagram of sample processing**
COPD=chronic obstructive pulmonary disease. ICS=inhaled corticosteroids.

Results

Between February, 2009, and March, 2012, brush samples from 574 individuals (360 patients with COPD and 214 healthy individuals) were obtained as part of the EvA consortium. Seven samples (three from patients with COPD and four from healthy individuals) were excluded from 16S rRNA and RNA sequencing because of insufficient amounts of DNA and RNA. On the basis of the sequencing depth cutoff, an additional 21 samples (18 from patients with COPD and three from healthy individuals) were excluded from 16S rRNA sequencing and 121 samples (100 from patients with COPD and 21 from healthy individuals) were excluded from host RNA sequencing, resulting in 546 samples (339 from patients with COPD, 207 from healthy individuals) used for 16S rRNA sequencing and 446 samples (257 from patients with COPD, 189 from healthy individuals) used for host RNA sequencing (figure 1). The study population included more men (234 [69%] of 339 with COPD and 129 [62%] of 207 who were healthy) than women (105 [31%] with COPD and 78 [38%] who were healthy), and the median age was 65 years (IQR 61–70) for patients with COPD and 60 years (52–66) for healthy individuals. Baseline and clinical characteristics of these patients are described in tables 1 and 2; all patients with COPD and 30 healthy individuals had a CT scan.

58 077 234 sequencing reads (geometric mean 73 391 reads per sample, SD 2·03) were generated for 16SrRNA analyses after quality control filtering and removal of potential human DNA contamination. In total, 4687 operational taxonomic units were assigned at 97% sequence identity across all the bronchial brush samples.

We observed no significant differences in the total bacterial burden between patients with COPD and healthy individuals or between patients with COPD with and without inhaled corticosteroids treatment (appendix p 6). Therefore, all subsequent analyses focused on microbiome-determined bacterial relative abundance. The evenness and richness of bacterial community compositions were compared between patients with COPD and healthy individuals (appendix p 7). The number of observed species or operational taxonomic units was significantly greater in healthy individuals (median 201, IQR 168–237) than in patients with COPD (181, 152–218; p<0·0001). The α diversity (microbial diversity within a sample) was reduced in patients with COPD versus healthy individuals (appendix p 19). These differences were observed after correction for age, sex, centre, batch effect, pack-year history, and GOLD grades (appendix p 19). No significant difference in α diversity was observed between patients with COPD with and without inhaled corticosteroids treatment (appendix p 8). The analysis of β diversity (microbial composition dissimilarity between samples) showed no significant difference between patients with COPD and healthy individuals or between patients with and without inhaled corticosteroids treatment after adjustment for age, sex, centre, batch effect, pack-year history, and GOLD grades (appendix pp 9–10).

Of the 26 phyla detected in samples of both patients with COPD and healthy individuals, the most abundant were Bacteroidetes (mean relative abundance 43·0%, median 46·7% [IQR 26·2–58·9]) followed by Firmicutes (24·5%, 21·7% [15·9–30·7]), Proteobacteria (20·3%, 13·9% [7·4–25·4]), Actinobacteria (6·6%, 3·0% [1·6–6·7]), and Fusobacteria (4·1%, 3·4% [1·6–5·7]), comprising approximately 99% of sequences (9852 of 10 000). Comparing patients with COPD and healthy individuals, the relative abundance of Bacteroidetes and Fusobacteria was significantly higher in healthy individuals, whereas Firmicutes, Proteobacteria, and Actinobacteria were more abundant in patients with COPD (figure 2A, appendix p 20). Among the phyla with less than 1% relative abundance, only Saccharibacteria (formerly known as TM7) and Spirochaetes (both higher in healthy individuals) were significantly different between patients with COPD and healthy individuals (appendix p 20). The relative abundances of two phyla were significantly different in patients with COPD with versus without inhaled corticosteroids treatment: Bacteroidetes (mean 34·6%, median 35·3% [IQR 15·3–50·4], *vs* 43·1%, 47·9% [24·5–58·8]; p=0·0036) and Proteobacteria (25·0%, 18·2% [7·8–33·4], *vs* 20·2%, 13·4% [7·6–24·9]; p=0·037; figure 2B, appendix p 25).

