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The Sputum Microbiome, Airway Inflammation and Mortality in Chronic Obstructive **Pulmonary Disease**

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The Sputum Microbiome, Airway Inflammation and Mortality in Chronic Obstructive Pulmonary Disease

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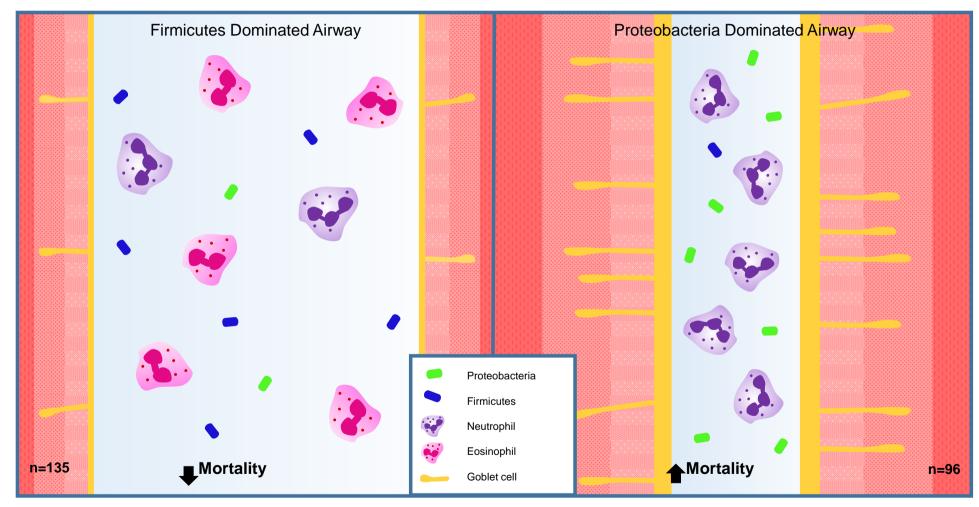
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Microbiome, Airway Inflammation and Mortality in COPD

n=253





1	The Sputum Microbiome, Airway Inflammation and Mortality in Chronic Obstructive Pulmonary
2	Disease
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13	
14	
15	Conflicts of interest
16	JDC reports grants from Glaxosmithkline (GSK) for this research. In addition, he reports grants and
17	personal fees from Glaxosmithkline, Boehringer-Ingelheim, AstraZeneca, Pfizer, Bayer Healthcare,
18	Grifols, Napp, Aradigm corporation, and Insmed outside the submitted work. HM and RTS were
19	employees of GSK at the time this study was conducted. RTS is a shareholder of GSK. BEM is an
20	employee and shareholder of GSK. The other authors declared no conflicts of interest.
21	
22	Word count: 3311

23	Abstract
24	Background: The sputum microbiome has a potential role in disease phenotyping and risk stratification
25	in chronic obstructive pulmonary disease but few large longitudinal cohort studies exist.
26 27	Objective: To investigate the COPD sputum microbiome and its association with inflammatory phenotypes and mortality.
28	Methods: 16S rRNA gene sequencing was performed on sputum from 253 clinically stable COPD
29	patients (4-years median follow-up). Samples were classified as Proteobacteria or Firmicutes (phylum
30	level) and Haemophilus or Streptococcus (genera level) dominant. Alpha diversity was measured using
31	Shannon-Wiener Diversity and Berger-Parker Dominance Indices. Survival was modelled using Cox
32	proportional hazards regression. A subset of 78 patients had label-free liquid chromatography/mass
33	spectrometry performed, with partial least square discriminant analysis integrating clinical, microbiome
34	and proteomics data.
35	Results: Proteobacteria dominance and lower diversity was associated with more severe COPD using the
36	GOLD classification system (p=0.0015), more frequent exacerbations (p=0.0042), blood eosinophils
37	\leq 100cells/ μ L (p<0.0001) and lower FEV ₁ (p=0.026). Blood eosinophil counts showed a positive
38	relationship with %Firmicutes and Streptococcus, and a negative association with %Proteobacteria and
39	Haemophilus. Proteobacteria dominance was associated with increased mortality compared to Firmicutes
40	dominated or balanced microbiome profiles (HR 2·58 95%CI 1·43-4·66, p=0·0017 and HR 7·47 95%CI
41	1.02-54.86, p=0.048 respectively). Integrated omics analysis showed significant associations between
42	Proteobacteria dominance and the neutrophil activation pathway in sputum.
43	Conclusion : The sputum microbiome is associated with clinical and inflammatory phenotypes in COPD.
44	Reduced microbiome diversity, associated with Proteobacteria (predominantly Haemophilus) dominance,
45	is associated with neutrophil associated protein profiles and an increased risk of mortality.
46	
47	Capsule summary: Microbiome analysis of sputum reveals distinct endotypes of COPD with different
48	inflammatory profiles and long term survival.
49	
50	Key Messages:

