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ORIGINAL ARTICLE

Haemophilus influenzae and *Moraxella catarrhalis* in sputum of severe asthma with inflammasome and neutrophil activation

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Abstract

Background: Because of altered airway microbiome in asthma, we analysed the bacterial species in sputum of patients with severe asthma.

Methods: Whole genome sequencing was performed on induced sputum from non-smoking (SAn) and current or ex-smoker (SAs/ex) severe asthma patients, mild/moderate asthma (MMA) and healthy controls (HC). Data were analysed by asthma severity, inflammatory status and transcriptome-associated clusters (TACs).

Results: α-diversity at the species level was lower in SAn and SAs/ex, with an increase in *Haemophilus influenzae* and *Moraxella catarrhalis*, and *Haemophilus influenzae* and *Tropheryma whipplei*, respectively, compared to HC. In neutrophilic asthma, there was greater abundance of *Haemophilus influenzae* and *Moraxella catarrhalis* and in eosinophilic asthma, *Tropheryma whipplei* was increased. There was a reduction in α-diversity in TAC1 and TAC2 that expressed high levels of *Haemophilus influenzae* and *Tropheryma whipplei*, and *Haemophilus influenzae* and *Moraxella catarrhalis*, respectively, compared to HC. Sputum neutrophils correlated positively with *Moraxella catarrhalis* and negatively with *Prevotella*, *Neisseria* and *Veillonella* species and *Haemophilus parainfluenzae*. Sputum eosinophils correlated positively with *Tropheryma whipplei* which correlated with pack-years of smoking. α- and β-diversities were stable at one year.

Conclusions: *Haemophilus influenzae* and *Moraxella catarrhalis* were more abundant in severe neutrophilic asthma and TAC2 linked to inflammasome and neutrophil activation, while *Haemophilus influenzae* and *Tropheryma whipplei* were highest in SAs/ex and in TAC1 associated with highest expression of IL-13 type 2 and ILC2 signatures

Abbreviations: EOS, eosinophilic; IL-33, interleukin 33; MIX, mixed granulocytic; NEU, neutrophilic; PAU, paucigranulocytic; TAC, transcriptomic-associated cluster; TSLP, thymic stromal lymphopoietin.

Ali Versi and Fransiskus Xaverius Ivan contributed equally to this study.

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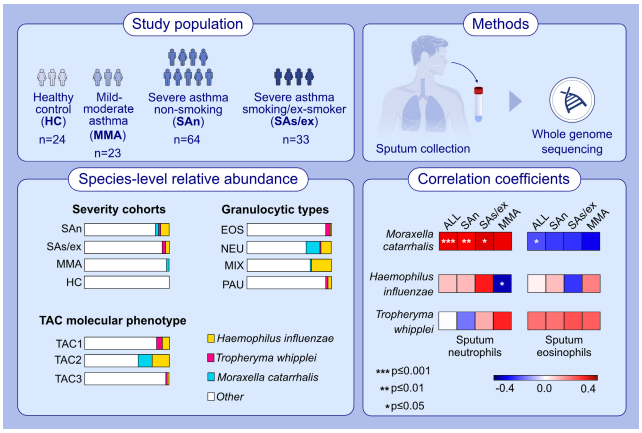
with the abundance of *Tropheryma whipplei* correlating positively with sputum eosinophils. Whether these bacterial species drive the inflammatory response in asthma needs evaluation.

KEYWORDS
Haemophilus influenzae, *Moraxella catarrhalis*, severe asthma, *Tropheryma whipplei*, α-diversity



GRAPHICAL ABSTRACT

Haemophilus influenzae and *Moraxella catarrhalis* were in highest abundance in non-smoking severe asthma, neutrophilic inflammation and TAC2 phenotype of inflammasome activation compared to healthy subjects, while *Haemophilus influenzae* and *Tropheryma whipplei* in smoking/ex-smoking severe asthma and TAC1 phenotype of TSLP and IL-33 receptors and Type 2 inflammation.

1 | INTRODUCTION

Severe asthma is a heterogeneous condition that can be differentiated into clinical and molecular inflammatory phenotypes.[1] The severe eosinophilic asthma cluster is the most established inflammatory phenotype linked to type 2 inflammation driven by cytokines such as IL-4, IL-5 and IL-13.[2] On the contrary, the neutrophilic inflammatory phenotype characterized by sputum neutrophilia is associated with type 1 inflammatory pathways and inflammasome activation.[3] While there has been a link between inflammasome activation and neutrophil airway recruitment in asthma,[4] the mechanism driving neutrophil recruitment and activation remains unclear. Because of the ability of airway microbial organisms to induce inflammatory responses, the complex lung microbiome may contribute to clinical and inflammatory phenotypes of asthma.[5,6]

Detailed analysis of the microbial community composition in asthma has been made possible by using culture-independent techniques such as 16S ribosomal RNA microarray and PCR. *Proteobacteria* families are enriched in airway microbiota of asthma patients[7] and have been associated with airway hyperresponsiveness and worsening asthma control.[8] *Streptococcus pneumoniae*, *Moraxella catarrhalis*[9,10] and *Haemophilus influenzae*[7,9,11] have been reported in patients with stable asthma. Neutrophilic asthma has been associated with enriched *Proteobacteria*,[12] including *Moraxella* and *Haemophilus*,[13] and in particular *Haemophilus influenzae*.[12] Reduced sputum bacterial diversity has been reported in neutrophilic asthma as compared with other asthma phenotypes.[12,13]

For the first time, we have undertaken a metagenomic whole genome sequencing approach of induced sputum from patients with asthma that has the added advantage of providing an in-depth characterization and insights into the microbiome down to the species level in order to understand the relationship between airway dysbiosis and airway inflammation. This has allowed us to examine the abundance of clinically relevant microbial species in patients with asthma. We have examined the effect of asthma severity and granulocytic inflammation on the abundance of microbial species. We also examined for the first time the relationship of the molecular phenotype of asthma to the bacterial species abundance that provides the potential links between these species and the host immunological and inflammatory responses.[14]

2 | METHODS

2.1 | Participants

The U-BIOPRED severe asthma cohort consisted of two groups of adult severe asthmatics: non-smokers (SAn), and smokers or ex-smokers (SAs/ex) together with non-smoking mild–moderate asthma (MMA) and non-smoking healthy volunteers (HC), as previously described.[15] Participants provided induced sputum samples that passed quality control for metagenomic analysis,[16] at baseline and at 12–16 months later. Ethics approval was obtained at the 14

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participating centres, and all participants gave written informed consent (ClinicalTrials.gov identifier: NCT01982162).

2.2 | Induction and initial sample handling

Sputum samples were collected between January 2012 and May 2013. Sputum induction was undertaken and processed as previously described.[15] All frozen sputum samples were sent to Second Genome (San Francisco, California, USA) for metagenomic sequencing in November 2014. Genomic DNA was extracted using the MoBio Tissue and Cells DNA Isolation Kit (Qiagen) at 250 μL input volume, performed in a BioSafety Cabinet in an ISO8 Cleanroom. Each batch included negative and positive controls, as described previously.[17] Extracted DNA was stored at −20°C. Samples were prepared for sequencing with the Illumina Nextera kit (Illumina) and quantified with Quant-iT dsDNA High Sensitivity assays (ThermoFisher Scientific). Libraries were pooled and run with 100 base-pairs paired-end sequencing protocols on the Illumina HiSeq 2500 platform.

