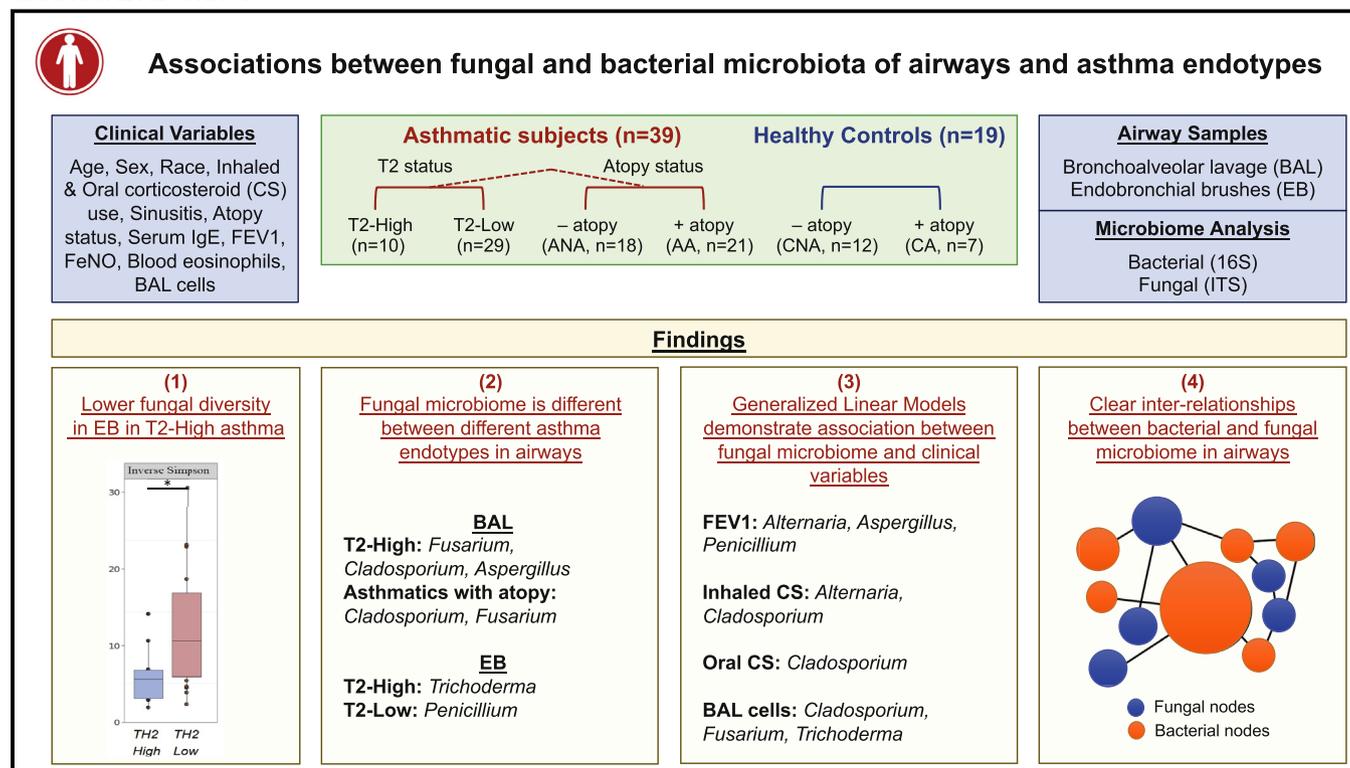


Associations between fungal and bacterial microbiota of airways and asthma endotypes

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GRAPHICAL ABSTRACT



Background: The relationship between asthma, atopy, and underlying type 2 (T2) airway inflammation is complex. Although the bacterial airway microbiota is known to differ in asthmatic patients, the fungal and bacterial markers that discriminate T2-high (eosinophilic) and T2-low (neutrophilic/

mixed-inflammation) asthma and atopy are still incompletely identified.

Objectives: The aim of this study was to demonstrate the fungal microbiota structure of airways in asthmatic patients associated with T2 inflammation, atopy, and key clinical parameters. **Methods:** We collected endobronchial brush (EB) and bronchoalveolar lavage (BAL) samples from 39 asthmatic patients and 19 healthy subjects followed by 16S gene and internal transcribed spacer-based microbiota sequencing. The microbial sequences were classified into exact sequence variants. The T2 phenotype was defined by using a blood eosinophil count with a threshold of 300 cells/ μ L.

