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T2 biomarkers and airway microbial composition in severe asthma

Relationship between inflammatory status and microbial composition in severe asthma and during exacerbation

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Authors' contribution

SD and KH undertook the clinical and microbiological data analysis and prepared the manuscript. PJM, FY, CB and RH undertook patient recruitment and data collection. VM, CM, VB and CC performed analysis of samples in the laboratory. MYR and MRB were involved in the interpretation of microbiological data. JB contributed significantly to data management and interpretation. TCH, JRA, PB, DC, AHM, SJF, JL, AMG, DSR, JGM, IDP and RC were core members of the RASP-UK consortium. CEB and LGH led the design of the study, data collection, data interpretation, data analysis and had full access to the data and were responsible for the integrity of the data and final decision to submit. All authors contributed to the study design, writing of the manuscript and have approved the final version for submission.

Conflicts of interest:

SD, KH, VM, CM, VB, CC, CB, RS, MYR, DC, SJF, JL, DSR and MRB have no relevant conflicts of interest to declare. **PJM** has received speakers fees and support to attend meetings from GlaxoSmithKline (GSK). **JB** declares personal fees from NuvoAir for attendance at advisory boards. **FY** has received support to attend educational meetings from

ABSTRACT

Background

In T2-mediated severe asthma, biologic therapies such as mepolizumab, are increasingly used to control disease. Current biomarkers can indicate adequate suppression of T2 inflammation, but it is unclear whether they provide information about airway microbial composition. We investigated the relationships between current T2 biomarkers and microbial profiles, characteristics associated with a Proteobacteria^{HIGH} microbial profile and the effects of mepolizumab on airway ecology.

Methods

Microbiota sequencing was performed on sputum samples obtained at stable and exacerbation state from 140 subjects with severe asthma participating in two clinical trials. Inflammatory subgroups were compared on the basis of biomarkers including FeNO and sputum and blood eosinophils. Proteobacteria^{HIGH} subjects were identified by Proteobacteria to Firmicutes ratio ≥ 0.485. Where paired sputum from stable visits was available we compared microbial composition at baseline and following ≥ 12 weeks of mepolizumab.

Results

Microbial composition was not related to inflammatory subgroup based on sputum or blood eosinophils. FeNO ≥ 50 ppb when stable and at exacerbation indicated a group with less dispersed microbial profiles characterised by high alpha-diversity and low Proteobacteria. Proteobacteria^{HIGH} subjects were neutrophilic and had a longer time from asthma diagnosis than Proteobacteria^{LOW} subjects. In those studied, mepolizumab did not alter airway bacterial load or lead to increased Proteobacteria.

Conclusion

High FeNO could indicate a subgroup of severe asthma less likely to benefit from antimicrobial strategies at exacerbation or in the context of poor control. Where FeNO is <50 ppb, biomarkers of microbial composition are required to identify those likely to respond to microbiome-directed strategies. We found no evidence that mepolizumab alters airway microbial composition.

KEYWORDS: asthma, biologics, biomarkers, infection

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Introduction

Characterisation of heterogeneous pathophysiological mechanisms within severe asthma[1] along with the development of multiple adjunctive therapies for the T2-mediated endotype have transformed the care of real-world severe asthma populations. Biologic agents targeting T2 cytokines, such as the anti-IL5 agent mepolizumab, have led to consistent improvements in asthma exacerbation rates[2,3] and reduced oral corticosteroid burden[4]. However, some patients demonstrate a suboptimal response to these therapies, with studies indicating that some 'loss-of-control' events are not mediated by T2 inflammation[5]. In a small proportion of asthmatics there is a persistent absence of T2 inflammation[6]. With no targeted therapies currently licensed in the T2-low endotype, identifying and understanding non-T2 drivers of disease remains an unmet clinical need.

In severe asthma, drivers of non-T2 disease include airway infection and dysbiosis[5,7], defined as a compositional or functional change in the airway microbiome compared to health. Molecular approaches to define the airway ecology typically include microbiomics and polymerase chain reaction (PCR) for specific airway pathogens. Previously we have proposed the ratio of the predominant phyla in the airway, Proteobacteria (including the genera *Haemophilus, Moraxella and Pseudomonas*) and Firmicutes (including *Streptococcus*), as a simple biomarker (P:F ratio) to reflect the overall pattern of the microbiome[7]. In subjects with asthma, there is heterogeneity in composition of the microbiome. Cluster analyses have demonstrated two microbiological groups, with a smaller subgroup of ~20-25% characterised by lower alpha diversity indices and predominance of potentially pathogenic organisms representing the Proteobacteria phylum (P:F high), especially *H. influenzae,* compared to the larger subgroup[8,9]. These differences could be important clinically as specific phyla or genera that differ between these groups are associated with adverse clinical features, such as

worsening symptoms[10] or bronchial hyperresponsiveness[11]. Additionally, airway microbial composition may impact on responsiveness to specific therapies such as corticosteroids[12] or macrolides[13]. Inflammatory indices also differ between these microbiological subgroups. Associations between airway inflammation and airway microbial composition are recognised[14-16]. However, the relationship between inflammation, as indicated by more widely accessible T2 biomarkers such as FeNO or blood eosinophils, and airway ecology is not fully established, especially in response to treatment. This has led to some concern that iatrogenic suppression of eosinophils could affect the immune response to pathogens[17,18] and increase the risk of clinical infection.

In this exploratory analysis of sputum samples collected in 2 trials previously published by the Medical Research Council Refractory Asthma Stratification Programme (RASP-UK) consortium[5,6] we hypothesised that levels of T2 biomarkers (FeNO, sputum and blood eosinophils) are associated with aspects of airway microbial composition. We also aimed to (1) provide further characterisation of microbiological subgroups and (2) study longitudinal samples, where available, to explore the impact of mepolizumab on the airway microbiome.

Methods

Study design and participants

All samples that were adequate for microbiota analysis, collected from two previously completed clinical trials (NCT03324230 and NCT02717689) were combined for the current analysis (**Figure S1**). Both trials recruited patients with GINA step 4/5 asthma[19], aged between 18 and 80 years, from specialist severe asthma centres across the UK participating in

the RASP-UK consortium. All participants provided written informed consent. Sputum induction was performed according to an established protocol[20].