These sequence data have been submitted to the NCBI under accession number PRJNA946921 and will be accessible on 2023-08-01 at the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA946921.

2.3 | Derivation of transcriptomic-associated clusters (TACs)

For TAC derivation as previously reported,[3] gene expression profiling was performed by Affymetrix microarray (GPL570 Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array) (Affymetrix) microarrays with RNA extracted from sputum cells (GSE76262). The three TACs were derived by applying hierarchical clustering based on the Euclidian distance of a reduced 508 set of genes from the sputum transcriptomics.[3] Consensus clustering was used to determine the optimum number of clusters where the optimum number *k* is chosen when the consensus matrix histogram reaches a bimodal distribution.[3] The nearest shrunken centroid was employed to determine the gene signatures for each TAC which are shown in Table S4.

2.4 | Quality control of metagenomic data and host reads removal

FastQC version 0.11.8[17] was used to check the quality of the original sequencing reads. Then, bowtie2 version 2.3.5.1 (with its default parameters)[18] was used to align the quality-filtered reads to the human reference genome (hg38 [https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.26/]). Unmapped, non-host reads were separated from host reads and sorted using samtools version 1.9.[19] The output files, which were in BAM format, were converted into fastq files using bedtools version 2.28[20] and then into fasta files using R/Bioconductor package ShortRead version 3.6.2.[21] Non-host metagenomic reads of each sputum sample were subjected to bioinformatics pipelines that generate microbiome profiles. We used MetaPhlAn2 version 2.7.7 and its marker database[22] to estimate microbiome profiles (viruses were excluded from the output).

2.5 | Statistical and computational analyses

The R languages' vegan package was used for calculating Shannon's index within sample diversity, α-diversity[23] and Bray–Curtis dissimilarity between microbiome sample pairs. Similarity between pairs of profiles was calculated as 1-day, where day is the associated Bray–Curtis dissimilarity. These dissimilarity matrices were used to evaluate sample-to sample diversity, β-diversity, between cohorts and timepoints using the permutational multivariate analysis of variance (PERMANOVA) implemented in vegan's ADONIS function, as well as to visualize the relationship between sample groups using the principal coordinate analysis implemented in R's phyloseq package.[24] When PERMANOVA analysis gave a significant result, the R's DESeq2 package[25] with test and fitType parameters set to 'Wald' and 'parametric', and with pseudocount of 1 addition to phylum-/genus-/species-level MetaPhlAn2 RPKM data, were used to identify differentially abundant microbiome taxa. The p-values following Wald test were corrected for multiple testing using the Benjamini–Hochberg method.

Kruskal–Wallis test was employed to assess differences between groups for alpha diversity. When a significant difference was obtained, the Mann–Whitney *U*-test with a Holm–Bonferroni correction was used for post hoc comparisons. Wilcoxon signed-rank test was used to test differences in alpha diversity over time using paired baseline and longitudinal follow-up. All statistical tests performed were two-tailed. Spearman correlations were used to determine the relationship between microbiome abundance and clinical traits and inflammatory biomarkers.

3 | RESULTS

Table S1 summarizes the clinical and physiological characteristics and Tables S2 and S3 the asthma participants according to granulocytic inflammation status and to molecular phenotype, respectively. Using MetaPhlAn2, out of 193 sputum samples, 189 were available for species-level analysis. Although 46 sputum samples were analysed at follow-up, there were paired samples for baseline and longitudinal analyses in only 33 subjects. We also analysed the results in terms of the granulocytic inflammation defined by the level of sputum eosinophils and neutrophils[16] (Table S2) and of the molecular phenotypes defined as transcriptomic-associated clusters (TACs) (Table S3).[14] The molecular pathways characterizing the three TACs are shown in Table S4.

3.1 | Metagenomic sequencing

The depth of metagenomic sequencing of the 190 sputum samples before host reads removal is shown in Figure S1A. Excluding one

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sample with a very low depth of 11,204 reads (left-most), the sequencing depth ranged from 9.1 million to 63.6 million reads, with a median and an interquartile range of 33.3 million and 17.2 million reads, respectively. Following host reads removal, the range of the depth of non-host sequencing reads had median of ~1,5 million reads. Airway microbiome data, excluding 5 samples whose reads were not assigned to any phylum by MetaPhlAn2, were assessed at phylum level (Figure S1B). The sequencing depth did not influence airway microbiome profile (Figure S1C). Four bacterial phyla, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*, accounted for >95% of the overall abundance (Figure S1D).

3.2 | α- and β-diversity

The species-level relative abundance across the severity cohorts are shown in Figure 1A. Difference in airway microbiome was observed across cohorts at species level in both α-diversity ($p = .001$, Figure 1B) and β-diversity ($p \leq .002$, Figure 1C). Although the levels of α-diversity in SAn and SAs/ex were comparable, SAs/ex exhibited lower α-diversity compared to MMA and HC. α-diversity of SAn was lower than that of MMA while there was no difference between MMA and HC (Figure 1B). α-diversity was inversely correlated with sputum neutrophils in SAn, neutrophilic and paucigranulocytic inflammation groups, and in TAC1 and TAC3 (Figure S3). There was no positive association between α-diversity and exacerbations in the previous year or pack-years of smoking, but there was a positive correlation between α-diversity, and FEV1 (% predicted) in neutrophilic inflammation and in TAC2 (Figure S3).

The species-level relative abundance across the granulocytic types are shown in Figure 2A. Compared to HC, α-diversity was reduced in eosinophilic ($p < .05$), neutrophilic ($p < .05$) and mixed granulocytic ($p < .001$) (Figure 2B). Differences in β-diversity based on Bray–Curtis dissimilarity were observed across the inflammatory groups using PERMANOVA ($p < .001$) and ordination using principal coordinate analysis (Figure 2C).

α-diversity in TAC1 and TAC2 was lower than in TAC3 and HC ($p < .001$) (Figure 3B). Differences in β-diversity based on Bray–Curtis dissimilarity were also observed across the TACs ($p < .001$) (Figure 3C).