Results: Fungal diversity was significantly lower in EB samples from patients with T2-high compared with T2-low inflammation; key fungal genera enriched in patients with T2-high inflammation included *Trichoderma* species, whereas *Penicillium* species was enriched in patients with atopy. In BAL fluid samples the dominant genera were *Cladosporium*, *Fusarium*, *Aspergillus*, and *Alternaria*. Using generalized linear models, we identified significant associations between specific fungal exact sequence variants and FEV₁, fraction of exhaled nitric oxide values, BAL fluid cell counts, and corticosteroid use.

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Investigation of interkingdom (bacterial-fungal) co-occurrence patterns revealed different topologies between asthmatic patients and healthy control subjects. Random forest models with fungal classifiers predicted asthma status with 75% accuracy for BAL fluid samples and 80% accuracy for EB samples.

Conclusions: We demonstrate clear differences in bacterial and fungal microbiota in asthma-associated phenotypes. Our study provides additional support for considering microbial signatures in delineating asthma phenotypes. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: Asthma, microbiota, corticosteroids, FEV₁, bacteria, fungi, airway inflammation, 16S ribosomal RNA

Asthma is a complex heterogenous disease characterized by airway inflammation, airflow obstruction, and bronchial hyper-responsiveness.¹ In recent years, an emphasis on delineating select phenotypes within asthma has led to recognition of 2 phenotypes defined by their association or lack thereof with markers of atopy and type 2 (T2) inflammation. In patients with “T2-high” asthma inhaled allergens and other stimulators activate antigen-presenting cells in the airway, leading to release of mediators from both dendritic cells and the airway epithelium.^{2,3} In turn, these elicit differentiation of T-helper lymphocytes into T_H2 cells and activation of type 2 innate lymphoid cells, with a downstream cascade of inflammatory mediators by cytokines, such as IL-4, IL-5, and IL-13.⁴ In contrast, the pathways that mediate “T2-low” asthma are less well established and are likely to be multiple; some of these might be regulated in part by neutrophils and T_H17 cells.⁵

Several recent investigations have shown significant associations of bronchial microbiota composition with these phenotypes.⁶⁻⁹ Denner et al⁹ demonstrated that the central airway microbiota of asthmatic patients differed significantly from that of the peripheral airways and that changes in microbial diversity and the presence of taxa, such as *Pseudomonas* species, was associated with worsening airflow obstruction and oral corticosteroid (OCS) use. More recently, Durack et al⁸ showed significant compositional differences in the bronchial bacterial community between asthmatic patients with atopy (AAs) and asthmatic patients without atopy (ANAs). These differences included enrichment of *Prevotella*, *Actinomyces*, and *Lactobacillus* species in AAs and enrichment of *Aggregatibacter*, *Haemophilus*, and *Actinobacteria* species in ANAs.

Durack et al⁸ also demonstrated that T2-high asthma was associated with a low bronchial bacterial burden. Although this could be related to the effect of eosinophilic inflammation on bacterial survival, another potential explanation would be the presence and influence of nonbacterial microbiota, such as fungi. Fungi, such as *Aspergillus* species, have been recognized as a significant allergen in the setting of both upper and lower respiratory tract atopic diseases, and there is evidence that fungal colonization can contribute to asthma.¹⁰⁻¹³ Microbial associations in asthmatic patients might not be restricted to each kingdom (bacteria, fungi, and viruses) independently but rather might involve an interplay between these domains that might participate in asthma exacerbations.^{14,15} Moreover, the role of the fungal microbiota in airways of patients with asthma and asthma-associated endotypes and phenotypes remains poorly understood.