The MEX trial[5] (NCT03324230) was a prospective observational trial performed across four UK severe asthma centres, using sputum samples to characterise exacerbations in subjects on mepolizumab. In this trial, an exacerbation was defined as severe asthma symptoms worsening outside of a patient's normal daily variation and occurring any time after the initial dose of mepolizumab, at which point participants attended their clinical site for assessment before commencing rescue treatment[5]. Induced or spontaneous sputum samples were collected preceding treatment with high dose oral corticosteroids or antibiotics in the context of an acute asthma exacerbation, whereas stable sputum samples, collected prior to mepolizumab initiation and when clinically stable after at least 12 weeks of treatment, were induced. All participants were deemed suitable to receive mepolizumab by a severe asthma multi-disciplinary team in accordance with UK clinical guidance, with eligibility criteria including both blood eosinophilia and either maintenance oral corticosteroid use or an annual exacerbation rate of ≥4 (supplementary methods and previously published in full[5]).

The RASP-UK biomarker stratification study[6] (NCT02717689) was a prospective parallel group trial across 12 UK severe asthma centres that compared strategies of corticosteroid adjustment; either using a composite score derived from three T2 biomarkers (blood eosinophils, fractional exhaled nitric oxide (FeNO) and periostin) or an algorithm based on symptoms, physiology and exacerbations. Participants were required to demonstrate a FeNO <45 ppb to enrich for a population in whom steroid reduction was possible. Sputum samples collected were induced at baseline and spontaneous at exacerbation (supplementary methods

and previously published in full[6]). In this trial, exacerbation sputum samples were obtained including if rescue treatment had been commenced.

Microbiological analysis

In NCT03324230, DNA was extracted from sputum using the Qiagen DNA Mini kit (Qiagen) and in NCT02717689, the MagNA Pure 96 DNA and Viral NA Small Volume Kit - Pathogen Universal 200 kit was used, both as per manufacturer's protocol. Sputum nucleic acid extracts underwent PCR using LightCycler 480 II instrumentation (Roche Molecular Diagnostics, Germany) to indicate the presence of a respiratory virus (influenza A or B, respiratory syncytial virus A or B, rhinovirus, metapneumovirus, adenovirus, parainfluenza 1–4, and coronavirus types OC43, NL63 and 229E). Bacterial loads were also measured by quantitative PCR (qPCR), using ThermoFisher Quantstudio 5 (Life Technologies, Paisley, UK), based on abundance of 16S ribosomal subunit encoding genes. Following DNA extraction the amplicon library was generated utilizing 28 PCR cycles and targeting the V4 hypervariable region of the 16S rRNA gene with 515F: 5′ GTGCCAGCMGCCGCGGTAA3', 806R: 5'GGACTACHVGGGTWTCTAAT3' primers. Pair-ended (250bp) sequencing was performed using the Illumina MiSeq platform. Sequencing runs included a commercial mock community DNA (ZymoBIOMICS microbial DNA standard) as a positive control and DNA extraction negative control (each batch of DNA extraction included a DNA extraction negative control and a single pooled aliquot was prepared from all controls for sequencing) and PCR negative control for reagent contamination check. PCR and DNA negative controls did not produce any reads. No differences in microbial composition were observed comparing the two DNA extraction methods. Processing of microbiome sequence data was performed using the Qiime2

(Quantitative Insights Into Microbial Ecology) pipeline[21]. Pre-processing of raw sequence reads to remove artefacts and chimeras, merge the paired ended reads and generate the amplicon sequence variants (ASV) feature table was done using DADA2 program[22]. All ASVs were aligned with mafft[23] (via q2-alignment) and used to construct a phylogeny with fasttree2[24] (via q2-phylogeny). Sample rarefication at 9700 read depth was performed as it showed good coverage of the bacterial diversity across our samples without any loss of samples. Post-rarefaction, alpha-diversity metrics for within sample comparison, beta diversity metrics using UniFrac distance measure[25] and Principle Coordinate Analysis (PCoA) were estimated using q2-diversity. Taxonomy was assigned to ASVs using the q2-feature-classifier[26] using the classify-sklearn naïve Bayes method against the Greengenes 13_8 99% OTUs reference sequences[27]. PERMANOVA and PERMDISP analysis[28] was applied to beta diversity measures to explore differences in between and within-group variation (dispersion) between the biomarker groups, respectively and FDR < 0.05 was considered significant for multiple group comparison.

Proteobacteria to Firmicutes (P:F) ratio was calculated for each individual sample using the sequencing data, by dividing the total proportion of the sample belonging to the phylum Proteobacteria, by the proportion belonging to the phylum Firmicutes. Sequence data are deposited at the National Center for Biotechnology Information Sequence Read Archive (Bioproject: PRJNA779201).

Statistical analysis

This was a secondary analysis of pre-existing data and hence no sample size calculation was conducted. Descriptive statistics are presented as means (standard error), medians (IQR) or

counts (%) unless otherwise stated. Microbial composition in biomarker subgroups were evaluated on the basis of FeNO ($\leq$20 ppb, 21-49 ppb and $\geq$50 ppb for low, mid and high FeNO subgroups respectively) and serum C-reactive protein (CRP) (>5 mg/L and $\leq$5 mg/L for high and low CRP subgroups respectively) in accordance with thresholds applied in the MEX trial[5], sputum eosinophils ($\geq$2% or <2% of total cells counted for eosinophilic and non-eosinophilic subgroups respectively)[19], sputum neutrophils ($\geq$65% or <65% for neutrophilic and non-neutrophilic subgroups respectively)[29] and blood eosinophils (<0.15 $\times 10^9$/L, 0.16-0.29 $\times 10^9$/L, $\geq$0.3 $\times 10^9$/L for low, mid and high subgroups respectively)[19,30]. Univariate analyses were conducted using paired and unpaired t-tests, Mann-Whitney U and Wilcoxon signed-rank test, and chi-square tests for parametric, non-parametric and nominal data respectively. ANOVA (parametric) and Kruskal-Wallis (non-parametric) tests were additionally used to compare across three FeNO subgroups. As data for assessed exacerbations was collected prospectively during exacerbations, accompanying clinical data where sputum samples were obtained was rarely missing. Therefore, we conducted all analyses under a complete-case framework. Analyses were conducted using SPSS (Version 26.0, Armonk, NY: IBM Corp).

