The upper-airway microbiome as a biomarker of asthma exacerbations despite inhaled corticosteroid treatment

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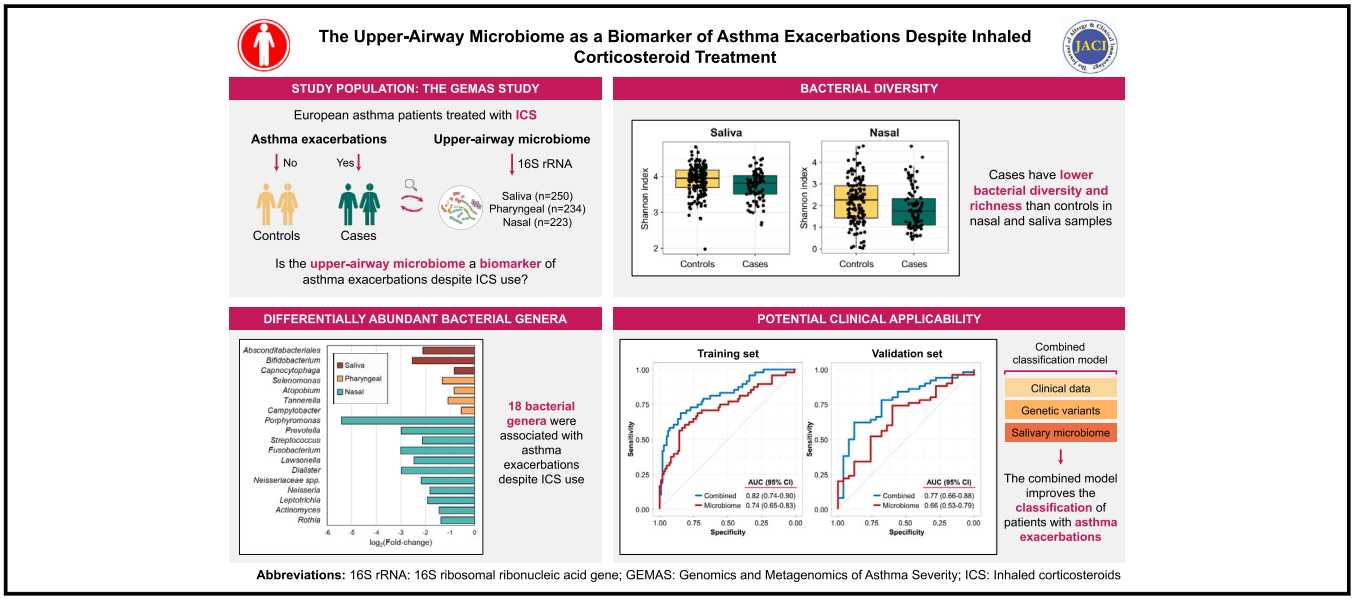
GRAPHICAL ABSTRACT



Abbreviations: 16S rRNA: 16S ribosomal ribonucleic acid gene; GEMAS: Genomics and Metagenomics of Asthma Severity; ICS: Inhaled corticosteroids

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The summary statistics of differential bacterial genera abundance analyses are openly available in the Zenodo repository (https://doi.org/10.5281/zenodo.6062007). Demultiplexed sequencing reads of the 16S rRNA gene generated in this study (biological samples, mock communities, and negatives controls) are publicly available in the Sequence Read Archive (SRA) database under accession no. PRJNA878647.

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Background: The response to inhaled corticosteroids (ICS) in asthma is affected by the interplay of several factors. Among these, the role of the upper-airway microbiome has been scarcely investigated. We aimed to evaluate the association between the salivary, pharyngeal, and nasal microbiome with asthma exacerbations despite receipt of ICS.

Methods: Samples from 250 asthma patients from the Genomics and Metagenomics of Asthma Severity (GEMAS) study treated with ICS were analyzed. Control/case subjects were defined by the absence/presence of asthma exacerbations in the past 6 months despite being treated with ICS. The bacterial microbiota was profiled by sequencing the V3-V4 region of the 16S rRNA gene. Differences between groups were assessed by PERMANOVA and regression models adjusted for potential confounders. A false discovery rate (FDR) of 5% was used to correct for multiple comparisons. Classification models of asthma exacerbations despite ICS treatment were built with machine learning approaches based on clinical, genetic, and microbiome data.

Results: In nasal and saliva samples, case subjects had lower bacterial diversity (Richness, Shannon, and Faith indices) than control subjects ($.007 \leq P \leq .037$). Asthma exacerbations accounted for 8% to 9% of the interindividual variation of the salivary and nasal microbiomes ($.003 \leq P \leq .046$). Three, 4, and 11 bacterial genera from the salivary, pharyngeal, and nasal microbiomes were differentially abundant between groups ($4.09 \times 10^{-12} \leq \text{FDR} \leq 0.047$). Integrating clinical, genetic, and microbiome data showed good discrimination for the development of asthma exacerbations despite receipt of ICS ($AUC_{training}$: 0.82 and $AUC_{validation}$: 0.77).

Conclusion: The diversity and composition of the upper-airway microbiome are associated with asthma exacerbations despite ICS treatment. The salivary microbiome has a potential application as a biomarker of asthma exacerbations despite receipt of ICS. (J Allergy Clin Immunol 2023;151:706-15.)