• The sputum microbiome in COPD is associated with clinical and inflammatory phenotypes

52	• Domir	nance and loss of microbial diversity are associated with increased mortality in COPD	
53	Microbiome and inflammatory profiles are linked, with Proteobacteria dominant profiles being		
54	associated with neutrophil activation markers, and Firmicutes dominant profiles being associated		
55	with ra	aised blood eosinophil counts.	
56			
57	Keywords: M	icrobiome, COPD, Eosinophil, Phenotype, Haemophilus,	
58			
59	Abbreviations	S	
60	aHR:	Adjusted hazards ratio	
61	BPDI:	Berger-Parker dominance index	
62	BMI:	Body mass index	
63	CAT:	COPD assessment test	
64	COPD:	Chronic Obstructive Pulmonary Disease	
65	DNA:	Deoxyribonucleic acid	
66	FDR:	False discovery rate	
67	FEV ₁ :	Forced expiratory volume in 1 second	
68	FVC:	Forced vital capacity	
69	GOLD:	Global initiative for chronic obstructive lung disease	
70	HR:	Hazard ratio	
71	ICS:	Inhaled corticosteroids	
72	LC-MS/MS:	Liquid chromatography with tandem mass spectrometry	
73	MRC:	Medical research council	
74	OTU:	Operational taxonomic unit	
75	QIIME:	Quantitative insights into microbial ecology	
76	RNA:	Ribonucleic acid	
77	SGRQ:	St Georges respiratory questionnaire	
78	SWDI:	Shannon-Wiener diversity index	
79	TARDIS:	Tayside allergic and respiratory disease information system	
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Introduction

Chronic Obstructive Pulmonary Disease (COPD) is recognized as a heterogeneous disease with patients manifesting multiple diverse phenotypes, endotypes and "treatable traits".(1-4) Recent therapeutic concepts in COPD have moved away from the previous "one size fits all" treatment approach with bronchodilators, inhaled corticosteroids (ICS) and other medications, towards a precision medicine approach in which clinical characteristics and biomarkers are used to direct treatments and to optimize their risk: benefit profile.(5-8) A recent example of this is the use of blood eosinophil counts to direct treatment with inhaled corticosteroids.(7, 8) The use of a blood biomarker to guide treatment has gained acceptance due to identification of a relationship between blood and sputum eosinophil counts. Randomized controlled trials have subsequently shown that patients with blood eosinophil counts less than 100cells/µL derive no significant benefit in terms of exacerbation reduction when treated with ICS, and may be at increased risk of pneumonia.(6, 9, 10)

Eosinophilic COPD is an example of an endotype, a subtype of a condition defined by a distinct pathophysiological mechanism.(2) Inflammation, however, is only one of the pathophysiological features

pathophysiological mechanism.(2) Inflammation, however, is only one of the pathophysiological features of COPD which is associated with airway structural damage and airway infection.(11, 12) Bacteria are thought to play a key role in COPD pathogenesis and recent data has brought a deeper understanding of the complex microbial communities present in the lung affected by COPD.(11, 13-16) Studies of the COPD "lung microbiome" to date have found that as COPD becomes more severe in terms of lower lung function, there is an increase in the relative abundance of Proteobacteria, and particularly the genus *Haemophilus*.(11, 13, 15) Lower microbiome diversity is also associated with increased severity of disease and neutrophilic inflammation including the formation of neutrophil extracellular traps.(11, 13, 15)

Exacerbations are key events in the natural history of COPD and recent work suggests no consistent pattern of altered microbial profiles at exacerbation compared to stable disease.(15) Previous work suggests there are at least four different exacerbation "endotypes" associated with distinct inflammatory profiles - bacterial exacerbations, eosinophilic exacerbations, virus predominant exacerbations and pauci-inflammatory events.(17) These events, defined by clinical and inflammatory criteria, may also have distinct microbiome profiles.(18)

Lower lung function and frequent exacerbations may relate to endotypes linked to greater mortality in COPD.(19-21) If changes in the microbiome loss of microbial diversity are indeed related to more severe disease and high risk patient subtypes, it is plausible that the respiratory microbiome may associate with

- long term mortality as has been observed in idiopathic pulmonary fibrosis.(22) No studies have assessed
- the association of the sputum microbiome profile with mortality in stable COPD.
- Here we report the results from a longitudinal cohort study of COPD patients designed to integrate data
- from the sputum microbiome and proteome with clinical phenotypes and long-term clinical outcomes, to
- provide further insights into the relevance of the respiratory microbiome in clinical practice.

