Sputum microbiome profiles identify severe asthma phenotypes of relative stability at 12 to 18 months

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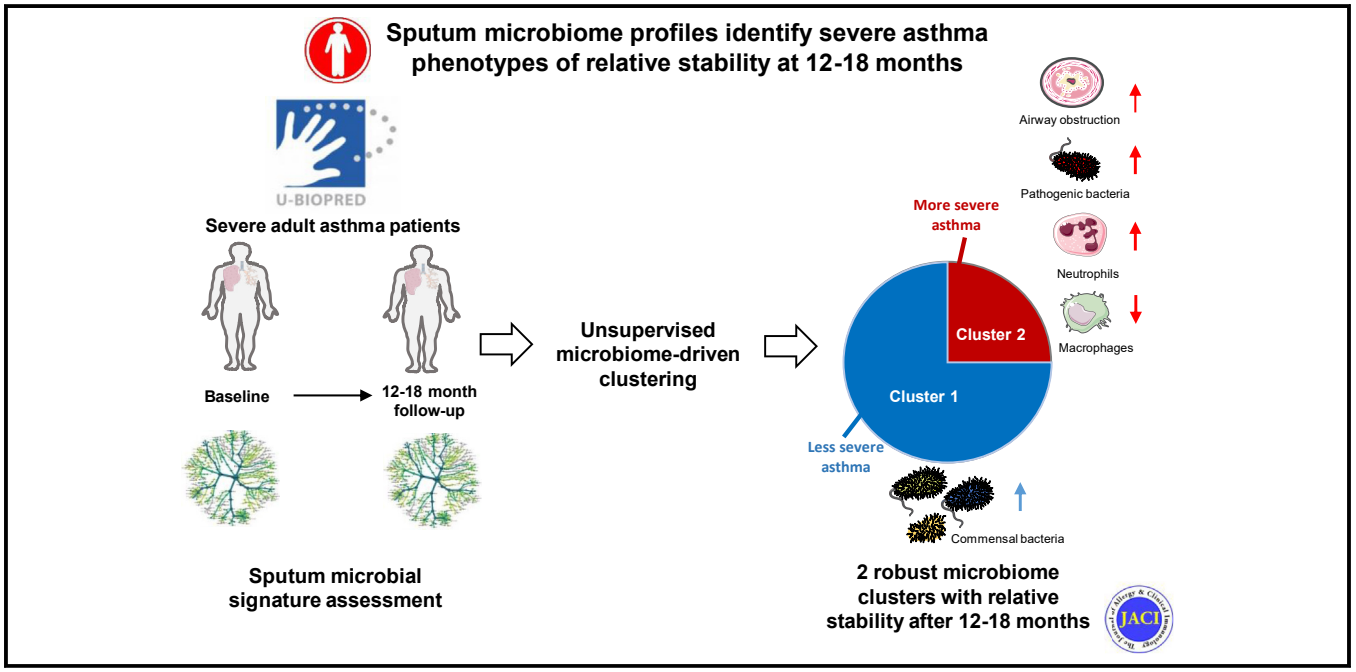
GRAPHICAL ABSTRACT



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Background: Asthma is a heterogeneous disease characterized by distinct phenotypes with associated microbial dysbiosis.

Objectives: Our aim was to identify severe asthma phenotypes based on sputum microbiome profiles and assess their stability after 12 to 18 months. A further aim was to evaluate clusters' robustness after inclusion of an independent cohort of patients with mild-to-moderate asthma.

Methods: In this longitudinal multicenter cohort study, sputum samples were collected for microbiome profiling from a subset of the Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes adult patient cohort at baseline and after 12 to 18 months of follow-up. Unsupervised hierarchical clustering was performed by using the Bray-Curtis β-diversity measure of microbial profiles. For internal validation, partitioning around medoids, consensus cluster distribution, bootstrapping, and topological data analysis were applied. Follow-up samples were studied to evaluate within-patient clustering stability in patients with severe asthma. Cluster robustness was evaluated by using an independent cohort of patients with mild-to-moderate asthma.

Results: Data were available for 100 subjects with severe asthma (median age 55 years; 42% males). Two microbiome-driven clusters were identified; they were characterized by differences in asthma onset, smoking status, residential locations, percentage of blood and/or sputum neutrophils and macrophages, lung spirometry results, and concurrent asthma medications (all *P* values < .05). The cluster 2 patients displayed a commensal-deficient bacterial profile that was associated with worse asthma outcomes than those of the cluster 1 patients. Longitudinal clusters revealed high relative stability after 12 to 18 months in those with severe asthma. Further inclusion of an independent cohort of 24 patients with mild-to-moderate asthma was consistent with the clustering assignments.

Conclusion: Unbiased microbiome-driven clustering revealed 2 distinct robust phenotypes of severe asthma that exhibited relative overtime stability. This suggests that the sputum microbiome may serve as a biomarker for better characterizing asthma phenotypes. (J Allergy Clin Immunol 2021;147:123-34.)

Key words: *Sputum microbiome, metagenomics, asthma phenotypes, unbiased clusters, follow-up, neutrophils, macrophages, lung function*

Abbreviations used
FDR: False discovery rate
PAM: Partition around the medoids
RI: Rand index
rRNA: Ribosomal ribonucleic acid
TDA: Topological data analysis
U-BIOPRED: Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes

Patients with severe asthma represent approximately 5% of the total population of individuals with asthma.[1] Severe asthma places substantial health and cost burdens on patients and health care communities.[2] It is a heterogeneous disease consisting of multiple phenotypes that show differences in clinical characteristics, inflammatory biomarkers, pathophysiologic processes, and therapeutic requirements. Better characterization of the population of patients with severe asthma should eventually lead to more effective tailoring of therapeutic decisions to meet patients' needs and thus improve outcomes and reduce burdens (precision medicine).

Asthma phenotyping aims to classify the population of individuals with asthma into subgroups based on clinical characteristics and/or biologic parameters,[3] underpinned by different pathophysiologic mechanisms that drive these phenotypes. Omics technologies utilize high throughput advanced analytic and computational tools to elucidate biologic pathways and/or highlight novel biomarkers that can improve diagnosis and therapeutic decisions.[4] Classifying cohorts of individuals with asthma by omics methods can be done by using a supervised or an unsupervised approach. The latter can be considered "unbiased" because it does not involve any *a priori* assumptions and is therefore the preferred option. By applying this principle, the Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes (U-BIOPRED) project published asthma phenotypes driven by sputum transcriptomics,[5] proteomics,[6] or breathomics.[7]

Over the past decade several studies have investigated the airway microbial dysbiosis in patients with asthma,[8-12] with some focusing on airway microbiome profiles in patients with different inflammatory phenotypes of asthma.[13-17] Inconsistencies in the reported results between the studies that might hinder direct clinical applicability have been observed.[18] In addition, most of the studies conducted have used amplicon sequencing of bacterial ribosomal RNA (rRNA), which has a limited ability to identify bacteria at the species level and therefore limits its clinical relevance.

Studying the induced sputum microbiome within the context of a large-scale multicenter asthma cohort study such as the U-BIOPRED and using both 16s rRNA sequencing and metagenomics could lead to more conclusive results. In this study, we hypothesized that the sputum microbiome, through its host and environment interaction, could reveal distinct phenotypes of severe asthma. Specifically, we aimed to (1) identify severe asthma phenotypes through unsupervised unbiased clustering of sputum microbiome profiles of patients with severe asthma, (2) assess within-patient longitudinal stability of the identified clinical clusters after 12 to 18 months, and (3) evaluate the robustness of the clinical clusters by subsequently analyzing the microbiome of patients from an independent cohort of patients with mild-to-moderate asthma in the analysis.