Key words: *16S rRNA, asthma, biomarker, exacerbations, inhaled corticosteroids, microbiota, nasal, pharyngeal, precision medicine, saliva*

Inhaled corticosteroids (ICS) are the main controller medication used to treat asthma, a chronic respiratory disease that affects 300 million people worldwide, with almost half a million associated annual deaths.[1,2] ICS are recommended to treat mild-to-severe asthma in children and adults because of their efficacy, safety, and low cost.[3] However, over 10% of patients develop refractory asthma with steroid resistance and asthma exacerbations despite being treated with high-dose corticosteroids.[2] To date, the best predictor of future asthma exacerbations is having a recent severe exacerbation.[4] Thus, searching for new biomarkers of treatment response is a priority area of research in asthma.[2,4]

The human microbiome of different body sites (eg, saliva, airways, and gut) has been closely related to the development of allergic diseases, including asthma.[5,6] The oral and nasopharyngeal microbiomes have been described as proxies of the dysbiosis in the lung microbiome that have an impact on asthma development.[7,8] Clinical interest in the human microbiome relates to its high dynamism and ability to be modulated by changes in lifestyle, diet, antibiotics, or probiotics. Thus, the microbiome has become a potential therapeutic target in precision medicine.[9]

Abbreviations used
AUC: Area under the receiver operating characteristic curve
FDR: False discovery rate
GEMAS: Genomics and Metagenomics of Asthma Severity
ICS: Inhaled corticosteroid

Nonetheless, despite its role in asthma,[5] only 2 studies have investigated its relationship with ICS response or asthma exacerbations despite ICS treatment.[10,11] Durack et al[10] studied bronchial brushing samples from 15 ICS-responder and 10 ICS-nonresponder patients with asthma and identified distinct bacterial communities between groups. Zhou et al[11] conducted a longitudinal study of the nasal bacterial microbiome in 214 children with asthma treated with low-dose ICS and reported a protective role against asthma exacerbations of nasal microbiomes with high bacterial richness dominated by *Corynebacterium* and *Dolosigranulum* compared to those dominated by *Staphylococcus, Streptococcus,* or *Moraxella*. However, the influence of the bacterial microbiome of several airway body sites on ICS response and asthma exacerbations remains unstudied.

We hypothesize that salivary, pharyngeal, and nasal bacterial microbiome diversity and composition are associated with asthma exacerbations despite ICS treatment in patients with asthma, with potential applicability as biomarkers of treatment response. We aimed to identify bacterial microbiome biomarkers of asthma exacerbations despite receipt of ICS and to assess their classification performance.

METHODS

We followed the Strengthening The Organizing and Reporting of Microbiome Studies (STORMS) guidelines to report this study (see Table E1 in this article's Online Repository at www.jacionline.org).[12] More information is provided in this article's Methods section in the Online Repository.

Study population

This study was conducted on a subset of individuals treated with ICS the previous year from the Genomics and Metagenomics of Asthma Severity (GEMAS) study (ClinicalTrials.gov NCT04501926 [https://clinicaltrials.gov/ct2/show/NCT04501926?term=NCT04501926&draw=2&rank=1]). A full description of the study design, eligibility criteria, and characteristics of enrolled subjects is reported elsewhere.[13] Briefly, European patients aged between 8 and 85 years with a diagnosis of asthma based on the Global Initiative for Asthma (GINA) 2020 guidelines[3] were recruited in several allergy and respiratory medicine hospital units in Spain. GEMAS was approved by the ethics committees of participant centers (approval nos. 29/17 and PI2019077). Saliva samples as well as nasal and pharyngeal swabs were collected from 263 patients during regular medical checkups. Furthermore, clinical and demographical data, as well as variables that may disturb the microbiome composition before sampling, were recorded in the same visit. Control and case subjects were retrospectively defined according to the absence or presence of asthma exacerbations, which were defined as receipt of systemic corticosteroids and/or hospitalizations due to asthma in the past 6 months, despite being treated with ICS.[14]

16S rRNA sequencing

Genomic DNA was isolated from 789 samples using the Pathogen Lysis Tubes S kit and the QIAamp UCP Pathogen Mini kit (Qiagen, Hilden, Germany). A TaqMan-based quantitative PCR assay was used to optimize sequencing libraries preparation. The human bacterial microbiome was profiled by targeted sequencing of the V3-V4 region of the 16S rRNA gene following the Illumina guidelines (Illumina, San Diego, Calif) and published protocols for amplicon-based microbiome profiling.[15] Paired-end sequences (300×2 bp) were obtained using the MiSeq platform (Illumina). We included a simulated mock community as a positive control. Negative controls and replicates of DNA extraction and PCR were also sequenced.

Human genome-wide genotyping

Genome-wide genotyping of human genetic variants is detailed in the Online Repository at www.jacionline.org and elsewhere.[16] Briefly, genotyping was performed using the Infinium Global Screening Array-24 kit v3.0 (Illumina). Standard quality control of genotype data was performed by PLINK 1.9, and imputation was carried out using the Trans-Omics for Precision Medicine (TOPMed) reference panel. Human genotype data were used to identify first- and second-degree relatives in our population (denoted by Pihat ≥ 0.2).

Microbiome analyses

Demultiplexed sequences were processed by QIIME2 and the 'phyloseq' R software package. Briefly, adapters were removed, and the DADA2 denoising pipeline was used for quality control of Illumina sequence data. Denoised reads were clustered into amplicon sequence variants (ASVs). Sequences were aligned against the reference SILVA rRNA database (v138) for the depletion of nonbacterial sequences and taxonomy assignation. A masked alignment of sequences was performed before the construction of rooted phylogenetic trees. The robustness of the sequencing libraries preparation protocol was assessed by correlating the observed and expected genera relative abundance distributions in positive controls and examining the correlation among biological replicates. Related individuals, samples with <10,000 reads, and contaminant amplicon sequence variants were discarded. Potential confounders of microbial communities' composition, including clinical, demographic, and technical variables, were assessed by PERMANOVA tests on beta diversity distances.