117 Methods

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118 **Study Design**

119 We performed a longitudinal observational study of patients with a diagnosis of COPD nested within a population based COPD registry (Tayside Allergic and Respiratory Disease Information System (TARDIS)) in the East of Scotland.(16, 20) Patients were invited to participate in a microbiome substudy and were included if they were >40 years, had a FEV₁/FVC ratio <70% at screening, and a clinical diagnosis of COPD. Exclusion criteria included the inability to give informed consent; primary diagnosis of asthma; and systemic immunosuppression (excluding prednisolone at 5mg or less daily). Additionally, patients needed to be clinically stable and free of antibiotic or oral corticosteroid therapy for > 4 weeks 126 prior to enrolment. All relevant medical history (comorbidities, current medications, significant past conditions, operations and diagnostic procedures) was recorded at screening. Participants provided induced sputum samples following induction with 3% hypertonic saline. Spirometry, St Georges Respiratory Questionnaire (SGRQ), COPD assessment test (CAT) and MRC dyspnoea scoring was performed at each visit. Exacerbations in the study period were defined as an increase in respiratory symptoms greater than day to day variation requiring a change in therapy; participants returned to the 132 clinic for assessment and were given a standardized treatment of antibiotics and corticosteroids. Patients 133 were enrolled from 2013-2015 and survival data was obtained from linked medical records within the TARDIS registry as described previously.(16)

135 Clinical phenotypes

- 136 A-priori, we identified clinical phenotypes that have been linked with response to therapies in COPD.
- 137 These were low blood eosinophil counts (examined using a cut-off of ≤100cells/µL according to recent
- 138 Global Initiative for Chronic Obstructive Lung Disease (GOLD) recommendations and linked to ICS
- 139 response),(23) chronic bronchitis (defined as daily sputum production at least three months of the year
- 140 over at least two years and linked to response to treatments including roflumilast) and, finally, the
- 141 frequent exacerbator phenotype, defined as patients with ≥ 2 exacerbations/year.(9, 24) Clinical
- 142 phenotypes were not mutually exclusive.

Sputum Microbiome

- 144 DNA was extracted from whole sputum as described in the online supplement, followed by 16S rRNA
- 145 gene sequencing on the Illumina MiSeq platform. Bioinformatic analysis and quality checking of the
- 146 resulting sequences was performed using QIIME (version 1.9.0) and R (version 3.4.0). Shannon-Wiener
- Diversity Index (SWDI, where a higher value indicates a sample is more diverse) and Berger-Parker 147

- Dominance Index (BPDI, which measures the proportion of the microbiome dominated by the most
- abundant taxa, with a value closer to 1 indicating a microbiome with greater dominance) were used as
- measures of alpha diversity of samples. See online supplement for full methods.

151 Characterization of microbiome subtypes of COPD

- Prior studies of the COPD sputum microbiome identified that Proteobacteria and Firmicutes were the
- dominant phyla and that inflammation and COPD severity were strongly associated with the presence of a
- single genera at >40% relative abundance in many patients.(11, 13, 18) Therefore *a-priori* we defined
- candidate microbiome subgroups. At the phylum level samples were classified as either Proteobacteria
- dominant or Firmicutes dominant based on >40% Observed Taxonomic Units (OTUs) of either phylum.
- For analysis at the genera level we classified samples into Haemophilus dominant and Streptococcus
- dominant (as the most abundant Firmicute); "balanced" microbiome profiles were defined by the absence
- of >40% OTUs of either Proteobacteria or Firmicutes at the phylum level or no individual OTU
- exceeding 40% at the genera level. The cut-off of 40% OTUs to define these groups was used based on
- our previous work.(11) Throughout the manuscript we refer to the respiratory microbiome to reflect that
- all assessments are made on sputum which includes contributions from the lower and upper respiratory
- 163 tracts.