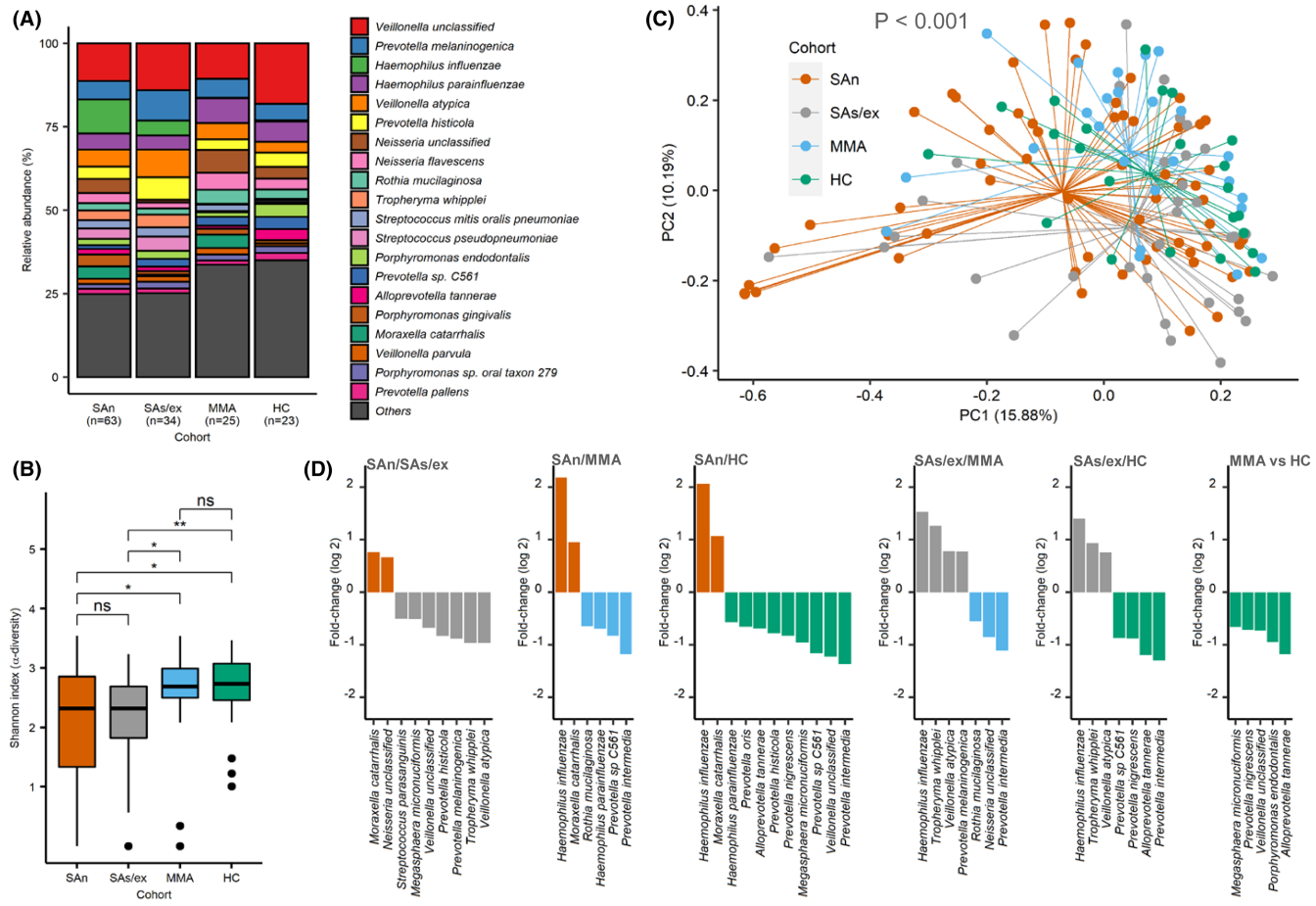

FIGURE 1 Airway of severe asthma subjects exhibits distinct species-level microbiome profiles. (A) Barplot showing the average species-level airway microbiome profiles across study cohorts. (B) Boxplot illustrating species-level α-diversity of airway microbiome across study cohorts. (C) Barplots showing log2 fold-change of significantly differentially abundant airway microbial species across study cohorts. (D) Barplots showing $\log_2$ fold-change of significantly differentially abundant airway microbial species between the four groups of San, SAs/ex, MMA and HC. Significance: ns (not significant, $p > .05$), *$p \leq .05$, **$p \leq .01$ and ***$p \leq .001$. Study cohorts: HC, healthy controls; MMA, non-smokers with mild or moderate asthma; SAn, non-smokers with severe asthma; SAs/ex, smokers or ex-smokers with severe asthma.

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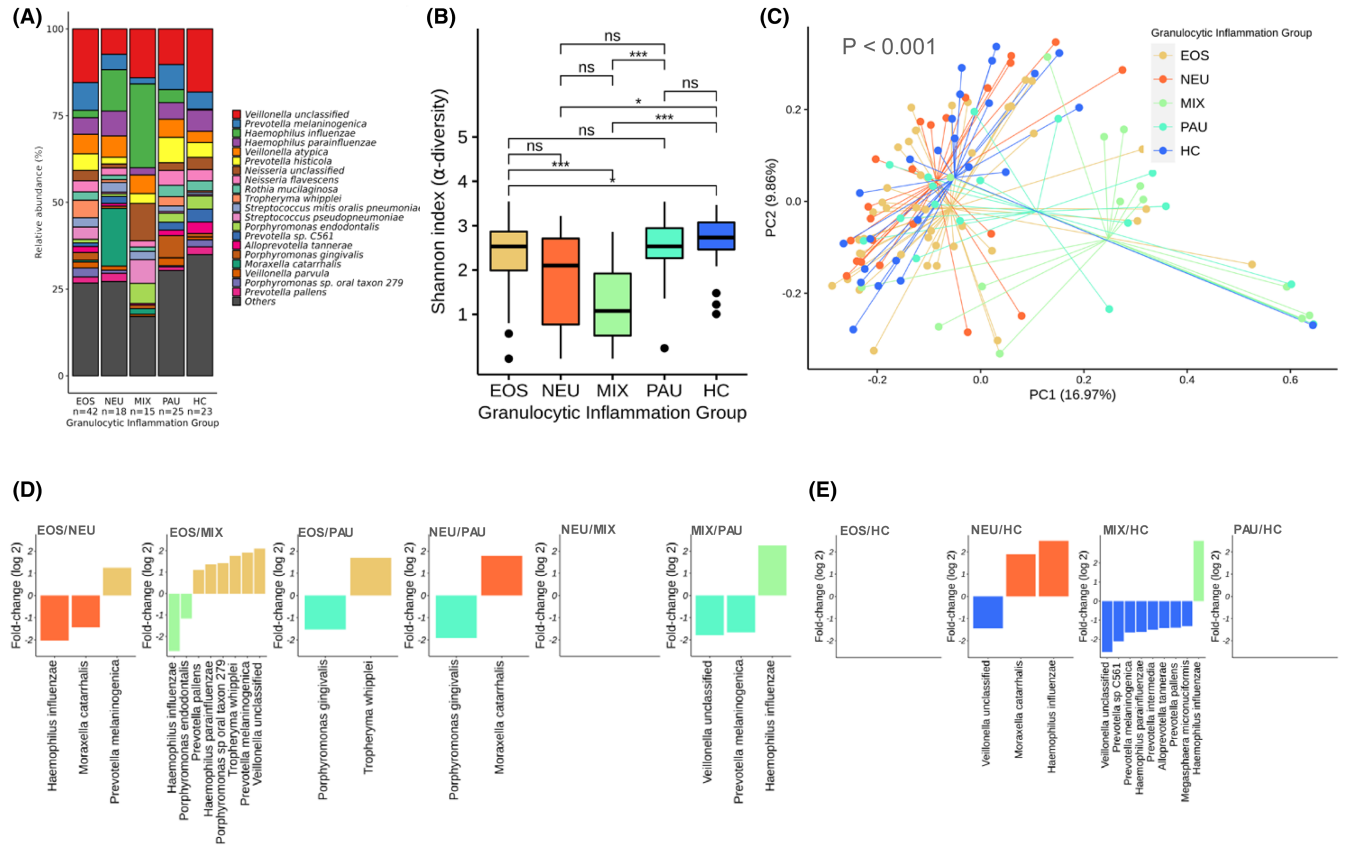