Of the 704 detected genera, *Prevotella* was the most abundant (mean 37·7%, median 38·4% [IQR 20·1–54·6]) and was significantly lower in patients with COPD (33·2%, 33·5% [14·5–49·4]) than in healthy individuals (45·3%, 47·7% [31·1–60·7]; p<0·0001), whereas *Streptococcus*, at mean 10·5% (median 6·9% [3·3–13·8]) of total reads, was more abundant in patients with COPD (12·2%, 8·6% [3·8–15·8]) than in healthy

individuals (7·7%, 5·3% [3·0–10·1]; p<0·0001). *Veillonella* (8·0%, 7·2% [3·9–11·0], of total reads) and *Haemophilus* (4·1%, 2·6% [1·1–4·8]) showed similar relative abundances in health and disease (figure 2C, E). Pairwise correlation of the top four genera in samples, as well as *Moraxella*, are presented in the appendix (p 11). We also included *Moraxella* because we observed that its relative abundance (1·60%, 0·04% [0·01–0·11], overall) was significantly different in patients with COPD (2·30%, 0·05% [0·02–0·14]) and healthy participants (0·39%, 0·02% [0–0·07]; p<0·0001). *Prevotella* abundance was negatively correlated with three genera, *Streptococcus*, *Haemophilus*, and *Moraxella*, each of which includes notable respiratory tract pathogens. In total, 50 genera were significantly differentially represented in patients with COPD versus healthy individuals, on the basis of the Wilcoxon-Mann-Whitney tests and FDR-corrected p values, with 17 more abundant in patients with COPD and 33 more abundant in healthy individuals (appendix pp 21–24). *Prevotella* and *Veillonella* were significantly more abundant in patients with COPD without inhaled corticosteroids treatment than in those treated with inhaled corticosteroids (figure 2D, appendix p 25).

We modelled phyla and genera that were significantly different between patients with COPD and healthy individuals or between patients with and without inhaled corticosteroids treatment, after controlling for possible confounding factors. All phyla except Spirochaetes remained significantly different between patients with COPD and healthy individuals (appendix p 20). Bacteroidetes was the only phylum that remained significantly lower in patients with COPD with inhaled corticosteroids treatment than in those without inhaled corticosteroids treatment (estimate 0·08, 95% CI 0·03–0·12; p=0·015; appendix p 25). Additionally, Bacteroidetes had the largest effect size among phyla (0·40 for patients with COPD *vs* healthy individuals, and 0·23 for patients receiving inhaled corticosteroids *vs* those not receiving inhaled corticosteroids; appendix pp 20, 25). Of 50 genera with a significant difference between patients with COPD and healthy individuals, 14 remained significant (nine were more abundant in patients with COPD; appendix pp 21–24). *Prevotella* was the only genus that remained significantly higher in patients with COPD without inhaled corticosteroids treatment than in those treated with inhaled corticosteroids (0·07, 0·03–0·11; p=0·021; appendix p 25). Additionally, *Prevotella* had the largest effect size among genera (0·40 for patients with COPD *vs* healthy individuals, and 0·23 for patients receiving inhaled corticosteroids *vs* those not receiving inhaled corticosteroids; appendix pp 21–25).

We did microbial community network analyses using samples of both patients with COPD and healthy individuals to investigate the co-occurrence or co-exclusion network of bacterial genera in the lung microbiome. Four distinct communities were obtained (appendix p 12). All genera were positively associated with each other apart from *Prevotella*, which was inversely related to *Streptococcus*, *Moraxella*, *Staphylococcus*, *Propionibacterium*, *Corynebacterium*, *Acinetobacter*, and *Pseudomonas* (all had

