

The airway microbiome in patients with severe asthma: Associations with disease features and severity

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Background: Asthma is heterogeneous, and airway dysbiosis is associated with clinical features in patients with mild-to-moderate asthma. Whether similar relationships exist among patients with severe asthma is unknown.

Objective: We sought to evaluate relationships between the bronchial microbiome and features of severe asthma.

Methods: Bronchial brushings from 40 participants in the Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma (BOBCAT) study were evaluated by using 16S ribosomal RNA-based methods.

Relationships to clinical and inflammatory features were analyzed among microbiome-profiled subjects. Secondarily, bacterial compositional profiles were compared between patients with

severe asthma and previously studied healthy control subjects (n = 7) and patients with mild-to-moderate asthma (n = 41). **Results:** In patients with severe asthma, bronchial bacterial composition was associated with several disease-related features, including body mass index ($P < .05$, Bray-Curtis distance-based permutational multivariate analysis of variance; PERMANOVA), changes in Asthma Control Questionnaire (ACQ) scores ($P < .01$), sputum total leukocyte values ($P = .06$), and bronchial biopsy eosinophil values (per square millimeter, $P = .07$). Bacterial communities associated with worsening ACQ scores and sputum total leukocyte values (predominantly Proteobacteria) differed markedly from those associated with body mass index (Bacteroidetes/Firmicutes). In contrast, improving/stable ACQ scores and bronchial epithelial gene expression of FK506 binding protein (*FKBP5*), an indicator of steroid responsiveness, correlated with Actinobacteria. Mostly negative correlations were observed between biopsy eosinophil values and Proteobacteria. No taxa were associated with a T_H2 -related epithelial gene expression signature, but expression of T_H17 -related genes was associated with Proteobacteria. Patients with severe asthma compared with healthy control subjects or patients with mild-to-moderate asthma were significantly enriched in Actinobacteria, although the largest differences observed involved a *Klebsiella* genus member (7.8-fold increase in patients with severe asthma, adjusted $P < .001$). **Conclusions:** Specific microbiota are associated with and may modulate inflammatory processes in patients with severe asthma and related phenotypes. Airway dysbiosis in patients with severe asthma appears to differ from that observed in those with milder asthma in the setting of inhaled corticosteroid use. (*J Allergy Clin Immunol* 2015;136:874-84.)

Key words: Microbiota, lung, inflammation, 16S ribosomal RNA, body mass index, asthma control, steroids, T_H2

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The spectrum of heterogeneity in asthmatic patients includes those with severe disease whose symptoms remain inadequately controlled despite treatment with current therapies.^{1,2} Importantly, heterogeneity within patients with severe asthma also has been described.^{3,4} Different pathologic mechanisms have been proposed to underlie various asthma phenotypes,⁵ including those meeting the criteria for severe disease.⁶

Recent studies have expanded considerations of the role of microbial infection or colonization in asthmatic patients.⁷⁻¹⁰ Reported observations include differences in lower airway bacterial composition between asthmatic and healthy subjects^{7-9,11} characterized by a greater prevalence in asthmatic patients of

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Abbreviations used

| | |
|------------|--|
| ACQ: | Asthma Control Questionnaire |
| BH: | Benjamini-Hochberg |
| BMI: | Body mass index |
| BOBCAT: | Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma |
| FKBP5: | FK506 binding protein |
| PB: | Protected brush |
| PERMANOVA: | Permutational multivariate analysis of variance |
| PICRUST: | Phylogenetic Investigation of Communities by Reconstruction of Unobserved States |
| qPCR: | Quantitative PCR |
| 16S rRNA: | 16S Ribosomal RNA |

Proteobacteria, a large phylum that includes many potential respiratory pathogens. This prevalence of Proteobacteria also has been observed among patients with mild asthma not taking regular inhaled corticosteroid therapies,⁷ suggesting that the presence of airway dysbiosis is associated with asthma itself and not simply a reflection of concurrent corticosteroid treatment.

In patients with mild-to-moderate asthma, relationships between the airway microbiome and clinical features have been observed.⁸ Whether similar relationships exist in patients with severe asthma are unknown. To address this knowledge gap, we examined the bacterial microbiome in protected bronchial brushings collected from patients with severe asthma in the Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma (BOBCAT) study.¹² Bacterial composition was analyzed by using 16S ribosomal RNA (16S rRNA)-based methods, followed by *in silico* predictive metagenomic analysis of bacterial groups of interest. We identified significant relationships between airway microbiota members and features of severe asthma. Additional analyses demonstrated differences in bronchial microbiota composition between this severe asthma cohort and healthy control subjects and patients with mild-to-moderate asthma, whose bronchial microbiomes were examined in an earlier study.⁸ Our primary findings invite speculation that features of the bronchial microbiome can contribute to endotypes of severe asthma.

METHODS

Patients with severe asthma and sample processing for microbiome analysis

As previously described,¹² the BOBCAT study was a multicenter, 3-visit study that enrolled 67 patients with severe asthma, which was defined as an FEV₁ of 40% to 80% of predicted value, an Asthma Control Questionnaire (ACQ) score of greater than 1.50, and a daily inhaled corticosteroid dose equivalent to 1000 µg of beclomethasone or greater. Subjects underwent sputum induction (visit 2), bronchoscopy (visit 3), and blood sampling. Medications were kept constant during the study; no corticosteroids or antibiotics were started or changed from 30 days before visit 1 through visit 3.

To ensure total DNA was extracted from the same number of protected brushes (PBs) for all evaluated subjects and on the basis of our prior experience⁸ that brush extractions yield variable amounts of DNA, we required at least 2 brushes to evaluate for microbiome analysis. Forty subjects fulfilled this criterion, and 2 PBs (stored in RNAlater at -80°C) were extracted. Subjects' characteristics were analyzed for differences between those whose samples were (n = 40) or were not (n = 27) evaluated for microbiome analysis by using nonparametric or parametric tests. Amplified 16S rRNA gene products (using universal primers 27F and 1492R¹³) were processed for profiling by using the 16S rRNA-based PhyloChip platform (Second Genome, South San Francisco, Calif), as previously described.^{8,14}

Data analysis

Raw array data were processed, as previously described,¹⁵⁻¹⁷ including scaling to spiked-in quantitative standards and normalization to the mean intensity of all samples. Bacterial taxa were identified based on 97% or greater identity in 16S rRNA gene sequences (Greengenes 2011 iteration¹⁸) by using published methods.^{14,15,19} Details are available in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Log₂-transformed fluorescence intensities, which are correlative with the relative abundance of taxa,²⁰ were used for all analyses in R software. Array data were normalized separately for exploratory analyses of microbiota differences between patients with severe asthma and previously studied⁸ healthy subjects and patients with mild-to-moderate asthma from whom PB-sampled bacterial composition had been profiled by the same platform (see data sets in [Table E1](#) in this article's Online Repository at www.jacionline.org). The healthy control data set included 2 subjects not in the earlier study⁸ but who had been evaluated at the University of California San Francisco by using the same criteria to exclude asthma.