Sequencing reads were rarefied for diversity analyses. Alpha diversity was measured using the richness (observed ASVs), Shannon, and Faith phylogenetic diversity indices. We tested for the association between alpha diversity and asthma exacerbations despite ICS treatment through logistic regression models. The effect of asthma exacerbations on beta diversity (measured by the unweighted UniFrac distance) was inspected by PERMANOVA test. Differential genera abundance analyses focused on the core measurable microbiome[17] were performed using DESeq2. Multiple comparison adjustment was performed using an FDR of 5%. DESeq2 results were further curated, as explained in the Online Repository at www.jacionline.org. All diversity and abundance analyses were adjusted for age, sex, and potential confounders associated with the bacterial composition of each biological sample. Sensitivity analyses were also conducted for antibiotic use.

Classification models of asthma exacerbations

A machine learning method[18] was applied to develop a classification model of asthma exacerbations despite ICS treatment on the bases of clinical, genetic, and bacterial microbiome data. Further details are provided in the Online Repository at www.jacionline.org. Briefly, we considered clinical features and single nucleotide polymorphisms previously associated in the literature with ICS response (see Table E2 in the Online Repository), as well as the abundances of core measurable microbiome and alpha diversity indices. Individuals were divided into 2 subgroups for training (65%) and validation (35%) of the model. An elastic net model was used for variable selection and regularization. The optimal model was selected on the basis of the area under the receiver operating characteristic curve (AUC) in the training set. Model performance was then validated in the independent data set. We examined the contribution of microbiome variables by comparing the AUCs between the combined model (clinical, genetic, and microbiome data) and the salivary microbiome based on the same data sets (ie, training and validation sets).

RESULTS

Study population

A total of 250 European individuals from the GEMAS study were retained after removing related individuals (n = 5) and those not treated with ICS during the past 12 months (n = 8). After removing samples with too low biomass and low sequencing depth (<10,000 reads), we analyzed a total of 250 salivary, 234 pharyngeal, and 223 nasal samples (see Fig E1 in the Online Repository at www.jacionline.org). From those, 162 individuals were classified as control and 88 as case subjects. Their main characteristics are summarized in Table I. Briefly, over 61% were female, with a median age of 46.5 years for control and 33.0 years for case subjects. Groups did not show significant differences in lung function measurement, body mass index, cavities, seasonal biological specimen collection, and other variables that may disturb the upper-airway microbiome before sampling ($P > .05$). However, case subjects were younger and had higher receipt of antibiotics in the past 2 months than control subjects (45.5% vs 19.9%, $P = 3.54 \times 10^{-5}$). Medication adherence was high in both groups; case subjects had more severe and uncontrolled asthma than control subjects.

Assessment of potential biases in microbiome analyses

The observed and expected microbial compositions of positive controls among sequencing pools showed a high correlation (r range 0.84-0.98, $P < 2.6 \times 10^{-3}$) (see Table E3 and Fig E2 in the Online Repository at www.jacionline.org). Both DNA extraction and PCR replicates of biological samples were highly correlated ($r > 0.97$, $P < 9 \times 10^{-44}$) (see Figs E3 and E4 in the Online Repository). The 13 sequenced negative controls had an average of 76 $\pm$ 46 denoised reads; only 5 contaminant amplicon sequence variants were identified. According to alpha diversity rarefaction plots, sequencing reads were rarefied to 10,000 reads for diversity analyses in all biological samples (see Fig E5 in the Online Repository). Univariate and multivariate PERMANOVA analyses identified the following clinical, demographic, and technical variables associated with microbiome composition: (1) age, receipt of antibiotics, sequencing pool, cavities, and chewing gum in the past 30 minutes in saliva samples; (2) age, receipt of antibiotics, cavities, and liquid intake in the past 30 minutes in pharyngeal samples; and (3) age, sex, sequencing pool, body mass index, season, and smoking in the past 30 minutes in nasal samples (see Table E4 in the Online Repository). Therefore, subsequent analyses were adjusted for age, sex, and all these potential confounders identified for each sample type.

Upper-airway bacterial diversity and asthma exacerbations despite ICS treatment

Individuals who developed asthma exacerbations despite ICS treatment had lower bacterial richness and diversity (Shannon and

TABLE I. Clinical and demographic characteristics of the study population

Characteristic	Sample size	Controls (n = 162)	Cases (n = 88)	*P* value
Age (years)	250	46.5 (23.0-60.0)	33.0 (12.0-56.2)	.016*
Sex (female)	250	99 (61.1)	54 (61.4)	1.000
Pre-FEV_1 (%)	235	84.6 (70.3-99.6)	86.7 (67.9-103.2)	.930
Pre-FVC (%)	234	88.6 (73.0-103.3)	93.6 (76.0-103.0)	.829
FEV_1/FVC (%)	234	79.0 (72.4-83.9)	80.6 (70.3-85.7)	.571
Receipt of antibiotic therapy	249	32 (19.9)	40 (45.5)	3.54×10^{-5}*
Body mass index category	236			.292
Normal weight		49 (31.4)	32 (40)	
Overweight		54 (34.6)	28 (35)	
Obesity		53 (34)	20 (25)	
Season	250			.053
Spring		4 (2.5)	9 (10.2)	
Summer		30 (18.5)	1921.6	
Autumn		28 (17.3)	15 (17)	
Winter		100 (61.7)	45 (51.1)	
Asthma severity	244			7.49×10^{-5}*
Mild		5 (3.2)	0	
Moderate	18 (11.5)	0		
Severe	133 (85.3)	88 (100)		
Asthma control	230			.005*
Well controlled		79 (51.0)	33 (44.0)	
Partially controlled	44 (28.4)	12 (16.0)		
Poorly controlled	32 (20.6)	30 (40.0)		
Medication adherence	25.0 (23.0-25.0)	25.0 (23.0-25.0)	0.821	
Cavities	249	34 (21.1)	16 (18.2)	.623
Smoke†	246	4 (2.5)	0	.301
Liquid intake†	249	11 (7.0)	2 (2.4)	.148
Chewing gum†	246	5 (3.1)	3 (3.5)	1.000

Descriptive statistics are represented by median (interquartile range) for continuous variables and count (proportion) for categorical variables.
*Differences between group were evaluated using the Mann–Whitney *U* test (continuous variables) and the Fisher Exact Test (categorical variables), as appropiate. $P < .05$ was used to declare significance association.
†Variables recorded in the 30 minutes before biological sample collection.