	Patients with COPD (n=339)	Healthy participants (n=207)	p value
Sex	..	..	0·11
Female	105 (31%)	78 (38%)	..
Male	234 (69%)	129 (62%)	..
Age, years	65 (61 to 70)	60 (52 to 66)	<0·0001
Body-mass index	27·94 (24·44 to 30·76)	27·73 (24·77 to 30·83)	0·78
Smoking status	..	..	NA
Never smoker	0	31 (15%)	..
Ex-smoker	339 (100%)	176 (85%)	..
Pack-year history	38·0 (26·1 to 54·0)	22·5 (8·7 to 35·0)	<0·0001
Physical function (walking ability), m	462 (390 to 520)	540 (471 to 610)	<0·0001
Post-bronchodilator FEV_1, L	1·92 (1·54 to 2·43)	3·21 (2·74 to 3·68)	<0·0001
Post-bronchodilator FEV_1, % predicted	71·48% (57·81 to 86·07)	108·20% (99·34 to 119·50)	<0·0001
Post-bronchodilator FEV_1/FVC ratio, %	58·17% (49·96 to 64·86)	78·98% (75·44 to 82·19)	<0·0001
Reversibility, mL	146 (70 to 240)	100 (20 to 180)	<0·0001
TLCO/VA	1·10 (0·87 to 1·35)	1·46 (1·32 to 1·64)	<0·0001
GOLD grades	..	..	NA
1	112 (33%)	NA	..
2	185 (55%)	NA	..
3	42 (12%)	NA	..
4	0	NA	..
Modified MRC dyspnoea scale, mode (range)	1 (4)	0 (2)	<0·0001
BODE index, mode (range)	1 (8)	0 (3)	<0·0001
Treatment	..	..	NA
LABA alone	14 (4%)	0	..
LAMA alone	29 (9%)	0	..
ICS alone	12 (4%)	0	..
ICS combined with LABA or LAMA	84 (25%)	0	..
Combined LABA, LAMA, and ICS	96 (28%)	0	..
Total cell count, × 10^6 cells per g sputum	1·28 (0·49 to 3·48)	0·75 (0·40 to 1·56)	0·0025
Sputum neutrophil count	77·00% (63·06 to 86·00)	55·13% (40·75 to 76·25)	<0·0001
Sputum eosinophil count	1·75% (0·31 to 5·00)	0·50% (0 to 1·75)	<0·0001
Blood white cell count, × 10^9 cells per L	7·20 (6·10 to 8·41)	6·40 (5·50 to 7·30)	<0·0001
Blood neutrophil count, × 10^9 cells per L	4·50 (3·60 to 5·63)	3·90 (3·10 to 4·80)	<0·0001
Blood eosinophil count, × 10^9 cells per L	0·16 (0·10 to 0·27)	0·13 (0·08 to 0·20)	<0·0001
Lung density, Perc15 HU	–918·4 (–931·9 to –906·3)	–907·0 (–914·4 to –890·9)	<0·0001
Wall area, %*	63·45 (59·27 to 68·21)	59·65 (54·95 to 65·93)	0·035

Data are n (%) or median (IQR), unless otherwise specified. BODE=body-mass index, airflow obstruction, dyspnoea, and exercise. COPD=chronic obstructive pulmonary disease. FEV_1=forced expiratory volume in 1 s. FVC=forced vital capacity. GOLD=Global Initiative for Chronic Obstructive Lung Disease. ICS=inhaled corticosteroids. LABA=long-acting β agonists. LAMA=long-acting muscarinic antagonists. MRC=Medical Research Council. NA=not applicable. Perc15 HU=15th percentile Hounsfield units. TLCO/VA=transfer factor for carbon monoxide/alveolar volume. *Data were available for only 25 healthy individuals.

Table 1: **Clinical characteristics of patients with COPD and healthy individuals**

	Patients treated with ICS (n=192)	Patients not treated with ICS (n=147)	p value
Sex	..	..	0·91
Female	59 (31%)	46 (31%)	..
Male	133 (69%)	101 (69%)	..
Age, years	67 (61 to 71)	64 (60 to 69)	0·031
Body-mass index	27·80 (24·46 to 31·12)	28·07 (24·38 to 30·64)	0·69
Smoking status	..	..	NA
Never smoker	0	0	..
Ex-smoker	192 (100%)	147 (100%)	..
Pack-year history	36·75 (23·81 to 49·88)	40·00 (28·00 to 56·00)	0·023
Physical function (walking ability), m	436·0 (360·8 to 500·5)	486·0 (425·0 to 550·0)	<0·0001
Post-bronchodilator FEV_1, L	1·78 (1·42 to 2·28)	2·09 (1·73 to 2·62)	<0·0001
Post-bronchodilator FEV_1, % predicted	67·48 (52·98 to 79·93)	76·07 (66·64 to 91·48)	<0·0001
Post-bronchodilator FEV_1/FVC ratio, %	55·60 (47·62 to 62·19)	61·76 (53·24 to 65·99)	<0·0001
Reversibility, mL	130 (70 to 220)	160 (70 to 270)	0·17
TLCO/VA	1·06 (0·85 to 1·30)	1·15 (0·94 to 1·40)	0·03
GOLD stages	..	..	0·0003
1	49 (26%)	63 (43%)	..
2	110 (57%)	75 (51%)	..
3	33 (17%)	9 (6%)	..
4	0	0	..
Modified MRC dyspnoea scale, mode (range)	1 (4)	1 (3)	<0·0001
BODE index, mode (range)	1 (8)	0 (8)	<0·0001
Treatment	..	..	NA
LABA alone	79 (41%)	14 (10%)	..
LAMA alone	5 (3%)	29 (20%)	..
ICS alone	12 (6%)	0	..
ICS combined with LABA or LAMA	84 (44%)	0	..
Combined LABA, LAMA, and ICS	96 (50%)	0	..
Total cell count, × 10^6 cells per g sputum	1·28 (0·44 to 3·60)	1·27 (0·57 to 3·41)	0·80
Sputum neutrophil count	77·00 (61·00 to 87·25)	77·00 (66·00 to 84·25)	0·87
Sputum eosinophil count	1·75 (0·25 to 5·00)	2·00 (0·50 to 5·00)	0·63
Blood white cell count, × 10^9 cells per L	7·22 (6·28 to 8·60)	7·05 (5·78 to 8·10)	0·036
Blood neutrophil count, × 10^9 cells per L	4·60 (3·66 to 5·81)	4·21 (3·35 to 5·53)	0·017
Blood eosinophil count, × 10^9 cells per L	0·16 (0·10 to 0·28)	0·16 (0·10 to 0·27)	0·53
Lung density, Perc15 HU	−920·6 (−934·7 to −909·0)	−912·5 (−925·3 to −900·0)	0·015
Wall area, %*	64·19 (59·30 to 68·37)	63·14 (58·26 to 67·45)	0·44