α-Diversity for each sample was calculated,^{21,22} including Shannon and inverse Simpson indices (number and relative distribution of taxa) and Faith phylogenetic diversity, which additionally weights phylogenetic relationships.²³ Bray-Curtis and UniFrac distance measures^{24,25} were calculated to evaluate between-sample compositional differences by means of ordination analyses and in PERMANOVA tests with clinical and inflammatory variables.²⁶ Correlation analyses or the Significance of Analysis of Microarrays method, based on a univariate penalized regression approach,²⁷ were used to identify specific taxa-to-variable relationships. Adjustments for multiple comparisons were applied (Benjamini-Hochberg [BH] correction or *q* values²⁸). For predictive metagenomic analyses, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST)²⁹ was used to predict gene functions and KEGG pathways.

Airway epithelial gene expression analysis

RNA extracted from a separate epithelial brush was amplified for Agilent (Santa Clara, Calif) 2-color Whole Human Genome 4x44k gene expression microarray analysis. Universal Human Reference RNA (Stratagene, La Jolla, Calif) was used for the reference channel. T_H2 and T_H17 scores were calculated by means of case-wise averaging of zero-centered gene expression data after annotation-based independent filtering³⁰; that is, if multiple probes correspond to an Entrez gene, the probe with the highest interquartile range was selected. *SERPIN2*, *CLCA1*, and *POSTN* were used as IL-13-responsive T_H2 signature genes, as described previously.³¹ *CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3* were used as IL-17-responsive T_H17 signature genes based on previous evidence that expression of these genes is involved in IL-17-driven pathways.³²⁻³⁴ The data have been deposited in the National Center for Biotechnology Information's NCBI GEO database (accession no. GSE65584).

Quantitative PCR studies

Total 16S rRNA gene copies, as well as copy number determinations to validate specific species of interest, were assessed by using quantitative PCR (qPCR). Further details are provided in the [Methods](#) section in this article's Online Repository.

RESULTS

Characteristics of patients with severe asthma

PBs from 40 of 67 subjects in the BOBCAT study underwent evaluation for bacterial microbiome analysis. Bacterial evaluation was not pursued in the other 27 subjects because they lacked the requisite number of 2 PBs for technical consistency in source DNA extractions. Of the evaluated subjects, 30 demonstrated sufficient amplified 16S rRNA product (prespecified threshold of ≥100 ng) for array-based profiling.

Characteristics of these subjects are summarized in [Table I](#). Mean FEV₁ percent predicted (average of visit 1 and visit 2) was 59%, with a mean ACQ score of 2.4, which is indicative of poor control. qPCR experiments confirmed that this group had

TABLE I. Characteristics of the 30 patients with severe asthma for whom bronchial microbiome analysis was performed

| | |
|--|----------------------------|
| Age (y) | 46 (20 to 63) |
| White race (%) | 24 (80) |
| Female sex, no. (%) | 11 (37) |
| BMI (kg/m ²) | 28.7 ± 6.9 (19.3 to 52.1) |
| FEV ₁ /FVC ratio | 0.73 (0.55 to 1.0) |
| FEV ₁ (% predicted), visit 1 | 59.9 (34.3 to 77.7) |
| FEV ₁ (% predicted), visit 2 | 58.3 (33.6 to 82.8) |
| Bronchodilator reversibility (%) | 13.7 (−8.6 to 38.9) |
| ACQ score, visit 1 | 2.4 ± 0.6 |
| ACQ score, visit 2 | 2.4 ± 0.8 |
| Prednisolone use, no. (%) | 6 (20) |
| FENO (ppb) | 42.8 ± 37.9 (9.7-141.5) |
| Sputum eosinophils (%) | 5.5 ± 5.9 (0.0 to 18.8) |
| Sputum neutrophils (%) | 52.4 ± 28.3 (0 to 88.3) |
| Blood absolute eosinophils (× 10 ³ /μL) | 0.29 ± 0.2 |
| Serum IgE (IU/mL) | 180.8 ± 202.9 (9 to 835.2) |
| Serum periostin (ng/mL) | 24.2 ± 7.3 |

Values are means ± SDs (percentages or ranges).

FENO, Fraction of exhaled nitric oxide representing the mean from visits 1 and 2; FVC, forced vital capacity.

significantly higher total 16S rRNA copy numbers compared with the other 10 evaluated subjects who had insufficient 16S rRNA amplicons (ie, low bacterial burden) for array-based profiling ($P < .05$, Wilcoxon rank sum test).

We examined whether characteristics differed among the evaluated subjects between those in whom bacterial compositional profiles were or were not successfully obtained. The subjects in whom microbiome analysis could be performed had significantly lower sputum eosinophil percentages ($5.5\% \pm 5.9\%$ vs $17.3\% \pm 21.1\%$, $P < .05$) and a trend toward lower bronchial biopsy eosinophil numbers (31.8 ± 33.7 vs 45.2 ± 19.3 eosinophils/mm², $P = .09$). We also analyzed whether characteristics differed between BOBCAT study participants whose samples were ($n = 40$) or were not ($n = 27$) evaluated for microbiome profiling. The evaluated group had lower mean body mass index (BMI; 29 vs 34 kg/m², $P < .01$) but higher numbers of biopsied IL-17A- and IL-17F-positive cells ($P < .001$ and $P < .05$, respectively). In both sets of analyses, there were no significant differences between the compared subgroups in other clinical and inflammatory parameters, including 3 examined gene expression patterns (FK506 binding protein [*FKBP5*], T_H2 , and T_H17).

Severe asthma features are associated with differences in airway bacterial composition

Distance-based PERMANOVA²⁶ analyses (Bray-Curtis or weighted UniFrac distances) were performed to identify factors that might explain variation in microbial community composition among samples (Table II). Identified factors included BMI ($P < .05$), ACQ scores at visit 2 ($P < .05$), and changes in ACQ scores during the study (visit 2 minus visit 1, $P < .01$). Associations that trended toward significance included sputum total leukocyte percentages ($P = .06$) and bronchial biopsy eosinophil numbers (per square millimeter, $P = .07$). These observations indicate that specific bacterial compositions are associated with particular disease features in this cohort. No associations with other assessed variables, including inflammatory measures from blood or bronchoalveolar lavage fluid, were found (results for all evaluated variables are available in Table E2 in this article's

TABLE II. Clinical and inflammatory features of patients with severe asthma demonstrating associations with differences in bacterial community composition based on Bray-Curtis distance measures

| Variable | Distance-based PERMANOVA result (P value) |
|---|---|
| BMI | .05 |
| ACQ score, visit 2 | .05 |
| ACQ score change (visit 2 – visit 1) | .01 |
| Sputum total leukocyte value | .06 |
| Biopsy eosinophil cells (mm ^{−2}) | .07 |