Faith indices) in the salivary and nasal microbiome compared to those who did not develop these events ($.007 \leq P \leq .037$). The same trend was observed in the pharyngeal microbiome, but the association was not significant ($.062 \leq P \leq .145$) (Fig 1, and see Table E5 in the Online Repository at www.jacionline.org). In addition, the development of asthma exacerbations despite receipt of ICS was associated with beta-diversity unweighted UniFrac distances in saliva and nasal samples. Asthma exacerbations were found to be responsible for 8.6% ($P_{PERMANOVA} = .003$) and 9.1% ($P_{PERMANOVA} = .046$) of the interindividual variation in the salivary and nasal microbiomes, respectively (Fig 2, and see Table E6 in the Online Repository). This result was not confounded by a heterogenous dispersion of the data ($P_{PERMDISPR} > .05$). Furthermore, sensitivity analyses adding receipt of antibiotics as a covariate showed the robustness of associations between alpha and beta diversity of the nasal microbiome, with asthma exacerbations despite receipt of ICS ($.004 \leq P \leq .019$, Tables E5 and E6).

Differential bacterial genera abundance analyses

A total of 55, 52, and 44 bacterial genera constituted the core measurable microbiome in the saliva, pharyngeal, and nasal microbiomes, respectively. The bacterial genera with an average relative abundance >1% are represented in Fig 3, *A*. The most abundant bacterial genera were *Streptococcus* (29.8%), *Prevotella* (10.9%), and *Neisseria* (7.3%) in the salivary microbiome; *Streptococcus* (32.9%), *Prevotella* (12.5%), and *Veillonella* (6.9%) in the pharyngeal microbiome; and *Corynebacterium* (30.4%), *Staphylococcus* (22.4%), and *Dolosigranulum* (11.5%) in the nasal microbiome. A total of 3, 4, and 12 bacterial genera from salivary, pharyngeal, and nasal samples, respectively, were associated with asthma exacerbations despite ICS treatment, with FDR ≤ 0.05 (Table II, Fig 3, *B*) after removing potential spurious results based on raw data (see Figs E6-E8 in the Online Repository at www.jacionline.org). All bacterial genera from the nasal microbiome remained significant in sensitivity analyses adjusting for receipt of antibiotics except for *Dolosigranulum* ($P = .182$; see Table E7 in the Online Repository at www.jacionline.org).

Classification models of asthma exacerbations despite receipt of ICS

A 15-variable model based on machine learning showed the best classification performance in the training subset. This model consisted of 2 clinical variables (asthma control and asthma severity), 2 single nucleotide polymorphisms (rs279728 and rs6467778), and 11 bacterial genera from the salivary microbiome (see Fig E9 in the Online Repository at www.jacionline.org). The combined trained model showed a good performance in classifying the development of asthma exacerbations despite receipt of ICS with an AUC of 0.82 (95% confidence interval, 0.74-0.90) (Fig 4, *A*). Additionally, the model was validated in the validation subset, reaching an AUC of 0.77 (95% confidence