METHODS

Study design

The U-BIOPRED project is a multicenter prospective observational pan-European cohort study, comprising 3 subcohorts of individuals with asthma defined by standard clinical criteria: nonsmoking patients with severe asthma (cohort A), previously or currently smoking patients with severe asthma (cohort B), and nonsmoking patients with mild-to-moderate asthma (cohort C), as described in detail previously.[19] All recruited participants provided written informed consent, and each study center obtained local medical ethics committee approval. The study was registered under the identifier NCT01976767 at ClinicalTrials.gov.

The study involved 2 research visits: screening and baseline visits for patients with mild-to-moderate and severe asthma and an additional

longitudinal visit (12-18 months after baseline) for the patients with severe asthma.[19] At the baseline and longitudinal visits, several questionnaires and biologic measurements were obtained from the recruited participants as described in detail elsewhere.[19]

Participants

A total of 100 adults with severe asthma and 24 adults with mild-to-moderate asthma from the U-BIOPRED cohort study provided induced sputum samples at baseline that passed quality control, with 46 of those with severe asthma providing single additional follow-up samples after 12 to 18 months (see Fig E1 in this article's Online Repository at www.jacionline.org); the participants were from 13 study centers spanning 9 different European countries. Individuals with mild-to-moderate or severe asthma were defined according to criteria of the Innovative Medicines Initiative and Global Initiative for Asthma guidelines.[20,21] The participants completed standard asthma control and quality of life questionnaires,[22,23] underwent spirometry,[24] and were assessed for inflammatory biomarkers[25,26] and for atopy[27] (for details, see the Online Supplement in this article's Online Repository at www.jacionline.org). All participants were considered nonsmokers if they had not smoked for at least the past 12 months and had less than a 5–pack year smoking history.

Sputum induction, 16s rRNA amplicon sequencing, and shotgun metagenomics processing

Sputum at baseline and at longitudinal visits was induced by inhalation of hypertonic (0.9%-4.5%) saline according to standardized protocols.[26,28] The induced sputum samples were prepared for microbiome and metagenomics profiling, as described in detail in the Online Supplement and elsewhere.[29]

Data analysis

The general data analysis workflow is shown in Fig E2 (in this article's Online Repository at www.jacionline.org) and is further described in the following sections.

Clustering protocol. Cluster benchmarking was based on the analysis performed by Brinkman et al,[7] with modifications to suit the microbiome data. To assess the patient variability in the microbiome profiles, the Bray-Curtis β-diversity dissimilarity measure was computed separately on the basis of the numeric count data of 16s rRNA microbiome operational taxonomic units and metagenomics species. Clustering was then performed by using hierarchical Ward2 agglomerative clustering on the Bray-Curtis measure.[30] The optimum number of clusters was determined on the basis of several indices, such as optimum average silhouette width,[31] total within-cluster sum of square,[32] and Calinski-Harabasz[33] indices. Cluster assignment of the patients was internally validated by using partition around the medoids (PAM).[34] Agreement in the clustering of patients' assignments between hierarchical Ward's clustering and PAM clustering was quantified by means of the Pearson chi-square or Fisher exact test and the Rand index (RI).[35] This quantification was also performed to assess whether the clustering would differ in 16s rRNA sequencing versus in the metagenomics approach. Clustering was further validated visually by using topological data analysis (TDA) with the Ayasdi workbench (version 7.15.0; Ayasdi, Menlo Park, Calif) as reported previously.[6,7] In TDA, visual depiction of the shape of patients' metagenomics data was performed, wherein nodes represent patients' points that are highly similar and connected by edges (lines) to nodes that have data in common. The neighborhood lenses (filter functions) 1 and 2 were used to generate a graph of patients' metagenomics data into 2-dimensional space by the k-nearest neighbor's algorithm. The TDA graph thus shows connections of each patient point to its nearest neighbors only by information driven from the metagenome.

Clusterwise stability was evaluated by consensus cluster distribution[36] and by resampling the data (1000 iterations) using bootstrapping, jittering, and replacement of points by noise with subsequent calculation of the Jaccard similarity indices.[37]

Metagenomics data were used to reveal the bacterial profiles of the identified clusters up to species level.

Cluster migration. The same clustering protocol was reperformed for the longitudinal samples. Longitudinal cluster migration and stability were assessed by cross-tabulating baseline and longitudinal patient clusters assignments and assessed visually by a Sankey diagram.

Cluster robustness. Robustness of the clustering was evaluated by including patients with mild-to-moderate asthmas (U-BIOPRED cohort C) to test whether their inclusion would fit the previous clustering solution or result in cluster disintegration.

Statistical analysis. Patient cluster distribution according to the inclusion country and season of sample collection was tested by using a chi-square test with Monte Carlo simulation (2000 permutations) and later visualized by principal coordinate analysis with the Bray-Curtis dissimilarity measure.

Differences in patients' demographic and clinical characteristics between the baseline and longitudinal visits and between the revealed clusters were compared by using the Wilcoxon signed rank and McNemar tests for paired data and the Pearson chi square, Fisher exact, or Mann-Whitney *U* tests for independent data as appropriate (2-tailed). Results are considered significant at an alpha level less than 0.05.

Differences in microbiome profiles between the clusters and between baseline and longitudinal visits were compared by using a Mann-Whitney *U* test and Wilcoxon signed rank test, respectively. P-values for metagenomics species differences were adjusted for multiple testing by using Benjamini-Hochberg false discovery rate correction (FDR).[38] Results are considered significant at an FDR alpha level less than 0.05.

A correlation heatmap (Spearman and point-biserial correlations) was depicted between bacterial species and asthma clinical characteristics that were found to be statistically significantly different between the baseline clusters.

All analyses were performed by using R studio (version 1.1.453) with R software (version 3.5.1) supported with the following software packages: phyloseq, vegan, stats, cluster, factoextra, ConsensusClusterPlus, fpc, fossil, metacoder, and SIAMCAT.

RESULTS

The baseline and follow-up characteristics for the included participants are summarized in Table I.

Unsupervised unbiased clustering of microbiome profiles of patients with severe asthma at baseline identified 2 main clusters

The 16s rRNA microbiome sequencing identified a total of 2777 operational taxonomic units, whereas the metagenomics approach identified a total of 251 bacterial species. Bray-Curtis beta microbiome diversity suggested 2 optimum clusters as evaluated by multiple indices (see Fig E3 in this article's Online Repository at www.jacionline.org). Applying hierarchical Ward's clustering revealed 2 groups of patients with severe asthma whose asthma was driven only by their microbiome profiles at baseline visit (Fig 1, *A*). PAM clustering also revealed 2 isolated clusters (Fig 1, *B*). Quantitative assessment of similarity in patients' assignment between hierarchical Ward's and PAM clustering was performed by means of the Pearson chi-square test ($\chi^2 = 51.85$; $P < 1 \times 10^{-11}$) and RI value (0.82), suggesting great similarity. The 16s rRNA microbiome-generated clusters were highly concordant with the metagenomics generated clusters as indicated by Pearson chi-square test ($\chi^2 = 63.659$; $P < 1.48 \times 10^{-15}$) and RI value (RI = 0.85). Visual representation by the TDA analysis showed that the 2 patient groups could be driven by the microbiome profiles (Fig 1, *C*) when the metagenomics species were mapped to the color coding of the 2 hierarchical clusters.