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Integration of microbiome, clinical and proteomic data

- A subset of 78 patients included in the primary study provided sufficient sputum samples for proteomics
- with the results integrated with their microbiome and clinical data. The total protein concentrations of
- sputum supernatants were quantified using Pierce 660 protein assays. Sputum protein (50µg) from each
- sample was added to an equal volume of acetonitrile before incubating at 100°C for 15mins. The samples
- were dried down in a centrifugal vacuum and resuspended with 50 mM ammonium bicarbonate (pH 8·5)
- to a final concentration of 1mg/mL. Samples were then reduced and alkylated before subjecting to nano-
- 171 flow-LC-MS/MS analysis. Protein identification and label-free quantification were carried out using
- Maxquant (version 1.4.1.2) against Uniprot-human database (version 2014-07-09). The fixed
- modification was carbamidomethylation on cysteine, and variable modifications include oxidation on
- methionine and N-terminal acetylation. False Discovery Rate (FDR) for protein identification was set to
- 175 1% at protein level.

Statistical analysis

- 177 Statistical analysis of data was carried out using R version 3.4.0, SPSS version 21 and GraphPad Prism
- 178 6.07. Continuous data are presented as median (interquartile range) and categorical data are presented as
- 179 N (%). Continuous parametric data were compared using unpaired T-test while continuous non-

parametric data were compared using the Mann-Whitney U test. Generalized linear models with binomial errors and a logistic link function were used to model the relationship between blood eosinophils and relative abundance. Principal component analysis was used to analyse microbiome beta diversity. Unadjusted survival was studied using Kaplan-Meier survival analysis. Cox Proportional Hazards models incorporating age, sex, baseline FEV₁ and exacerbation frequency in the previous year were used to model survival with the proportional hazards assumption checked using log-minus-log plots. Integrated omics analysis was performed using partial least square discriminant analysis with FDR correction using the Benjamini-Hochberg method, followed by a separate univariate analysis to confirm the results and to avoid overfitting. Statistical significance was set at P<0·05 for all other analyses.

189	Results		
190	296 participants were enrolled in the study; after excluding 44 patients whose baseline sputum samples		
191	failed DNA extraction and sequencing quality controls the final cohort was 252 participants (Figure 1)		
192	with the baseline characteristics of the cohort shown in Table 1. The predominant phyla observed in the		
193	microbiome were Proteobacteria and Firmicutes, with fewer OTUs identified as Bacteroidetes,		
194	Actinobacteria and Fusobacteria (Figure 2A). At the genus level the most abundant genera were		
195	Haemophilus, Streptococcus, Neisseria, Veillonella, and Prevotella, whilst some individual samples		
196	additionally showed a predominance of genera such as Pseudomonas, Stenotrophomonans, Pasteurella,		
197	Unknown Enterobacteriaceae, and Moraxella (Figure 2B).		
198	The non-eosinophilic phenotype has lower alpha diversity and increased relative abundance of		
199	Proteobacteria		
200	We observed a clear relationship between microbiome and clinical phenotypes. Proteobacteria dominated		
201	microbiomes (n=96) were associated with poorer lung function and more frequent exacerbations.		
202	Significantly more <i>Streptoccocus</i> dysbiotic microbiomes (n=56) were GOLD group D (p=0·011) and had		
203	a worse mMRC score compared to balanced genera (p=0·0017) (Figure S2).		
204	The low blood eosinophil subgroup (n=62) had lower microbiome diversity as measured by SWDI		
205	(p<0.0001) whilst the BPDI was higher in the low blood eosinophil subgroup (p<0.0001). These		
206	differences appeared to be associated with a higher relative abundance of Proteobacteria at the phylum		
207	level (p=0.0001) and $Hae mophilus$ at the genus level (p=0.046, Figure 3B). We observed a statistically		
208	significant negative correlation between % Proteobacteria and eosinophil count in peripheral blood		
209	(Spearman r correlation, p<0.0001, Figure 3C) and a positive correlation between $\%$ Firmicutes and		
210	eosinophil count (p<0.0001, Figure 3C). Further data are shown in Figure S3 online.		
211	Differences at both the phylum and genus level were observed when comparing patients who frequently		
212	exacerbate compared to non-frequent exacerbators (defined as those with ≤ 2 exacerbations/year) and		
213	comparing patients with chronic bronchitis compared to those patients without chronic bronchitis (Figure		
214	S4). Principle component analysis suggested that there was no clear clustering of microbiome based on		
215	either blood eosinophil group, chronic bronchitic status or exacerbation frequency.		
216	Microbiome profiles are linked to long term mortality		
217	50 participants ($19.8%$ of patients enrolled) died during a median follow-up of four years. At the genera		
218	level, $\textit{Haemophilus}$ dominated profiles (HR 2·53 95%CI 1·08-5·94, p=0·032) were associated with		
219	increased mortality compared to balanced profiles. Streptococcus dominated profiles were associated with		