We investigated the fungal composition of bronchial samples in 39 asthmatic patients and 19 healthy control subjects in whom we

Abbreviations used

AA:	Asthmatic patient with atopy
ABC:	ATP-binding cassette
ANA:	Asthmatic patient without atopy
ANCOM:	Analysis of composition of microbiomes
BMI:	Body mass index
CA:	Control subject with atopy
CNA:	Control subject without atopy
BAL:	Bronchoalveolar lavage
BH-FDR:	Benjamini-Hochberg false discovery rate
EB:	Endobronchial brush
ESV:	Exact sequence variant
FENO:	Fraction of exhaled nitric oxide
GLM:	Generalized linear regression model
ICS:	Inhaled corticosteroid
ITS:	Internal transcribed spacer
NMDS:	Nonmetric multidimensional scaling
OCS:	Oral corticosteroid
OOB:	Out-Of-Bag
PERMANOVA:	Permutational multivariate analysis of variance
PICRUS:	Phylogenetic Reconstruction of Unobserved States
T2:	Type 2
WGCNA:	Weighted correlation network analysis

have previously reported differences in the bacterial microbiota in both peripheral (bronchoalveolar lavage [BAL]) and central (EB) airways.⁹ Here we compared the fungal microbiota between (1) AAs and ANAs with that between control subjects with atopy (CAs) and control subjects without atopy (CNAs) and (2) among asthmatic patients with the T2-high and T2-low inflammatory phenotypes, as defined by the number of blood eosinophils. We demonstrated that patients with T2-high asthma were characterized by low bronchial fungal diversity (eg, bacteria) and that the fungal microbiota correlates with clinical parameters, such as FEV₁, fraction of exhaled nitric oxide (FENO) values, IgE levels, OCS use, and sinusitis. Using interkingdom co-occurrence networks, we were able to demonstrate coregulated networks between bacterial and fungal genera associated with different asthmatic patients and healthy control subjects. These results suggested that in the future both fungal and bacterial microbiota can potentially be used for diagnosis of select asthma phenotypes, as well as for direct treatment.

Additionally, we found that predicted bacterial functions correlate with asthma-associated phenotypes and clinical parameters, including associations between predicted nitrogen and bile-acid metabolism, calcium signaling, and clinical parameters, including corticosteroid use and FEV₁ and FENO values. Our findings show compositional differences in the bacterial and fungal microbiota of groups created based on the following: (1) atopy status in asthmatic patients (ie, AAs vs ANAs) and (2) T2 inflammation (ie, patients with T2-high vs those with T2-low asthma), with evidence for potential association of these microbial differences with different clinical parameters.

METHODS

Study subjects and sample collection

This is a cross-sectional study.⁹ We used samples that were collected from adult asthmatic patients and control subjects who participated in previous asthma genetic and airway biology studies originally conducted from 2011

to 2015. Approval for use of samples generated from these subjects was obtained from the Institutional Review Board at the University of Chicago. All subjects provided written informed consent at the time of their recruitment. Asthmatic patients and control subjects without known lung disease were recruited from the Chicago-area community. Subjects with mild persistent asthma met the criteria for step 1 or step 2 asthma, and patients with severe persistent asthma met the criteria for step 4 through step 6 asthma, as defined by the Expert Panel Report 3 guidelines on asthma.¹⁶ Confirmation of airway reactivity in asthmatic patients was done by using methacholine challenge when baseline FEV₁ was 60% or more of predicted value; when this could not be done, measurement of reversibility before and after albuterol inhalation was done per the guidelines of the American Thoracic Society.¹⁷ Control subjects all had a less than 16% reduction in FEV₁ after inhalation of 25 mg/mL methacholine. Subjects with a smoking history of 10 or more pack-years, who had a history of chronic obstructive pulmonary disease, or who had medical contraindications to bronchoscopy were excluded. Subjects underwent collection of clinical and demographic data, venipuncture, spirometry, and flexible video bronchoscopy at a time of clinical stability and at least 8 weeks after any known asthma exacerbation or respiratory viral illness. For details on bronchoscopy and BAL fluid collection, please refer to Denner et al⁹ and the **Methods** section in this article's Online Repository at www.jacionline.org.