TABLE I. Baseline and longitudinal patient characteristics for the patients with severe and mild-to-moderate asthma

Characteristic	Patients with severe asthma			Patients with mild-to-moderate asthma
	Baseline (n = 100)	Longitudinal (n = 46)	*P* value*	Baseline (n = 24)
Median age, y (IQR)	55 (46.0-62.0)	57 (51.3-63.5)	4.3×10^{-11}	40.50 (25.75-51.00)
Median age of onset, y (IQR)	27 (7-46)	37 (14-49)	NA	9 (3-22)
Females, n (%)	58 (58.0%)	25 (54.3%)	NA	11 (45.8%)
Median BMI, kg/m^2 (IQR)	27.72 (24.67-32.45)	27.71 (24.37-31.04)	NA	24.02 (21.80-30.18)
White race, n (%)	92 (92.0%)	43 (93.5%)	NA	23 (95.8%)
Residential location, n (%)			NA	
● Rural	26 (26.0%)	10 (21.7%)		6 (25%)
● Suburban	25 (25.0%)	8 (17.4%)		8 (33.3%)
● Urban	49 (49.0%)	28 (60.9%)		10 (41.7%)
Atopy, n (%)	70 (70.0%)	31 (67.4%)	NA	23 (95.8%)
Nonsmoking patients, n (%)	66 (66.0%)	30 (65.2%)	NA	24 (100%)
Median percent of eosinophils in sputum (IQR)	2.75 (0.37-19.27)	2.19 (0.76-17.12)	.838	0.72 (0.21-1.81)
Median percent of neutrophils in sputum (IQR)	57.98 (39.59-81.98)	62.95 (51.19-78.24)	.096	42.17 (26.16-75.18)
Median percent of macrophages in sputum (IQR)	22.82 (10.15-39.55)	21.57 (10.01-37.84)	.068	42.97 (21.62-66.69)
Median percent of eosinophils in blood (IQR)	3.43 (1.58-6.51)	3.50 (1.86-4.97)	.024	3.55 (1.81-4.46)
Median percent of neutrophils in blood (IQR)	58.48 (53.96-67.45)	60.55 (54.88-76.83)	.190	58.75 (53.35-64.82)
Median FEV_1 percent predicted before salbutamol (IQR)	62.78 (45.31-74.16)	61.42 (49.03-74.75)	.642	92.00 (87.34-104.45)
Median FEV_1 percent predicted after salbutamol (IQR)	73.08 (52.77-86.92)	72.79 (55.24-85.61)	.468	103.13 (88.51-114.07)
Median FEV_1/FVC percent predicted before salbutamol (IQR)	73.46 (61.03-83.66)	75.21 (61.35-83.33)	.282	89.99 (79.80-96.54)
Median FEV_1/FVC percent predicted after salbutamol (IQR)	76.49 (65.03-87.28)	78.70 (64.03-85.90)	.461	96.91 (87.42-100.83)
Median F_{ENO} in ppb (IQR)	23.50 (13.25-45.00)	27.50 (16.90-50.00)	.708	30.25 (19.50-58.13)
Median exacerbations per year (IQR)	2 (1-3)	2 (0-3)	.737	0 (0-1)
Median ACQ5 score average (IQR)	2.3 (1.60-3.20)	1.9 (0.95-3.00)	.589	1.00 (0.45-1.55)
Median AQLQ score average (IQR)	4.46 (3.50-5.50)	4.59 (3.67-5.60)	.245	5.59 (4.84-6.56)
Current asthma medication used, n (%)				
● ICS	100 (100%)	42 (91.3%)	.125	24 (100%)
● SABA	63 (63.0%)	27 (58.7%)	.999	19 (79.2%)
● LABA	99 (99.0%)	43 (93.5%)	.250	1 (4.2%)
● OCS	87 (87.0%)	14 (30.4%)	.070	0 (0.0%)
● Short-acting anticholinergic	9 (9.0%)	3 (6.5%)	.999	1 (4.2%)
● Long-acting anticholinergic	29 (29.0%)	15 (32.6%)	.999	0 (0.0%)
● Leukotriene antagonists	43 (43.0%)	19 (41.3%)	.999	0 (0.0%)
● Theophylline	21 (21.0%)	7 (15.2%)	.999	0 (0.0%)
Antibiotic use, n (%)				
● Current intake	18 (18.0%)	7 (15.2%)	.500	0 (0.0%)
● Current and previous (ever) intake	22 (22.0%)	26 (56.5%)	<.001	1 (4.2%)

ACQ5, 5-Item Asthma Control Questionnaire; *AQLQ*, Asthma Quality of Life Questionnaire; *BMI*, body mass index; *F_{ENO}*, fraction of exhaled nitric oxide; *FVC*, forced vital capacity; *ICS*, inhaled corticosteroid; *IQR*, interquartile range; *LABA*, long-acting β-agonist; *NA*, not applicable; *OCS*, oral corticosteroid; *SABA*, short-acting β-agonist.
**P* values were computed for paired differences only for patients who have both baseline and longitudinal sputum samples.

There were no statistically significant associations between the patients' clusters assignments and the 9 countries from which samples were collected (the *P* values were .110 and .229 for hierarchical and PAM clustering, respectively) or the season of sample collection (the *P* values were .633 and .702 for hierarchical and PAM clustering, respectively). This was visually confirmed by running principal coordinate analysis on Bray-Curtis dissimilarity measure, showing random patient allocations (see Fig E4 in this article's Online Repository at www.jacionline.org).

Baseline clusters show distinct demographic and clinical characteristics

Table II shows the demographic and clinical characteristics of the 2 baseline clusters of patients with severe asthma. Cluster 1 represents 75 % of the patients with severe asthma (n = 75). More than half of them lived in urban areas. As compared with the cluster 2 patients, the cluster 1 patients had significantly lower percentages of sputum neutrophils, higher percentages of sputum macrophages, and higher values for FEV_1 and FEV_1/FVC percent predicted before and after salbutamol administration (see Fig E5, *A* in this article's Online Repository at www.jacionline.org). In contrast, the cluster 2 patients had a significantly younger age of asthma onset, were mostly nonsmokers (84%), and were more likely to live in suburban areas. In addition, a higher percentage of cluster 2 patients were prescribed theophylline.

Microbial profiles of the 2 baseline clusters of patients with severe asthma

The cluster 2 patients had lower microbial richness and alpha diversity indices when compared with the cluster 1 patients (Fig 2). Fig E6 (in this article's Online Repository at www.jacionline.org) shows the bacterial phylogenetic map of the 2 baseline clusters. Statistical testing showed that a total of

