The sputum microbiome is distinct between COPD and health, independent of smoking history

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Abstract

Background: Airway bacterial dysbiosis is a feature of chronic obstructive pulmonary disease (COPD). However, there is limited comparative data of the lung microbiome between healthy smokers, non-smokers and COPD. Methods: We compared the 16S rRNA gene-based sputum microbiome generated from pair-ended Illumina sequencing of 124 healthy subjects (28 smokers and 96 non-smokers with normal lung function), with single stable samples from 218 COPD subjects collected from three UK clinical centres as part of the COPDMAP consortium. Results: In healthy subjects Firmicutes, Bacteroidetes and Actinobacteria were the major phyla constituting 88% of the total reads, and Streptococcus, Veillonella, Prevotella, Actinomyces and Rothia were the dominant genera. Haemophilus formed only 3% of the healthy microbiome. In contrast, Proteobacteria was the most dominant phylum accounting for 50% of the microbiome in COPD subjects, with Haemophilus and Moraxella at genus level contributing 25 and 3% respectively. There were no differences in the microbiome profile within healthy and COPD subgroups when stratified based on smoking history. Principal coordinate analysis on operational taxonomic units showed two distinct clusters, representative of healthy and COPD subjects (PERMANOVA, p = 0.001). Conclusion: The healthy and COPD sputum microbiomes are distinct and independent of smoking history. Our results underline the important role for Gammaproteobacteria in COPD.

Keywords: COPD, Healthy airway, Microbiome, Haemophilus, Proteobacteria

Background

Chronic obstructive pulmonary disease (COPD) is characterised by inflammation and irreversible airflow obstruction. Before the advent of culture-independent DNA profiling methods, the healthy lung was deemed a sterile niche while COPD samples would frequently culture Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis and Pseudomonas aeruginosa. It is considered that pathogenic bacteria gain a foothold in damaged airways contributing to further lung pathology through release of noxious bacterial products and provocation of host inflammation [1, 2].

In recent years, use of high-throughput 16S rRNA gene based sequencing has demonstrated that rich, complex bacterial communities exist in the airways of both health and COPD, with overlapping bacterial communities. Use of these methods demonstrated a distinct microbiome in COPD patients compared to health volunteers.

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composition observed [3, 4]. In COPD aerobic, facultative and anaerobic organisms colonise the airways [3, 5], with *Proteobacteria* and *Firmicutes* being the two major phyla reported in the microbiome and *Haemophilus* and *Streptococcus*, the respective dominant genera [3, 4, 6, 7]. At exacerbation, shifts in bacterial composition, characterised by a relative increase in *Proteobacteria* that falls in response to antibiotics has been observed, suggesting an association with the aetiology of COPD exacerbations [8, 9]. Furthermore, the ratio of *Gammaproteobacteria* to *Firmicutes* identifies the subgroup with dynamic changes in their microbiome during exacerbation, suggesting a potential use of this ratio as a biomarker for targeting antimicrobial treatment [10].

While *Proteobacteria* have been associated with COPD exacerbation events, the role of the microbiome in the stable state and important differences in composition with health are unclear [3, 4, 6]. Furthermore, changes in the microbiome that may associate with development of COPD in smokers are unclear. Differences have been shown in the nasal and oropharyngeal microbiome between smokers and non-smokers [11] but microbiome data from healthy smokers and non-smokers is limited making the findings inconclusive to contextualize the pathological basis of the observations in COPD.

To address this, we have used sputum collected from a substantial number of well characterised healthy volunteers to investigate the impact of smoking on healthy lower airway microbiome; to explore if there are differences in microbiome between health and COPD and its association with smoking in health.

**Methods**

**Subjects and study samples**

Healthy volunteers (*n* = 251) (excluded participants with asthma, COPD or bronchiectasis) were selected from the Extended Cohort for E-health, Environment and DNA (EXCEED) cohort and assessed at a single centre, Glenfield Hospital, Leicester. Participants with ≥10 pack year (PY) smoking were grouped as healthy smokers and remaining as occasional / never smokers (< 10 PY) [12]. Participant demographics and clinical characteristics including lung function, blood and sputum cell differentials were recorded. The comparator COPD group comprised of a single stable (non-exacerbation) visit sputum collected from 218 subjects (included ex- and current smokers) at three centres, Leicester, London and Manchester, as part of the COPDMAP consortium ([www.copdmaph.org](http://www.copdmaph.org); NCT01620645) [13]. All the participants in both studies had a minimum of 6 weeks antibiotic free period before their sample collection. Sputum induction was undertaken if an adequate spontaneous sample was not produced. In most of the healthy participants induced sputum was collected. Both cohort studies had ethical approval and all subjects gave written informed consent before the performance of any study-related assessments.