FIGURE 2 Sputum inflammation type shows distinct species-level microbiome profiles. (A) Barplot showing the average species-level airway microbiome profiles according to granulocytic sputum inflammation type. (B) Boxplot illustrating species-level α-diversity of airway microbiome according to granulocytic sputum inflammation type. (C) Principle coordinate analysis (PCoA) based on Bray–Curtis dissimilarity illustrating species-level β-diversity of airway microbiomes in the 4 granulocytic sputum inflammation types and HC. (D) Barplots showing $\log_2$ fold-change of significantly differentially abundant airway microbial species according to granulocytic sputum inflammation type. (E) Barplots showing $\log_2$ fold-change of significantly differentially abundant airway microbial species between each of the sputum granulocytic types and healthy control. Significance: ns (not significant, $p > .05$), $^{*}p \leq .05$, $^{**}p \leq .01$ and $^{***}p \leq .001$. Granulocytic sputum inflammation type: EOS, eosinophilic high (sputum eosinophil % ≥1.49%); NEU, neutrophilic high (sputum neutrophil count % ≥73.6%); MIX, both neutrophilic and eosinophilic high; PAU, paucigranulocytic (sputum eosinophil % <1.49% and sputum neutrophil count % <73.6%); HC, healthy controls.

3.3 | Differentially abundant genera and species by asthma grouping

Prevotella, *Veillonella*, *Haemophilus*, *Neisseria*, *Streptococcus*, *Porphyromonas*, *Rothia*, *Alloprevotella* and *Tropheryma* were the major genera present in healthy subjects (Figure S2A). *Moraxella* and *Tropheryma* abundance was higher in SAn and SAs/ex compared to HC, respectively (Figure S2C).

The most abundant microbes included *Haemophilus influenzae* and *Tropheryma whipplei* (Figure 1A). The species abundance in SAn compared to MMA and HC was enhanced with *Haemophilus influenzae* and *Moraxella catarrhalis*, while in SAs/ex, *Haemophilus influenzae*, *Tropheryma whipplei* and *Veilloneilla atypica* were enriched compared to both MMA and HC (Figure 1D). In an analysis of differences between current and ex-smokers within the SAs/ex cohort, current smokers had a lower abundance of *Haemophilus parainfluenzae* and *Neisseiria flava* compared to ex-smokers (Figure S4).

We analysed the effect of being on daily oral corticosteroid (OCS) therapy. While there was no difference in both α- and β-diversity between those on OCS and not on OCS in SAn and SAs/ex (Figure S5), those on OCS in SAn had lower *Haemophilus influenzae*, *Moraxella catarrhalis*, *Porphyromonas gingivalis and Porphyromonas endodontalis*, and in SAs/ex, lower *Haemophilus parainfluenzae* (Figure S5).

3.4 | Species abundance and clinical and inflammatory features

We examined the abundance of the top 16 species with clinical and inflammatory features in the whole group of asthmatics and also in the 3 subgroups of SAn, SAs/ex and MMA (Figure 4).

3.4.1 | *Airflow obstruction*

Species including those of *Veillonella* and *Prevotella*, *Rothia mucilaginosa* and *Haemophilus parainfluenzae* were positively correlated with FEV1 as an index of airflow obstruction. There was a positive

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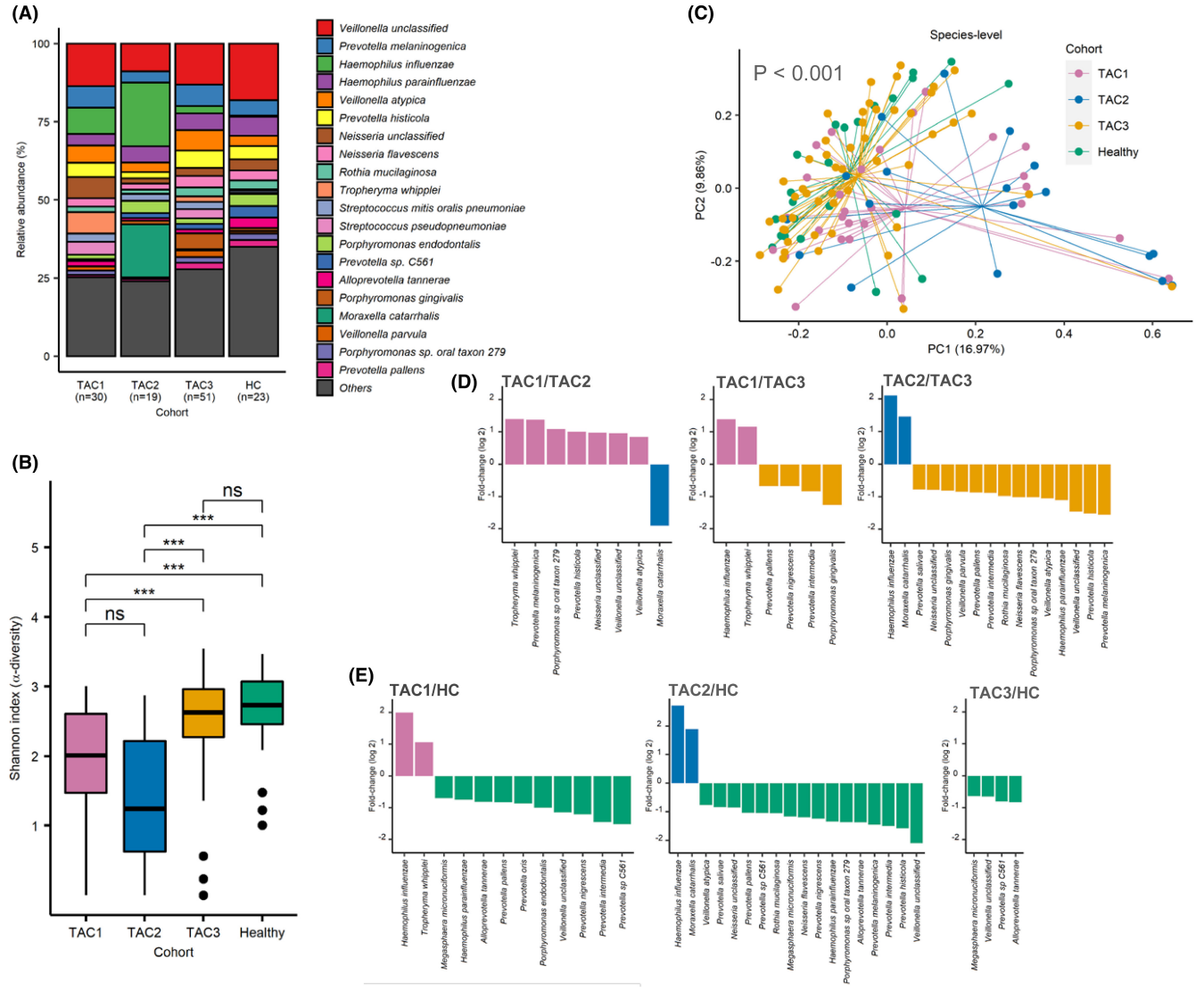