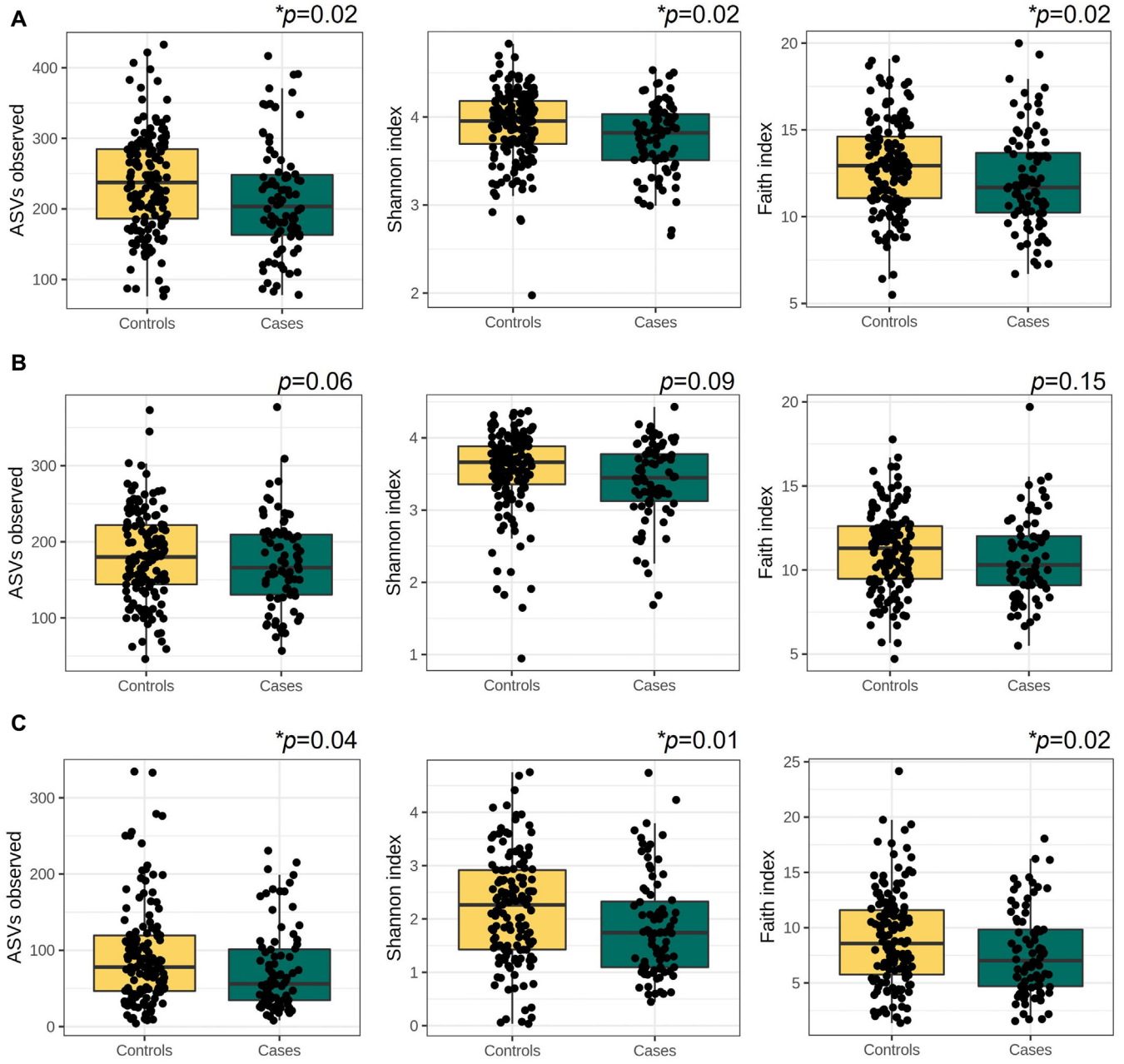

FIG 1. Box plots of alpha diversity indices for control *(yellow)* and case *(green)* subjects in **(A)** saliva, **(B)**, pharyngeal, and **(C)** nasal samples. *P* values correspond to the association between asthma exacerbations despite ICS treatment and alpha diversity adjusted by all potential confounders. *Significant *P* values.

interval, 0.66-0.88) (Fig 4, *B*). Moreover, after removing clinical and genetic variables, a model based only on the salivary microbiome had an AUC of 0.74 (95% confidence interval, 0.65-0.83) (Fig 4, *A*) and was validated with an AUC of 0.66 (95% confidence interval, 0.53-0.79) (Fig 4, *B*). Thus, the integration of the clinical, genetic, and bacterial microbiome data showed the best AUCs in both the training ($AUC_{combined}$: 0.84 vs $AUC_{microbiome}$: 0.74, $P = 5.36 \times 10^{-5}$) and validation sets ($AUC_{combined}$: 0.77 vs $AUC_{microbiome}$: 0.66, $P = .027$). The diagnostic test performance measures from all models are shown in Table E8 in the Online Repository at www.jacionline.org. In contrast to the salivary microbiome, the pharyngeal and nasal microbiome were not able to generate validated classification models (see Fig E10 in the Online Repository).

DISCUSSION

To our knowledge, this is the largest microbiome study in asthma evaluating the influence of upper-airway microbiota on asthma exacerbations despite ICS treatment in 707 biological samples from 250 European patients, and the first study assessing the role of the salivary microbiome on asthma exacerbations despite receipt of ICS. We found that asthma patients who