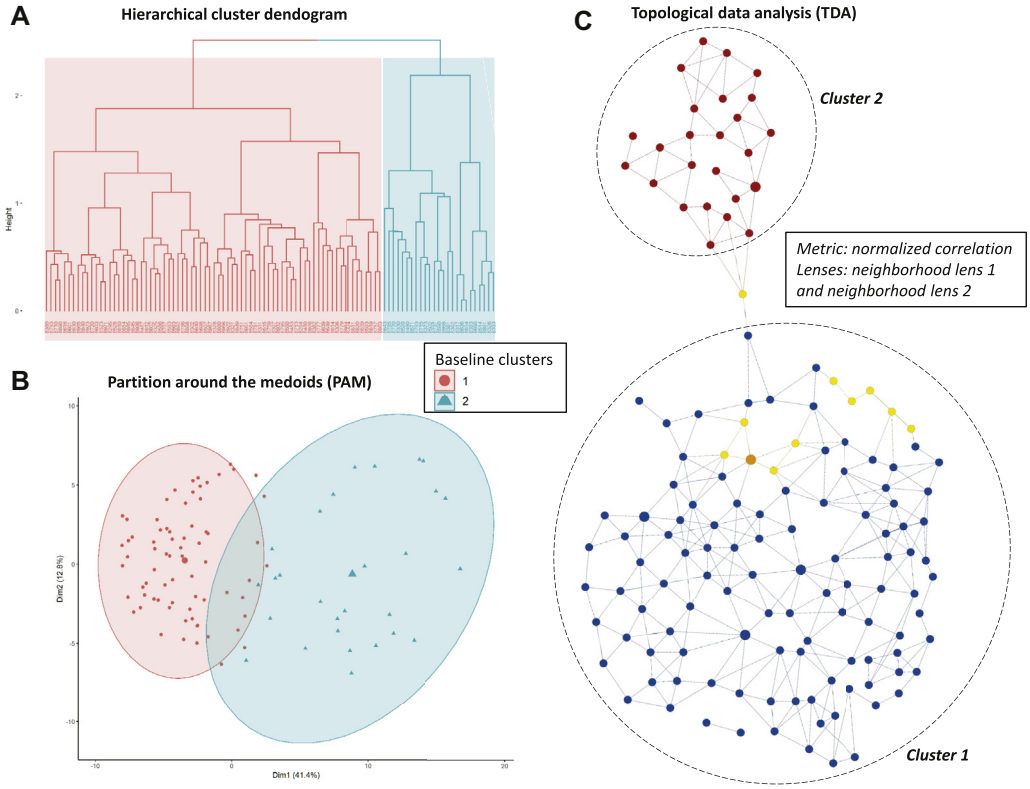

FIG 1. A, Hierarchical cluster dendogram in a tree-like structure in which patients' nodes (*leaves at the bottom of the dendogram*) that are statistically closely connected are joined together by edges (*small branches*). The small branches are further joined by larger branches (*bottom-up*) to the upper part of the dendogram representing the 2 main branches (*clusters*) originating from the severe asthma cohort. **B,** PAM clustering show 2 relatively detached ellipses. Similarity in patients' assignment between the 2 clustering algorithms was assessed by Pearson chi-square test ($\chi^2 = 51.85$, $P < 1 \times 10^{-11}$) and RI value (0.802), suggesting great similarity. Bootstrapping, jittering, and replacement of points by noise schemes (1000 iterations) resulted in Jaccard similarity indices ranged from 0.82 to 1 for both clusters by either hierarchical clustering or PAM, suggesting highly stable clusters. **C,** TDA graph, in which nodes are colored in accordance with baseline hierarchical clustering of patients. Two distinct patient clusters based on the metagenome profile are observed; blue nodes represent cluster 1 patients, whereas red nodes represent cluster 2 patients. Yellow nodes represent less matched patient cluster assignment by TDA compared with when hierarchical clustering is used.

28 species remained significantly different between the 2 clusters after FDR correction. All of them were more abundant in the patients in cluster 1 than in the patients in cluster 2 (Figs 3 and 4). Those species were related to 3 dominant phyla, *Firmicutes, Bacteriodetes,* and *Actinobacteria,* and to a lesser extent, to the phylum Proteobacteria, which comprises the following main genera: *Veillonella, Prevotella, Alloprevotella, Streptococcus, Porphyromonas, Rothia, Haemophilus, Neisseria, Megasphaera,* and a few others. In contrast, there was a trend toward increased relative abundance of a few pathogenic species, such as *Haemophilus influenza*, *Moraxella catarrhalis,* and *Streptococcus pseudopneumoniae* in cluster 2 patients compared with in cluster 1 patients; however, these results were not statistically significant (see Fig E7 in this article's Online Repository at www.jacionline.org). Correlations between individual bacterial species and asthma characteristics were of weak or moderate ($r < 0.50$) strength (see Fig E8 in this article's Online Repository at www.jacionline.org) and reflected the findings revealed by the clustering.

Unsupervised clustering of the microbiome profiles of patients with severe asthma at follow-up (longitudinal clusters)

Microbiome data were available for 46 of the 100 patients with severe asthma after 12 to 18 months from baseline inclusion. Similar to the values at the baseline visit, different indices suggested that the microbiome profiles of patients with severe asthma at the longitudinal visit allocated the patients into 2 main clusters (see Fig E9 in this article's Online Repository at www.jacionline.org). Hierarchical Ward's clustering had a great similarity to PAM (see Fig E10 in this article's Online Repository at www.jacionline.org) as quantified by the Fisher exact test ($P < 1 \times 10^{-6}$) and RI value (0.875).

Demographic and clinical characteristics of the longitudinal sputum microbiome clusters

Table III shows the demographic and clinical characteristics of the 2 longitudinal clusters of patients with severe asthma. The

TABLE II. Demographic and clinical characteristics of the baseline clusters.

Characteristics	Baseline		*P* value
	Cluster 1 (n = 75)	Cluster 2 (n = 25)	
Median age, y (IQR)	55 (46-62)	57 (49-63)	.327
Median age of onset, y (IQR)	30.50 (14.00-48.25)	16.00 (5.00-33.50)	**.012**
Females, n (%)	44 (58.7%)	14 (44.0%)	.815
Median BMI, kg/m^2 (IQR)	27.73 (24.60-32.61)	27.47 (24.48-30.82)	.591
White race, n (%)	70 (93.3%)	22 (88.0%)	.409
Residential location, n (%)			**.009**
● Rural	21 (28.0%)	5 (20.0%)	
● Suburban	13 (17.3%)	12 (48.0%)	
● Urban	41 (54.7%)	8 (32.0%)	
Atopy, n (%)	54 (72.0%)	16 (64.0%)	.359
Nonsmokers, n (%)	45 (60.0%)	21 (84.0%)	**.028**
Eosinophils % in sputum, median (IQR)	3.81 (0.19-21.92)	2.28 (0.39-7.08)	.446
Median percent of neutrophils in sputum (IQR)	53.40 (32.40-70.74)	86.90 (57.32-92.73)	**<.0001**
Median percent of macrophages in sputum (IQR)	26.69 (14.60-47.30)	9.21 (3.89-19.49)	**<.0001**
Median percent of eosinophils in blood (IQR)	3.50 (1.52-6.53)	3.28 (1.74-6.42)	.987
Median percent of neutrophils in blood (IQR)	58.16 (53.93-66.54)	58.80 (53.48-70.57)	.678
Median FEV_1 percent predicted before salbutamol (IQR)	65.24 (52.38-74.15)	47.66 (38.36-74.18)	**.035**
Median FEV_1 percent predicted after salbutamol (IQR)	74.88 (61.52-86.94)	51.44 (42.90-87.62)	**.009**
Median FEV_1/FVC percent predicted before salbutamol (IQR)	74.62 (65.60-83.97)	65.99 (51.82-77.86)	**.030**
Median FEV_1/FVC percent predicted after salbutamol (IQR)	79.25 (67.02-89.11)	68.19 (52.31-83.86)	**.012**
Median FENO in ppb (IQR)	26.25 (12.63-53.00)	22.00 (14.00-26.75)	.328
Median exacerbations per year (IQR)	2 (1-3)	2 (1-4)	.416
Median ACQ5 score average (IQR)	2.40 (1.40-3.20)	2.20 (1.65-3.15)	.783
Median AQLQ score average (IQR)	4.53 (3.52-5.53)	4.09 (3.33-5.14)	.417
Current asthma medication use, n (%)			
● ICS	75 (100%)	25 (100%)	NA
● SABA	45 (60.0%)	18 (72.0%)	.282
● LABA	74 (98.7%)	25 (100%)	.999
● OCS	33 (44.0%)	14 (56.0%)	.298
● Short-acting anticholinergics	8 (10.7%)	1 (4.0%)	.444
● Long-acting anticholinergics	20 (26.7%)	9 (36.0%)	.373
● Leukotriene antagonists	32 (42.7%)	11 (44.0%)	.907
● Theophylline	11 (14.7%)	10 (40.0%)	**.007**
Median OCS normalized dose, mg (IQR)	10.00 (8.44-16.25) (n = 33)	10.00 (5.00-14.38) (n = 14)	.363
Antibiotic use, n (%)			
● Current intake	14 (18.7%)	4 (16.0%)	.999
● Current and previous (ever) intake	18 (24%)	4 (16.0%)	.403

ACQ5, 5-Item Asthma Control Questionnaire; *AQLQ*, Asthma Quality of Life Questionnaire; *BMI*, body mass index; *FENO*, fraction of exhaled nitric oxide; *FVC*, forced vital capacity; *ICS*, inhaled corticosteroid; *IQR*, interquartile range; *LABA*, long-acting β-agonist; *NA*, not applicable; *OCS*, oral corticosteroid; *SABA*, short-acting β-agonist.
P values for testing statistical significance between the 2 longitudinal clusters were calculated by using the Pearson chi-square or Fisher exact tests as appropriate for categoric variables and the Mann-Whitney *U* test for continuous variables. Entries with statistically significant *P* values are boldface.

patients in longitudinal cluster 2 were more likely to live in rural areas (55.6%) than were the cluster 1 patients, who were more likely to live in urban areas (70.3%). As in the baseline analysis, the patients in longitudinal cluster 2 had higher percentages of sputum and blood neutrophils, lower percentages of sputum macrophages, and lower values for FEV_1 percent predicted before and after administration of salbutamol (see Fig E5, *B*). In addition, a higher percentage of cluster 2 patients than cluster 1 patients were prescribed long-acting anticholinergics.