FIGURE 3 Molecular phenotypes of asthma exhibit distinct microbiome profiles. (A) Barplot showing the average species-level airway microbiome profiles across the three transcriptome-associated molecular phenotypes (TACs) and in healthy controls (HC). (B) Boxplot illustrating species-level α-diversity of airway microbiome across the TACs and HC. (C) Principle coordinate analysis (PCoA) based on Bray–Curtis dissimilarity illustrating species-level β-diversity of airway microbiomes in the three TACs and HC (D) Barplots showing $\log_2$ fold-change of significantly differentially abundant airway microbial species between the three TACs (E) Barplots showing $\log_2$ fold-change of significantly differentially abundant airway microbial species between each TAC and HCs. Significance: ns (not significant, $p > 0.05$), *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

correlation between *Prevotella intermedia*, *Prevotella melaninogenica*, *Veillonella unclassified* and *Veillonella atypica* in SAn, between *Veillonella atypica* and *Prevotella melaninogenica* in SAs/ex, and between *Prevotella* sp C561 in MMA with FEV_1 (% predicted) (Figure 4). In SAs/ex, there was a negative correlation of FEV1 (% predicted) and *Haemophilus influenzae*, *Neisseria sicca* and *Trophemyra whipplei*.

3.4.2 | *Exacerbations*

Exacerbations in the previous year were negatively correlated with *Prevotella melaninogenica and Prevotella intermedia* in the whole group. This negative correlation was found with *Prevotella melaninogenica* only in SAn (Figure 4). In SAs/ex, there was a negative correlation with *Neisseria sicca* and a positive correlation with *Rothia mucilaginosa*.

3.4.3 | *Sputum neutrophils*

In the whole group, there was a strong negative correlation of sputum neutrophils with *Prevotella intermedia*, *Prevotella melaninogenica*, *Prevotella nigrescens*, *Alloprevotella unclassified*, *Haemophilus parainfluenzae*, *Neisseria flavescence*, *Neisseria sicca*, *Streptococcus pseudopneumoniae*, *Porphyromonas endodontalis*, *Rothia mucilaginosa*, *Veillonella unclassified and Veillonella atypica* but a positive correlation

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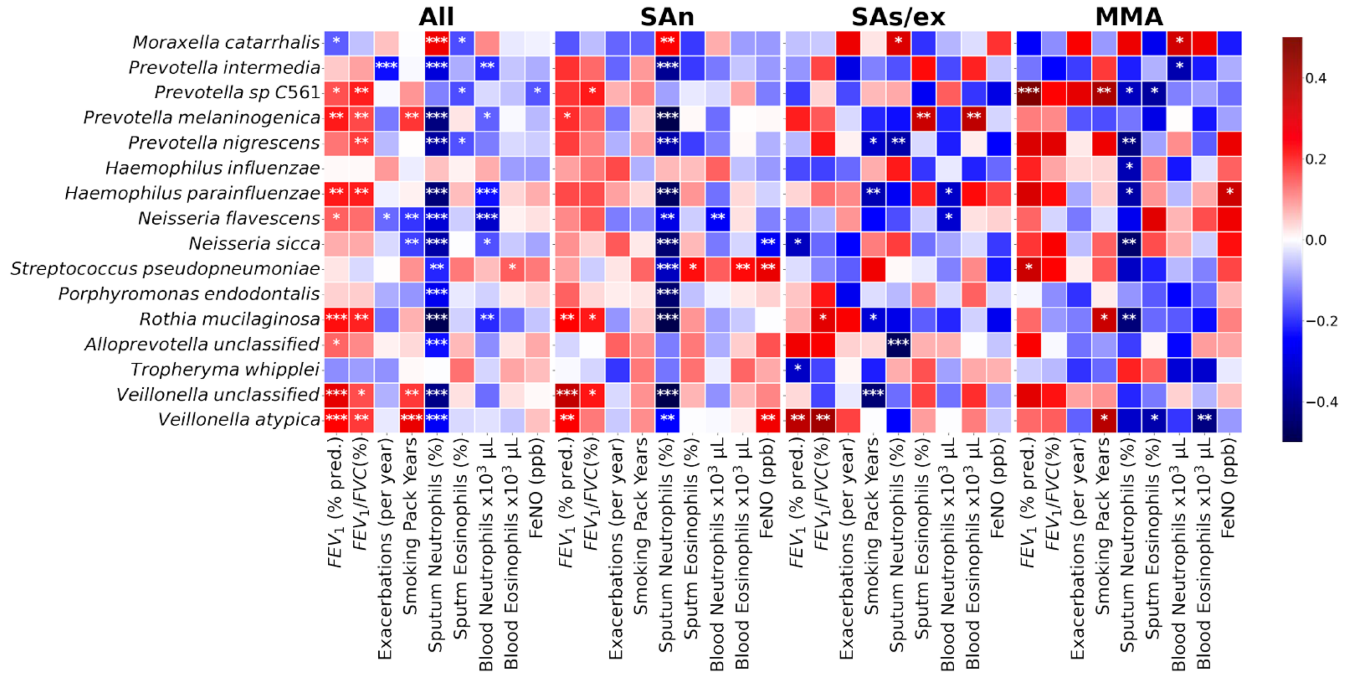

FIGURE 4 Heatmap of Spearman's correlation coefficients between FEV1 (% predicted), FEV1/FVC ratio, exacerbations in previous year, sputum neutrophil and eosinophil cell counts (%), blood neutrophil and eosinophil counts, and fractional exhaled nitric oxide (FeNO) levels against the abundance of top bacterial species for the three asthma cohorts. Significance of Spearman coefficient correlation: ns—not significant (adjusted $p > .05$), with clear box; *adjusted $p \leq .05$; **adjusted $p \leq .01$; ***adjusted $p \leq .001$. HC, healthy non-smoking and non-asthmatic; MMA, mild–moderate non-smoking asthma; SAn, non-smokers with severe asthma; SAs/ex, smokers or ex-smokers with severe asthma.

with *Moraxella catarrhalis*. This was similar in SAn apart from the lack of correlation with *Alloprevotella unclassified*. In SAs/ex, there was negative correlation between *Prevotella nigrescens* and *Alloprovotella unclassified*, but a positive correlation between *Moraxella catarrhalis* and *Neisseria sicca* with sputum neutrophils. In MMA, a negative correlation was seen with *Prevotella* sp *C561*, *Prevotella nigrescens*, *Streptococcus pseudopneumoniae*, *Neisseria flavescens*, *Neisseria sicca*, *Rothia mucilaginosa*, *Haemophilus parainfluenzae* and *Haemophilus influenzae* with sputum neutrophils.