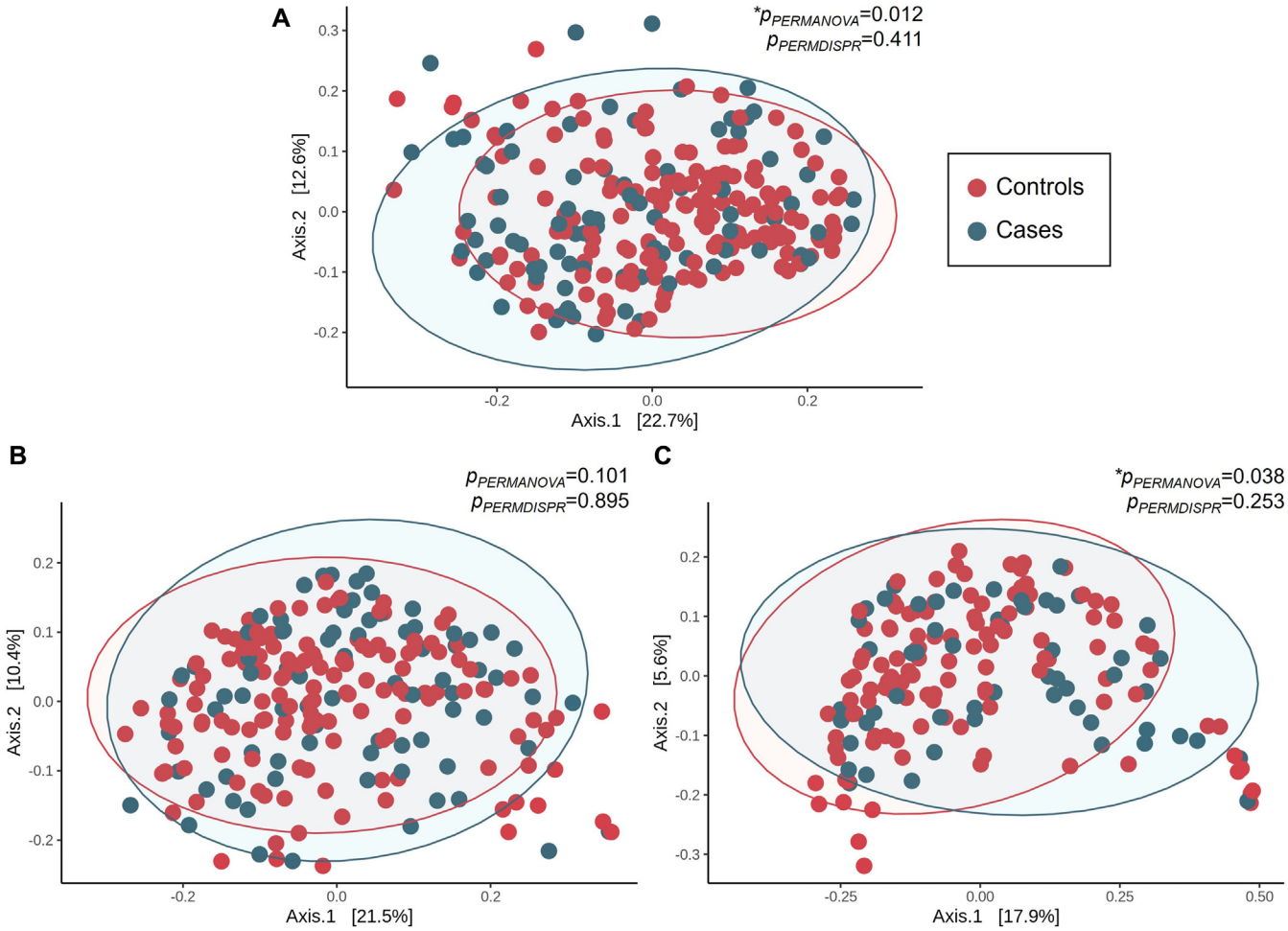

FIG 2. Principal coordinates analysis plot of beta diversity (unweighted UniFrac distances) in **(A)** saliva, **(B)** pharyngeal, and **(C)** nasal samples. X- and y-axes represent first and second principal coordinates, respectively, and the percentage of variance explained by each. Each *dot* corresponds to 1 individual (control subjects indicated by *red* and case subjects by *blue*). The $P_{PERMANOVA}$ correspond to the PERMANOVA test and $P_{PERMDISPR}$ to the multivariate homogeneity of group dispersion test. *Significant *P* values.

developed asthma exacerbations despite being treated with ICS had a less diverse salivary and nasal microbiome than those without exacerbations, and also showed interindividual differences in bacterial microbiome composition. A total of 18 bacterial genera from the salivary, pharyngeal, and nasal microbiomes were found to be differentially abundant between case and control subjects. Finally, we developed a classification model of asthma exacerbations despite ICS treatment that integrates bacterial microbiome, genetics, and clinical data, showing that the salivary microbiome improved the classification performance of the development of asthma exacerbations of clinical and genetic models.

Our study revealed for the first time the relationship between the salivary microbiome and asthma exacerbations despite ICS treatment. We found that control subjects had higher salivary bacterial richness and diversity than case subjects, a finding in agreement with the protective effect of high bacterial diversity previously described for allergies and asthma.[19,20] We identified that *Bifidobacterium, Capnocytophaga,* and *Absconditabacteriales* were more abundant in the salivary microbiome from control subjects. The most differentially abundant bacteria in control subjects was *Bifidobacterium,* which is a commensal bacteria extensively recognized to protect against allergies, given its role in the maturation of the gut microbiome in early-life stages.[21] *Bifidobacterium*-based probiotics have been shown to alleviate asthma symptoms, reduce nitric oxide levels, and improve lung function.[22-24] Interestingly, *Bifidobacterium* adjuvant therapy has been reported to improve the anti-inflammatory effects of ICS, suggesting it to be as effective as budesonide in controlling asthma.[24,25] The protective effect has also been described in murine models of steroid-resistant severe asthma by suppressing neutrophil and eosinophil lung infiltration and airway inflammation.[26] Additionally, here we also found a protective effect of *Capnocytophaga* in asthma exacerbations despite receipt of ICS in noninvasive samples such as saliva, which may reflect the lung dysbiosis described in the literature. *Capnocytophaga* abundance in lower airways has been previously associated with improved asthma control and reduced severe asthma risk.[27,28] The salivary abundance of *Capnocytophaga* has also shown a protective effect on asthma susceptibility.[19]

Although the pharyngeal microbiome had not been previously related to asthma exacerbations,[29] we detected novel associations of *Selenomonas, Atopobium, Tannerella,* and *Campylobacter*