All patients provided written informed consent using protocols approved by the local Ethics Committees at each site (London- 11/L0/1630; Manchester- 10/H/1003/108; Leicester- 07/H0406/157).

**16S rRNA gene sequencing**

As a part of standard routine, for both studies, sputum plugs were separated from the salivary contents to minimise the oral bacterial contamination in samples. Samples with only salivary contents were not processed for microbial work. Sputum plugs, were stored (~ 80 °C) and processed for high-throughput sequencing similar to the COPD cohort samples [13]. Briefly, bacterial genomic DNA was extracted from the homogenised (0.1% dithiothreitol) plugs using the lysozyme-based lysis procedure from Qiagen DNA Mini kit (Qiagen, CA, USA) as per manufacturer’s protocol. Out of 251 samples only 137 had adequate DNA concentration for microbiome analysis. Amplicon library was generated utilizing 28 PCR cycles and targeting the V4 hypervariable region of the 16S rRNA gene with 515F: 5′ GTGCCAGCMGCC GCGGTAA3′, 806R: 5′GGACTACHVGGGTWTCTTAAT3′ primers, including Illumina sequencing adapters and a 12 bp Golay barcode sequence attached to forward primer. Pair-ended sequencing was performed using multiplex libraries on the Illumina MiSeq platform. Sequencing run included a commercial mock community DNA (ZymoBIOMICS microbial DNA standard) as a positive control and DNA extraction negative control (each batch of healthy sample DNA extraction included a DNA extraction negative control and a single pooled aliquot was prepared from all of these controls for sequencing) and PCR negative control for reagent contamination check. PCR negative controls didn’t produce any reads and DNA extraction negative control only produced 8 raw reads and therefore were not processed further.

COPDMAP single stable samples, utilized here for comparative analysis with healthy samples, had their sequence data generated as part of that study along with appropriate sequencing controls [13] and the sequence data are deposited at the National Centre for Biotechnology Information Sequence Read Archive (SRP102480).

**Microbiome analysis**

Reads were processed using QIIME pipeline version 1.9.1 [14] after adaptor trimming and removing low quality reads with Trimmomatic 0.36 [14, 15]. Pair-ended sequences were joined using fastq-join with a minimum 10 bp overlap [16]. The joined sequences were filtered with a Phred score ≥ 20 and processed to remove
contaminating sequences and chimeras using UCHIME [17]. Sequence reads are deposited at the National Centre for Biotechnology Information (SRA accession: PRJNA491861) https://www.ncbi.nlm.nih.gov/bioproject/PRJNA491861/.

Based on rarefaction curves, healthy and COPDMAP samples, were normalised to 11,000 reads sequencing depth leaving 124 samples in healthy and 218 samples in COPD MAP for microbiome analysis. Operational taxonomic units were generated at 97% sequence identity using close reference (OTU) method and Greengenes database (version 13.8) and assigned taxonomic identities with the RDP classifier [18]. Alpha diversity index was generated based on the number of OTUs (oberved_OTUs and Chao1-richness measure) and their distribution (Shannon index- diversity measure) within a sample. Beta diversity index was based on weighted UniFrac distance measure [19] (phylogenetic distance-based) between OTUs present in each sample and visualized using PCoA plots.

Microbial function prediction through 16S rRNA gene sequences
Predictive microbial functional profile was generated using the PICRUSt software (v1.0.0) which infers the pathway content of the microbiome by assigning bacterial functional genes for the OTUs, normalised for 16S rRNA gene copy number, using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [20]. Statistical analysis and visualisation plots were generated using Statistical Analysis of Metagenomic Profiles (STAMP) software [21].

Statistical analysis
Univariate statistical analyses were performed using GraphPad Prism (Version 7, San Diego, CA). Parametric and nonparametric data are presented as mean (SEM) and median (interquartile range) respectively. Most of the dataset did not meet the normal distribution criteria (Shapiro-wilk test). Therefore, non-parametric Mann-Whitney test and Kruskal-Wallis (KW) test were performed for between-group comparison of two or greater than two groups respectively. For the KW test, Dunn’s multiple comparison test was used to determine statistical significance of pair-wise comparisons. For parametric data, equivalent parametric statistical tests were performed. For categorical data, Chi-square test was performed.