- a similar mortality risk to *Haemophilus* when compared to balanced profiles (HR 2.05 95% CI 0.80-5.20,
- 221 p=0·13 Figure 4). The adjusted hazard ratios (aHR) were similar to the primary analysis (aHR 2·17
- 222 95%CI 0.89-5.27, p=0.09 and aHR 1.67 95%CI 0.64-4.37, p=0.30
- for *Haemophilus* and *Streptococcus* dominated profiles respectively).
- 224 At the phylum level, Proteobacteria dominance was associated with increased mortality compared to both
- Firmicutes dominated and balanced microbiome profiles (Hazard ratios (HR) 2.58 95%CI 1.43-4.67,
- 226 p=0.0017 and 7.47 95%CI 1.02-54.86, p=0.048, respectively). The relationship
- between Proteobacteria dominance and survival compared to Firmicutes persisted after multivariate
- 228 adjustment for age, sex, FEV₁ and previous exacerbation frequency, aHR 2·35 95%CI 1·27-4·32,
- p=0.006. Firmicutes dominated and balanced profiles were more similar to each other (HR 2.85 95%CI
- 230 0.38-21.43, p=0.31) (Figure 4). As *Streptococcus* dominated profiles appeared to behave differently to
- 231 others in the Firmicutes phylum, a sensitivity analysis was conducted comparing Proteobacteria
- dominated profiles to Firmicutes dominated profiles with Streptococcus excluded (Figure S5). This did
- 233 not greatly affect the results, with a clearly worse mortality associated with Proteobacteria dominance
- 234 (HR 2·93 95%CI 1.22-7·06 p=0.017, aHR 2·80 95%CI 1·13-6·90 p=0·026.
- 235 Lower alpha diversity was also strongly associated with long term outcome. The median SWDI for all
- samples was 3.36. Patients with values below this level had a markedly increased mortality (HR 4.05
- 95%CI 2·07-7·93, p<0·0001) which persisted after multivariate adjustment (aHR 3·08 95%CI 1·57-6·09,
- p=0.001). The median BPDI was 0.41 and values above this level were similarly predictive of mortality
- 239 (HR 3·81 95%CI 1·99-7·30, p<0·0001), aHR 3·36 95%CI 1·73-6·51, p=0·0003) (see online Figure S6).
- 240 Integration of clinical, microbiome and proteomic data shows that Proteobacteria dominated
- 241 microbiome profiles are associated with neutrophilic inflammation
- To investigate how microbiome profiles influence pathophysiology of COPD, we further investigated the
- sputum protein profiles by nano-flow-LC-MS/MS from a subset of patients (n=78) with three predefined
- 244 (Proteobacteria dominated (33), balanced (15), Firmicutes dominated (30)) sputum microbiome types. All
- patients with Proteobacteria and Firmicutes dominated profiles had greater than 50% OTUs of these phyla
- in their sputum sample. The characteristics of these patients are shown in Supplementary Table S1.
- 247 The combined dataset consisted of 113 taxa identified through the microbiome analysis, 21 clinical
- variables and 613 proteins. Partial least square discriminant analysis of the combined dataset revealed
- separation between Proteobacteria, balanced and Firmicutes microbiome profiles (R²X=0·17,
- 250 R²Y=0.73, Figure 5A). The loadings plot (Figure 5B, used to determine what variable(s) in the dataset

drive the separation between the different microbiome profiles) indicated that the Proteobacteria dominated microbiome cluster was associated with multiple proteins including myeloperoxidase, catalase, matrix metalloproteinase 9 and 8, and neutrophil elastase, all of which may be associated with neutrophilic inflammation. Further pathway analysis indicated that significantly upregulated proteins (Supplementary Table S2) associated with the Proteobacteria group are over-represented within the "neutrophil activation" pathway (p=2·2E-14, FDR corrected) adding further evidence to a possible association between Proteobacteria and neutrophilic inflammation. In contrast, the Firmicutes dominated microbiome cluster was associated with proteins such as Cystatin B (CSTB), Folate Receptor 1(FOLR1), Small Proline Rich Protein 3(SPRR3), Golgi Membrane Protein 1 (GOLM1), and Clusterin (CLU) (Figure 5B, black labels). Pathway analysis of significantly upregulated proteins (Supplementary Table S2) in the Firmicutes group showed an over-representation of the "negative regulation of peptidase activity" pathway (p=3·7E-4, FDR corrected) including cystatin B, Cysteine-S, alpha 1 antitrypsin, serpin B3 and WAP four disulphide core domain protein 2.