Microbial profiles of the longitudinal clusters of patients with severe asthma

The patients in longitudinal cluster 2 had lower microbial richness and diversity than did the patients in longitudinal cluster 1, as estimated by multiple indices (as shown in Fig E11 in this article's Online Repository at www.jacionline.org). Fig E12 (in this article's Online Repository at www.jacionline.org) shows the bacterial phylogenetic map of the 2 longitudinal clusters. A total of 13 species remained significantly different between the 2 clusters after FDR correction. All of them were more abundant in the patients in cluster 1 than in the patients in cluster 2 (see Figs E13 and E14 in this article's Online Repository at www.jacionline.org). Those species were related to the following main genera: *Veillonella, Prevotella, Streptococcus, Rothia, Haemophilus,* and *Neisseria*.

Stability of the longitudinal clusters over time in patients with severe asthma

Patients who had both baseline and longitudinal microbiome samples were cross-tabulated to check similarity in the clusters' assignment between the 2 visits (Fig 5). Of the 46 patients, 39 (84.7%) remained cluster stable longitudinally. Quantitative assessment resulted in a statistically significant Fisher exact test value ($P < .01$) and relatively high RI value (0.74), suggesting

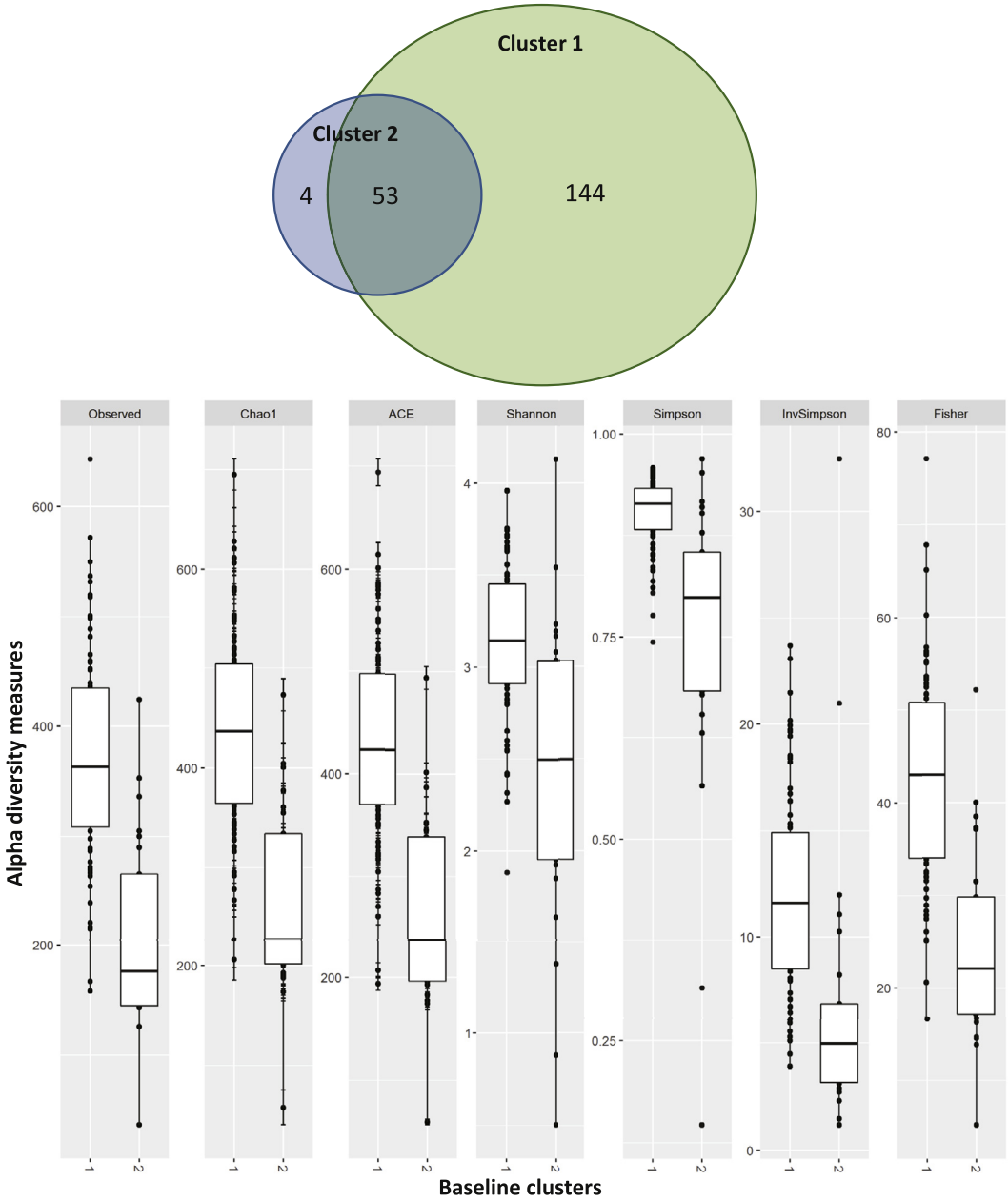

FIG 2. Venn diagram representing the metagenomics species distribution between the 2 clusters (*upper panel*). Different alpha diversity measures reveal that the cluster 2 patients had much lower microbial diversity than did the cluster 1 patients (all *P* values < .0001) (*lower panel*).

relative cluster stability after 12 to 18 months. No significant differences in microbial richness and diversity (see Fig E15 in this article's Online Repository at www.jacionline.org) or in species' relative abundance after FDR correction between the baseline and longitudinal visits were observed. Fig E16 (in this article's Online Repository at www.jacionline.org) shows a bar chart of the mean percentage in relative abundance of bacterial taxa at the baseline and longitudinal visits.