Data are n (%) or median (IQR), unless otherwise specified. BODE=body-mass index, airflow obstruction, dyspnoea, and exercise. COPD=chronic obstructive pulmonary disease. FEV_1=forced expiratory volume in 1 s. FVC=forced vital capacity. GOLD=Global Initiative for Chronic Obstructive Lung Disease. ICS=inhaled corticosteroids. LABA=long-acting β agonists. LAMA=long-acting muscarinic antagonists. MRC=Medical Research Council. NA=not applicable. Perc15 HU=15th percentile Hounsfield units. TLCO/VA=transfer factor for carbon monoxide/alveolar volume. *Data were available for only 25 healthy individuals.

Table 2: **Clinical characteristics of patients with COPD who were or not treated with ICS**

significantly higher relative abundance in patients with COPD than in healthy individuals). The co-exclusion pattern of *Streptococcus* and *Moraxella* with *Prevotella* remained significant when microbial community network analyses were done by use of samples from either patients with COPD or healthy individuals alone (data not shown).

We used linear discriminant analysis to identify which bacterial groups best distinguished the microbiomes from healthy individuals and patients with COPD (appendix p 13). Among those genera significantly differentially detected, *Prevotella*, *Streptococcus*, and *Moraxella* contributed the greatest differences between patients and controls (figure 3A) and between patients with COPD with and without inhaled corticosteroids treatment (figure 3B).

We investigated the association of *Prevotella* with clinical characteristics using generalised linear mixed models after adjusting for age, sex, centre, batch effect, and pack-year history. The relative abundance of *Prevotella* was positively associated with post-bronchodilator FEV_1, post-bronchodilator FEV_1/FVC ratio, and 6-min walk distance but negatively associated with modified MRC dyspnoea scale and BODE index in all patients with COPD (appendix p 14). Similar associations between *Prevotella* and clinical characteristics were observed in patients with COPD treated with inhaled corticosteroids (appendix p 14). By contrast, we observed no significant associations between *Prevotella* abundance and clinical characteristics when patients with COPD not treated with inhaled corticosteroids were considered alone (appendix p 14). Neither *Moraxella* nor *Streptococcus* abundance were significantly associated with the clinical characteristics associated with *Prevotella* (data not shown). Modified MRC dyspnoea scale and BODE index scores for all participants are shown in the appendix (p 15).

Of the 546 bronchial brush samples used for microbiome analysis, 446 also had assessable RNA-Seq data, including data from 257 patients with COPD (136 with and 121 without inhaled corticosteroids treatment) and 189 healthy individuals. 7399 lung genes were differentially expressed (and met FDR criteria) between samples of patients with COPD and those of healthy individuals (3813 genes upregulated in patients with COPD) after correction for age, sex, centre, batch effect, pack-year history, and GOLD grades (data not shown). Pathway analysis of these differentially expressed genes revealed that 60 genes (45 genes upregulated in patients with COPD) were involved in TNF signalling pathways (appendix p 26).

472 genes were differentially expressed between samples of patients with COPD treated with inhaled corticosteroids and those of patients not treated with inhaled corticosteroids (230 upregulated in patients treated with inhaled corticosteroids) by use of the same corrections and FDR criteria previously applied (appendix pp 27–32). However, common pathways for these genes were not identified.