PERMANOVA [22] was performed to test if the overall healthy microbiome was different to COPD using weighted UniFrac distance measure as input. The individual OTUs contributing to differences in health and COPD were identified by performing a non-parametric group-significance test and a corrected p-value of ≤0.05 for multiple testing conditions was considered statistically significant. To rank the discriminating taxonomic groups between health and COPD, linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed [23], which detects taxonomies with differential abundance (p ≤ 0.05) using the KW test and then assigns a LDA based effect size score. A threshold of ≥3.6 LDA score was used instead of default value of 2 to feature the most discriminant bacterial groups [24]. To determine if any individual microbiome constituents were associated with any subject metadata, MaAsLin was performed [25] and Benjamini-Hochberg (FDR) corrected q < 0.05 (FDR-adjusted P value) for multiple comparisons was considered statistically significant.

Results
Table 1 summarizes the demographics and clinical features of the healthy and COPD cohorts (refer to ‘Methods’ section for more details on both the cohort selection criteria).

Healthy sputum microbiome
A total of 1424 OTUs at 97% sequence identity were observed in 124 healthy samples after rarefaction. Most OTUs belonged to Firmicutes (55% ± 13%) followed by Bacteroidetes (21% ± 11%) and Actinobacteria (12% ± 6%) phyla (Fig. 1a). Streptococcus (30% ± 13%) was the most abundant genus followed by Veillonella (17% ± 9%), Prevotella (16% ± 10%), Actinomyces (6% ± 5%), Rothia (5% ± 4%) and Granulicatella (3% ± 3%) (Fig. 1b). The Proteobacteria phylum constituted 7% (± 7%) of the bacterial community with Haemophilus (3% ± 5%) as its dominant genus.

Compared to participants with a smoking history of <10 pack year (PY), the subgroup with ≥10PY showed a higher proportion of Firmicutes and a lower proportion of Bacteroidetes at phylum level (Fig. 1a); followed by higher Streptococcus and lower Prevotella at the genus level but these differences did not reach statistical significance (Fig. 1b). Beta diversity based principal coordinate analysis (PCoA) plots did not reveal distinct microbiome clusters for the two smoking subgroups (Fig. 1c). Shannon index was higher (p < 0.01) in healthy subjects with <10 PY smoking history compared with ≥10 PY, suggesting a more diverse microbiome in the former (Fig. 1d) but there was no significant difference in observed_OTUs or Chao1 index.

COPD MAP stable sputum microbiome
A total of 2329 OTUs at 97% sequence identity were observed in 124 stable COPD samples after rarefaction. The Proteobacteria (51% ± 12%) phylum constituted half of the COPD microbiome with Haemophilus (25% ± 8%) as its most abundant genus followed by Erwinia (7% ±
### Table 1 Healthy and COPD subject characteristics