Cluster robustness when including patients with mild-to-moderate asthma in the analysis

Repeating the baseline clustering with inclusion of both the data from the patients with severe asthma (n = 100) and the data from those with mild-to-moderate asthma (n = 24) also resulted in 2 main clusters (see Fig E17 in this article's Online Repository at www.jacionline.org); 23 of the 24 patients with mild-to-moderate asthma (95.8%) were assigned to cluster 1, with only 1 patient with mild-to-moderate asthma being assigned to cluster 2 when hierarchical clustering was used. This patient displayed clinical characteristics similar to those of patients with severe asthma in cluster 2 with respect to young age of asthma onset, sputum neutrophilia, and decreased percentage of sputum macrophages and FEV_1 values (Table E1 in this article's Online Repository at www.jacionline.org). A cross-table to assess whether the initial cluster assignment of the patients with severe asthma had changed after inclusion of the group of patients with mild-to-moderate asthma is shown in Table E2 (in this article's

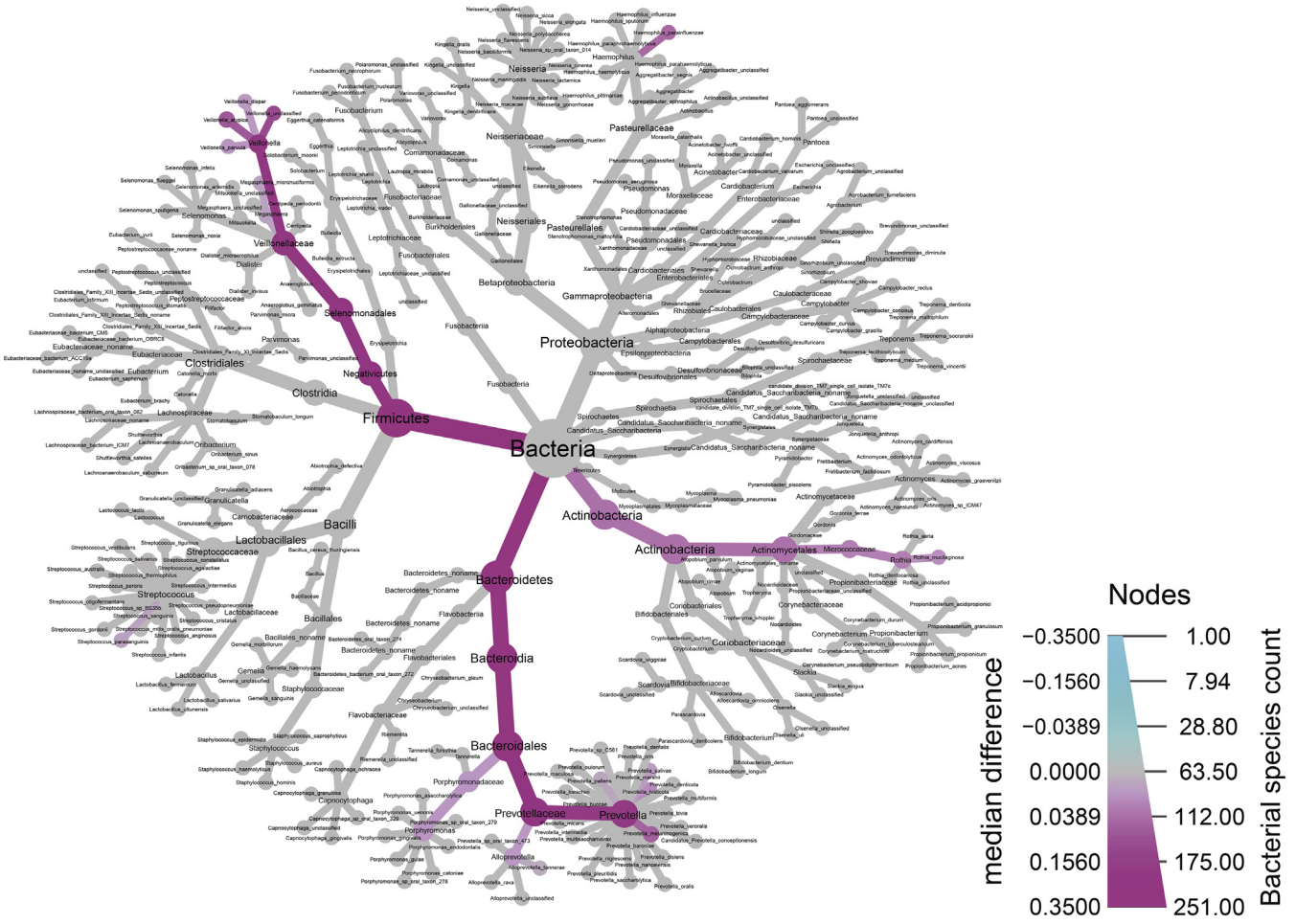

FIG 3. Metagenomics phylogenetic map shows statistically significantly differential bacterial taxa between the 2 baseline clusters after FDR correction. Nodes' color corresponds to the median difference in relative abundances of the bacterial taxa. The darker the color of the phylogenetic branches, the higher median differences, whereas gray nodes and branches indicate no significant differences. Magenta indicates that all significant taxa were more abundant in cluster 1 compared with in cluster 2, whereas absence of cyan in the phylogenetic map indicates that no significant taxa were more abundant in cluster 2 as compared with cluster 1.

Online Repository at www.jacionline.org). High cluster stability is indicated by the Pearson chi-square test result ($\chi^2 = 73.397$, $P < 1 \times 10^{-15}$) and RI value (0.904), suggesting robustness of the clustering model.

DISCUSSION

This is the first study to investigate microbiome-driven phenotypes and their stability over time in patients with severe asthma. Using unbiased clustering based on microbiome profiles, we have shown that patients with severe asthma can be stratified into 2 phenotypic clusters. These clusters differed significantly by age of asthma onset, patient residential locations, smoking history, percentage of blood and/or sputum neutrophils and macrophages, spirometry results, and asthma medications used. At both the baseline and longitudinal visits, the patients with severe asthma in cluster 2 had worse lung function, with associated blood and/or sputum neutrophilia and decreased sputum macrophages, than did the cluster 1 patients. In addition, they were more likely to receive add-on asthma medications, such as theophylline or long-acting anticholinergics, possibly indicating greater need to control their more severe airway obstruction.

The 2 phenotypic clusters were associated with markedly distinct microbiome profiles; cluster 1 had higher bacterial richness and diversity than did cluster 2, both at the baseline and longitudinal visits. Cluster 2 was characterized by a clear deficiency of several bacterial species, including the genera *Veillonella, Prevotella, Alloprevotella, Streptococcus, Porphyromonas, Rothia, Haemophilus, Neisseria,* and *Megasphaera.* Most of these species are considered commensals inhabiting the oropharyngeal region and the airways. This microbial dysbiosis was associated with blood and/or sputum neutrophilia, deceased sputum macrophages, and worse lung function outcomes at the baseline and longitudinal visits. A study by the Severe Asthma Research Program showed that sputum neutrophilia (with or without eosinophilia) is a characteristic of more severe asthma phenotypes.[39] In our study, the neutrophilia in cluster 2 could be attributed to the presence of either "subclinical infection" or modulation of the airway