Discussion

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To the authors' knowledge, this is the first study to demonstrate that the sputum microbiome in stable COPD patients is associated with long term survival in a prospective cohort of patients; a recent study has linked the microbiome (reduced alpha diversity, increased Staphylococcus and reduced Veillonella) at acute exacerbation of COPD requiring hospitalization with increased mortality. (25) Taken together these studies suggest that the respiratory microbiome is associated with long term outcomes in COPD. In our study, sputum microbiome profiles at baseline were linked with clinical phenotypes, with exacerbation subtypes and ultimately with long term outcomes. Proteobacteria dysbiosis, defined by the dominance of one or more organisms including well recognized COPD pathogenic genera such as Haemophilus, Moraxella and Pseudomonas, was more frequent in patients with low blood eosinophil counts, chronic bronchitis symptoms and patients with frequent exacerbations. (26-28) These patients had worse lung function and ultimately increased mortality. In contrast, patients with Firmicutes dominance had milder disease, apart from those patients with dominance of the genera Streptococcus. Patients with dominance due to Streptococcus had a high level of disease severity with impairment of quality of life, lung function impairment and had equivalent mortality to patients with Haemophilus dominance. Neither Haemophilus nor Streptococcus was significantly associated with mortality compared to balanced profiles after adjustment for age, sex, FEV₁ and exacerbation history. In the case of *Haemophilus*, the HR was still greater than 2 after multivariable adjustment suggesting our study may have been underpowered. IWe, and others, have previously shown an association between Proteobacteria, Haemophilus and neutrophilic inflammation.(11, 29) We have now extended this observation by conducting an integrated analysis of microbiome, proteomic and clinical data which demonstrates a clear statistically significant association between Proteobacteria and neutrophilic inflammatory markers such as neutrophil elastase, myeloperoxidase and matrix metalloproteinases which are released from neutrophils during inflammatory responses. Although the pathway analysis and prior work suggests these are neutrophil associated, other cells such as macrophages may contribute to the release of matrix metalloproteinases and myeloperoxidase for example. Our data therefore suggests that while there is significant overlap in the phenotypic characteristics of patients with COPD, at each extreme of the spectrum are patients with Proteobacteria dominant profiles with eosinopenia, chronic bronchitis, neutrophil dominated inflammation, frequent exacerbations, poor lung function and reduced survival, and conversely Streptococcus dominant profiles associated with raised blood eosinophil counts, the absence of chronic bronchitis and frequent exacerbations. Whether these microbiome profiles link to response to treatments such as inhaled corticosteroids or anti-inflammatory drugs remains to be established but neutrophilic disease is established to be less responsive to inhaled corticosteroids.(12)

It is not possible from our analysis to disentangle cause and effect when considering the impact of the microbiome on clinical phenotype and clinical outcomes. Proteobacteria dysbiosis may be associated with a more rapid decline in FEV₁, or changes in the lung during remodelling may predispose to Proteobacteria dominance, or both statements may be true. Similarly, patients with raised eosinophil counts have an excess of Streptococcus. This may suggest that eosinophilic inflammation predisposes to Streptococcus infection/ colonisation, as has been demonstrated for other Firmicutes such as Staphylococcus, (30) or some members of the Streptococcus genera may provoke an eosinophilic response, or changes in the microbiome may be the result of external factors such as antibiotic treatment, inhaled or oral corticosteroid therapy or interactions with the upper airway and gut microbiomes.(31) Further mechanistic studies will be required to test these above hypotheses and to determine the cause or effect of the association between eosinophil count and Streptococcus. The cross-sectional nature of our study makes causal inference impossible. Antibiotic treatment in particular is an important potential confounder. The relationship between diversity and the frequent exacerbator phenotype may reflect the effect of repeated antibiotic courses in patients with frequent exacerbations. Some aspects of our survival analysis nevertheless suggest that dominance and loss of microbial diversity themselves may be harmful and contribute to disease progression. The strength of association with survival was striking with a more than 2-fold increased risk of death among individuals with Proteobacteria or *Haemophilus* dominance. While this could reflect these patients simply having more severe disease, adjustment for age, sex, and FEV₁ and exacerbation frequency, did not significantly modify the association of Proteobacteria with mortality. We have previously demonstrated that *Haemophilus* dysbiosis is associated with an increased frequency of exacerbations and increased airway neutrophil extracellular trap formation.(11) NET formation exposes the airway to increased concentrations of toxic proteases and antimicrobial peptides such as neutrophil elastase and matrix metalloproteinases which are linked to disease progression in emphysema and COPD, and which were linked to Proteobacteria dominance in this study. (32) There is therefore a clear causal pathway through which bacterial infection could lead to disease progression and increased mortality. Despite the strength of our findings the number of events during follow-up was relatively small and confidence intervals wide. Our findings should ideally be replicated in future longitudinal microbiome studies.