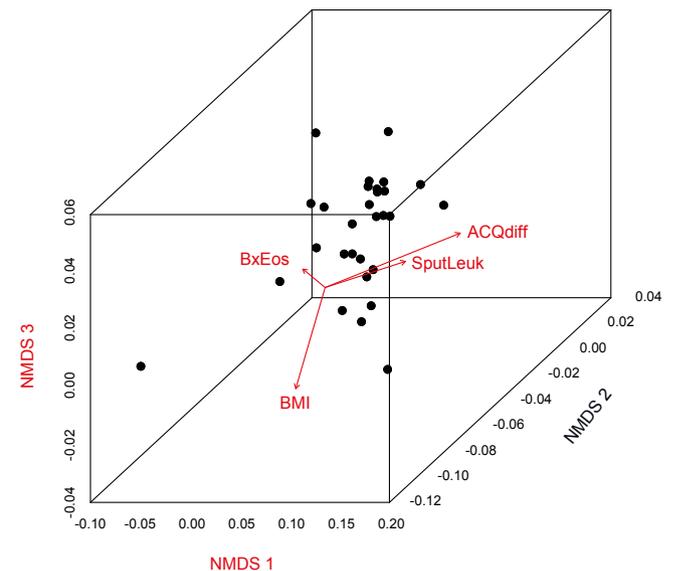


FIG 1. Nonmetric multidimensional scaling analysis (NMDS) based on Bray-Curtis distances showing differences in bronchial bacterial composition among 30 patients with severe asthma. Each dot represents the overall bacterial community in each subject. Vectors indicate linear regression-based fits for variables found by using independent distance-based PERMANOVA testing to be associated with distinct bacterial compositions. Vector directions indicate that the specific communities associated with differences in ACQ scores (*ACQdiff*) and sputum total leukocyte values (*SputLeuk*) are similar to each other and differ from specific communities associated with BMI and biopsy eosinophil values (*BxEos*).

Online Repository at www.jacionline.org). To visualize these relationships, ordination analysis was used (nonmetric multidimensional scaling, Fig 1). Regression-based linear fits to this ordination indicated that change in ACQ score and sputum total leukocyte values were associated with microbiome compositions distinct from those associated with BMI and biopsy eosinophil numbers.

Distinct microbiota members are associated with different features of severe asthma

Taxon-level correlation analyses were performed between the relative abundance of each detected taxon and each variable across all subjects to identify specific bacterial taxa associated with these variables of interest. Results confirmed the ordination analyses in that taxa significantly associated with BMI were distinct from those associated with the other features.

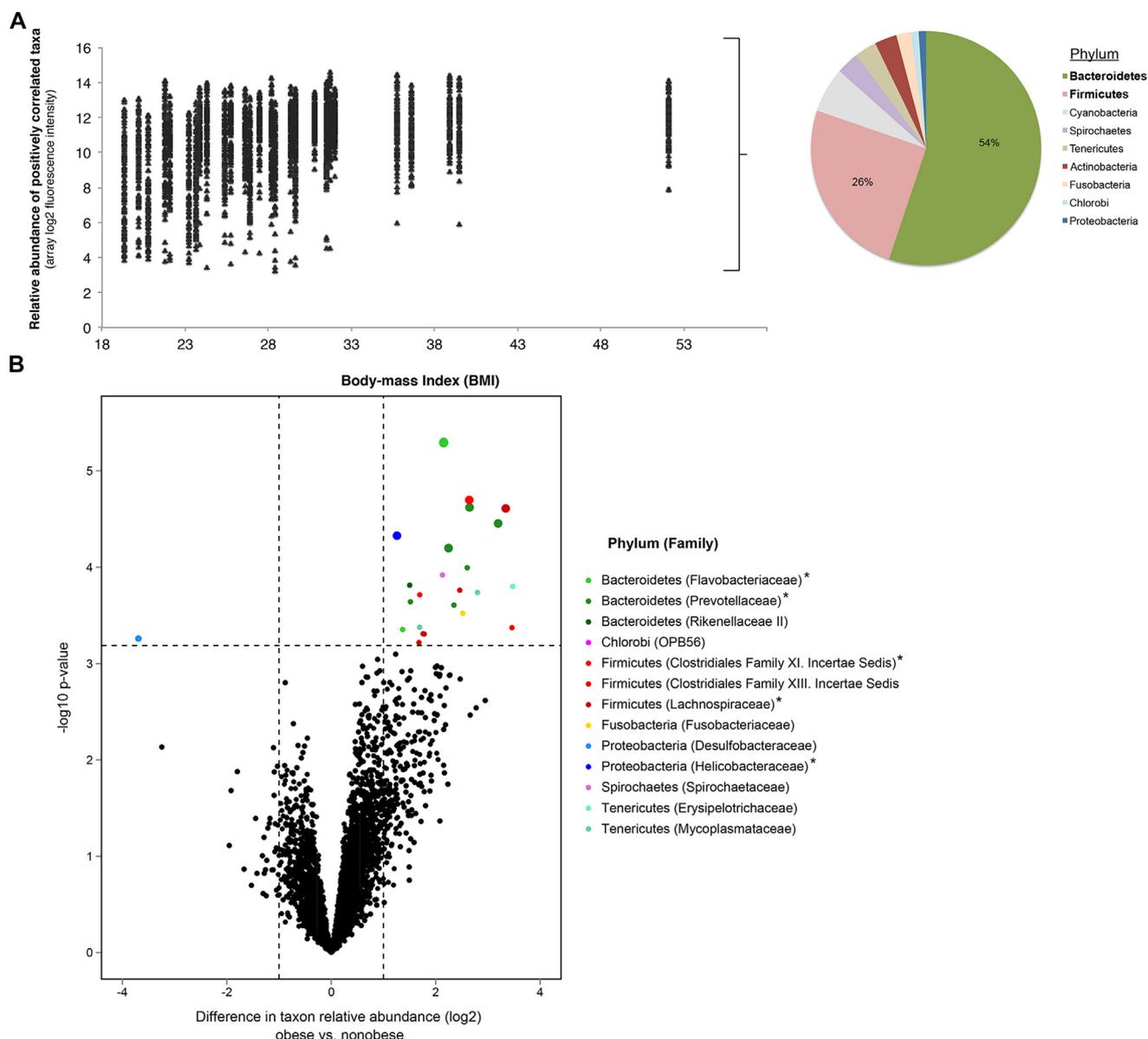


FIG 2. A, Relative abundance plotted against BMI for the 98 taxa found to be significantly correlated with BMI among the patients with severe asthma studied ($R = 0.5-0.8$, BH-adjusted $P < .05$). The distribution of bacterial phyla representing these taxa are shown, which are mainly Bacteroidetes and Firmicutes. **B**, Differences in the relative abundance of all detected taxa between obese (BMI > 30 kg/m², $n = 10$) and nonobese ($n = 19$) patients with severe asthma. Taxa significantly enriched among obese subjects (2-fold, adjusted $P < .10$) include members of the Bacteroidetes and Firmicutes phyla, such as *Prevotella* species. Dashed lines indicate a 2-fold difference ($\log_2 = 1.0$) and false discovery rate significance level of 10%, respectively. Asterisks indicate taxa most significantly greater in abundance among obese subjects (adjusted $P < .05$), which are also represented by larger circles in the plot.

Bacteroidetes and Firmicutes comprised 54% and 26%, respectively, of the 98 taxa significantly correlated with BMI (Pearson $R = 0.5-0.8$; BH-adjusted $P < .05$; Fig 2, A). This contrasted with the predominant representation of Proteobacteria among taxa uncorrelated with BMI ($P < .01$, χ^2 test). Further analysis for specific compositional differences between obese (BMI ≥ 30 kg/m²) and nonobese subjects identified several taxa more abundant among obese subjects (R package *limma* moderated t test, BH-adjusted $P < .05$; Fig 2, B). These included members of the Prevotellaceae, Mycoplasmataceae, Lachnospiraceae (*Clostridium* species), and Spirochaetaceae (*Treponema* species; see Table E3 in this article's

Online Repository at www.jacionline.org). Given this, we explored whether obese and nonobese subjects in this microbiome-profiled group differed in other characteristics. The only significant finding was that obese subjects had fewer biopsy eosinophils than nonobese subjects (mean biopsy eosinophil numbers, 11.9/mm² in subjects with high BMI vs 45.3/mm² in subjects with low BMI; $P < .01$).