|                      | Healthy control (n = 124) | Healthy control PY ≥ 10 (n = 28) | Healthy control PY < 10 (n = 96) | COPD (n = 218) | p-value* |
|----------------------|---------------------------|----------------------------------|----------------------------------|---------------|----------|
| Gender female (n)    | 73                        | 15                               | 58                               | 60            | <0.0001  |
| Age (years)          | 61 (54, 67)               | 65 (58, 69)                      | 59 (53, 67)                      | 69 (64, 74)   | <0.0001  |
| BMI                  | 27.7 (24.3, 31.2)         | 29.3 (25.8, 34.4)                | 27.3 (24.2, 29.9)                | 26.7 (23.2, 30.06) | 0.0325  |
| Smoking history (Pack years) | 0 (0, 8)     | 29 (17.3, 45.8)                 | 0 (0, 0)                         | 46 (34.64)    | <0.0001  |
| MRC Dyspnoea scale   | 0 (0, 1)                  | 0 (0, 1)                         | 0 (0, 1)                         | 2 (1.2)       | <0.0001  |
| VAS dyspnoea (mm scale) | 0 (0, 2)       | 0 (0, 2)                         | 0 (0, 2)                         | 30 (15.49)    | <0.0001  |
| Post BD FEV1 (L)     | 2.87 (2.43, 3.37)         | 2.82 (2.33, 3.24)                | 2.88 (2.46, 3.40)                | 1.45 (1.03, 1.86) | <0.0001  |
| Post BD FEV1% Predicted | 109 (100, 118)  | 105 (92, 118)                   | 110 (100, 118)                   | 57 (42, 69)   | <0.0001  |
| Post BD FEV1/FVC %   | 80 (77, 83)               | 78 (75,82)                       | 80 (78,83)                       | 51 (41,59)    | <0.0001  |
| Blood White cell count (×10^9/L) | 5.8 (4.9, 7.3) | 6.4 (5.1, 7.5)                 | 5.7 (4.8, 7.25)                  | 7.2 (6.3,8.8) | <0.0001  |
| Blood Neutrophil count (×10^9/L) | 3.3 (2.7, 4.2) | 3.8 (2.8, 4.3)                 | 3.2 (2.6, 4.1)                   | 4.7 (3.9, 5.7) | <0.0001  |
| Blood Eosinophil count (×10^9/L) | 0.18 (0.09, 0.23) | 0.14 (0.09, 0.22)             | 0.21 (0.14, 0.27)               | <0.0001      |
| Sputum TCC (×10^6/g) | 0.24 (1.1, 4.2)           | 2.19 (16.5, 9.0)                | 2.2 (1.3, 6.0)                   | 10.5 (4.7,13.0) | <0.0001  |
| Sputum eosinophils % | 0.25 (0.075)              | 0.25 (0.18, 2.38)               | 0.25 (0.075)                     | 0.75 (0.2)    | 0.0029   |
| Sputum neutrophil (%) | 60 (43, 74)               | 65 (42, 75)                      | 56 (43, 73)                      | 75 (40, 89)   | 0.0011   |

Data is expressed as median (IQR). BMI body mass index, Sputum TCC sputum total cell count, FEV1 forced expiratory volume in 1 s, Post BD FEV1 spirometry recording post bronchodilator; FVC forced vital capacity. * = represents Kruskal-Wallis test comparing between healthy (PY ≥ 10), healthy (PY < 10) and COPD smokers with ≥10 PY smoking history showed a trend towards a higher ratio of Streptococcus to Prevotella.
Fig. 1 Microbiome profile of Healthy volunteers based on smoking pack year history. a Relative abundance of major phyla between all healthy (n = 124) represented in the outer ring followed by healthy < 10 PY smoking history subgroup (n = 96) in the middle ring and innermost ring representing healthy ≥10 PY history subgroup (n = 28). b Relative abundance of major genera between all healthy (n = 124) represented in the outer ring followed by healthy < 10 PY (n = 96) in the middle ring and innermost ring representing healthy ≥10 PY history (n = 28). c Principal coordinate analysis (PCoA) analysis of weighted unifrac distance measures relative to pack year history. d Alpha diversity indices comparison between < 10 PY and ≥10 PY smoking sub-groups. Chao1 and observed_ots are represented as bar chart as mean and standard deviation; Shannon index is represented by box whisker plot showing median, interquartile range and minimum and maximum. ** P < 0.01
Existing comparative respiratory microbiome data are divided, with some studies reporting an overlapping microbial composition between health and COPD [3, 4, 26] while others have shown the COPD microbiome to be distinct [5, 6, 27]. These discordant outcomes likely reflect the underlying heterogeneity in COPD groups and small sample sizes of healthy individuals (< 20), undermining the strength of these studies [3–6, 26, 27]. However, similar to our observation, higher levels of Proteobacteria, especially Haemophilus, in COPD [3–5] and relatively higher proportion of Prevotella, Veillonella and Actinomyces species in health have been observed [5, 6] but differences did not reach significance. Contrary to our observations, most studies have reported similar or a
higher abundance of *Firmicutes* and especially *Streptococcus* in COPD compared with health [4, 6]. One reason for this might be that composition varies between samples depending upon the type of treatment received, disease severity and inflammation. Previous studies have shown association between very severe COPD and eosinophilic phenotypes with dominance of *Firmicutes*, while *Proteobacteria* are predominant in moderate COPD and the bacterial related phenotype [9, 28]. The COPD cohort analysed here was mainly of moderate-to-severe severity with high neutrophil counts.