A linear mixed model analysis of the lung transcriptional signal from bronchial brushings versus *Prevotella* abundance in combined samples of patients

with COPD and healthy individuals revealed no significant associations with use of the same corrections and FDR criteria (data not shown). Similarly, we observed no significant associations between gene expression and *Prevotella* abundance when patients with COPD and healthy individuals were analysed separately (data

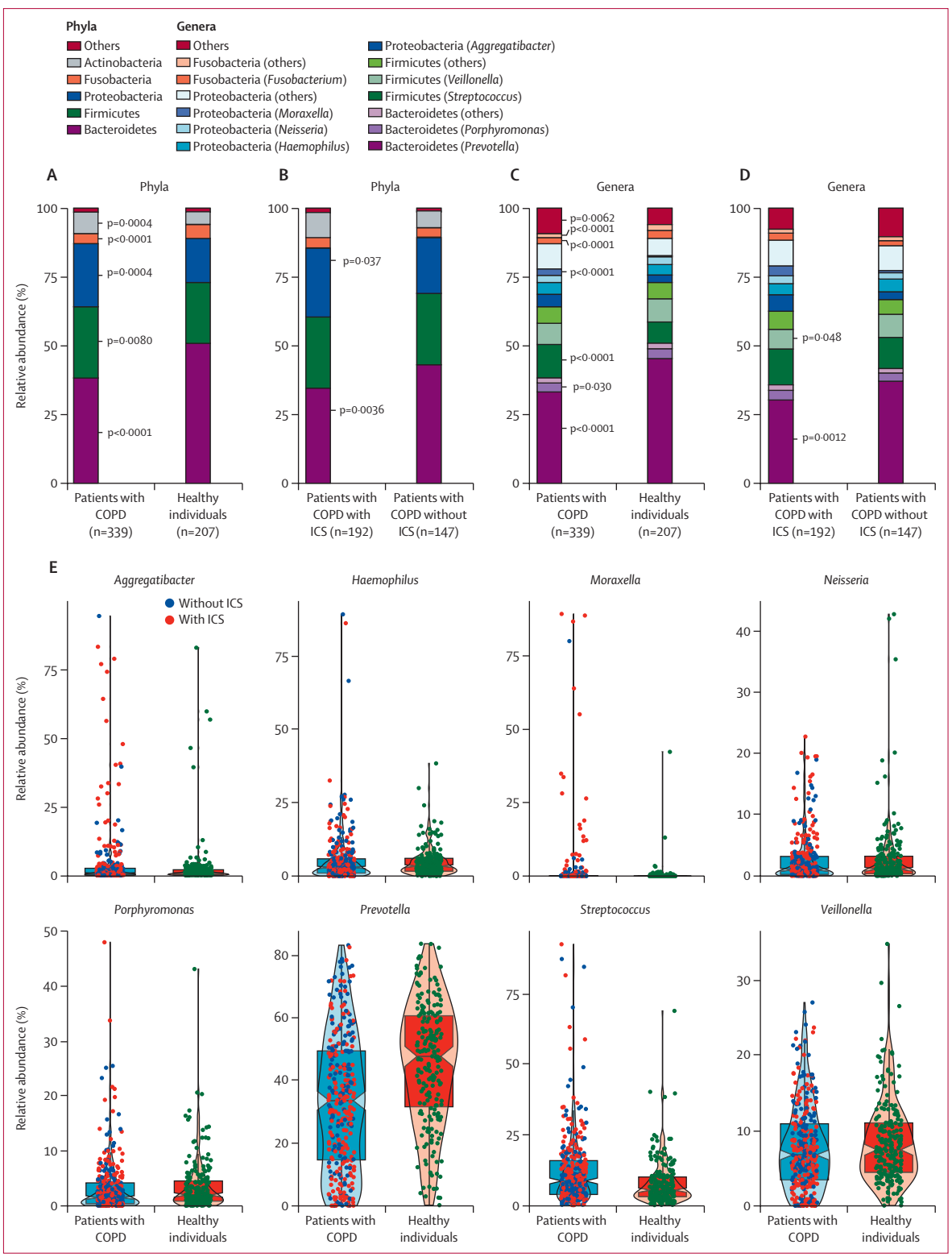
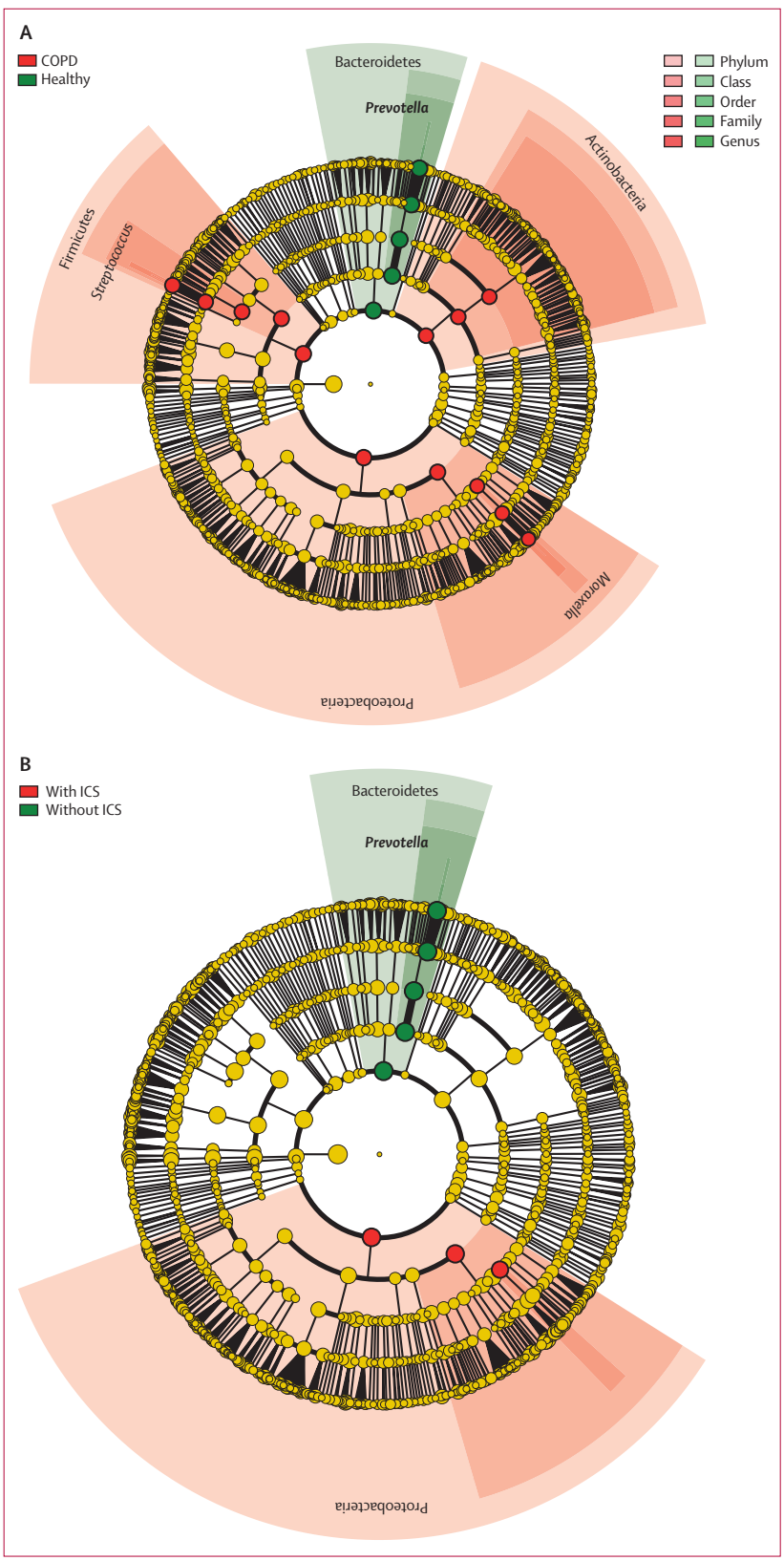