In contrast to BMI, Proteobacteria represented the majority of taxa ($>90\%$) whose relative abundance correlated with worsening ACQ scores (visit 2 minus visit 1 scores; $R = 0.5-0.7$, BH-adjusted $P < .05$; Fig 3, A). These included families representing

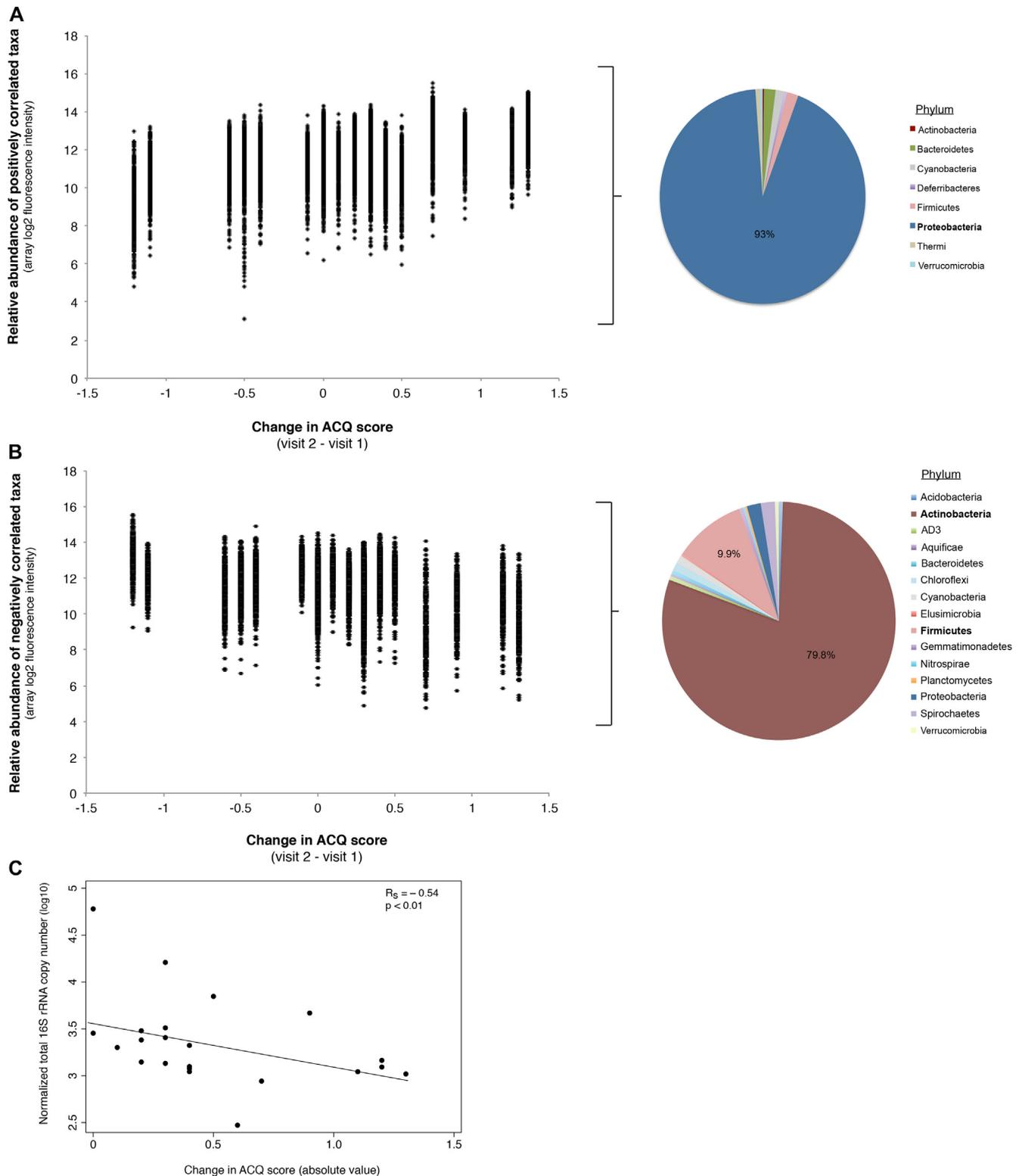


FIG 3. **A**, Relative abundance plotted against changes in ACQ scores for the bacterial taxa ($n = 448$) found to be positively correlated with this parameter ($R = 0.5-0.7$, BH-adjusted $P < .05$). Increasing values of the change in ACQ score are associated with greater relative abundance predominantly of Proteobacteria members. **B**, Relative abundance plotted against change in ACQ score for the bacterial taxa ($n = 362$) found to be negatively correlated with changes in ACQ scores ($R = -0.5$ to -0.8 , BH-adjusted $P < .05$). Decreasing values of the change in ACQ score are associated with greater relative abundance of predominantly members of the Actinobacteria, followed by Firmicutes, phyla. **C**, Total 16S rRNA copy numbers, a proxy for bacterial burden, are inversely correlated with absolute values of the change in ACQ score (Spearman $\rho = -0.54$, $P < .01$).

known respiratory pathogens, such as Pasteurellaceae, Enterobacteriaceae, Neisseriaceae, Burkholderiaceae, and Pseudomonadaceae. Similar results were observed among communities significantly correlated with higher visit 2 ACQ scores (see Fig E1 in this article's Online Repository at www.jacionline.org). In contrast, taxa correlated with improving or stable ACQ scores were primarily Actinobacteria (80%; Fig 3, B), such as Streptomycetaceae, Nocardiaceae, and Mycobacteriaceae. Analyses with absolute values of the change in ACQ score, presumably a reflection of instability in asthma control, also showed Proteobacteria to most represent positively correlated communities (40/91 taxa; $R = 0.4-0.6$, $q < 0.15$). Total 16S rRNA copy numbers demonstrated an inverse relationship to absolute values of the change in ACQ score (Spearman $\rho = -0.54$, $P < .01$; Fig 3, C), with a similar trend observed with inverse Simpson diversity ($R = -0.37$, $P = .09$), suggesting that greater bacterial burden in general is associated with less variation in asthma control.

Proteobacteria also constituted the majority of taxa (71% [73/103] of taxa) significantly correlated with sputum total leukocyte numbers (mean \pm SD, 812 ± 678 cells/mL). Families with members demonstrating strong correlations with this parameter ($R = 0.6-0.8$, BH-adjusted $P < .05$) included the Enterobacteriaceae and Moraxellaceae. Total 16S rRNA copy numbers significantly correlated with sputum total leukocyte numbers ($R = 0.4$, $P < .05$), which indicates that bacterial burden is associated with increased numbers of inflammatory cells.