Amplicon library preparation and sequence analyses

The V4 region of the 16S rRNA gene (515F-806R) was amplified with region-specific primers that included the Illumina flow cell adapter sequences (Illumina, San Diego, Calif) and a 12-base barcode sequence.¹⁸ For details on library preparation, please see the **Methods** section in this article's Online Repository. The internal transcribed spacer (ITS) data ($n = 1,639,213$ reads) were quality filtered and demultiplexed with the same QIIME 1.9.1 scripts (ie, `join_paired_ends.py` and `split_libraries_fastq.py`). The chimeras were removed by using VSEARCH.¹⁹

Exact sequence variant (ESV) picking was done by using Deblur²⁰ with UNITE database (alpha version 7.2) for ITS.²¹ ESVs present in less than 10 samples were removed from analyses in the phyloseq package for an increased confidence of analyses.²² The final BIOM file containing samples from 39 asthmatic patients and 19 healthy subjects, with 2144 ESVs after strict quality control, was further used for diversity analyses. For the 16S rRNA analysis, 1.8 million paired-end reads generated were joined by using `join_paired_ends.py` script, followed by quality filtering and demultiplexing with `split_libraries_fastq.py` script in QIIME 1.9.1.²³

The final set of demultiplexed sequences were then selected for ESV picking by using the Deblur pipeline.²⁰ In the pipeline *de novo* chimeras and artifacts were removed, and ESVs present in less than 10 samples were removed with the phyloseq package.²² The final BIOM file²⁴ comprised of 4,327 ESVs with samples from 39 asthmatic patients and 19 healthy subjects was then used for analyses. Random forest supervised learning models were used to estimate the predictive power of microbial community profiles for 16S and ITS to determine atopy and T2 status.²⁵ For parameter details, please see the **Methods** section in this article's Online Repository.

Statistical analyses

Nonmetric multidimensional scaling (NMDS) plots were used to reveal β -diversity variations based on the Bray-Curtis matrix²⁶ for ITS data and the Weighted UniFrac index²⁷ for 16S ESV data in the phyloseq package. Shannon analysis and the inverse Simpson index²⁸ were used to estimate α -diversity, and variation between groups (β -diversity) was statistically tested by using permutational multivariate analysis of variance (PERMANOVA).²⁹ Analysis of composition of microbiomes (ANCOM) was used to identify differentially abundant bacterial and fungal ESVs between the groups at a P value cutoff of .05 with Benjamini-Hochberg false discovery rate (BH-DR) correction.³⁰ Phylogenetic Reconstruction of Unobserved States (PICRUSt) was used to annotate bacterial ESVs for predicted bacterial encoded

functions.³¹ The weighted correlation network analysis (WGCNA) package in R software was used to identify bacterial functional clusters (modules) of significantly correlated pathways.³²⁻³⁴

To minimize spurious associations during module identification, we transformed the adjacency into a Topological Overlap Matrix and calculate the corresponding dissimilarity.³⁵ The bacterially encoded functions were organized into modules by using this topological overlap measure as a robust measure of interconnectedness in a hierarchical cluster analysis.

To relate modules to clinical parameters, we used an eigengene network methodology to identify potential significant associations (BH-FDR-corrected $P < .05$).³⁶ Co-occurrence networks were generated by calculating Spearman correlations between the abundance of ESVs with Hmisc in R software (<https://cran.r-project.org/web/packages/Hmisc/index.html>). The significant results (BH-FDR-corrected P value $< .05$) were exported as GML format network files using igraph in R software (<https://cran.r-project.org/web/packages/igraph/index.html>). Modularity analyses and keystone node identification were performed by using Gephi software.³⁷

Generalized linear regression models (GLMs) were run to test for correlations between select ESVs (significantly enriched in each asthma-associated phenotype) and clinical variables. GLMs were implemented in the `glm()` package using counts data for the genera with Poisson regression and "log" link without making assumptions about the data distribution. Significance for each correlation for clinical parameters was evaluated by using ANOVA to compare nested models using χ^2 tests.³⁸ For linear models, standardized β -coefficients (division by 2 SDs) were plotted to overcome the bias introduced because of varying scales (units) for the clinical parameters.³⁹

RESULTS

Demographic and clinical characteristics of study subjects

We collected endobronchial brush (EB) samples from 39 asthmatic patients and 19 healthy control subjects and generated technical control samples comprising 5 brush controls and 7 negative reagent controls.⁹ Among the 39 asthmatic patients, we defined 2 comparison groups: (1) 18 ANAs versus 21 AAs based on atopy status and (2) 10 patients with T2-high asthma (defined as a peripheral blood absolute eosinophil count $\geq 300/\mu\text{L}$) versus 29 patients with T2-low asthma (peripheral blood absolute eosinophil count $< 300/\mu\text{L}$).⁴⁰ Subjects were considered atopic if they had evidence of allergic sensitization to 2 or more allergens based on either a skin prick test or an immunosorbent assay.⁴¹

Study cohort characteristics are shown in **Table I**. As expected, FENO values, blood eosinophil counts, and serum IgE levels were greater in AAs compared with ANAs and in asthmatic patients compared healthy control subjects (**Table I**). Among healthy control subjects, serum IgE concentrations, FENO concentrations, and blood eosinophil counts were significantly greater in CAs versus CNAs (**Table I**).