***Figure 2*: Comparison of relative abundance (%) of major phyla and genera within the lung microbiome**
p values were computed with two-sided Wilcoxon-Mann-Whitney tests. (A and C) Comparison between patients with COPD and healthy individuals. (B and D) Comparison between patients with COPD treated with ICS and without ICS. (E) Inter-subject variation of relative abundance of major genera in healthy individuals and patients with COPD treated with ICS and without ICS; darker areas with horizontal line represent median (IQR), and lighter areas represent the distribution of the relative abundance of genera across samples. COPD=chronic obstructive pulmonary disease. ICS=inhaled corticosteroids.



not shown). A list of the top 10 upregulated and down-regulated genes linearly associated with increasing relative abundance of *Prevotella* in combined samples of patients with COPD and healthy individuals is shown in the appendix (p 33).

We observed 327 positive and 202 negative associations between lung gene expression and *Prevotella* abundance in samples from patients with COPD treated with inhaled corticosteroids, using the same corrections and FDR criteria (appendix pp 34–40). The top 100 genes with significant positive and negative associations with *Prevotella* abundance are shown in the appendix (p 16). Again, no significant associations were observed between bronchial transcriptional signals and *Prevotella* abundance when patients with COPD without inhaled corticosteroids treatment were considered separately (data not shown).

We did a pathway analysis of genes showing significant association with *Prevotella* in samples from patients with COPD treated with inhaled corticosteroids. 64 genes (53 downregulated and 11 upregulated) were involved in eight KEGG-defined pathways, including metabolic pathways, protein processing in endoplasm, biosynthesis of antibiotics, carbon metabolism, tight junction, biosynthesis of amino acids, glycolysis and gluconeogenesis, and glycosphingolipid biosynthesis (appendix p 17).

Similar linear mixed model and pathway analyses were done with *Streptococcus* and *Moraxella* abundance versus lung transcriptional signals from bronchial brushings. No significant associations were found with *Streptococcus* (data not shown). However, 192 positive and 35 negative associations (appendix pp 41–43) were observed with *Moraxella* in samples from patient with COPD with inhaled corticosteroids treatment; of these, 15 genes were enriched in TNF and IL-17 signalling pathways (appendix p 18).

We observed no significant associations between each of the genera presented in the microbial network and the lung transcriptional signal from bronchial brushings (data not shown).

Discussion

To our knowledge, this study is the first to characterise airway host–microbiome relationship in COPD using host RNA and microbial DNA isolated from the same

Figure 3: **Bacterial groups with the most influence on distinguishing healthy from COPD microbiome (A) and the microbiome of patients with COPD treated with ICS from those without ICS (B)**
Each of the circles in the cladogram represents a bacterial taxa, and each ring a taxonomy level starting with kingdom in the innermost circle, followed by phylum, class, order, family, and genus, in the outermost circle. Green and red circles and zones represent differentially enriched bacterial taxa based on LDA score (>4). Yellow circles represent bacterial taxa with no significant differential enrichment between groups (LDA score <4). Different shades of colour intensity indicate different taxonomy levels. Each circle's diameter is proportional to the taxon's abundance and correlated with LDA score. COPD=chronic obstructive pulmonary disease. ICS=inhaled corticosteroids. LDA=linear discriminant analysis.

bronchial brush samples. The main bacterial genera differentiating the airway microbiomes between patients with mild-to-moderate COPD and healthy individuals were *Prevotella*, *Streptococcus,* and *Moraxella*. The relative abundance of *Prevotella* had a significant negative association with *Streptococcus* and *Moraxella*. *Prevotella* abundance was significantly greater in healthy individuals, and its abundance was significantly associated with better lung function and reduced symptoms in patients with COPD. By contrast, *Streptococcus* and *Moraxella* abundance was significantly higher in patients with COPD than in healthy individuals and was not significantly associated with clinical characteristics. Significant associations were observed between host lung gene expression profile and *Prevotella* and *Moraxella*, but not *Streptococcus*. Among these bacterial genera, *Prevotella* abundance was much greater in the airway microbiome, and thus was the genus that had the most discriminatory power between the healthy and COPD microbiome. Likewise, *Prevotella* was the only genus that significantly distinguished between patients with COPD with and without inhaled corticosteroids treatment.

The decrease in *Prevotella* abundance in patients treated with inhaled corticosteroids was associated with increased severity of COPD. The effect of inhaled corticosteroids on the lung microbiome of patients with COPD is poorly understood; however, growing evidence exists that inhaled corticosteroids can alter airway microbiome in individuals with asthma.[13,14] Our findings support the view that clinical characteristics of COPD and gene expression are related to microbial dysbiosis. We cannot say whether changes in the airway microbiome precede or are a consequence of worsening lung disease, or what contribution inhaled corticosteroids make to this balance. Nonetheless, dynamic host–microbiota interactions are likely to be important and might amplify airway inflammation, reduce resistance to acute and chronic infection, and potentiate lung damage.