Results

We analysed all sputum samples that were adequate for microbiota analysis with paired biomarker data. The number of subjects and samples included in the analysis are shown in **Figure S1**. Clinical characteristics for the subjects with stable severe asthma and those studied during exacerbation are demonstrated in **Table S1**. Clinical characteristics of the participants included from each study are compared in **Table S2**. We compared microbial composition in those receiving or not receiving maintenance oral corticosteroids, with no difference in beta-diversity between these groups (**Figure S2**).

Microbial diversity and composition in biomarker subgroups at stable state

The number of subjects and samples for the biomarker subgroup analyses is shown in **Table S3**.

FeNO was available in 115 subjects with sequencing data at stable state and deemed to be low (≤20 ppb), mid (21-49 ppb) or high (≥50 ppb)[5]. Clinical characteristics are demonstrated in **Table 1.** Subjects in the low FeNO subgroup had higher asthma control questionnaire (ACQ)-5 scores and the highest proportion of childhood asthma diagnosis, whilst the high FeNO subgroup had a history of more frequent exacerbations and higher proportions were receiving mepolizumab**.** Although there was no significant difference in the microbial composition between the three FeNO subgroups (Permanova = 0.077) samples within the low and mid FeNO groups showed significantly higher dispersion (differences in microbial composition between samples) (Permdisp = 0.027; pairwise comparison low vs high 0.011, full details of Permdisp analyses provided in supplement, Tables S4A-B) compared to samples within the high FeNO group (**Figure 1A-B**). The high FeNO subgroup had a lower

mean relative abundance of Proteobacteria (0.07±0.07) compared to the low (0.21±0.29) and mid (0.15±0.25) FeNO subgroups, and lower *Haemophilus* (the most dominant Proteobacteria genus) (0.02±0.02) versus the low (0.11±0.23) and mid (0.08±0.23) FeNO subgroups (**Figure 1D**).

95 subjects with sequencing data were able to provide sputum for differential cell count at stable state. There were 42 eosinophilic and 62 non-eosinophilic samples based on sputum eosinophil count ≥2% or <2% of total cells counted. There was no difference in UniFrac distance to centroid between sputum eosinophil subgroups. Alpha diversity measures or microbial composition at phylum or genus level did not differ between subgroups (**Figure S3**). There were no notable differences in microbial composition according to the stable blood eosinophil-defined biomarker subgroups: low (<0.15 x10^9/L, n=62), mid (0.16-0.29 x10^9/L, n=35), high (≥0.3 x10^9/L, n=31) (**Figure S4**).

Microbial diversity and composition in biomarker subgroups at exacerbation

The same low (≤20 ppb), mid (21-49 ppb) and high (≥50 ppb) FeNO classification was applied to FeNO measurements made at exacerbation. Clinical characteristics are presented in **Table 2**, with lowest symptom scores occurring in subjects with high FeNO exacerbations, all of whom were treated with mepolizumab. There was no between group difference in total bacterial load or sputum neutrophils, however the high FeNO group had significantly fewer subjects positive for a respiratory virus, and significantly fewer positive for either a viral or bacterial respiratory pathogen on qPCR testing. As in stable state, the high FeNO group demonstrated less dispersed microbial profiles (Permdisp = 0.027; pairwise comparison high versus low 0.031, and high versus mid 0.012, **Tables S4C-D**) and measures of alpha-

diversity were higher (**Figure 2**). The high FeNO subgroup had a lower mean relative abundance of Proteobacteria (0.12±0.15) compared to the low (0.30±0.34) and mid (0.33±0.37) FeNO subgroups, and lower *Haemophilus* (the most dominant Proteobacteria genus) (0.02±0.02) versus the low (0.21±0.35) and mid (0.17±0.29) FeNO subgroups. The Proteobacteria:Firmicutes ratio (P:F) is a proposed biomarker reflecting airway microbial composition that has previously been demonstrated to suggest a subgroup in COPD who might respond better to antibiotics[31]. The major phyla driving changes in microbial composition across all biomarker subgroups in this analysis were Proteobacteria and Firmicutes, therefore the P:F ratio was used to evaluate changes in the microbiota according to clinical status. We compared P:F ratio from stable state to exacerbation and found no significant change in any of the three FeNO subgroups (data not shown).

41 subjects provided sputum samples adequate for differential cell count at exacerbation, with 20 eosinophilic (sputum eosinophils ≥2%) and 27 non-eosinophilic (sputum eosinophils <2%) samples sequenced. Inter-subject variability was not different between the 2 exacerbation subgroups, however the Shannon index and number of observed OTUs were higher in those with eosinophilic exacerbations. Microbial composition at phylum or genus level was not different (**Figure S5**). Subjects with low, mid and high blood eosinophils at exacerbation had no differences in microbial diversity or composition (**Figure S6**).

Microbial profiles were also compared between subjects with neutrophilic (sputum neutrophils ≥65%) and non-neutrophilic (sputum neutrophils <65%) exacerbations. There was no difference in beta-diversity between these 2 subgroups. Serum CRP level at

exacerbation was available in 45 subjects and, similarly, there was no difference in beta-diversity between those with CRP >5mg/L versus CRP ≤5mg/L (**Figure S7**).

Clinical characteristics associated with a Proteobacteria-dominant microbial profile

The heterogeneity (dispersion) in microbial composition in the low and mid FeNO subgroups was driven by an increased Proteobacteria and reduced Firmicutes dominance in a number of samples in these subgroups. Therefore we utilized the P:F ratio to determine the Proteobacteria$^{\text{HIGH}}$ samples and their associated clinical characteristics in the stable FeNO subgroups. The 75$^{\text{th}}$ quartile value in the low FeNO group was used as a threshold to identify Proteobacteria$^{\text{HIGH}}$ samples (**Figure S8**) with a value of 0.485.

Proteobacteria$^{\text{HIGH}}$ subjects all had FeNO <50ppb, however still represented a minority within the combined mid and low FeNO subgroups (20/97, 20.6%). Clinical characteristics were compared across those who were Proteobacteria$^{\text{HIGH}}$ and Proteobacteria$^{\text{LOW}}$ as shown in **Table S5**. The Proteobacteria$^{\text{HIGH}}$ subjects had a significantly longer time from asthma diagnosis and more severe airflow obstruction but there were no other defining clinical characteristics. Sputum neutrophils were higher, and sputum eosinophils lower, in those who were Proteobacteria$^{\text{HIGH}}$. The proportion of subjects using antimicrobials at the time of sampling was similar in both groups.