3.4.4 | *Sputum eosinophils*

There was a negative correlation between *Moraxella catarrhalis*, *Prevotella* sp *C561*, *Prevotella nigrescens* and sputum eosinophils, but a positive correlation between *Tropheryma whipplei* and sputum eosinophils. In SAs/ex, sputum eosinophils were negatively correlated with *Rothia mucilaginosa*, but positively with *Tropheryma whipplei*, while in SAn, with *Streptococcus pseudopneumoniae* and *Alloprevotella unclassified* (Figure 4).

3.5 | Species abundance according to granulocytic inflammation

The neutrophilic group had more *Haemophilus influenzae* and *Moraxella catarrhalis* than the eosinophilic and paucigranulocytic groups, and more *Moraxella catarrhalis* compared to the mixed granulocytic group. Interestingly, the eosinophilic group showed more abundant *Tropheryma whipplei* compared to the paucigranulocytic group and HC while it had more abundant *Tropheryma whipplei*, *Haemophilus parainfluenzae*, and other species including *Prevotella pallens*, *Prevotella melaninogenica*, *Porphyromonas* sp *oral taxon 279 and Veillonella unclassified* compared to the mixed granulocytic group (Figure 2C).

3.6 | Species abundance according to TAC molecular phenotypes

As previously described,[14] TAC1 is characterized by the expression of immune receptors IL-33R, CCR3R and TSLPR, TAC2 by TNF- and IFN-associated genes, and TAC3 by expression of high glucose and succinate metabolism genes (Table S4). There was an increase in relative abundance of *Tropheryma whipplei*, *Prevotella melaninogenica*, *Porphyromonas sp oral taxon 279*, *Prevotella histicola*, *Neiseirria unclassified*, *Veillonella unclassified* and *Veillonella atypica* but decrease in relative abundance of *Moraxella catarrhalis* in TAC1 compared to TAC2. An increase in relative abundance of *Haemophilus influenzae* and *Tropheryma whipplei* in TAC1 compared to TAC3, and in *Haemophilus influenzae* and *Moraxella catarrhalis* in TAC2 compared to TAC3 (Figure 3D). Of note, *Moraxella catarrhalis* was not observable in the TAC1 group. By comparison to healthy controls, TAC1 which has the highest expression score for an IL-13 Th2 and an ILC-2

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signatures had an excess of *Haemophilus influenzae* and *Tropheryma whipplei* while TAC2 which had the highest expression scores for inflammasome and neutrophil activation an excess of *Haemophilus influenzae* and *Moraxella catarrhalis*.

3.7 | Longitudinal stability of microbiome according to asthma severity

The severe asthmatics who came for follow-up visit at one year are shown in Table S1. The dominance of *Haemophilus influenzae* or *Tropheryma whipplei* persisted in the severe asthma subjects: 3 out of 4 subjects with *Haemophilus influenzae* dominance at baseline and 2 out of 3 subjects with *Tropheryma whipplei* dominance at baseline maintained this prevalence at longitudinal follow-up (Figure 5). The same trend was observed at the genus level (Figure S6A).

There was no shift in α-diversity (Figure 5B) or β-diversity (Figure 5C) of airway microbiome in severe asthma cases analysed at the species or genus level. Similar results were seen at the genus level in α-diversity (Figure S6B) or β-diversity (Figure S6C). Moreover, Mann–Whitney *U*-test with Holm correction between paired and non-paired groups showed that genus- and species-level (Figure 5D and Figure S6D) microbiome compositional similarity between paired samples that were from the same subject was significantly higher compared to that from other possible sample pairings.

4 | DISCUSSION

This study provides a better understanding of the microbial species in severe asthma. The novelty of our analysis is being able to pin down to the microbial species level differences at the severity, inflammatory and molecular phenotype level of severe asthma using sputum metagenomic sequencing in one of the largest cohort of patients with severe asthma. This study allowed us to focus on 3 pathogenic microbial species, namely *Haemophilus influenzae*, *Moraxella catarrhalis* and *Tropheryma whipplei* because of their potential pathogenecity in respiratory diseases. We found reduced bacterial α-diversity of microbial species in the 2 severe asthma groups where there was higher abundance of *Haemophilus influenzae* and *Moraxella catarrhalis* in SAn compared to SAs/ex, MMA and HC, and of *Haemophilus influenzae* and *Tropheryma whipplei* in SAs/ex compared to MMA. In terms of inflammatory status, α-diversity was lowest in the mixed granulocytic group followed by a slight reduction in the neutrophilic group, with neutrophilic inflammation associated with higher abundance of *Haemophilus influenzae* and *Moraxella catarrhalis*, while eosinophilic inflammation was associated with high abundance of *Tropheryma whipplei*. For the first time, we have examined the abundance of microbial species with molecular phenotypes. Thus, *Haemophilus influenzae* was most abundant in TAC1 and TAC2, accompanied by an increase in *Tropheryma whipplei* in TAC1 and in *Moraxella catarrhalis* in TAC2. The abundance of *Moraxella catarrhalis* in TAC1 and TAC3 was very low. The increased abundance of *Haemophilus influenzae* in both TAC1, an eosinophilic phenotype, and in TAC2, a neutrophilic phenotype, but not in TAC3, a paucigranulocytic phenotype, is of interest. One possibility is that this increased abundance may be due to the reduced phagocytosis of *Haemophilus influenzae* by lung macrophages from patients with severe asthma.[26] *Haemophilus influenzae* can induce the release of IL1α, IL1β, IL-6, IL-8, MCP-1 and TNFα from human tracheal epithelial cells,[27] through the toll-like receptor, TLR2, activation,[28] that could lead to neutrophil activation and inflammation. However, the increased abundance of *Haemophilus influenzae* in both TAC1 and TAC2 remain unclear and was not particularly associated with oral corticosteroid therapy.