Contrary to most studies, we found a higher alpha diversity in COPD compared to health [3, 6]. Although the COPD sample reads were reanalysed with the healthy at a normalised sequence depth, they were sequenced as part of COPDMAP study which involved a much larger
sample size [13], including different disease stages, and this may have contributed higher COPD diversity. Moreover, our COPD cohort was older than the healthy group and from moderate to severe GOLD stage. Higher diversity has been associated with both increasing disease severity and age in COPD [4, 27, 29].

Although a strong association exists between smoking and both airway inflammation and COPD, the determinants for developing COPD in smokers are not clear. We hypothesised that smoking associated microbiome changes in health would help in understanding the role of microbes in transition from health towards COPD. Consistent with previous studies, we found no significant difference between the microbiome of smokers with ≥10PY history and the < 10 PY group [3, 4, 6, 30]. However, similar to Morris and colleagues [30], a trend towards lower proportions of both Bacteroidetes and Proteobacteria in smokers with ≥10PY history was observed, suggesting subtle effects of smoking on the airway microbiome. Other pathological factors may therefore be important in shaping the microbiome in COPD. Hypoxia and chronic systemic inflammation related factors, which are features of COPD, have been reported to be associated with the airway microbiome [31] and may be relevant to the differences observed in our COPD cohort.

PICRUSt analysis showed relatively higher lipopolysaccharide biosynthesis products in COPD. Lipopolysaccharides are present in the outer membrane of Proteobacteria and together with pathogen-associated
molecular patterns, induce strong and damaging pro-inflammatory responses. In keeping with this, our previous study showed that sputum chemokine interleukin-8, known to play a key role in COPD inflammation, is positively correlated with *Haemophilus* and *Moraxella*, suggesting these bacteria trigger the excessive production of this chemokine [9]. Moreover, *Haemophilus* has been implicated in a dysbiotic role by co-inclusion of its related phylotypes and depletion of *Firmicutes, Bacteroidetes* and *Actinobacteria* that are involved in pathways for production of anti-inflammatory compounds [8, 9].

Although antibiotic treatment has been associated with suppression of *Proteobacteria* in COPD [8, 9], it is not true for all cases [10]. With the increasing urgency for effective antibiotic stewardship, research is needed to better understand the impact of both acute and long term antimicrobial therapy on the COPD microbiome. In this respect, alternate therapeutic strategies such as *H. influenzae* vaccination, or highly selective antimicrobial approaches such as phage therapy may effectively reverse some dysbiotic with prognostic benefit.

A limitation of this study is that the lung microbiome has been analysed from sputum samples which can be contaminated with the microbiome of the oropharynx. However, we emphasise that this effect will have been limited by sputum plug selection for the analysis. We did not perform longitudinal sampling to demonstrate reproducibility of the sputum microbiome over time in healthy participants. For COPD we have previously demonstrated that the sputum microbiome is comparable between time-points when sampling at their stable state [32]. The effects on the microbiome of using sputum induction as the predominant sampling technique in the healthy control group are also not known, but it is noteworthy that the predominant bacterial constituents of our healthy microbiome are consistent with the respiratory microbiome detected by investigating BAL and bronchial samples reported in previous studies [3, 6]. This suggests that our observations are robust and representative of the bacterial composition of the lung microbiome. A major incentive to work with sputum is its compatibility with routine clinical practice as any findings are therefore more readily translated into established care pathways. In this study we have not characterized the viral and fungal communities, and this will be important to understand their role in health and disease.

**Conclusions**

In summary, clear and significant differences exist between the lung microbiome in health and COPD, with dysbiosis in COPD characterised by increased abundance of *Proteobacteria* especially *Haemophilus*. The changes observed in COPD are distinct from the microbiome in smokers without COPD, suggesting an association between airway damage and dysbiosis. The
pathogenesis and pathological significance of dysbiosis in COPD remains unclear. Longitudinal studies are needed to determine whether, and to what extent, the onset and progression of COPD are attributable to an altered lung microbiome.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12931-020-01448-3.

Fig. 5 Predictive functional profiling shows distinct clustering of COPD and Healthy sputum microbiome. a PCA analysis of functional groups inferred from 16S rDNA microbial community. b lists the top 19 functional groups which were significantly different (p < 0.05, multiple comparison corrected) and had > 0.2% difference between COPD and healthy subjects.