The effect of mepolizumab on airway microbial composition

Paired sputum samples before initiation of mepolizumab and after ≥12 weeks of treatment were available for 13 subjects from the MEX trial. Clinical characteristics are presented in

Table 3, along with baseline and follow up symptom scores, physiology and biomarkers, demonstrating a reduction in blood eosinophils consistent with the known drug effect. From baseline to follow up visit there was no change in total bacterial load as measured by total 16S rRNA gene copy numbers.

Genus-level microbial composition data and FeNO subgroup is shown for the 13 individuals in **Figure 3.** There was no significant increase in pathogenic organisms, specifically *Haemophilus* or *Moraxella*, in response to mepolizumab. Similarly, there was no significant change in P:F ratio, and no cases in which a baseline Proteobacteria^{LOW} subject became Proteobacteria^{HIGH} at follow up.

Discussion

In this analysis, the airway microbiome was evaluated in a well-characterised, real-life severe asthma population. There was no marked difference in microbial composition comparing subgroups with high or low sputum or blood eosinophils, however high FeNO level both when stable and at exacerbation indicated a Proteobacteria-low microbial profile with high alpha-diversity and low intersubject variability. Proteobacteria[HIGH] individuals tended to have a longer duration of asthma but no other defining clinical characteristics. Additionally, we were able to demonstrate, in a limited subset, that mepolizumab treatment did not lead to an increase in airway bacterial load or relative abundance of pathogenic organisms.

Previous studies have demonstrated an association between inflammatory phenotype and airway microbiome, with the greatest differences observed between neutrophilic and eosinophilic phenotypes[14]. Lower alpha-diversity is widely reported in neutrophilic asthma, with a relative abundance of pathogenic organisms including *Haemophilus* and *Moraxella*[15,16,32]. Associations between microbial composition and eosinophilic inflammation are less clear and appear to differ in relation to disease severity[10,14,33,34]. Overall, our cohort reflect that reported by Zhang et al.[34], with high relative abundance of Firmicutes in subjects with severe asthma characterised by high blood and sputum eosinophils. The relationships between T2-mediated, eosinophilic inflammation and microbial composition have been evaluated in a number of studies, defining T2 status on the basis of indices including FeNO, blood eosinophils and T2 gene expression signatures[35-38]. FeNO is an attractive biomarker of T2 inflammation in view of its wide accessibility and relative ease of measurement, and has recently demonstrated potential utility in determining inflammatory phenotype at acute exacerbation in severe asthma[5]. FeNO has previously been shown to correlate with the alpha-

diversity of the mycobiome, but was unrelated to the microbiome, in asthma[38]. In our study, a FeNO ≥50ppb indicated a subgroup with low intersubject variability in microbial profiles, with low relative abundance of pathogenic organisms, including *Haemophilus*; increased abundance of which has been shown to predict response to macrolide therapy[13]. None of the high FeNO exacerbations we assessed were positive for a respiratory virus, and the frequency of respiratory bacterial or viral pathogen detection was significantly lower in this group. This reinforces the potential application of FeNO measurement at the point of exacerbation to indicate a group with absence of infectious drivers in whom therapies such as antimicrobials may not be beneficial. Similarly consistently high FeNO at stable state could indicate a group with lower probability of chronic airway infection as a cause for suboptimal control. FeNO demonstrated a stronger association with microbiome composition than blood or sputum eosinophils. In view of the site of production, it is expected that FeNO may reflect changes in the airway environment more so than blood eosinophils, which are potentially susceptible to the influence of other systemic factors. Additionally, FeNO may indicate activity in other inflammatory pathways influenced by the airway ecology. *H. influenzae* is known to drive IL17 mediated neutrophilic inflammation[39] and in studies of T2 and T17 epithelial gene expression clusters, FeNO varied more between clusters than blood, sputum and tissue eosinophils, although between-cluster differences in sputum eosinophils were also significant[40]. High quality samples for measurement of sputum eosinophils are more challenging to obtain and this could influence the strength of the relationships observed, therefore it is important to study biomarker-microbiome associations in larger populations in future. An additional, established role of FeNO measurement is to identify non-adherence to inhaled corticosteroids[41,42]. Given the reported effects of inhaled steroids on airway ecology[43-45], it is plausible that the lower proportions of Proteobacteria and low rates of viral infection observed in our high FeNO subgroup relate to a lower airway corticosteroid burden,

despite similar reported doses across the subgroups. Direct, objective measures of inhaled corticosteroid load in individual subjects, such as via serum[46] or breath[47] monitoring, would facilitate further exploration of this in future trials. In contrast to the high FeNO subgroup, microbial profiles in those with lower T2-biomarkers (FeNO ≤50ppb) were highly dispersed, with only ~20% meeting our definition of Proteobacteria[HIGH]. This suggests that T2-low asthma encompasses various pathobiological mechanisms, with the best strategies to identify and target these remaining unclear.

Characterisation of microbial profiles in poorly controlled asthmatics is increasingly important in the pursuit of precision medicine, as evidence suggests that these may influence the response to specific therapeutic strategies such as low dose macrolides[13]. As observed in other studies, our results indicate that a Proteobacteria-dominant microbial profile was associated with sputum neutrophilia[14,15], but also a longer duration of disease. In a previous cluster analysis, subjects with more pathogenic organisms at stable state had a younger age at asthma diagnosis[8] and in other airways conditions such as cystic fibrosis, age impacts on microbial composition, with reduced diversity and predominance of single taxa in older age groups[48]. In asthma this may indicate the impact of chronic inflammation or mucus hypersecretion, or relate to use of antibiotics and anti-inflammatories over the course of disease. In studies of the COPD microbiome, antibiotics have been shown to reduce proportions of Proteobacteria at acute exacerbation[49] whilst in asthma, low dose azithromycin reduces airway load of *Haemophilus influenzae*[50]. In our analysis, a number of subjects were receiving macrolides or other antimicrobials at the time of sampling which may have altered their microbial composition and affected the proportion found to be Proteobacteria[HIGH]. The microbiological subgroups in our study are consistent with those identified in other analyses[8,9], however further longitudinal study is required to characterise the impact of

antimicrobial agents on airway ecology in severe asthma, both at stable state and during exacerbation. The lack of association between a Proteobacteria[HIGH] microbial composition and a particular clinical phenotype in our study, and in previous analyses[8,9], indicates that specific biomarkers reflecting an increase in the proportions of relevant phyla or genera are required. We identified no differences in microbial composition in subgroups dichotomised on the basis of serum CRP, or sputum neutrophils. There is a paucity of data relating to CRP as a biomarker of acute asthma, and the lack of an observed relationship here may represent the threshold applied to define subgroups or the sensitivity of the assay used. Synthesis of CRP is induced by IL6 and whilst sputum IL6 was significantly higher in bacterial exacerbations in a previous study of exacerbation phenotypes[7], serum IL6 level did not differentiate between subgroups, suggesting that serum biomarkers may be less reflective of inflammatory changes in the airway compartment.