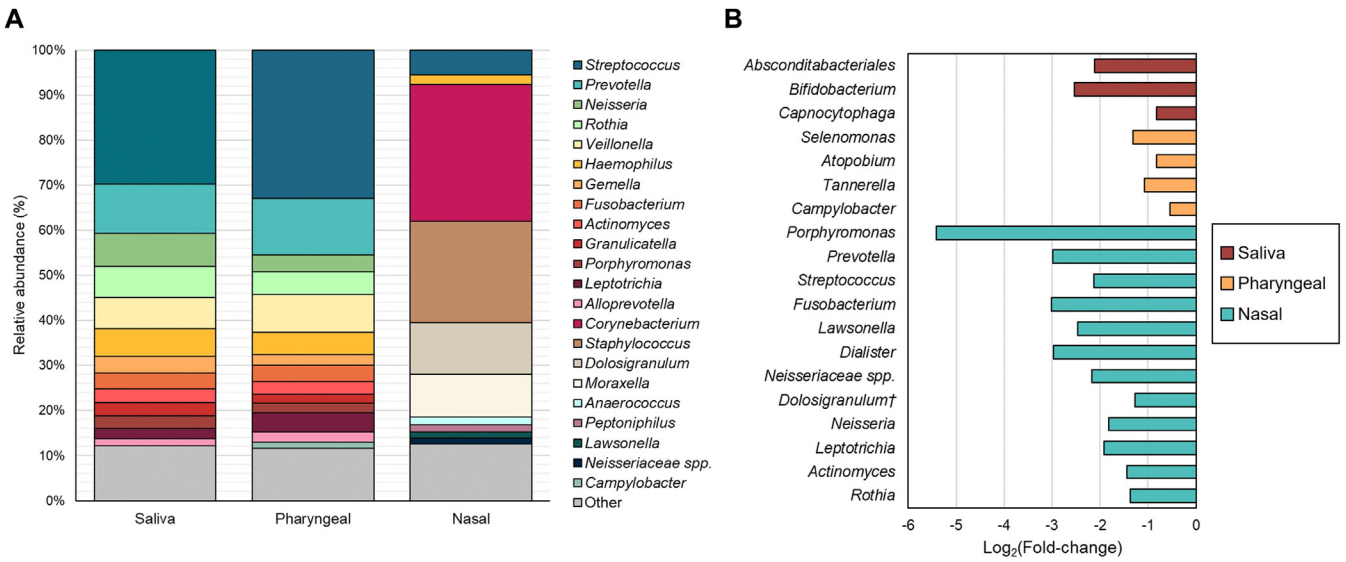

FIG 3. **(A)** Taxa bar plots of bacterial genera with an average relative abundance >1% in the salivary, pharyngeal, and nasal microbiomes. Y-axis shows relative abundance (%) of each genus. **(B)** Summary results of the bacterial genera differentially abundant between control and case subjects with FDR ≤ 0.05. X-axis shows $\log_2$ (fold change) of differential abundance for each genus. †Association did not remain significant in the sensitivity analysis.

TABLE II. Bacterial genera associated with asthma exacerbations despite receipt of ICS with FDR < 0.05

Sample and genus	$\log_2$ (fold change)	SE	*P* value	FDR
Saliva				
Absconditabacteriales	−2.11	0.53	7.47×10^{-5}	.004
Bifidobacterium	−2.53	0.67	1.66×10^{-4}	.005
Capnocytophaga	−0.83	0.26	1.62×10^{-3}	.030
Pharyngeal				
Selenomonas	−1.31	0.29	7.52×10^{-6}	3.61×10^{-4}
Atopobium	−0.83	0.25	8.56×10^{-4}	.021
Tannerella	−1.08	0.35	1.80×10^{-3}	.029
Campylobacter	−0.54	0.19	3.96×10^{-3}	.038
Nasal				
Porphyromonas	−5.42	0.73	9.31×10^{-14}	4.09×10^{-12}
Prevotella	−2.99	0.60	7.47×10^{-7}	1.64×10^{-5}
Streptococcus	−2.13	0.47	6.14×10^{-6}	9.00×10^{-5}
Fusobacterium	−3.01	0.77	1.01×10^{-4}	8.92×10^{-4}
Lawsonella	−2.47	0.68	2.52×10^{-4}	1.85×10^{-3}
Dialister	−2.97	1.01	3.42×10^{-3}	.019
Neisseriaceae spp	−2.17	0.76	4.31×10^{-3}	.021
*Dolosigranulum**	−1.27	0.46	5.82×10^{-3}	.026
Neisseria	−1.82	0.68	7.15×10^{-3}	.029
Leptotrichia	−1.92	0.75	.010	.032
Actinomyces	−1.44	0.59	.014	.041
Rothia	−1.37	0.57	.017	.047

*The association did not remain significant in the sensitivity analysis.

abundances with asthma exacerbations despite receipt of ICS. All these bacterial genera have been previously reported to interact with the gut microbiome and protect against the development of atopy in children with airway allergies.[30] Furthermore, reduced levels of *Selenomonas, Atopobium,* and *Campylobacter* have been observed in sputum samples from patients with severe asthma.[31] In addition, *Selenomonas*-based therapy in murine models of asthma decreased airway hyperreactivity and levels of lung inflammatory cells and cytokines.[31] In our study, *Selenomonas* had the strongest protective effect on asthma exacerbations despite ICS therapy, supporting its potential assessment through less invasive samples of the upper airway. In addition, *Campylobacter* and *Atopobium* have been negatively associated with metabolites involved in high risk of atopy, supporting their protective effect.[32]

Regarding the nasal microbiome, we identified 11 bacterial genera related to asthma exacerbations despite being treated with

