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Clinical endotypes of exacerbation are associated with differences in microbial composition and diversity in COPD

Holly R Keir<sup>\*1</sup>, Alison Dicker<sup>\*1</sup>, Mike Lonergan<sup>1</sup>, Megan Crichton<sup>1</sup>, Bruce E Miller<sup>2</sup> Ruth Tal-Singer<sup>2</sup>, James D Chalmers<sup>1</sup>

**Scottish Centre for Respiratory Research, University of Dundee, United Kingdom<sup>1</sup>;**

**Medical Innovation, GSK Pharmaceuticals R&D, Value Evidence & Outcomes, Collegeville, PA, USA<sup>2</sup>**

\*these authors contributed equally.

Corresponding author: James D Chalmers, Scottish Centre for Respiratory Research, University of Dundee,

Ninewells Hospital and Medical School, Dundee, DD1 9SY, [jchalmers@dundee.ac.uk](mailto:jchalmers@dundee.ac.uk)

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Introduction: COPD exacerbations are associated with worsening symptoms including cough, shortness of breath, sputum production and worsening airflow obstruction. Increased number of exacerbations is associated with morbidity and mortality (1). Exacerbations are classically believed to be associated with viral or bacterial infection, although they may also be associated with non-infectious stimuli including eosinophilic inflammation or pauci-granulocytic exacerbation such as the worsening of cardiovascular disease (2). The mainstay treatment for exacerbation of COPD is antibiotics and corticosteroids, however treatment with these is not always successful and there is a need for more personalised management of exacerbations (3).

One of the largest studies of the sputum microbiome in COPD compared the microbiome at stability and exacerbation in 161 exacerbations in 78 patients and found no consistent changes in the microbiome with no overall difference in Shannon diversity index between the groups (4). The most striking finding of this study was that COPD exacerbations could be classified into bacterial, viral and eosinophilic and patients showed remarkable consistency of their exacerbation phenotype over time. A number of studies have previously supported these defined phenotypes of exacerbation in COPD (2).

We therefore aimed in this study to examine whether we could observe changes in the microbiome from stability to COPD exacerbation within these 3 subtypes of clinically defined events.

Methods: Patients were invited to participate in the study and were included if they were >40 years, had a FEV<sub>1</sub>/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). Patients were required to be clinically stable and 4 weeks free from antibiotic or corticosteroid treatment at baseline for enrolment. Patients provided an induced sputum sample at baseline and attended for a further induced sputum sample at exacerbation if one occurred within 6 months. Details of this cohort have been previously reported.(5) Exacerbations were classified as bacterial (positive sputum culture), eosinophilic or viral/paucigranulocytic as previously described (2, 4). Bacterial/viral co-infections were analysed as bacterial for the purposes of this analysis. DNA was extracted from sputum followed by 16S rRNA gene sequencing on the Illumina MiSeq platform. Bioinformatic analysis and quality checking of the resulting sequences was performed using QIIME (version 1.9.0). Appropriate negative (water) extraction and sequencing controls were included. Shannon-Wiener Diversity Index (SWDI) was used as a measure of alpha diversity within samples. Beta-diversity was analysed using principle coordinates analysis and groups were compared using Permutational multivariate analysis of variance based on the Bray Curtis similarity index. Differences between two groups were further evaluated using Linear discriminant analysis Effect Size (LEfSe) with

false discovery rate (FDR) adjustment for multiple testing. Paired tests were used when comparing paired stable and exacerbation samples, while exacerbation subgroups were analysed as independent groups. Detailed methods have been previously described (5) and all sequences generated are available in the NCBI Sequence Read Archive under the Bioproject accession numbers PRJNA539959 and PRJNA316126.

Results: We studied 46 participants who experienced a total of 73 exacerbations. Mean age 70.8 years (standard deviation  $\pm 7.0$ ), 27 male (58.7%), 69.6% were receiving inhaled corticosteroids, mean BMI 27.3 (SD  $\pm 4.8$ ), Mean MRC dyspnoea score 2.9 (SD  $\pm 1.3$ ). The mean FEV1 was 1.45L (SD  $\pm 0.54$ ) equating to 63.3% predicted (SD  $\pm 22$ ). Mean baseline SGRQ total score was 40.3 (SD  $\pm 26.6$ ) and CAT score was 17.2 (10.7). Using the GOLD classification system 5 participants were GOLD A, 5 were GOLD B, 2 were GOLD C and 34 were classified as GOLD D.

Using the exacerbation endotype classification, 33 exacerbations were bacterial, 19 eosinophilic and 21 viral. For bacterial events the most frequently identified bacteria was *Haemophilus influenzae* in 54.5% of cases, followed by *Moraxella catarrhalis* (27.2%). *Pseudomonas aeruginosa* was identified in 3 cases (9.1%).

Visualising stable and exacerbation samples by principle coordinate analysis there was no obvious clustering of stable and exacerbation samples (figure 1A). Consistent with this observation there were no significant differences between groups by PERMANOVA ( $p=0.08$ ). LEfSe identified reduced levels of *Megasphaera* (LDA score 2.1, FDR adjusted P value 0.014), *Leptotrichia* (LDA score 2.22, FDR adjusted p-value 0.043), *Veillonella* (LDA score 3.32, FDR adjusted p-value=0.043 and *Campylobacter* (LDA score 2.0, FDR adjusted p-value 0.043) at exacerbation. *Prevotella* was not significantly different after FDR adjustment (LDA score 2.78,  $p=0.059$ ) (figure 1B). ICS use was not found to be associated with microbiome composition, in terms of either alpha-diversity ( $p=0.77$ ) or beta-diversity ( $p=0.86$ ).