Notwithstanding the limited sample size, we found that mepolizumab treatment was not associated with an increase in total airway bacterial load or increased relative abundance of potentially pathogenic organisms from baseline to ≥12 weeks. The impact of corticosteroid treatment on airway bacterial loads, the microbiome and subsequent infection risk is well recognised[44,49,51], and avoidance of these effects is one of multiple reasons supporting the use of targeted biologic agents in airways disease. However, it has also been suggested that biologic-induced airway eosinopenia could predispose to airway infection in view of the recognised pathogen-killing mechanisms attributed to eosinophils, and their suggested role in bacterial, fungal and viral immunity[52,53]. Whilst there is little data supporting an association between increased airway infection and mepolizumab, this association has been reported in subjects receiving benralizumab for severe asthma[54], perhaps due to the different mechanism of action demonstrated in the rapid and near complete blood eosinophil depletion observed

with benralizumab, but not mepolizumab, following initiation of treatment, which is similar to the effect observed with oral prednisolone[55]. In COPD, benralizumab actually reduced airway bacterial loads with *in vitro* work failing to implicate eosinophils in the immune response to *Streptococcus pneumoniae* or *Haemophilus influenzae*[56]. Reassuringly, safety outcomes from multiple randomised controlled trials in asthma and COPD have not highlighted any increase in rates of clinical infection in the intervention groups compared to placebo[2,57-59]. Whilst this analysis demonstrates no significant increase in pathogenic organisms in subjects receiving mepolizumab, considering the limited number of subjects and relatively short time period studied, the effects of biologic agents on airway infection in severe asthma warrants further study in larger populations, as do the mechanistic relationships between changes in airway microbiota and development of clinical infection. The P:F ratio is not used in clinical practice nor included routinely in research studies. It can be determined by qPCR for rapid testing and is thus scalable[31]. Like the microbiome, it reflects relative rather than absolute abundance. Although it presents a simple biomarker that reduces the complexity of the microbiome to a single measure, it is restricted to the commonest phyla excluding the full spectrum of organisms in the airway ecology. Further studies are required to determine if this biomarker has wider utility.

A particular strength of our analysis is the inclusion of a relatively large number of subjects representing a real-world severe asthma population, with various levels of T2 inflammation as indicated by the widely used T2 biomarkers, FeNO and blood eosinophils. Targeting this particular population at the time of data collection meant that a majority of the subjects included were receiving oral corticosteroid treatment. The impact of systemic steroid treatment on bacterial host defence is well recognised and it would be important to compare our results with future data as increasing access to biologic agents reduces the burden of

corticosteroid use in severe asthma populations. It is also the first study to our knowledge, albeit in a limited subset, to report the effects of mepolizumab on the airway microbiome. We acknowledge a number of limitations. We used a combination of induced and spontaneous sputum to evaluate the microbiome. These have been shown to be comparable[60] therefore combining sampling methods are unlikely to affect our results, however we recognise the potential for acquisition bias with the use of sputum, and the potential for certain phenotypes, such as those with mucus hypersecretion, to be overrepresented. Our sample size was limited. Sample numbers could be improved through the use of a more reliable sampling method, whilst longitudinal sampling over a prolonged period would allow us to evaluate the effects of steroid reduction on the microbiome and assess the impact of mepolizumab on rates of clinical infection. Practically, larger sample size with full characterisation of potential confounders would support more complex statistical analyses such as logistic regression. A longer or more invasive visit and sampling schedule would likely, however, have discouraged participation in this real-world severe asthma population. Finally, whilst subjects with clinically important bronchiectasis were excluded from participating in both trials, we did not collect computed tomography data relating to airway structure or presence of airway dilatation. Whether comorbid bronchiectasis is a major risk factor for Proteobacteria predominance is an important question for future analyses.

In conclusion, our analysis has demonstrated that, whilst microbial composition in severe asthma is heterogeneous, biomarkers such as FeNO, when elevated, can indicate a subgroup with microbial profiles characterised by high alpha-diversity and low relative abundance of Proteobacteria. Where FeNO is mid or low range however, microbial profiles are highly dispersed with only ~1 in 5 subjects demonstrating a Proteobacteria-dominant microbial composition. Therefore, well-validated, reliable biomarkers are required to allow clinicians to

identify and target microbiome-directed, precision medicine strategies to responsive individuals. Finally, we have shown, in a small subset, that mepolizumab treatment reduced blood eosinophils without affecting microbial composition or airway bacterial load; the impact of this finding on rates of clinical infection requires further study.

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Table 1. Clinical characteristics across the 3 stable FeNO subgroups: low (FeNO ≤20ppb), mid (FeNO 21-49ppb) and high (FeNO ≥50ppb).