Abbreviations

COPD: Chronic obstructive pulmonary Disease; EXCEED: Extended Cohort for E-health, Environment and DNA; KW: Kruskal-Wallis; MaAsLin: Multivariate analysis by linear models; OTU: Operational taxonomic unit; PY: Pack year smoking history; PCoA: Principal coordinate analysis; PERM ANOVA: Permutation multivariate analysis of variance; SEM: Standard error mean; LefSe: Linear discriminant effect size

Acknowledgements

The EXCEED study gratefully acknowledges the support of all participants and staff who have contributed to the study. The study has been supported by the University of Leicester, Leicester City Council, the NIHR Leicester Biomedical Research Centre, the NIHR Clinical Research Network East Midlands, and the Medical Research Council (grant G0902313 to MDT) and the Wellcome Trust (grant 202849 to MDT).
Authors’ contributions
KH undertook the data analysis and statistical analysis. LG and VM undertook patient recruitment, data collection, and were involved in data analysis. KH and MRB led the microbiological assessments. Samples and analyses were contributed by LG, CJ, NFR and MT [EXCEED], and ZW, JRB, DS, JAW, GCD, BEM and RTS [COPDMAP] study. RF and A.J.W were responsible for data management. CEB led the design of the study, data collection, data interpretation, data analysis and had full access to the data and was responsible for the integrity of the data and final decision to submit. All the authors contributed to drafting the manuscript or revising it critically for important intellectual content; all authors gave final approval of the version to be published; and all authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding
This study was part supported by the MRC [COPDMAP], National Institute for Health Research Leicester Biomedical Research Centre, UK, AirPROM (FP7 – 270194). MDT is in receipt of a Wellcome Trust Investigator Award (202849). CJ holds a MRC Clinical Research Training Fellowship (MR/P00167X/1). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Availability of data and materials

Exceed healthy sample 16 rRNA gene sequence reads are deposited at the National Centre for Biotechnology Information (SRA accession: PRJNA491861) https://www.ncbi.nlm.nih.gov/bioproject/PRJNA491861. COPDMAP 16S rRNA gene sequence data are deposited at the National Centre for Biotechnology Information Sequence Read Archive (SRP102480) and associated data available from (https://www.ncbi.nlm.nih.gov/search/all/?term=SRP102480).

Ethics approval and consent to participate
All patients provided written informed consent using protocols approved by the local Ethics Committees at each site (London- 11/L0/1630; Manchester- 10/H1/003/108; Leicester- 07/H0406/157) for COPDMAP study and the EXCEED study ethics number is: 13/EM/0226.

Consent for publication
Not applicable.

Competing interests
KH, LG, ZW, VM, MYR, RFC, NFR, AWJ, AJB, MRB have nothing to declare. JRB, BEM and RTS are employees and shareholders of GSK; GCD reports grants and personal fees from AstraZeneca and grants from Microm Ltd and American Thoracic Society; MDT reports grants from GSK and other from Orion; JAW reports grants from GSK, grants from Johnson and Johnson, other from Novartis, other from Boehringer Ingelheim, other from Astra Zeneca, other from GSK, grants from GSK, grants from Astra Zeneca, grants from Boehringer Ingelheim, grants from Novartis; DS reports grants and personal fees from GlaxoSmithKline, grants and personal fees from AstraZeneca, grants and personal fees from Boehringer Ingelheim, grants and personal fees from Chiesi, personal fees from Cipla, personal fees from Menarini, grants and personal fees from Mundipharma, grants and personal fees from Novartis, personal fees from PepiniNovatec, grants and personal fees from Pfizer, grants and personal fees from Fulmatis, grants and personal fees from Theraveance, grants and personal fees from Verona; CEB reports grants and personal fees from GSK, grants and personal fees from Novartis, grants and personal fees from Genentech, grants and personal fees from GSK, grants and personal fees from Chiesi, personal fees from Sanoft/Regeneron, grants and personal fees from 4DPharma, grants and personal fees from Bi, grants and personal fees from Molicongs, grants and personal fees from Gossamer, grants and personal fees from AZ/MedImmune, grants and personal fees from TEVA, outside the submitted work. CEB reports grants from MRC [COPDMAP], grants from AirPROM (FP7 – 270194), during the conduct of the study.

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Received: 9 April 2020 Accepted: 8 July 2020
Published online: 14 July 2020

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