We conclude there are no large differences between stable disease and exacerbation at the overall community level. We therefore evaluated whether there were differences between exacerbation endotypes. Classifying exacerbations as bacterial, eosinophilic or viral/other resulted in significant differences by PERMANOVA,  $p=0.003$  (figure 1C). Differences in alpha-diversity were observed across the 3 exacerbation groups (ANOVA,  $p=0.0005$ ) with a lower Shannon-wiener diversity index particularly in the bacterial exacerbation group (t-test,  $p=0.0003$ ). There was no significant difference in Shannon diversity index between stable and exacerbation for the other groups ( $p=0.66$  for eosinophilic and

p=0.053 for viral/other by paired t tests). Similar results were observed with different alpha-diversity metrics, with between group differences in Chao index (p=0.019) and Simpsons index (p=0.0024) with lower diversity again demonstrated in those exacerbations associated with positive bacterial culture.

Higher CAT scores indicate more severe symptoms during exacerbations. The mean CAT scores for the 3 exacerbation subtypes were bacterial 24.2 (SD  $\pm$ 6.7), eosinophilic 17.6 (SD  $\pm$ 8.5), viral/other 21.1 (SD  $\pm$ 7.0). The difference between bacterial and eosinophilic was statistically significant (t-test, p=0.0067).

Discussion: Our analysis demonstrates that subgrouping exacerbations into bacterial, eosinophilic and viral/other endotypes results in significant differences in microbiome composition and identifies more symptomatic exacerbations as measured by the COPD assessment test. We found, as reported by others, that there are no significant difference between stable and exacerbation microbiome profiles as a whole (6-8). This adds to the growing literature suggesting that COPD exacerbations are heterogeneous events with diverse aetiologies and likely to require different treatments (9). LEfse shows that the taxa associated with a stable or less severe microbiome, which are predominantly oral taxa, constitute the largest change across the whole of the group. This suggests that a loss of these organisms, as opposed to increases of traditionally regarded pathogenic taxa like Haemophilus or Pseudomonas is the most common change observed at exacerbation. This is highly consistent with the findings from AERIS where no consistent changes in Haemophilus or Streptococcus were observed comparing stability to exacerbation [4].

We show bacterial events are associated with a significant loss of diversity and divergent changes in the overall composition of the microbiome, unlike subjects in the 'eosinophilic exacerbation' endotype who do not experience significant changes in microbiome composition. Additionally, higher CAT scores were observed in the bacterial exacerbation subgroup overall and were significantly different from the eosinophilic subgroup. This seems to confirm that bacterial and eosinophilic exacerbations are different and underscores the importance of a personalised approach to treatment.

A limitation of this study was the relatively small sample size (n=46), however the results were concordant with the larger AERIS study (n=78) and COPD-MAP cohort (4, 6). In particular COPD-MAP using different analysis methods identified a reduction in Veillonella at exacerbation which replicated here (6). As this was a study that included patients experiencing an exacerbation, there was a certain bias towards GOLD D patients and so may be reflective of a more severe COPD population than other studies. We allowed up to 2 exacerbations per patient, which may introduce bias but analysis limited to one exacerbation per subject produced similar results (not shown). Although 16s rRNA sequencing is a

powerful tool in providing a comprehensive description of the lung microbiome, it does have certain limitations, including bias towards certain bacterial taxa, exclusion of mycobacteria and viruses, and risk of contamination (6). This study used sputum, which are typically high biomass samples and included appropriate negative controls to minimise contamination risk. Nevertheless sputum is regarded as an intermediate sample between the upper and lower airway microbiome and so different results may be seen with studies utilising bronchoalveolar lavage (10).

Taken together, recent 16s rRNA sequencing data highlight the importance of phenotyping and endotyping exacerbations. Although the microbiome is not currently an easily accessible technology for clinical practice, our analysis demonstrates that using easily available biomarkers like bacterial culture or potentially a rapid PCR test for pathogens, blood eosinophils or biomarker surrogates may be practical options and may allow more personalized treatment of exacerbations.

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#### Figure legend

Figure 1. A: PCoA showing clustering of stable and exacerbation samples in the study. No differences were observed by PERMANOVA between the two groups ( $p=0.075$ ). B: Linear discriminant analysis Effect Size. Features with an LDA score greater than 1 are shown. Those that reach statistical significance after FDR adjustment are described in the text. Blue indicates taxa that are more abundant in stable samples while red indicates samples more abundant in exacerbation samples. C: PCoA as in (A) but with samples labelled 0-3 where 0= stable, 1=bacterial exacerbation, 2=eosinophilic exacerbation 3= Viral exacerbation or unexplained exacerbation. The ellipses are coloured according to group. The differences between exacerbation groups were statistically significant by PERMANOVA ( $p=0.003$ ). D: Differences between stable and exacerbation subgroups using the Shannon diversity index as an alpha-diversity metric. E: Mean CAT scores for each subgroup of exacerbation, 1=bacterial, 2=eosinophilic, 3=viral ( $p=0.0067$ ).