	Low FeNO (≤20 ppb) N=51	Mid FeNO (21-49 ppb) N=46	High FeNO (≥50 ppb) N=18	p
Age, yr	55.9 (1.7)	58.4 (1.6)	58.8 (2.3)	0.46
Duration of asthma, yr	32.8 (2.6)	28.1 (2.5)	25.1 (4.7)	0.23
Childhood asthma, n (%)	26 (51.0)	13 (28.3)	5 (27.8)	0.04
Male, n (%)	21 (41.2)	25 (54.3)	13 (72.2)	0.07
Ethnicity, n (%)				0.42
Caucasian	46 (90.2)	44 (95.7)	17 (94.4)	
Asian	2 (3.9)	2 (4.3)	1 (5.6)	
Other	3 (5.9)	0	0	
Never smoked, n (%)	37 (72.5)	35 (76.1)	11 (61.1)	0.48
Pack years	11.5 (5.0 – 40.8)	8.0 (2.0 – 14.0)	8.0 (5.0 – 12.0)	0.32
BMI, kg/m²	32.0 (1.0)	30.6 (0.9)	31.3 (1.5)	0.62
Exacerbations/yr †	3.0 (1.0 – 4.0)	2.0 (1.0 – 4.0)	4.5 (2.0 – 7.0)	0.04
ICS, mcg BDP	2000 (2000 – 2000)	2000 (2000 – 2000)	2000 (1600 – 2000)	0.38
Oral corticosteroid, n (%)	30 (58.8)	28 (60.9)	13 (72.2)	0.42
Prednisolone dose, mg	10.0 (10.0 – 15.0)	10.0 (8.0 – 10.0)	10.0 (8.0 – 12.5)	0.16
Other controllers, n (%)				
LABA	51 (100.0)	46 (100.0)	18 (100.0)	-
LAMA	31 (60.8)	23 (50.0)	10 (55.6)	0.57
LTRA	25 (49.0)	29 (63.0)	10 (55.6)	0.38
Xanthine	15 (29.4)	11 (23.9)	4 (22.2)	0.76
Mepolizumab treated, n (%)	7 (13.7)	9 (19.6)	8 (44.4)	0.02
Antimicrobial treated§, n (%)	12 (23.5)	6 (13.0)	2 (11.1)	0.30
ACQ-5	2.5 (0.2)	1.8 (0.2)	1.9 (0.3)	0.02
FEV₁, L	2.03 (0.12)	2.23 (0.10)	2.17 (0.13)	0.34
FEV₁, % predicted	69.5 (2.7)	76.3 (2.9)	69.5 (3.9)	0.17
FVC, L	3.19 (0.15)	3.43 (0.12)	3.34 (0.18)	0.49
FVC, % predicted	86.4 (2.5)	92.0 (2.5)	83.0 (3.4)	0.13

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FEV$_1$/FVC	0.64 (0.02)	0.65 (0.02)	0.65 (0.02)	0.74
FeNO, ppb	13.0 (10.0 – 16.0)	27.0 (24.0 – 35.0)	64.0 (56.5 – 118.8)	<0.001
Blood eosinophils, x10^9/L‡	0.10 (0.07 – 0.14)	0.15 (0.10 – 0.24)	0.12 (0.07 – 0.22)	0.35
Total 16S, x 10^9 copies/ml‡	N=48	N=46	N=18	
	0.60 (0.34 – 1.05)	0.71 (0.39 – 1.30)	1.19 (0.71 – 2.00)	0.42
Sputum cell counts	N=38	N=33	N=15	
Eosinophils, %‡	1.07 (0.65 – 1.75)	2.22 (1.06 – 4.64)	2.47 (0.77 – 7.90)	0.17
Neutrophils, %	63.7 (4.5)	51.9 (4.9)	49.3 (7.6)	0.13

Data is shown as mean (SEM), median (IQR) and n (%) unless otherwise stated. ‡Geometric mean (95% CI). In these cases, statistical tests applied were t-test (including for geometric mean), Kruskal-Wallis, and chi-squared test respectively.

†Pre-mepolizumab for those on mepolizumab treatment.

§Both trials permitted inclusion of subjects on background macrolide (or other antimicrobial) therapy.

Abbreviations: ACQ-5, asthma control questionnaire-5; BDP, beclometasone diproprionate equivalent; BMI, body mass index; FEV$_1$, forced expiratory volume in 1 second; FeNO, fractional exhaled nitric oxide; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long acting beta-agonist; LAMA, long acting muscarinic-antagonist; LTRA, leukotriene receptor antagonist.

Table 2. Clinical characteristics compared across exacerbation FeNO subgroups.

	Low FeNO (≤20 ppb) N=17	Mid FeNO (21-49 ppb) N=21	High FeNO (≥50 ppb) N=11	p
Age, yr	54.7 (2.3)	55.9 (1.9)	55.4 (3.8)	0.94
Duration of asthma, yr	28.9 (4.5)	28.1 (4.1)	22.1 (5.5)	0.60
Childhood asthma, n (%)	7 (41.2)	9 (42.9)	4 (36.4)	0.94
Male, n (%)	5 (29.4)	10 (47.6)	7 (63.6)	0.20
Ethnicity, n (%)				0.55
Caucasian	15 (88.2)	19 (90.5)	11 (100.0)	
Asian	2 (11.8)	1 (4.8)	0	
Other	0	1 (4.8)	0	
Never smoked, n (%)	12 (70.6)	13 (61.9)	10 (90.9)	0.23
Pack years	10.0 (2.0 – 26.0)	10.0 (6.5 – 18.8)	-	0.43
BMI, kg/m²	34.4 (2.5)	33.7 (1.5)	28.2 (0.9)	0.10
ICS, mcg BDP	2000 (2000 – 2000)	2000 (2000 – 2000)	2000 (2000 – 2000)	0.50
Oral corticosteroid, n (%)	9 (52.9)	14 (66.7)	8 (72.7)	0.66
Mepolizumab treated, n (%)	7 (41.2)	10 (47.6)	11 (100.0)	0.005
Antimicrobial treated‡, n (%)	9 (52.9)	6 (28.6)	2 (18.2)	0.12
ACQ-5	3.68 (0.29)	3.68 (0.21)	2.72 (0.26)	0.04
Change from baseline	1.52 (0.25)	1.27 (0.20)	1.52 (0.25)	
FEV₁, L	1.81 (0.19)	1.72 (0.17)	1.93 (0.19)	0.74
Change from baseline	-0.23 (0.10)	-0.36 (0.08)	-0.36 (0.10)	
FEV₁, % predicted	63.9 (6.6)	58.7 (5.3)	62.2 (5.5)	0.78
Change from baseline	-8.1 (3.4)	-12.1 (2.3)	-12.4 (2.4)	
FEV₁/FVC	0.61 (0.03)	0.61 (0.03)	0.60 (0.04)	0.99
Change from baseline	-0.01 (0.01)	-0.03 (0.01)	-0.07 (0.02)	
FeNO, ppb	9.0 (5.5 – 11.5)	28.0 (23.5 – 37.5)	72.0 (57.0 – 102.0)	<0.001
Change from baseline	-8.0 (-13.5 to -3.5)	7.0 (-1.0 to +12.0)	29.0 (-1.0 to +50.0)	
Blood eosinophils, x10⁹/L†	0.05 (0.03 – 0.10)	0.05 (0.03 – 0.10)	0.05 (0.03 – 0.10)	0.27
Change from baseline (ratio)	0.52 (0.30 – 0.92)	1.40 (0.64 – 3.06)	0.88 (0.45 – 1.75)	
Total 16S, x 10⁹ copies/ml†	N=15	N=19	N=11	0.86
	0.78 (0.40 – 1.53)	0.53 (0.14 – 2.04)	0.64 (0.36 – 1.14)	