In contrast, significant but predominantly negative associations were seen between biopsy eosinophil numbers and the relative abundance of specific bacteria (71 taxa, $R = -0.5$ to -0.74 , $q < 0.10$). These negatively correlated communities were mostly Proteobacteria (64% [45/71] of taxa), followed by Firmicutes, and included members of the Moraxellaceae and Helicobacteraceae (see Table E4 in this article's Online Repository at www.jacionline.org). Only 2 taxa demonstrated a significant positive correlation with biopsy eosinophil numbers, both Actinobacteria (*Streptomyces* and *Propionicimonas* species). Total 16S rRNA copy numbers were also inversely correlated with biopsy eosinophil numbers ($R = -0.50$, $P < .01$; Fig 4, A), suggesting that higher bacterial burden is associated with less eosinophil infiltration in bronchial tissue. We did not observe relationships between bacterial composition and other indicators of eosinophilic inflammation (eg, sputum or blood eosinophil, fraction of exhaled nitric oxide, and periostin values).

Bacterial microbiota are associated with specific airway epithelial gene expression signatures

For 33 of the 40 subjects evaluated for bacterial profiling, matched airway epithelial gene expression microarray analysis was performed (23/30 microbiome-profiled and 10/10 non-microbiome-profiled subjects). Brushes analyzed for epithelial gene expression were collected concurrently with those evaluated for microbiome profiling, presenting a unique opportunity to explore contemporaneous relationships between the microbiome and local host epithelial gene expression. We specifically explored relationships to 3 gene expression patterns identified by using microarray analysis: a marker of steroid response (*FKBP5*), T_H2 -associated inflammation (*POSTN*, *CLCA1*, and *SERPINB2*),⁵ and T_H17 -associated inflammation involving genes previously shown to be involved in IL-17-driven pathways (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL8*, and *CSF3*).³²⁻³⁴

Positive associations were observed between *FKBP5* expression and the relative abundance of 83 taxa ($q < 0.10$, Significance of Analysis of Microarrays), largely Actinobacteria (58%) and Proteobacteria (27%; Fig 4, B). Bacterial diversity measures were also significantly correlated with *FKBP5* expression ($R = 0.5$, $P < .01$; Fig 4, C), indicating that transcriptional evidence of steroid response is associated with increased community diversity.

No significant associations were observed between microbiota members and the T_H2 -related gene signature (at false discovery rate thresholds of up to 0.20). At a higher cutoff ($q < 0.25$), 16 taxa demonstrated negative associations with this gene signature. These included members of the Moraxellaceae and Helicobacteraceae, which were also represented among taxa negatively correlated with biopsy eosinophil numbers (see Table E4).

Positive correlations with the T_H17 -associated gene expression pattern were observed for a number of taxa. These communities were mainly Proteobacteria (72% [80/110] of taxa; $R = 0.5-0.7$, $P < .04$, $q < 0.20$), such as Pasteurellaceae, Enterobacteriaceae, and Bacillaceae (see Table E5 in this article's Online Repository at www.jacionline.org). Although Proteobacteria also represented a proportion of taxa correlated with *FKBP5* expression, the particular families largely did not overlap with those Proteobacteria families positively associated with the T_H17 expression pattern. Only 11 Proteobacteria families were represented in both gene signatures among correlated taxa, which is in contrast to the many more Proteobacteria families associated with either signature (41 families represented only among *FKBP5*-correlated taxa and 57 families represented only among T_H17 -correlated taxa). The compositional dissimilarity of taxa correlated with these 2 expression patterns was also evident by using nonmetric multidimensional scaling analysis (Fig 5). This suggests particular airway microbiota might promote T_H17 inflammation, resulting in neutrophil recruitment and contributing to neutrophilic airway inflammation independent of steroid-induced effects.

Predicted community functions of specific taxa related to severe asthma features

Shotgun metagenomic sequencing approaches can provide insight into the functional capacities of microbiota but to do so at high resolution is challenging. To explore the predicted functional capacity of identified taxa of interest in this study, we applied an alternative, informatics-based approach called PICRUSt,²⁹ which uses 16S rRNA sequence information to infer bacterial metagenomes and was shown to recapitulate results of actual metagenomic sequencing from the Human Microbiome Project.

Representative 16S rRNA sequences for taxa demonstrating significant associations (positive and negative) with variables of interest in this study were inputted into PICRUSt. The outputs are predicted functions defined as KEGG gene orthologs and represented as KEGG pathways where these functions are known to be involved. For example, predicted functions present among taxa correlated with BMI (see Fig E2 in this article's Online Repository at www.jacionline.org) included pathways in carbohydrate digestion and absorption, insulin signaling, and microbially related signaling and inflammation (eg, NOD-like receptor signaling, bacterial toxins and epithelial cell signaling in *Helicobacter* species infection). This suggests that particular airway-associated microbiota might incite inflammatory responses among overweight/