In both SAn and SAs/ex, the species most positively correlated with sputum neutrophils was *Moraxella catarrhalis* while in MMA, *Haemophilus influenzae* was inversely correlated. *Moraxella catarrhalis* is a known airway pathogen that causes respiratory infections linked to neutrophilic airway inflammation in severe or poorly controlled asthma.[9,12] *Moraxella catarrhalis* was also most abundant in the TAC2 molecular phenotype characterized by neutrophilic and inflammasome activation, supporting the possibility that it may be associated with or be responsible for the induction of neutrophilic inflammation in severe asthma. This link between *Moraxella catarrhalis* and neutrophilic inflammation is further strengthened by the highest abundance of *Moraxella catarrhalis* in TAC2 where it was positively correlated with sputum neutrophilia. This potential activating role of *Moraxella catarrhalis* is supported by its activation of TLR 2, 4 and 9,[29] induction of IL-6, IL-8 and prostaglandin E2 release through NF-κB and activation in lung epithelial cells.[30,31]

Although *Tropheryma whipplei* is known to cause Whipple's disease with gastrointestinal symptoms, it has also been implicated as a cause of aspiration, ventilator-associated and community-acquired pneumonia.[32] *Tropheryma whipplei* in bronchoalveolar lavage fluid has been isolated in asymptomatic immunosuppressed patients suffering from Human Immunodeficiency virus, which was reduced by anti-retroviral therapy.[33] The increased abundance of *Tropheryma whipplei* in SAs/ex was positively associated with sputum eosinophilia and also with pack years of smoking in the TAC1 and TAC2 phenotypes. A previous study has reported the presence of *Tropheryma whipplei* by PCR in sputum samples of severe asthma patients who were mainly eosinophilic.[12] We found that *Tropheryma whipplei* was most prominent in severe asthmatics who were either current smokers or ex-smokers. These differences between NSA and SAs/ex severe asthma groups in terms of the sputum metagenome are demonstrated here for the first time. Previous studies using 16S ribosomal RNA microarray comparing the lung microbiome of smokers with non-smokers have reported either no differences,[34,35] or a higher abundance of *Veillonella* and *Megasphaera* with a decrease in *Haemophilus*.[36] The reasons why *Tropheryma whipplei* should be increased in smoking/ex-smoking severe eosinophilic asthma and its pathogenic role of, if any, remain unclear.

Other bacterial species were also noted to be differentially abundant, being mainly reduced in the severe asthma groups of SAn and SAs/ex when compared to MMA and/ or HC. These include species of *Prevotella*, *Veillonella*, *Neisseria* and *Rothia*, *of* which we know little

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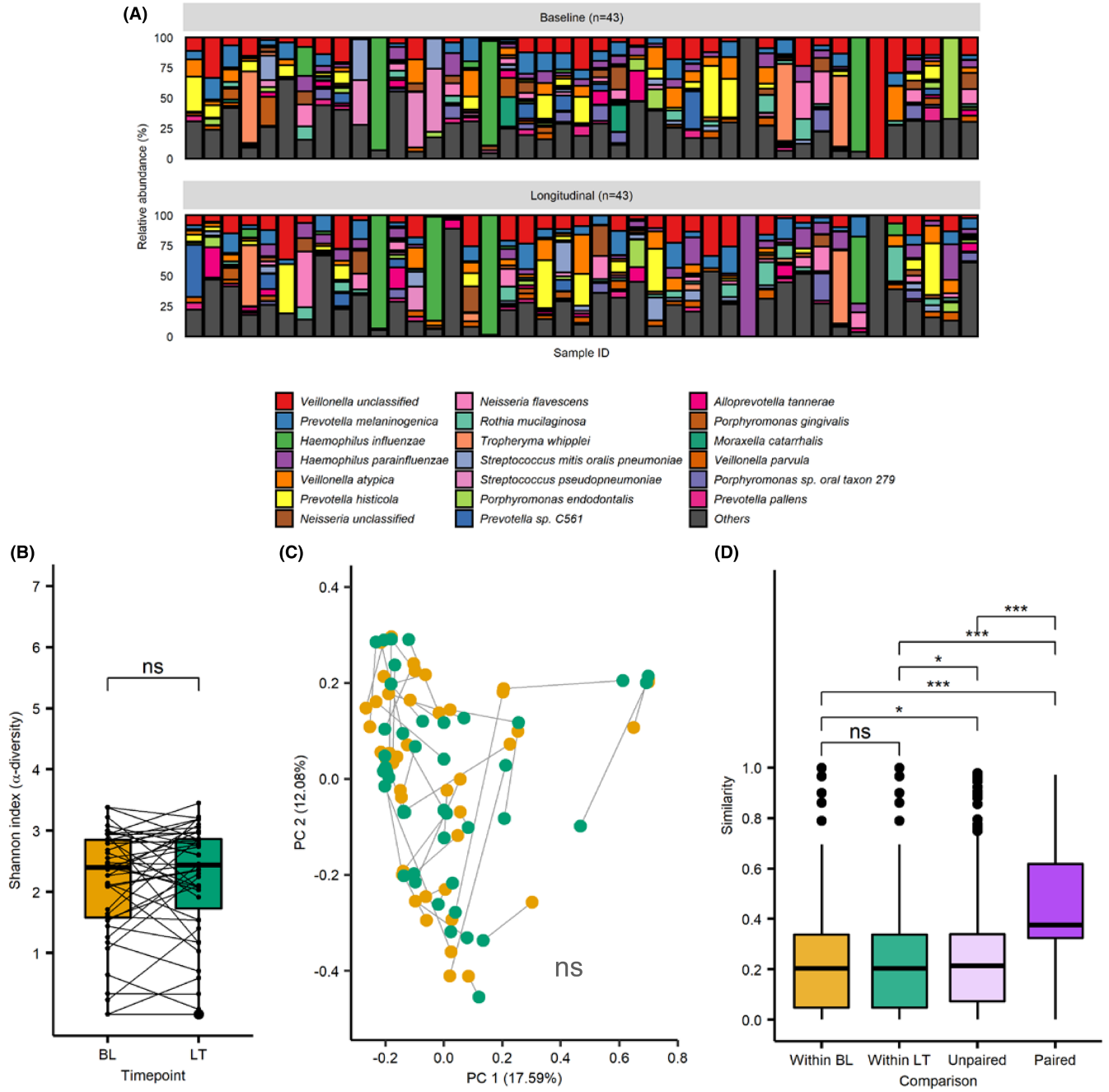

FIGURE 5 Airway of severe asthma subjects exhibits temporal stability in species-level microbiome profiles. (A) Barplots showing the baseline and longitudinal follow-up of species-level airway microbiome profiles in subjects with severe asthma, using the same order of subject identifiers. (B) Paired boxplot illustrating species-level α-diversity of airway microbiomes across in severe asthma cases. (C) Principle coordinate analysis (PCoA) based on Bray–Curtis dissimilarity illustrating species-level β-diversity of airway microbiomes at baseline or longitudinal follow-up. Paired samples that are from the same subject are connected by a line. (D) Boxplot showing the species-level microbial compositional similarity across possible sample pairing: Within BL (pairing between any two baseline samples), within LT (pairing between any two longitudinal follow-up samples), unpaired (pairing between baseline and longitudinal follow-up samples from different subjects), and paired (pairing between baseline and longitudinal samples from the same subject). Timepoint: BL (baseline) and LT (longitudinal follow-up). Significance: ns (not significant, $p > .05$), *$p \leq .05$, **$p \leq .01$ and ***$p \leq .001$.

in terms of their potential lung pathogenecity. SAn and SAS/ex were different from MMA with a decreased abundance of *Prevotella intermedia* and *Rothia mucilaginosa* consistent with the previous report of decreased abundance of the genus *Prevotella* in asthma compared to non-asthmatic individuals.[7,37] Most of these species were negatively correlated with sputum neutrophilia. Also, *Prevotella melaninogenica* and *Prevotella intermedia* abundance were inversely correlated with exacerbations in the whole asthma group, and some *Prevotella* species were reduced in abundance in non-smoking severe asthma compared to healthy controls and mild–moderate asthmatics, which has

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been previously reported.[7] However, the role of *Prevotella* species in the lungs remain less well-studied, but *Prevotella* has been associated with the activation of Th-17mediated mucosal inflammation.[38]

One bacterial species, *Haemophilus parainfluenzae*, was reduced in abundance in SAn and in TAC1 and TAC2, with negative correlation with sputum neutrophilia in SAn and MMA, and in TAC1 and TAC3. Our data indicate that *Haemophilus parainfluenzae* is decreased in current smokers compared to ex-smokers; however, since this same species is reduced also in the smoking/ex-smoking group on OCS compared to those not on OCS, it may be related to OCS therapy. *Haemophilus parainfluenzae* has been reported[39] to increase IL-8 expression with activation of p38 MAPK and inhibit corticosteroid responses in alveolar macrophages from corticosteroid-resistant asthma patients, thus may be involved in induction of corticosteroid resistance.