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Sputum cell counts	N=15	N=17	N=9	
Eosinophils, %[†]	0.76 (0.31 – 1.90)	2.97 (1.30 – 6.77)	2.85 (0.36 – 22.73)	0.11
Neutrophils, %	67.5 (6.1)	68.3 (7.4)	54.8 (9.6)	0.46
Any virus positive	N=17	N=21	N=11	0.003
	10 (58.8)	12 (57.1)	0 (0.0)	
Any bacteria positive	N=16	N=21	N=11	0.053
	10 (58.8)	12 (57.1)	2 (18.2)	
Any virus or bacteria positive	N=17	N=21	N=11	<0.001
	14 (82.4)	18 (85.7)	2 (18.2)	

Data is shown as mean (SEM), median (IQR) and n (%) unless otherwise stated. [†]Geometric mean (95% CI). In these cases, statistical tests applied were t-test (including for geometric mean), Kruskal-Wallis, and chi-squared test respectively. Where subjects experienced more than one exacerbation, data is shown for the earliest exacerbation episode only.

A positive respiratory virus result was defined by a dichotomous result from the panel of respiratory viruses tested (influenza A or B, respiratory syncytial virus A or B, rhinovirus, metapneumovirus, adenovirus, parainfluenza 1–4, and coronavirus types OC43, NL63 and 229E). A positive bacterial result was defined as >10^6 genome copies/ml of *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* as measured by quantitative PCR.

[‡]RASP-UK biostratification trial allowed sputum samples to be collected after antimicrobial treatment had been initiated, whilst all exacerbation samples in the MEX trial were collected before rescue therapy was commenced. Both trials allowed inclusion of subjects on long term macrolide therapy. Both background and acute treatment with antimicrobials are included in these numbers.

Abbreviations: ACQ-5, asthma control questionnaire-5; BDP, beclometasone diproprionate equivalent; BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FeNO, fractional exhaled nitric oxide; FVC, forced vital capacity; ICS, inhaled corticosteroid.

Table 3. Baseline demographics and clinical characteristics for subjects with paired stable sputum samples adequate for microbiome sequencing (a) before starting mepolizumab and, (b) after ≥12 weeks of treatment.

	Paired data available (n=13)
Age, yr	55.8 (3.6)
Duration of asthma, yr	26.6 (4.3)
Male, n (%)	10 (76.9)
Ethnicity, n (%)	
Caucasian	13 (100.0)
Never smoked, n (%)	10 (76.9)
BMI, kg/m^2	31.7 (1.6)
Exacerbations/yr	4.0 (1.0 – 4.8)
ICS, mcg BDP	2000 (1600 – 2000)
Oral corticosteroid, n (%)	11 (84.6)
Other controllers, n (%)	
LABA	13 (100.0)
LAMA	7 (53.8)
LTRA	7 (53.8)
Xanthine	5 (38.5)
Macrolide treated, n (%)	1 (7.7)

	Baseline	Mepolizumab ≥12 weeks	p
ACQ-5	2.8 (0.35)	1.9 (0.37)	0.009
Prednisolone dose, mg	10 (10 – 15)	8 (8 – 10)	0.026
FEV$_1$, L	2.26 (0.16)	2.41 (0.15)	0.124
FEV$_1$, % predicted	71.0 (5.34)	76.2 (5.27)	0.102
FVC, L	3.60 (0.19)	3.81 (0.19)	0.086
FVC, % predicted	89.6 (4.68)	94.9 (5.16)	0.077
FEV$_1$/FVC	0.62 (0.02)	0.63 (0.02)	0.518
FeNO, ppb[†]	40.2 (22.7 – 70.9)	37.2 (17.1 – 80.9)	0.638
FeNO class, n (%)			
Low <20 ppb	3 (23.1)	5 (38.5)	
Mid 20-50 ppb	6 (46.2)	2 (15.4)	
High >50 ppb	4 (30.8)	5 (38.5)	
Unable		1 (7.7)	
Blood eosinophils, x10^9/L[†]	0.36 (0.19 – 0.68)	0.06 (0.03 – 0.09)	<0.001
Sputum eosinophils, %[†] (n=8)	1.46 (0.09 – 24.04)	0.48 (0.03 – 8.14)	0.077
Sputum neutrophils, % (n=8)	65.7 (9.52)	52.5 (7.54)	0.328
Total 16S, x 10^9 copies/ml[†]	1.40 (0.56 – 3.55)	1.54 (0.58 – 4.12)	0.839

Values are mean (SEM) or median (IQR) unless otherwise stated. [†]Geometric mean (95% CI). In these cases, statistical tests used were paired t-tests or Wilcoxon signed-rank tests respectively.

Abbreviations: ACQ-5, asthma control questionnaire-5; BDP, beclometasone diproprionate equivalent; BMI, body mass index; FEV$_1$, forced expiratory volume in 1 second; FeNO, fractional exhaled nitric oxide; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long acting beta-agonist; LAMA, long acting muscarinic-antagonist; LTRA, leukotriene receptor antagonist.

Figures

Figure 1. Samples collected from subjects with high FeNO when stable showed a more even microbiome composition, with lower relative abundance of Proteobacteria.

Panel A shows a principle component analysis plot based on weighted UniFrac distance measure of samples in the FeNO subgroups, low (≤20 ppb, green), mid (21-49 ppb, orange), high (≥50 ppb, purple). The centroids are labelled according to subgroup with ellipses (1 standard deviation). Panel B shows boxplots representing the dispersion of samples in each subgroup. Boxplots comparing the alpha-diversity indexes (within-group microbiome composition differences) are shown in panel C. Panel D shows the taxonomic distribution of microbiome at genus level.