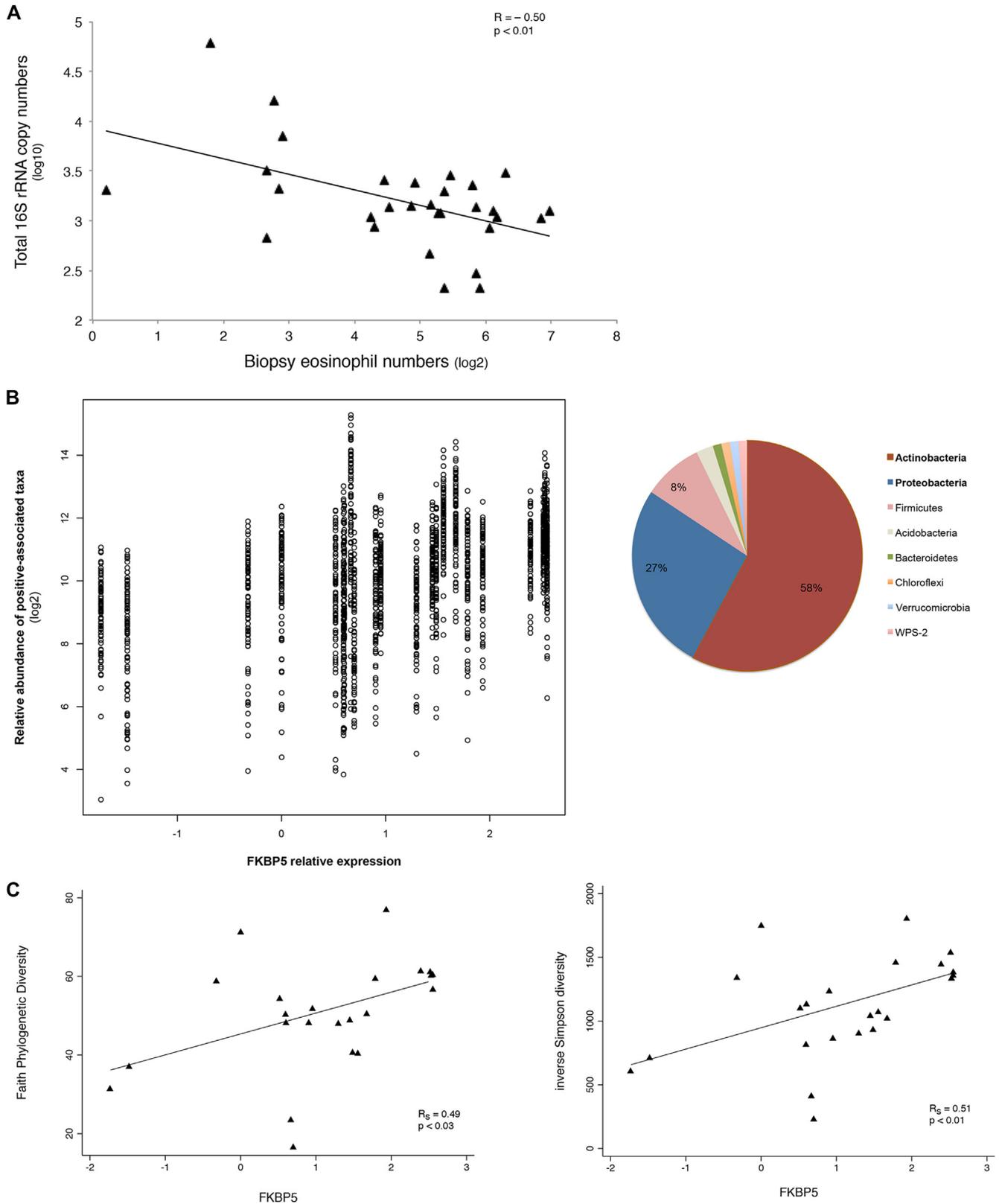


FIG 4. **A**, Total 16S rRNA copy numbers, a proxy for bacterial burden, are inversely correlated with biopsy eosinophil cell numbers ($R = -0.50$, $P < .01$). Data shown represent 29 of 40 subjects, including the subjects in whom array-based bacterial community profiling could not be performed because of low bacterial content. **B**, The relative abundance of bacterial taxa ($n = 83$, $q < .10$) associated with *FKBP5* expression plotted against the relative expression values for this gene. Phylum-level classification of these correlated communities is shown. **C**, Strongly significant correlations are seen between *FKBP5* expression and 2 different measures of bacterial community diversity: Faith phylogenetic diversity and the inverse Simpson diversity index. The former weights phylogenetic relationships among communities in the diversity determination, whereas the latter reflects primarily community richness and evenness.

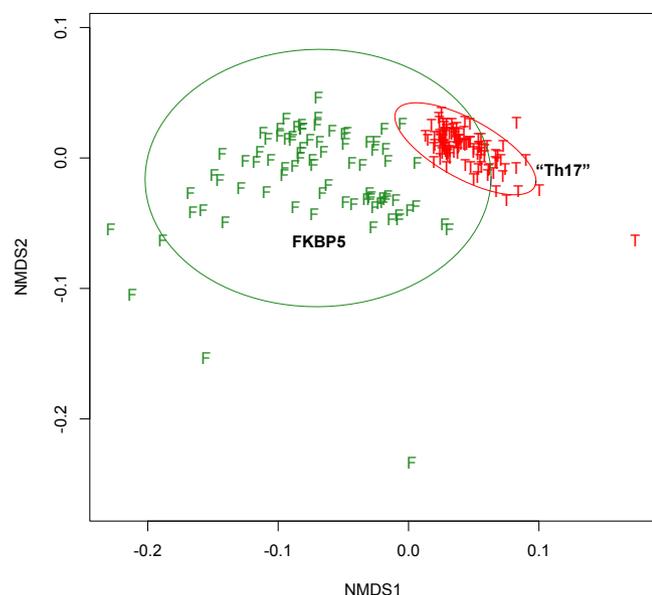


FIG 5. Compositionally distinct bacterial taxa are positively associated with *FKBP5* expression (green Fs; $n = 83$; 58% Actinobacteria) and the T_H17 -related gene expression pattern (red Ts; $n = 110$; 72% Proteobacteria). The nonmetric multidimensional scaling (NMDS) ordination is based on a Bray-Curtis distance matrix for all associated taxa in both expression patterns. Ellipses represent the 95% CIs for the SDs of the distances for each group.

obese asthmatic patients in this cohort. Analysis results for predicted functions among taxa associated with asthma control (ACQ score change) and with *FKBP5* and T_H17 -related gene expression pattern are shown in Figs E3 and E4 in this article's Online Repository at www.jacionline.org.

Bronchial microbiota differences between patients with severe asthma, healthy control subjects, and patients with mild-to-moderate asthma

We explored whether bronchial bacterial composition differs in patients with severe asthma from that profiled from previously studied healthy control subjects ($n = 7$) or patients with mild-to-moderate asthma who were receiving low-dose inhaled steroids ($n = 41$, 88 μg of fluticasone propionate twice daily).⁸ Compared with healthy control subjects (Fig 6, A, and see Table E6 in this article's Online Repository at www.jacionline.org), patients with severe asthma were enriched in several taxa, with the largest fold difference seen in a *Klebsiella* species (moderated t statistic linear models, R package *limma*, BH-adjusted $P < .001$). Compared with patients with mild-to-moderate asthma (Fig 6, B, and see Table E6), patients with severe asthma also were significantly enriched in 53 taxa, with the majority being Actinobacteria (47/53) and the remaining 5 taxa classifying to Gammaproteobacteria (all Enterobacteriaceae). The latter included a *Klebsiella* species that, as in the comparison with healthy control subjects, exhibited the highest fold difference in relative abundance (7.9-fold increase in patients with severe asthma, BH-adjusted $P < .0001$). Conversely, 42 taxa were more abundant in patients with mild-to-moderate asthma relative to the group with severe asthma. These taxa represented members from multiple phyla, with several different families of Proteobacteria (19 taxa) being most represented. This contrasted from the reverse

comparison wherein Enterobacteriaceae was the sole family of Proteobacteria with enriched members in patients with severe asthma.

qPCR studies

qPCR with primers specific for *Haemophilus influenzae*,¹⁴ a Pasteurellaceae member associated with worsening ACQ scores and expression of T_H17 -related genes, confirmed a significant correlation between β -actin-normalized log copies of *H influenzae* and fluorescence intensities for the *H influenzae*-represented taxon ($\rho = 0.67$, $P < .02$). qPCR also confirmed a strong correlation between *Prevotella* species abundance in samples and increased BMI ($\rho = 0.61$, $P < .05$; see Fig E5 in this article's Online Repository at www.jacionline.org) by using primers targeting specific *Prevotella* species represented in the BMI-associated taxa. *Prevotella* species copy numbers were also significantly higher in obese versus nonobese subjects ($P < .05$).

DISCUSSION

In this cohort of adults with severe asthma, we observed significant relationships between the bronchial airway microbiome and clinical and inflammatory features. The findings suggest that specific members of the airway microbiota are associated with putative phenotypes of severe asthma, such as those characterized by obesity-associated disease, corticosteroid-responsive disease, or T_H17 -associated airway inflammation. Interestingly, microbiome-associated clinical features in this study (eg, obesity and poor symptom control) are characteristics previously described among patients with some severe asthma phenotypes.⁴ A key strength of this study was the capacity to explore microbiome relationships to severe asthma-related clinical and inflammatory measures in different compartments, including blood and the lower airways. This enabled a comprehensive examination of possible microbiota relationships to underlying pathophysiologic processes reflected in these measures. Also, we explored and identified differences in bronchial bacterial composition between patients with severe asthma and healthy control subjects and patients with mild-to-moderate asthma whose bronchial microbiota had previously been characterized.⁸ Overall, our findings invite speculation that patterns of airway dysbiosis can contribute to putative endotypes of severe asthma or even asthma in general.