Host gene expression and microbiome associations have been investigated in several studies.[6,15–18] Previous studies using bronchial brush samples have analysed either lung transcriptome or microbiome profile independently.[9,19–21] In our study, we examined both host transcriptome and microbiome interaction from the same bronchial brush samples of patients with COPD. *Prevotella* and *Moraxella* were significantly associated with host airway transcriptome profiles, particularly genes involved in immunity and inflammation, suggesting that these bacterial genera might play a leading role in airway host–microbiome interaction in COPD. The pathway analysis suggests that *Prevotella* might promote innate immunity and reduce lung epithelial cell permeability by modulating the expression of tight junction protein. On one hand, the promotion of tight junction function observed with *Prevotella* could be due to a direct effect of *Prevotella* upon the epithelium, or through immunomodulatory response[22] due to upregulation of toll-like receptors (TLRs). On the other, *Moraxella* abundance was associated with an epithelial cell-derived signature of IL-17 and TNF inflammation[23] that is likely to be due to the effect of lipopolysaccharides on TLRs. The reciprocal relationship between *Prevotella* and *Moraxella* might be due to their differential abundance and the different types of lipopolysaccharides they produce, which have dissimilar TLR stimulating capacity.[24] Further investigation, for example by use of air-liquid interface epithelial cultures with *Prevotella* and *Moraxella*, is required to understand the microbial effect on epithelial repair.

Associations between Proteobacteria abundance and COPD severity and exacerbations have been reported previously.[3,8] However, a potential homoeostatic function for *Prevotella* in the healthy airway has not yet been shown. A few strains of *Prevotella* are known to cause opportunistic endogenous infections,[25] but they are generally considered commensal bacteria. The high abundance of *Prevotella* in healthy individuals in our study suggests that a mutualistic relationship has co-evolved between humans and *Prevotella*, where colonisation by these bacteria is tolerable to the respiratory immune system. Yadava and colleagues[26] have used inhaled lipopolysaccharides and elastase to induce a chronic lung inflammation, resembling COPD, in mice; this resulted in reduced abundance of *Prevotella* and an increase in *Pseudomonas* and *Lactobacillus*. This finding suggests that chronic inflammation might have a direct effect upon *Prevotella*, reducing its abundance by forming a hostile microenvironment. Microaspiration is thought to cause the seeding of healthy lungs with upper respiratory tract microbiota, such as *Prevotella*.[27] The weak TLR stimulating capacity of *Prevotella* mediates a low-grade inflammatory process that will cause *Prevotella* removal but might also protect the lung from invasion by respiratory pathogens and chronic disease under homoeostatic conditions.

Our study is a large multicentre study, but some limitations need to be highlighted. We used protected bronchial brushes, which minimise upper airway contamination,[28,29] but we cannot exclude the possibility of minor contamination from the oropharynx. We did not record the frequency of exacerbations or the specific inhaled corticosteroids formulations, and these factors could influence the microbial composition and transcriptomic patterns. Our findings are only associative and cannot show causality. Similar to many other studies, we considered COPD as a single disease entity. However, COPD is a heterogeneous disease, in which the diverse components of the microbiota could be involved in different disease phenotypes. We did not investigate protein expression in the bronchoscopic samples; evaluation of both the transcriptome and proteome, along with the use of other so-called omic approaches, could enhance our understanding of the host–microbiota interface and its role in disease pathogenesis.

In summary, our bronchial brush data indicate that *Prevotella* was the genus that most robustly distinguished

samples of patients with mild-to-moderate COPD from those of healthy individuals. *Prevotella* was associated with a distinct host bronchial gene expression, which involved promotion of tight junction function. Further insights into the biological mechanisms by which the microbiome influences alterations in host transcriptome profile, or vice versa, will inform disease understanding and the development of novel therapeutic strategies for COPD.

Contributors

MYR, LZ-H, MRB, DS, and CEB conceived and designed the study. All authors participated in the acquisition of data. MYR, KH, MR, AE-C, SH, IG, JAM, LZ-H, MRB, DS, and CEB analysed and interpreted the data. MYR, MRB, DS, and CEB drafted or revised the manuscript. MYR, MR, DS, and CEB verified the underlying data. All authors gave final approval of the manuscript, had full access to all the data in the study, and had final responsibility for the decision to submit for publication.

Declaration of interests

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Data sharing

For the **Sequencing Read Archive** see https://www.ncbi.nlm.nih.gov/sra

For the **European genome-phenome archive** see https://www.ebi.ac.uk/ega

Microbiome sequencing data have been submitted to the Sequence Read Archive of the US National Center for Biotechnology Information (BioProject ID: PRJNA632472). The RNA-Seq was uploaded to European genome-phenome archive: EGAD00001002003 and EGAD00001002004.

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