A series of microbiome studies of varying designs, including cross-sectional and longitudinal cohorts have now provided remarkably consistent results. The dominance of *Haemophilus* and *Streptococcus* as key taxa in COPD, distinct microbiome subtypes of exacerbation, the association with lower diversity and poor lung function and more severe disease are all highly consistent across multiple cohorts.(11, 13, 15, 18) This requires a consideration of how the microbiome could be therapeutically targeted. Here there is an absence of evidence. *Haemophilus influenzae*, the most frequently identified species on culture in

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those with *Haemophilus* dominance by sequencing, may be amenable to treatment with antibiotics but the microbiome has been shown to be remarkably resistant to short term disruption with antibiotics.(11, 15) Long term antibiotic treatment such as with macrolides has been shown to modify the microbiome, but in a study by Rogers *et al*, in patients with bronchiectasis, a reduced relative abundance of *Haemophilus* was associated with an increased relative abundance of other Proteobacteria including *Pseudomonas*.(33) Therefore it is not certain that antibiotic treatment would produce a positive change in the microbiome. Vaccination, pulmonary or gastrointestinal probiotics or discontinuation of immunosuppressive medications such as corticosteroids have all been considered but interventional studies are now required to establish if treatments can modify the microbiome in a meaningful way.

Limitations of our study should be acknowledged. Our study was enrolled from a single UK region and results may not be generalizable to other settings worldwide. Our study included a high proportion of patients with severe COPD and frequent exacerbations and consequently a high frequency of use of preventative therapies such as inhaled corticosteroids and antibiotics. CT scanning can identify additional phenotypic characteristics in COPD such as those patients with dominant bronchiectasis and emphysema and the absence of systematic CT scanning in our study is an important limitation. We reported all-cause mortality but did not have sufficient data to examine associations with respiratory specific mortality. We used 16S rRNA sequencing to characterize the respiratory microbiome. This is the most widely used method in the literature but has inherent biases which must be considered in interpretation of the results. The most relevant limitation in this case is the lack of resolution to determine organism identity to species level. It is therefore not known which Streptococcal species are being identified in the Streptococcus cluster; use of species specific PCR could resolve the identity to species level in future studies.(34) It should also be noted that sputum, while widely used in studies of the airway microbiome, has limitations in that it is often intermediate between bronchoalveolar lavage and upper airway swabs, therefore containing contributions from the upper and lower airway. Since our study aims to test the prognostic and phenotypic utility of the sputum microbiome, we make no inferences about lung ecology. Whilst different studies have utilized various cut-offs to investigate associations between microbiome profiles and blood eosinophil counts (e.g. 2%), we used 100cells/µL to define eosinophilia, based on the recent GOLD 2019 clinical guidelines. Our generalised linear models show a continuous relationship between microbiome profiles and blood eosinophil counts and did not suggest a true "cut-off" which is consistent with current thinking within the COPD field as a whole. Our proteomic analysis was only performed in a subset of patients. While the characteristics of these patients were similar to the overall cohort this introduces a risk of selection bias. Our study has many strengths including the large sample size compared to other COPD microbiome studies, the inclusion of a representative cohort including patients across the spectrum of COPD and the availability of long-term microbiome samples and follow-up data.

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365	In conclusion, we have identified microbiome associated subtypes of COPD associated with clinical
366	phenotypes and increased mortality. Our results support a personalized medicine approach to therapy in
367	COPD.
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372	Study design: AJD, HM, BEM, RTS, JDC
373	Data collection: AJD, SF, JDC
374	Laboratory analysis: AJD, JTJH, HRK, CJF, BT, AJC, SF
375	Data analysis: AJD, JTJH, ML, BEM, RTS, JDC
376	Data interpretation: AJD, JTJH, ML, BM, RTS, JDC
377	Drafting the manuscript: AJD, JTJH, BM, RTS, JDC
378	Revising the manuscript and approval for submission: All authors