Among patients with severe asthma, BMI was strongly correlated with particular microbiota members, specifically Bacteroidetes and Firmicutes. This pattern is in striking contrast to the increased prevalence of Proteobacteria observed in patients with milder asthma⁷⁻⁹ and other feature correlations observed in this study. Obese patients with severe asthma also had fewer biopsy eosinophil numbers than subjects with low BMI. This invites speculation about whether members of the Bacteroidetes and Firmicutes might influence the degree of eosinophilic inflammation seen in airway biopsy specimens or, alternatively, stimulate non-eosinophil-associated immune responses. These 2 phyla also represent gastrointestinal microbiota, suggesting the possibility of aspiration. Whether the observed BMI-associated taxa contribute to asthmatic airway inflammation cannot be answered from this study, but other lines of evidence suggest they could. Species of *Prevotella* species represented among these taxa are potential pathogens previously associated with periodontitis and arthritis,^{35,36} whereas Lachnospiraceae belong to a Clostridia

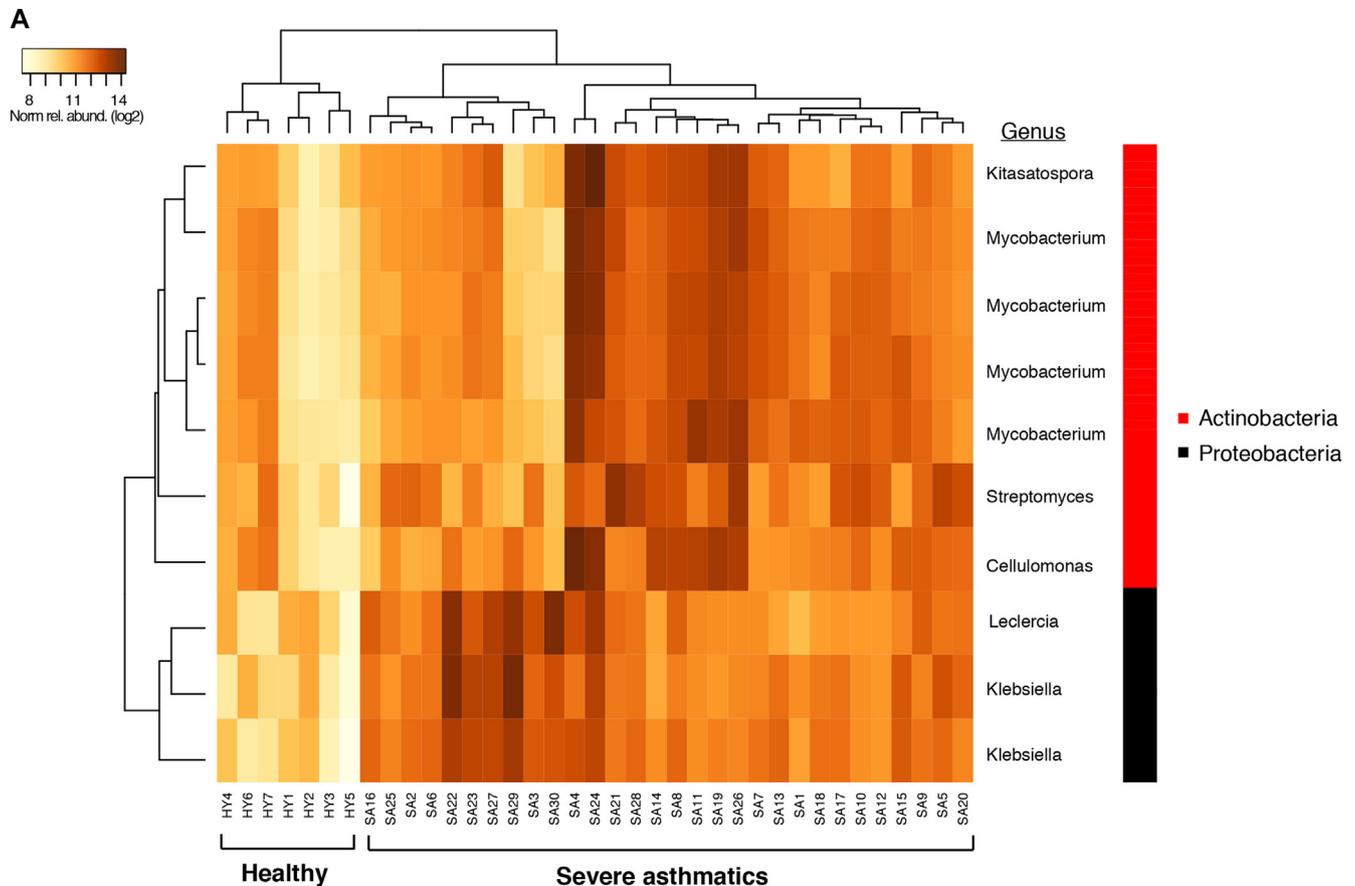


FIG 6. A, Heat map of the 10 taxa found to be significantly enriched among patients with severe asthma ($n = 30$) compared with healthy control subjects ($n = 7$; BH-adjusted $P < .15$; R package *limma*). **B**, Heat map of the 95 total taxa found to significantly differ in relative abundance between patients with severe asthma ($n = 30$) and those with mild-to-moderate asthma ($n = 41$; ≥ 2 -fold difference; BH-adjusted $P < .01$; R package *limma*). Relative to the other group, patients with severe asthma were enriched in 53 taxa, whereas patients with mild-to-moderate asthma were conversely enriched in 42 taxa.

clade known to induce colonic CD4⁺ forkhead box protein 3-positive regulatory T cells.^{37,38} Thus there is precedent for the capacity of these organisms to potentially promote or regulate inflammation in the respiratory tract. This paralleled results from our predictive metagenomic analysis, suggesting the inflammation-eliciting potential of these communities.

Worsening ACQ scores among patients with severe asthma were associated with greater relative abundance of Proteobacteria (eg, Enterobacteriaceae, Neisseriaceae, and Pasteurellaceae, families representing potential respiratory pathogens), whereas improving/stable ACQ scores were associated with Actinobacteria. Actinobacteria are metabolically diverse and prolific producers of secondary metabolites, including many with antimicrobial and anti-inflammatory properties.³⁹ Interestingly, Actinobacteria also were the main group of organisms associated with molecular evidence of response to steroids (*FKBP5* expression). This suggests the possibility of certain airway microbiota being indicative of asthma responsive to steroids, adherence to prescribed therapy, or even their involvement in mechanisms of steroid response.