Overall, the microbiome was stable when repeated at one year in severe asthma with no shift in α- or β-diversity. In an unbiased microbiome-driven clustering to identify severe asthma phenotypes using the same microbial data, we found 2 distinct robust phenotypes that exhibited relative overtime stability.[40] This stability does not exclude the possibility of changes in the microbiome during acute exacerbations of asthma as occurs in children where an increase in gram-negative microbes have been detected in induced sputum.[41] Out of these 2 phenotypes,[40] one had worse asthma outcomes, more sputum neutrophilia and greater enrichment of the gammaproteobacteria, *Haemophilus influenza*e and *Moraxella catarrhalis*. Further analysis of this phenotype has led to its association with differentially expressed genes, in particular TNFα and related regulatory genes, the IL-1 family of interleukins, Toll-like receptors and inflammasomes.[42]

We highlight some technical issues in our analysis. First, the sequencing technology of using paired-end 100 base-pairs at high sampling depth provides less accurate taxonomy profiling compared to reads of 150 base-pairs or longer at lower depth. However, we have used MetaPhlAn2 because it provides a more sensible taxonomic composition for respiratory microbiome experts compared to a protein-based method such as Kaiju.[43] Secondly, the use of a algorithmic marker-based approach employed by MetaPhlAn2 for taxonomic assignment may lead to exclusion of samples for downstream analysis due to their lack of marker genes. This is likely due to sputum having high amounts of host DNA, contributed by neutrophils and neutrophil extracellular traps resulting in low level of microbial reads, with lack of marker genes for estimating taxonomic composition, thus excluding some samples in the downstream analysis. Finally, for differential analysis, DESeq2 was used because it took into account the compositional nature of the metagenomic data, with the addition of pseudocount to overcome lack of taxa shared by all samples. Finally, medication with previous and current antibiotics and of oral corticosteroid therapy may affect the microbiome. One study of unstable asthmatics treated with the macrolide antibiotic, azithromycin, showed that the bacterial load of *Haemophilus Influenzae* but not of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Moraxella catarrhalis* was significantly decreased.[44] There were only a few participants who were on antibiotic therapy at the time of the first or follow-up visit but we had no information on their previous antibiotic usage. Regarding corticosteroid therapy, those on OCS in non-smoking severe asthma had lower abundance of *Haemophilus Influenzae* and *Moraxella catarrhalis*, and therefore, being on oral corticosteroids could be another factor influencing the microbiome.

In conclusion, this sputum metagenomic study of severe asthma patients during a stable state has revealed an important association of reduced bacterial α-diversity at the species level to neutrophilic airway inflammation and neutrophil and inflammasome activation where *Haemophilus influenzae* and *Moraxella catarrhalis* were in greater abundance. On the contrary, in eosinophilic inflammation, both *Haemophilus influenzae* and *Tropheryma whipplei* were most abundant with no *Moraxella catarrhalis* found. *Tropheryma whipplei* was mostly linked to the smoking/ex-smoking severe asthma and to eosinophilic inflammation. Our analytical process has only allowed us to surmise these associations but raises the possibility that certain specific bacterial species may be activating specific inflammatory pathways or conversely, certain inflammatory pathways may be conducive to the proliferation of some species over others. Irrespective of these interactions, these bacterial species once established may play an important role in influencing the severity and inflammatory phenotype of severe asthma, particularly linked to neutrophilic inflammation. Further studies such as an analysis of the lung microbiome in asthma patients being treated with therapies targeting pathways linked to type 2 or non-type 2 inflammatory pathways will be needed to confirm the importance and role of these various bacterial species in these processes.

AUTHOR CONTRIBUTIONS

IMA, PJS and KFC conceived the idea; IMA, KFC, PJS and RD obtained the funding for U-BIOPRED project; SB, JR and PH obtained the funding for the metagenomic analysis; SB, JR, PH, IMA, SHC, AV and KFC discussed the approach to data analysis; AV, FXI, MIA and NZK analysed the data; AV, FXI and KFC wrote the manuscript; all authors contributed to its finalization and agreed with the final version for submission.

All authors gave final approval of the manuscript, had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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CONFLICT OF INTEREST STATEMENT

Dr Chotirmall has received lecture fees from Astra-Zeneca, serves on advisory boards for Boehringer-Ingelheim, CSL Behring and Pneumagen Ltd. and is on Data and Safety Monitoring Boards (DSMB) for Inovio Pharmaceuticals and Imam Abdulrahman Bin Faisal University all outside of the submitted work. Dr Maitland-van der Zee is the PI of a P4O2 (Precision Medicine for more Oxygen) public private partnership sponsored by Health Holland involving many private partners that contribute in cash and/or in kind (Boehringer Ingelheim, Breathomix, Fluidda, Ortec Logiqcare, Philips, Quantib-U, Smartfish, SODAQ, Thirona, TopMD and Novartis), she received unrestricted research grants from GSK, Boehringer Ingelheim, AbbVie and Vertex, and she received consulting fees paid to her institution from Boehringer Ingelheim and AstraZeneca; outside the submitted work. Dr. Dahlén reports personal fees from AZ, Cayman Chemicals, GSK, Novartis, Regeneron, Sanofi, TEVA, outside the submitted work. Dr Baribaud owns stock options from his former and current employer. Dr Sterk is scientific advisor and has an officially non-substantial share in the SME Breathomix that produces eNoses. Dr Chung has received honoraria for participating in Advisory Board meetings of Roche, Merck, Shionogi and Rickett-Beckinson and has also been renumerated for speaking engagements for Novartis and AZ. Dr Riley worked for and had shares in GSK. Dr. Bates reports to be an employee of Johnson & Johnson and to have previously worked and holds stock in GSK. Dr Djukanovic declares consulting fees from Synairgen, Sanofi and Galapagos, lecture fees from GSK, AZ and Airways Vista and he holds shares from Synairgen. Dr Howarth is an employee of GSK. Dr Montuschi, Dr Kermani, Dr Adcock, Dr Ivan, Dr Abdel-Aziz and Mr Versi have nothing to declare.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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