Figure 2. Samples collected from subjects with high FeNO at exacerbation showed a more even microbiome composition, with lower relative abundance of Proteobacteria.

Panel A shows a principle component analysis plot based on weighted UniFrac distance measure of samples in the FeNO subgroups, low (≤20 ppb, green), mid (21-49 ppb, orange), high (≥50 ppb, purple). The centroids are labelled according to subgroup with ellipses (1 standard deviation). Panel B shows boxplots representing the dispersion of samples in each subgroup. Boxplots comparing alpha-diversity indexes (within-group microbiome composition differences) are shown in panel C. Panel D shows the taxonomic distribution of microbiome at genus level.

Figure 3. In subjects with paired samples, microbiome composition and Proteobacteria:Firmicutes ratio did not change following the introduction of mepolizumab treatment (from baseline to ≥12 weeks).
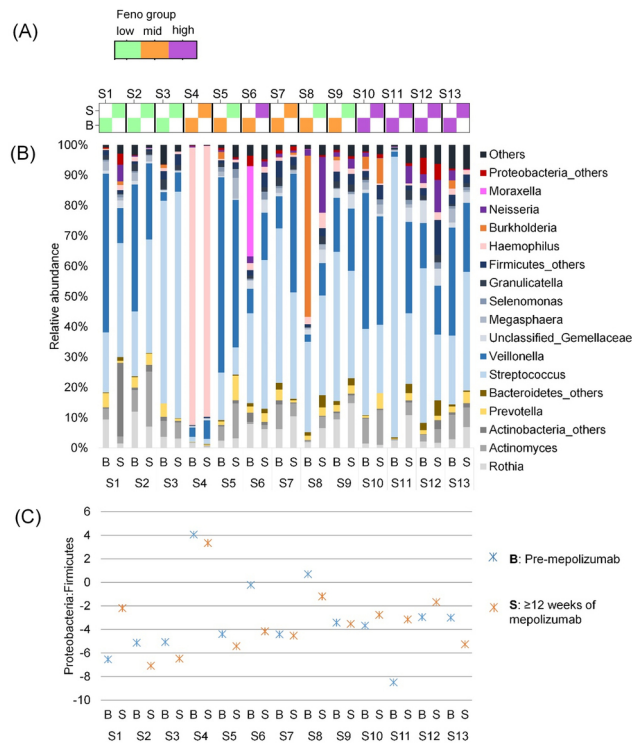
13 subjects provided paired samples at stable state before starting mepolizumab treatment (B) and after ≥12 weeks of treatment (S). Panels A-C show: FeNO subgroup at time of sampling where low FeNO ≤20ppb, mid FeNO 21-49ppb and high FeNO ≥50ppb, microbiome composition at genus level and Proteobacteria:Firmicutes (P:F) ratio for all samples included in the analysis.
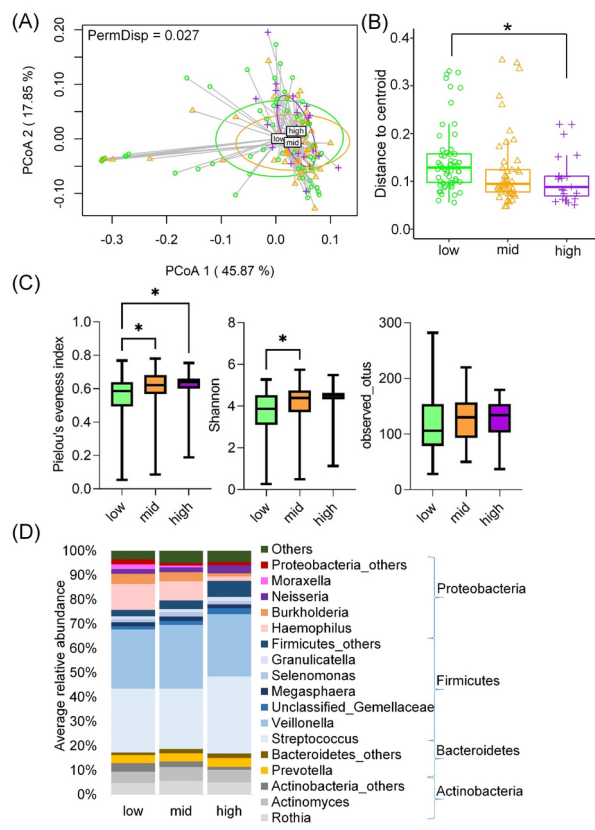
ALL_15425_Figure1.jpg
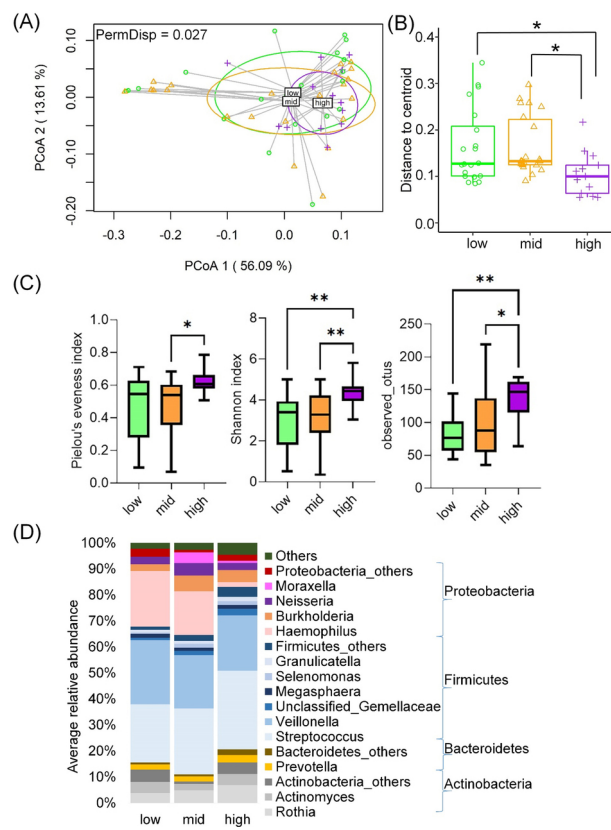
ALL_15425_Figure2.jpg

ALL_15425_Figure3.jpg