Study limitations include the site and timing of PB collection and the exclusion of other parent study subjects. PBs evaluated for microbiome analysis sampled at least 2 mucosal sites, with concurrently collected brushes used for gene expression studies, a

strength of this study. Although bronchoscopy followed earlier visit measurements of variables considered in our analyses (eg, ACQ score), there were no respiratory illnesses or medication changes to have influenced the bacteria identified. We cannot completely rule out possible biases related to the exclusion, for technical reasons, of other parent study subjects. However, a systematic analysis found only significant differences between evaluated and nonevaluated BOBCAT study subjects in BMI and biopsied numbers of IL-17⁺ cells. Finally, the primary aim of our study was to examine bronchial microbiome relationships to features of patients with severe asthma. No control group was enrolled and identically studied in the BOBCAT study. Therefore we performed a focused comparison of bronchial bacterial composition in patients with severe asthma with that profiled previously by using the same methods from PBs of healthy control subjects and patients with mild-to-moderate asthma studied earlier.⁸

Despite study differences and other potential biases that cannot be completely controlled, our findings suggest the following. First, airway dysbiosis is a feature of severe asthma and differs from that seen in patients with milder asthma receiving lower doses of inhaled corticosteroids. Second, different microbiota, even within the same bacterial phylum (eg, Proteobacteria), family, or even genus are implicated in different phenotypic

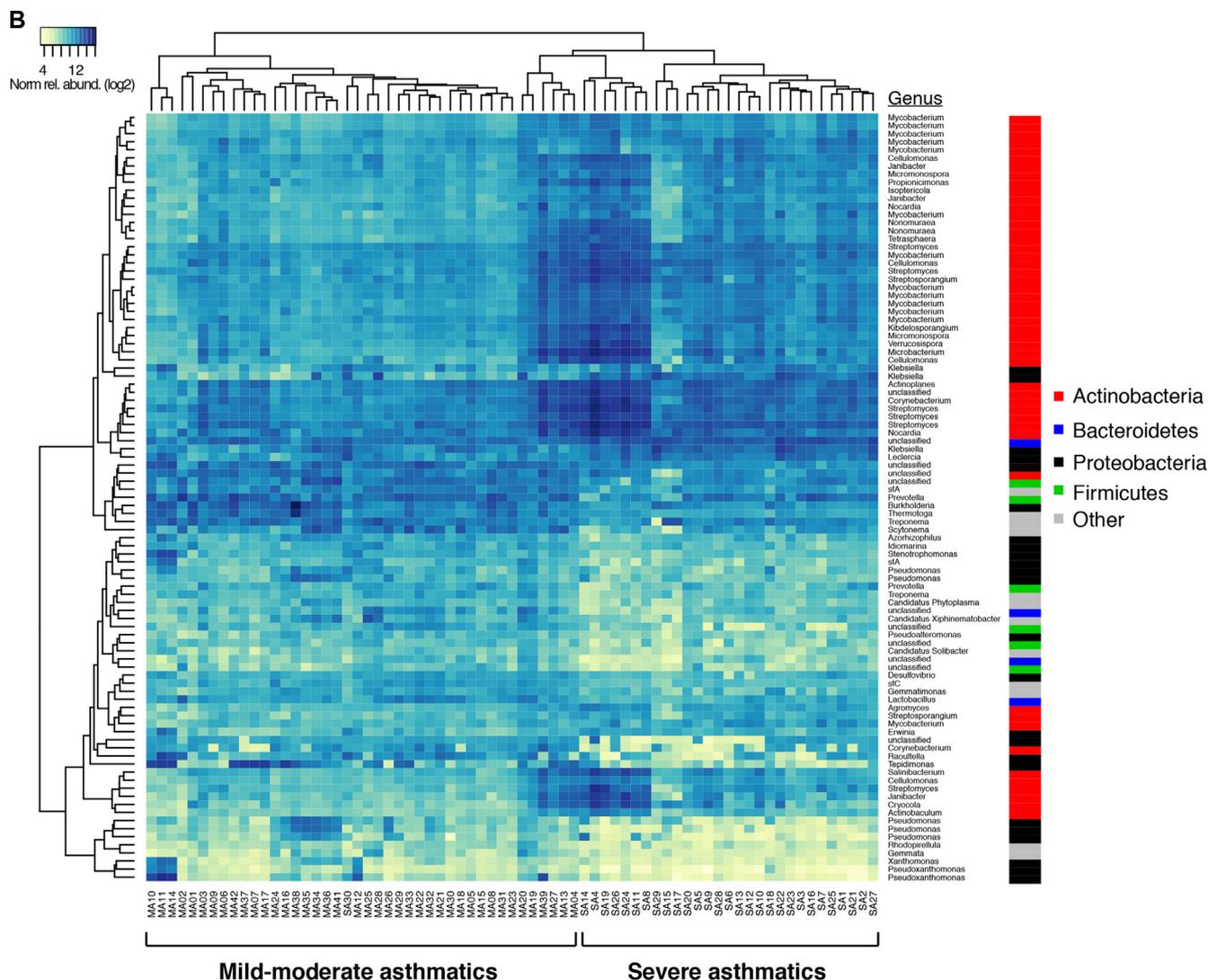


FIG 6. Continued

features of asthma, from putative disease-driven mechanisms (eg, T_H17-related or non-type 2 airway inflammation) to potential modulation of treatment responses (eg, corticosteroids).

A surprising observation was the overall absence of positive relationships between bacterial composition and indicators of type 2–driven inflammation, from epithelial expression of type 2–responsive genes to various markers of eosinophil-related inflammation. Indeed, only biopsy eosinophil values demonstrated significant but predominantly negative correlations with bacterial microbiota members. The use of high-dose inhaled corticosteroids could suppress these measures, although the mean sputum eosinophil percentage was increased among subjects in whom microbiome profiling could be performed and these percentages correlated with relative expression of the type 2–responsive epithelial gene signature (Spearman $\rho = 0.53$, $P < .05$). Other markers (eg, fraction of exhaled nitric oxide, periostin, and blood or sputum eosinophil values) also did not demonstrate relationships to bacterial composition. Antibacterial effects of eosinophils are a consideration for these findings. However, because DNA-based detection methods were used, we expect that any DNA present from killed bacteria should still be detectable. Other possibilities include that heterogeneity within the cohort masked

bacterial associations with type 2–related inflammation or that other microbial kingdoms, such as fungi, which were previously observed to be reciprocally related to bacterial abundance,⁴⁰ play a larger role in driving these responses.

Finally, we identified a group of mainly Proteobacteria significantly associated with epithelial expression of T_H17 inflammation–related genes.⁴¹ T_H17 cells, through IL-17A and IL-17F, promote neutrophil recruitment and have been invoked as a noneosinophilic/non-type 2 pathway in asthmatic patients.⁴² Asthmatic patients without evident type 2–driven inflammation are less responsive to inhaled corticosteroids.⁵ In mice T_H17 cell–mediated airway inflammation is resistant to steroid treatment.⁴³ Our finding suggests that neutrophil-predominant airway inflammation in asthmatic patients could also represent responses to bacterial infection and not solely reflect steroidal effects. Although we did not identify specific taxa associated with sputum neutrophil values, this is not entirely surprising for in this cohort, sputum neutrophil percentages were not found to be concordant with other measures of airway inflammation.⁴⁴

In summary, results of this study suggest the airway microbiome might be highly pertinent to non-type 2 pathways in patients with severe asthma and influence manifestations of

particular disease features. Further dissection of the microbiome's role in asthma-related immune responses will require additional study of asthmatic patients with varying disease severities and phenotypes and consideration of other microbiota (eg, fungi). Our findings also suggest future mechanism-oriented investigations of specific microbial targets. Future investigations involving larger cohorts of patients with well-characterized asthma will likely be necessary to dissect the complex relationships between the airway microbiome and distinct asthma phenotypes.

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Clinical implications: In patients with severe asthma, specific airway bacterial microbiota are associated with clinical and inflammatory features, including obesity and asthma control, but not markers of eosinophilic or type 2-related airway inflammation.

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