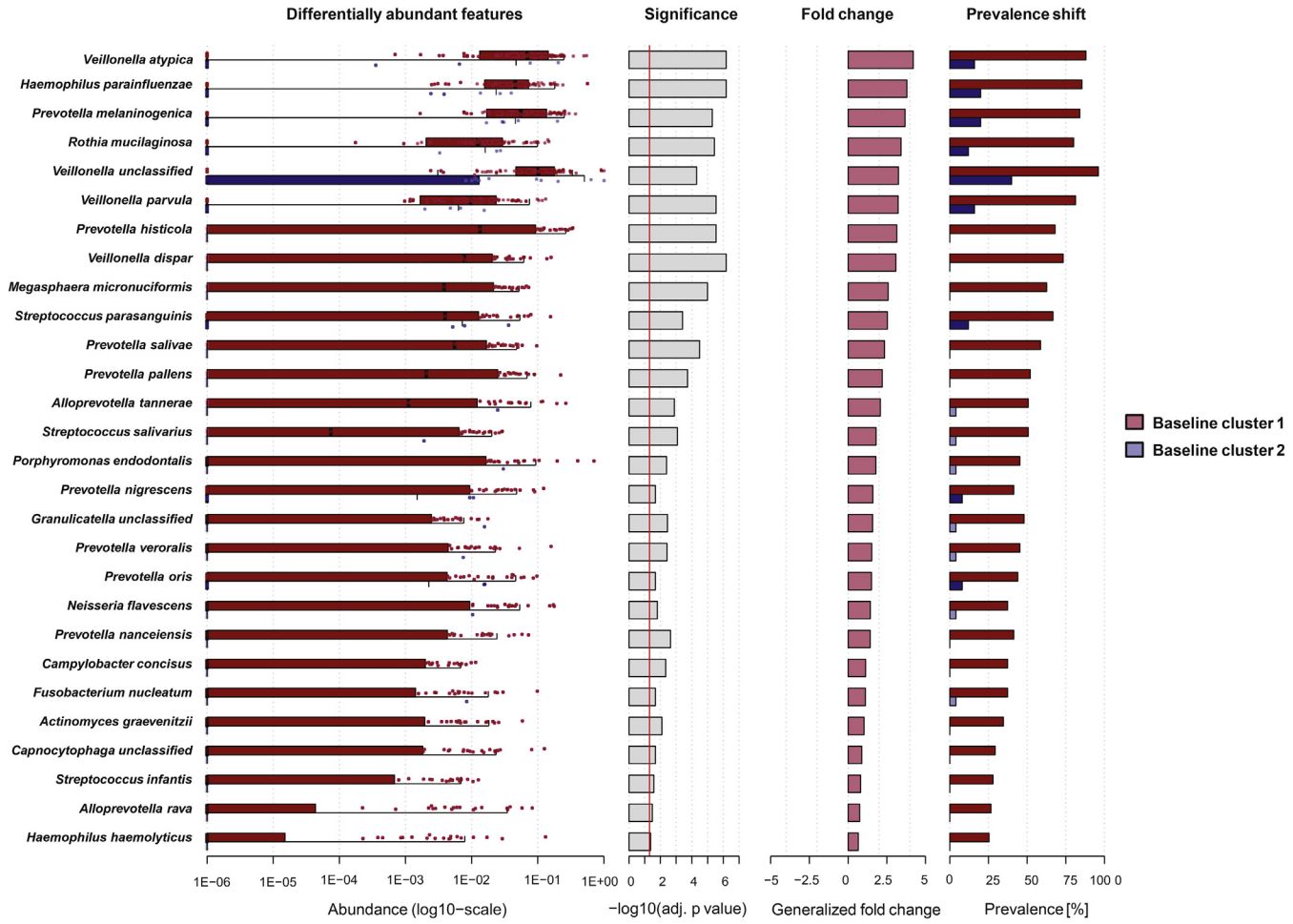

FIG 4. Statistically significant differentially abundant species after FDR correction between baseline cluster 1 and cluster 2 patients by metagenomics. All of them were more abundant in cluster 1 patients than in cluster 2 patients.

"immune tone" by the microbiota. Thus, deficiency of commensal bacteria leads to increased risk of infections with pathogenic ones, as manifested by increased abundance of *H influenza*, *M catarrhalis,* and *S pseudopneumoniae*. Consequently, these bacteria could be responsible for the observed blood and/or sputum neutrophilia. Our results are complementary to those of another U-BIOPRED study showing that adult patients with severe asthma had lower sputum microbiome alpha diversity than did patients with mild-to-moderate asthma and healthy controls, and this diversity was inversely correlated with sputum neutrophils.[29] This finding was in line with the findings of previously reported studies showing that neutrophilic asthma is characterized by low airway bacterial diversity and high abundance of the phylum Proteobacteria, especially the genera *Moraxella* and *Haemophilus*.[14-16] In addition, a decrease in the percentage of sputum macrophages may suggest a defective innate immune response with impaired macrophage phagocytosis of these pathogenic bacteria. This finding is in agreement with the findings of a previous study showing that patients with severe asthma have a reduced macrophage phagocytic capacity for certain pathogenic bacteria such as *H influenzae* compared with in patients with nonsevere asthma and healthy subjects.[40]

A study in patients with chronic obstructive pulmonary disease showed that the survivors with 1-year mortality had a higher relative abundance of *Veillonella* than the nonsurvivors did.[41] This was partly in agreement with the finding that the patients in cluster 1 with less severe asthma had a higher relative abundance of *Veillonella* bacteria than did the patients in cluster 2, which may suggest that these bacteria might have a protective role in chronic respiratory diseases. Although a few species, such as *Haemophilus parainfluenzae*, can become opportunistic pathogens in some situations, their increased abundance in the cluster 1 patients was not associated with asthma severity characteristics such as exacerbation frequency. These findings imply that the crosstalk of several species within the airway microbial community and their interplay with innate immunity may provide a better clinical relevance than does looking at the roles of one or a few bacterial species.

A striking finding in our study is that approximately 85% of the patients remained cluster stable after 12 to 18 months. In addition, the bacterial dysbiosis was not related to either current or previous (ever) antibiotic intake, suggesting that the microbial profiles of these patients were not a main consequence of short-term intake of antibiotics. Rather, they were probably shaped over a long

TABLE III. Demographic and clinical characteristics of the longitudinal clusters.

Characteristics	Longitudinal		*P* value
	Cluster 1 (n = 37)	Cluster 2 (n = 9)	
Median age, y (IQR)	57 (48-64)	55 (53-65)	.957
Median age of onset, y (IQR)	37.00 (17.50-48.50)	29.50 (3.50-49.75)	.716
Females, n (%)	22 (59.5%)	3 (33.3%)	.264
Median BMI, kg/m^2 (IQR)	27.96 (24.25-31.87)	29.56 (24.83-30.36)	.744
White race, n (%)	35 (94.6%)	8 (88.9%)	.488
Residential location, n (%)			**.012**
• Rural	5 (13.5%)	5 (55.6%)	
• Suburban	6 (16.2%)	2 (22.2%)	
• Urban	26 (70.3%)	2 (22.2%)	
Atopy, n (%)	25 (67.6%)	6 (66.7%)	.872
Nonsmokers, n (%)	23 (62.2%)	7 (77.8%)	.463
Median percent of eosinophils in sputum (IQR)	2.65 (0.88-16.45)	0.99 (0.51-18.69)	.651
Median percent of neutrophils in sputum (IQR)	59.40 (45.03-72.64)	83.76 (72.58-95.66)	**.002**
Median percent of macrophages in sputum (IQR)	27.24 (15.23-38.99)	5.68 (1.37-12.45)	**<.0001**
Median percent of eosinophils in blood (IQR)	3.65 (2.07-5.29)	2.07 (0.81-4.13)	.103
Median percent of neutrophils in blood (IQR)	59.81 (53.72-65.82)	76.61 (65.31-82.98)	**.023**
Median FEV_1 percent predicted before salbutamol (IQR)	63.76 (50.55-77.23)	56.17 (40.00-62.28)	**.036**
Median FEV_1 percent predicted after salbutamol (IQR)	74.33 (63.23-86.27)	64.22 (44.64-78.08)	**.036**
Median FEV_1/FVC percent predicted before salbutamol (IQR)	77.35 (62.08-83.38)	65.50 (51.22-76.02)	.116
Median FEV_1/FVC percent predicted after salbutamol (IQR)	80.40 (67.68-86.22)	65.04 (50.82-78.93)	.081
Median F_{ENO} in ppb (IQR)	25.00 (15.90-54.50)	28.25 (19.00-35.88)	.999
Median exacerbations per year (IQR)	2 (0-3)	2 (0-3)	.663
Median ACQ5 score average (IQR)	2.0 (0.9-3.1)	1.8 (1.0-2.7)	.957
Median AQLQ score average (IQR)	4.59 (3.86-5.53)	4.48 (3.44-6.09)	.807
Current medication use, n (%)			
• ICS	33 (89.2%)	9 (100%)	.571
• SABA	20 (54.1%)	7 (77.8%)	.270
• LABA	34 (91.9%)	9 (100%)	.999
• OCS	10 (27.0%)	4 (44.4%)	.423
• Short-acting anticholinergics	2 (5.4%)	1 (11.1%)	.488
• Long-acting anticholinergics	7 (18.9%)	8 (88.9%)	**<.001**
• Leukotriene antagonists	15 (40.5%)	4 (44.4%)	.999
• Theophylline	4 (10.8%)	3 (33.3%)	.124
Median OCS normalized dose, mg (IQR)	10.00 (8.13-12.50) (n = 10)	13.75 (5.63-20.00) (n = 4)	.999
Antibiotics use, n (%)			
• Current intake	4 (10.8%)	3 (33.3%)	.124
• Current and previous (ever) intake	20 (54.1%)	6 (66.7%)	.711