We also analyzed the potential confounding effect of age, sex, and body mass index (BMI), whereby only BMI was identified as a confounding variable. Hence, to rule out the confounding effect, we adjusted for BMI in all ANCOM analyses (discussed below).⁴² The remaining asthma-associated clinical variables were treated as variables of interest, and their association with microbial vectors was investigated by using GLMs (as discussed in later sections).

Fungal community differences in the lower airways between asthma-associated phenotypes

In addition to samples from 39 asthmatic patients and 19 healthy control subjects, we sequenced 5 brush control samples

TABLE I. Demographic and clinical characteristics of study subjects

Variable	AAs (n = 21)	ANAs (n = 18)	T2-high patients (n = 10)	T2-low patients (n = 29)	CAs (n = 7)	CNAs (n = 12)
Age (y), mean ± SE	41.7 ± 1.8	44.4 ± 2.2	42.5 ± 3.1	42.9 ± 1.4	33.6 ± 2.5	34 ± 2.3
Sex (male/female)	9/12	5/13	3/7	9/20	3/4	4/8
Race (European ancestry/African ancestry/other)	11/10/0	11/6/1	6/2/2	16/13/0	2/4/1	4/7/1
ICS use (yes/no)	16/5	13/5	7/3	22/7	0/0	0/0
OCS use (yes/no)	11/10	9/9	7/3	15/14	0/0	0/0
Sinusitis (yes/no)	6/15	9/9	5/5	9/20	0/0	0/0
FEV ₁ (L), mean ± SE	2.52 ± 0.13	2.26 ± 0.11	2.58 ± 0.37	2.38 ± 0.08	3.15 ± 0.11†	4.05 ± 0.12†
FENO (ppb), mean ± SE	33.3 ± 4.4	30.2 ± 4.8	43.2 ± 11.6*	29.8 ± 3.1*	21.7 ± 3.1†	11.4 ± 0.9†
BAL fluid neutrophil count (%), mean ± SE	0.052 ± 0.002	0.057 ± 0.003	0.055 ± 0.005	0.054 ± 0.002	0.046 ± 0.003	0.051 ± 0.005
BAL fluid eosinophil count (%), mean ± SE	0.052 ± 0.004‡	0.034 ± 0.003‡	0.054 ± 0.091*	0.043 ± 0.002*	0	0.002 ± 0.001†
Blood absolute eosinophil count (number/nL), mean ± SE	0.157 ± 0.023‡	0.213 ± 0.061‡	0.593 ± 0.124*	0.104 ± 0.009*	0.131 ± 0.021	0.093 ± 0.013
Serum IgE (U/mL), mean ± SE	376 ± 88‡	155 ± 59‡	790 ± 183*	183 ± 49*	144 ± 40†	46 ± 10†

Statistical significance was determined by using the Mann-Whitney test (2 groups).

*Significant *P* values (*P* < .05) for the 2-group comparisons (ie, T2-high vs T2-low patients).

†Significant *P* values (*P* < .05) for the 2-group comparisons (ie, CAs vs CNAs).

‡Significant *P* values (*P* < .05) for the 2-group comparisons (ie, AAs vs ANAs).

and 7 reagent control samples. None of the 7 reagent control samples provided more than 500 sequence reads per sample, likely because each sample had less than 1 ng/μL DNA. From this, we concluded that no reagent contamination contributed to sample sequencing.⁴³ Of 5 brush control samples, one generated fewer than 500 reads, whereas the other 4 samples averaged 5000 reads per sample. We identified 20, 10, 18, and 12 unique ESVs in brush controls 1, 2, 3, and 4, respectively. The large majority of these potentially contaminating ESVs (93.2%) could only be assigned at the kingdom level (ie, fungi with no further reliable taxonomic assignments). The remaining ESVs included *Saccharomyces*, *Phlebia*, and *Pluteus* species. All these potential contaminants had a proportion of less than 0.003% and therefore were not removed from further analysis.