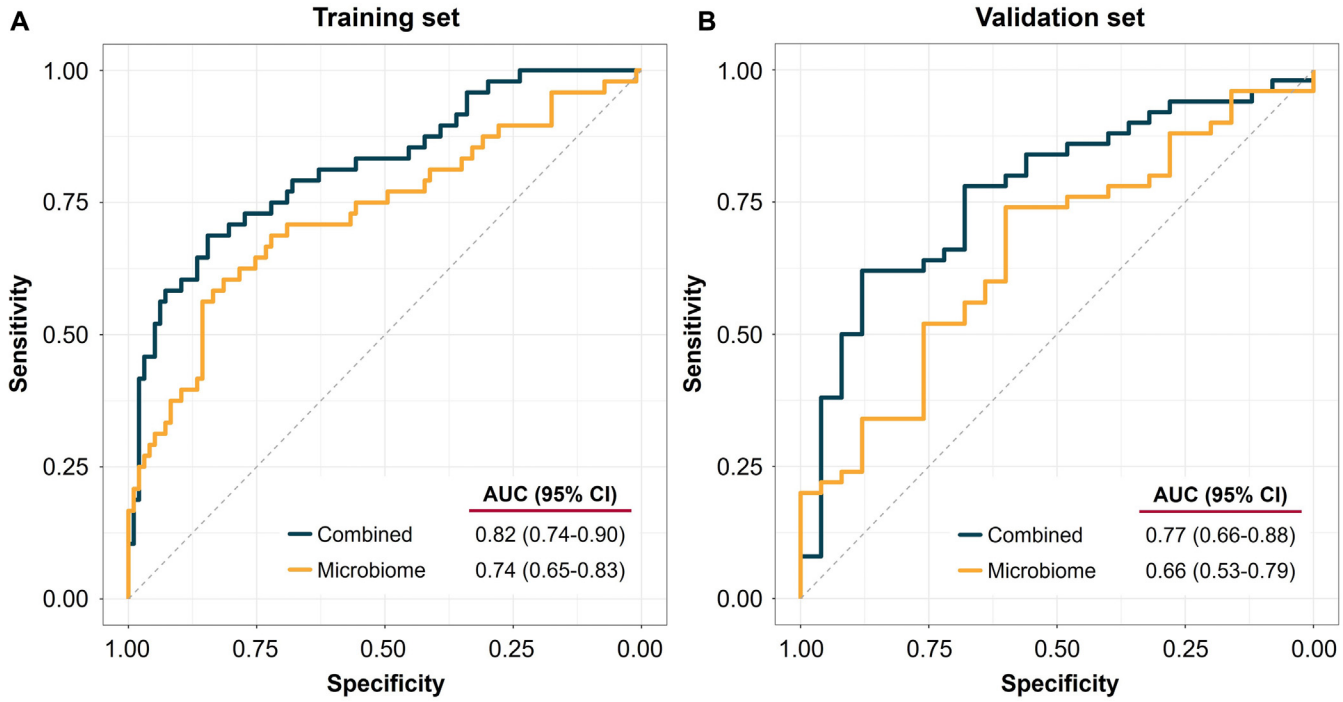

FIG 4. Receiver operating characteristic curves of classification models of asthma exacerbations despite ICS treatment based on a combined model of clinical, genetic, and salivary microbiome data (combined) and a model including only the salivary microbiome (microbiome). **(A)** Receiver operating characteristic (ROC) curves in training set. **(B)** ROC curves in validation set.

ICS. Most association studies of the human microbiome with ICS response and/or asthma exacerbations have been focused on the nasal microbiome.[10,11,14,29,33,34] Here, we confirmed the protective effects of *Rothia* and *Dialister* on asthma exacerbations.[34,35] These bacterial genera had a protective effect against the development of allergies and asthma, and *Rothia*-based probiotics have been shown to reduce airway inflammation in murine models.[36,37] Of note, the airway anti-inflammatory effect of *Rothia,* a common resident of the oral cavity, is mediated by inhibition of nuclear factor kappa–light-chain enhancer of activated B cells (aka NF-κB) pathways, similar to the effect of ICS.[38] In addition, we reported *Actinomyces* and *Porphyromonas* to be associated with asthma exacerbations despite receipt of ICS—2 bacterial genera that protect against bronchial inflammation pathways involved in asthma exacerbations.[35,39]

To date, genetic variants have not shown good performance in predicting ICS responsiveness (AUC < 0.67), and the best clinical classification models of asthma exacerbations still include as predictors the presence of recent exacerbations.[40,41] Here we report that the salivary microbiome has the potential to improve clinical and genetic classification models of asthma exacerbations, and could even be combined with other promising -omic data.[42,43] Because saliva is a noninvasive and stable sample for obtaining human and bacterial genomic DNA, even in infants with asthma,[44] further research should study the applicability of the salivary microbiome in assessing asthma treatment response and predicting asthma exacerbations.

We acknowledge several strengths of this study. First, we analyzed the largest bacterial microbiome data set of adults and children with asthma on asthma exacerbations despite ICS treatment, including noninvasive samples of the airways. Second, we ensured the robustness of bacterial microbiome profiling by including sequencing controls and considering clinical, demographic, and technical confounders in our analyses. Third, we integrated clinical, genetic, and microbiome data to develop a classification model of asthma exacerbations despite receipt of ICS validated in an independent subset. However, we also recognize some limitations. First, although we adjusted our analyses for receipt of antibiotic therapy, its effects vary significantly between individuals.[45] Furthermore, although this limitation is inherent to the definition of asthma exacerbations, our results may also reflect the effect of systemic corticosteroid therapy on the human microbiome during asthma exacerbations treatment. Second, our classification model requires further replication in other cohorts from different ancestries to validate its clinical applicability and generalizability. Moreover, all patients from GEMAS were enrolled in hospital units, which may not generalize our findings to mild asthma patients managed in primary-care health centers. Third, we used a targeted sequencing approach that does not allow us to investigate the specific bacterial species and other microorganisms involved in asthma exacerbations, or to assess the potential functionality of these microorganisms. Fourth, the cross-sectional nature of this study allows only identifying the association between the upper-airway microbiome and asthma exacerbations despite ICS treatment, but it does not provide information about causality. In addition, asthma exacerbations despite receipt of ICS are one of the main phenotypes used to measure ICS responsiveness.[46] Thus, our results could not only be related to asthma exacerbations but also to pharmacologic resistance to ICS. We encourage further research addressing the difficulties of measuring

ICS response to discover the role of the upper-airway microbiome on treatment response.[46]

In conclusion, we identified that the diversity and composition of the upper-airway microbiome are associated with asthma exacerbations despite ICS treatment. Moreover, our findings support the potential applicability of the salivary microbiome as a biomarker of asthma exacerbations despite receipt of ICS, moving toward precision medicine in asthma.

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Key messages

- **The salivary, nasal, and pharyngeal bacterial microbiome diversity and composition are associated with asthma exacerbations despite ICS therapy.**
- **The salivary microbiome is a potential biomarker of asthma exacerbations despite ICS treatment that may improve current clinical models in asthma precision medicine.**

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