ACQ5, 5-Item Asthma Control Questionnaire; *AQLQ*, Asthma Quality of Life Questionnaire; *BMI*, body mass index; *F_{ENO}*, fraction of exhaled nitric oxide; *FVC*, forced vital capacity; *ICS*, inhaled corticosteroid; *IQR*, interquartile range; *LABA*, long-acting β-agonist; *OCS*, oral corticosteroid; *SABA*, short-acting β-agonist.
P values for testing statistical significance between the 2 longitudinal clusters were calculated by using Pearson chi-square or Fisher exact tests as appropriate for categoric variables and the Mann-Whitney *U* test for continuous variables. Entries with statistically significant *P* values are boldface.

period and might have a genetic background[42] and/or might have resulted from a lifelong exposure to environmental factors.[43]

Tobacco smoking has been reported previously to be associated with sputum microbiome diversity of patients with asthma[44]; in addition, it has been reported to induce neutrophilia in chronic airway disease, including asthma.[45,46] In our study, however, the cluster 2 patients with more severe asthma were more likely to be nonsmokers (84.0%). Because microbial dysbiosis and neutrophilia were more often observed in cluster 2 patients, it seems unlikely that this is mainly attributed to smoking, possibly denoting the interplay between microbial dysbiosis and innate immunity altering the airway immune tone. In another study investigating the airway microbiome in patients with asthma, neutrophilia was the strongest predictor for microbiota variance, whereas smoking was not a predictor[14] supporting the findings observed in cluster 2. This warrants further investigation to gain more insight on the trajectories of neutrophilic asthma and possible overlap/delineation between microbial-associated and smoking-associated neutrophilic asthma. Cluster 2 patients were more likely to live in suburban or rural areas, in contrast to cluster 1 patients, who were more likely to live in urban areas. We could speculate that there is relatively more traffic in or around suburbs and driving times are longer on account of commuting to work. Therefore, more exposure to car gases and/or particulate air pollution may influence the airway microbiome profiles.[47] In addition, exposure to fine particulate air pollution can increase neutrophils,[48] which may contribute to the neutrophilia that we see in cluster 2 patients.

The relative cluster stability and robustness that we found in our study is an ideal criterion for personalized diagnostic or therapeutic decisions in patients with asthma. In addition, the inclusion of the group with mild-to-moderate asthma fitted well with our previous clustering scheme, whereas most of this patient group (>95 %) was assigned to the cluster of those with "milder"

	Cluster 1 Baseline	Cluster 2 Baseline	p-value	Rand index
Cluster 1 Longitudinal	**33 (91.7%)**	4 (40%)	.0014	0.736
Cluster 2 Longitudinal	3 (8.3%)	**6 (60%)**		

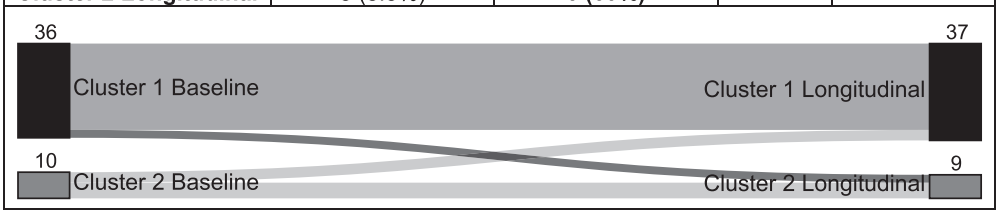

FIG 5. Cross-tabulated and Sankey diagram of patient cluster assignments among 46 patients with both baseline and follow-up visits: 39 patients are cluster stable (in boldface), and 7 patients migrate between clusters. Cells described in terms of number and percentage of baseline clusters. *P* value was generated by using the Fisher exact test.

asthma, except for 1 patient who also showed characteristics close to those of the cluster 2 patients with severe asthma (eg, young age of asthma onset, neutrophilia, and low values of FEV_1). This suggests that using the criteria of the observed microbiome profiles may enable better diagnosis and/or prediction of more severe phenotypes and hence tailor therapeutic decisions. In addition, the relative stability of the clusters after 12 to 18 months indicates that management approaches aiming at correcting the pathophysiology of these patients should be sought instead of just symptom control, which is not sufficient. This may include approaches that aim to reshape the lung microbiome (eg, using long-term prebiotics, probiotics, or synbiotics). Moreover, therapeutic strategies directed toward the neutrophilic corticosteroid-resistant asthma phenotype and innate immunity may be tried in these patients; this includes use of compounds, such as macrolide antibiotics,[49,50] low-dose theophylline,[51] and other antineutrophilic compounds.[52,53]

Our study has many strengths. First, the analysis protocol is unbiased and its clinical significance is driven only by the microbiome. Second, the pan-European nature of this study makes our results more generalizable and valid than those of previously reported single-country or single-center studies with lower sample sizes. Third, the utilization of both 16s rRNA sequencing and metagenomics further increase the reliability and generalizability to other studies. In addition, the limited but affordable 16s rRNA method provided clustering results that are highly concordant with those of the metagenomics approach, which further extends its potential applicability in clinical practice in settings in which metagenomics could not be performed. Fourth, we internally validated our finding by applying different clustering algorithms that were highly consistent. Although adding an independent cohort of patients with mild-to-moderate asthma is not considered a validation, its perfect harmonization with the clustering solution prove the robustness and clinical relevance of the clustering technique used. Fifth, shotgun metagenomics allowed us to reveal bacterial associations up to the species level and hence increase the clinical relevance, unlike in most previous studies that used 16s rRNA–based methods (which are limited in identifying bacterial species). Finally, this study is regarded as one of the first attempts to uncover microbiome-driven phenotypes and elaborate targets for precision medicine within the group of patients with severe asthma.

However, there are also limitations. First, we sampled only 1 airway compartment (induced sputum). Whether other sampling compartments would provide additional microbiome-related information needs to be determined. Second, only 2 time points were measured over a follow-up design of 12 to 18 months, which may not be sufficient to adequately assess longitudinal shifts of microbiome clusters in patients with asthma. Third, some patients were lost to follow-up, which may create further bias in the assessment. However, we are the first study to investigate the microbiome clusters in patients with severe asthma, which might serve as a basis for future investigations. Finally, using an external cohort outside the U-BIOPRED cohort for validation is still needed to confirm our findings.

In conclusion, our study has shown that microbiome-driven clustering can be used as an unbiased way to detect phenotypes in patients with severe asthma. Furthermore, they are relatively stable after 12 to 18 months of follow-up and demonstrated robustness after inclusion of an independent cohort of patients with asthma. Patients with asthma with the more severe, microbiome-driven phenotype comprised approximately 25% of those with severe asthma and need to be considered targets for personalized medicine decisions targeting the airway microbiome.

Some of the drawn objects in the graphical abstract were adapted from Servier Medical Art (https://smart.servier.com) in accordance with a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

Key messages

- **Using unbiased clustering based on sputum microbiome profiles showed that severe asthma can be stratified into 2 phenotypic clusters.**
- **The patients in these clusters differed significantly by age of asthma onset, patients' residential locations, smoking history, percentage of blood and/or sputum neutrophils and macrophages, spirometry results, and asthma medications used.**

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