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473 Figure Legends

- Figure 1. Flow chart of the patients and samples used in this study. Baseline characteristics of the cohort
- who provided sufficient sputum for microbiome sequencing (n=252) is shown in Table 1
- 476 **Figure 2. A:** Microbiome profiles of all stable samples at the phylum level. **B:** Patient microbiome
- profiles in COPD at the genus level. The samples are sorted by percentage *Haemophilus* and percentage
- 478 *Streptococcus* to emphasize the dominant populations.
- 479 Figure 3. Relationship between clinical phenotypes and microbiome composition A: Alpha diversity
- 480 measured by Shannon-Wiener Diversity Index and Berger-Parker Dominance Index, is associated with
- 481 the peripheral blood eosinophil count, p-value from paired T-test, 62 patients were identified as having a
- 482 peripheral blood eosinophil count of ≤100 cells/μL, 190 had a count of >100 cells/μL. **B:** Average %
- OTUs at the phyla and genera level for non-eosinophilic patients compared to eosinophilic patients. C: %
- Proteobacteria is negatively correlated with blood eosinophil counts. % Firmicutes is positively correlated
- with blood eosinophil count. Generalized linear models with binomial errors and a logistic link function
- were used to show how expected proportions of these phyla are related to blood eosinophil levels.
- 487 **Figure 4.** Relationship between baseline microbiome profiles and long-term survival. **A:** Microbiome
- 488 subgroups identified at the phylum level, for clarity only samples classed as Firmicutes or Proteobacteria
- dysbiotic or balanced phyla are shown. **B:** Microbiome subgroups identified at the genera level, only
- 490 samples classed as *Haemophilus* or *Streptococcus* dysbiotic or balanced genera are shown.
- 491 **Figure 5.** Proteobacteria dominated microbiome profiles are associated with neutrophilic inflammation
- $492\,$ in patients with COPD. 747 variables (consisting of 613 proteome, 113 microbiome and 21 clinical
- 493 variables) from n=78 patients were subjected to partial least square discriminant analysis based on three
- 494 pre-defined microbiome groups. **A:** The scores plot reveals the separation between the three
- 495 microbiome groups, Firmicutes (n=30, blue dots), balanced (n=15, yellow dots) and Proteobacteria
- 496 (n=33, red dots), ($R^2X=0.17$, $R^2Y=0.73$). **B**: Loadings plot showing which key variables (those closest to the
- 497 grouping reference points (black dots) drive the separation. Microbiome variables are labelled in red
- 498 with orange dots, proteins in black with green dots and clinical variables in blue with blue dots. A
- 499 separate univariate analysis was carried out to confirm the results and to avoid overfitting
- 500 (Supplementary Table S2).

Table 1. Baseline characteristics of the study population (n=252 unique subjects). Abbreviations: ICS= inhaled corticosteroids, BMI= body mass index, MRC= Medical Research Council, FEV_1 = forced expiratory volume in 1 second, FVC= forced vital capacity, SGRQ= St Georges Respiratory Questionnaire, CAT= COPD assessment test. *includes medications used in combination with other bronchodilators or inhaled steroids.

Characteristics	Median (IQR) or n (%)
N	252
Age	71 (66-78)
Male Gender	153 (60·71%)
BMI	26.47 (24.0-31.02)
ICS use* Long acting beta-agonists* Long acting muscarinic antagonist* Short acting bronchodilators only Oral antibiotics	163 (64·4%) 201 (79·4%) 186 (73·8%) 14 (5·6%) 51 (20.24%)
MRC dyspnoea score	3 (2-4)
Current smokers Ex-smokers	65 (25·79%) 184 (73·02%)
Pack years <10 10-20 20-40 40 or more Missing	12 (4·76%) 37 (14·68%) 94 (37·30%) 107 (42·46%) 2 (0·79%)
Exacerbation frequency (year prior to the study) 0 1 2 3 or more Severe exacerbation requiring hospitalization (year prior to the study)	52 (20·63%) 48 (19·0%) 40 (15·8%) 112 (44·3%) 57 (22·62%)
Daily Sputum volume (mL)	10.0 (4.75-20.0)
Spirometry FEV ₁ (L) FEV ₁ (% predicted) FVC FEV ₁ /FVC	1·50 (1·06-1·95) 65·35 (49·75-80·00) 2·87 (2·11-3·61) 53·00 (44·75-61·02)
GOLD 2017 A B C	58 (23·02%) 32 (12·6%) 52 (20·6%)

D	110 (43.5%)
SGRQ total score	47·82 (31·93-64·58)
CAT total score	19 (14-25)

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