Two α-diversity indices, including Shannon (which includes evenness) and inverse Simpson (diversity) analyses, differed significantly between the different asthma and control groups for EB samples (Fig 1, A). Although there were no significant differences observed between BAL fluid samples from healthy control subjects and asthmatic patients, we found a greater diversity of fungi in healthy control subjects compared with asthmatic patients (*P* < .05, Shannon and inverse Simpson; Fig 1, A). Within each sample type (BAL fluid and EB), α-diversity was not significantly different between atopy subgroups (see Fig E1, A, in this article's Online Repository at www.jacionline.org). However, asthmatic patients with T2-low inflammation had significantly greater fungal diversity (*P* < .05) compared with the T2-high group (Fig 1, B). Healthy control subjects also had significantly greater fungal α-diversity (*P* < .01) compared with patients with T2-high asthma (Fig 1, B). There were no significant differences in diversity between control subjects and patients with T2-low or T2-high asthma in BAL fluid samples (Fig 1, B).

No significant difference in β-diversity (Bray-Curtis dissimilarity) was observed in either BAL fluid or EB samples between asthmatic patients and healthy control subjects (BAL fluid, *P*_{PERMANOVA} = .1; EB, *P*_{PERMANOVA} = .07) or between atopic subjects (ie, AAs and CAs) and nonatopic subjects (ie, ANAs and CNAs; BAL fluid, *P*_{PERMANOVA} = .4; EB, *P*_{PERMANOVA} = .06; Fig 1, C and D). However, β-diversity was significantly different

between patients with T2-high/low asthma and healthy control subjects (BAL fluid, *P*_{PERMANOVA} = .001; EB, *P*_{PERMANOVA} = .03; Fig 1, C and D). BAL fluid samples maintained a distinct ordination pattern with the T2-high group in a tight cluster compared with patients with T2-low asthma and healthy control subjects, which were scattered (NMDS; Fig 1, C). This pattern could also be due the smaller sample size for patients with T2-high (n = 10) compared with T2-low (n = 29) asthma. For EB samples, healthy control samples formed a distinct cluster (EB, *P*_{PERMANOVA} = .03) compared with both patients with T2-high asthma and those with T2-low asthma (Fig 1, D). Interestingly, in healthy control subjects there was no significant difference in β-diversity between BAL fluid and EB samples, whereas in asthmatic patients BAL fluid and EB samples were significantly (*P* < .05) different (see Fig E1, B).

ANCOM (BH-FDR-corrected *P* < .05) analyses in BAL fluid identified 11 ESVs that were significantly differentially abundant between asthmatic patients and healthy control subjects. ESVs associated with *Trichoderma*, *Alternaria*, *Cladosporium*, and *Fusarium* species were significantly enriched in asthmatic patients (*P*_{FDR} < .05 in each analysis), whereas *Blumeria* species, *Mycosphaerella* species, and different *Fusarium* species ESVs were enriched in healthy control subjects (Fig 2, A). Additionally, 12 ESVs differed between patients with T2-high and those with T2-low asthma, including *Fusarium*, *Aspergillus*, *Cladosporium*, and *Alternaria* species, which were significantly enriched in patients with T2-high asthma (Fig 2, B), and a single ESV, *Mycosphaerella* species, was enriched in T2-low samples. Similarly, ESVs from *Cladosporium* and *Fusarium* species were enriched in AAs, whereas *Mycosphaerella* species, the Aspergillaceae, and a different *Cladosporium* species ESV were significantly enriched in ANAs (Fig 2, C).

Penicillium species were significantly enriched in asthmatic patients compared with healthy control subjects (*P*_{FDR} < .05 in each analysis), and *Trichoderma* species values were greater in patients with T2-high compared with T2-low asthma (Fig 2, C and D). Interestingly, the same *Penicillium* species ESV was enriched (*P*_{FDR} < .05) in AAs compared with ANAs (Fig 2, E). Overall, we observed a consistent fungal signature, especially with

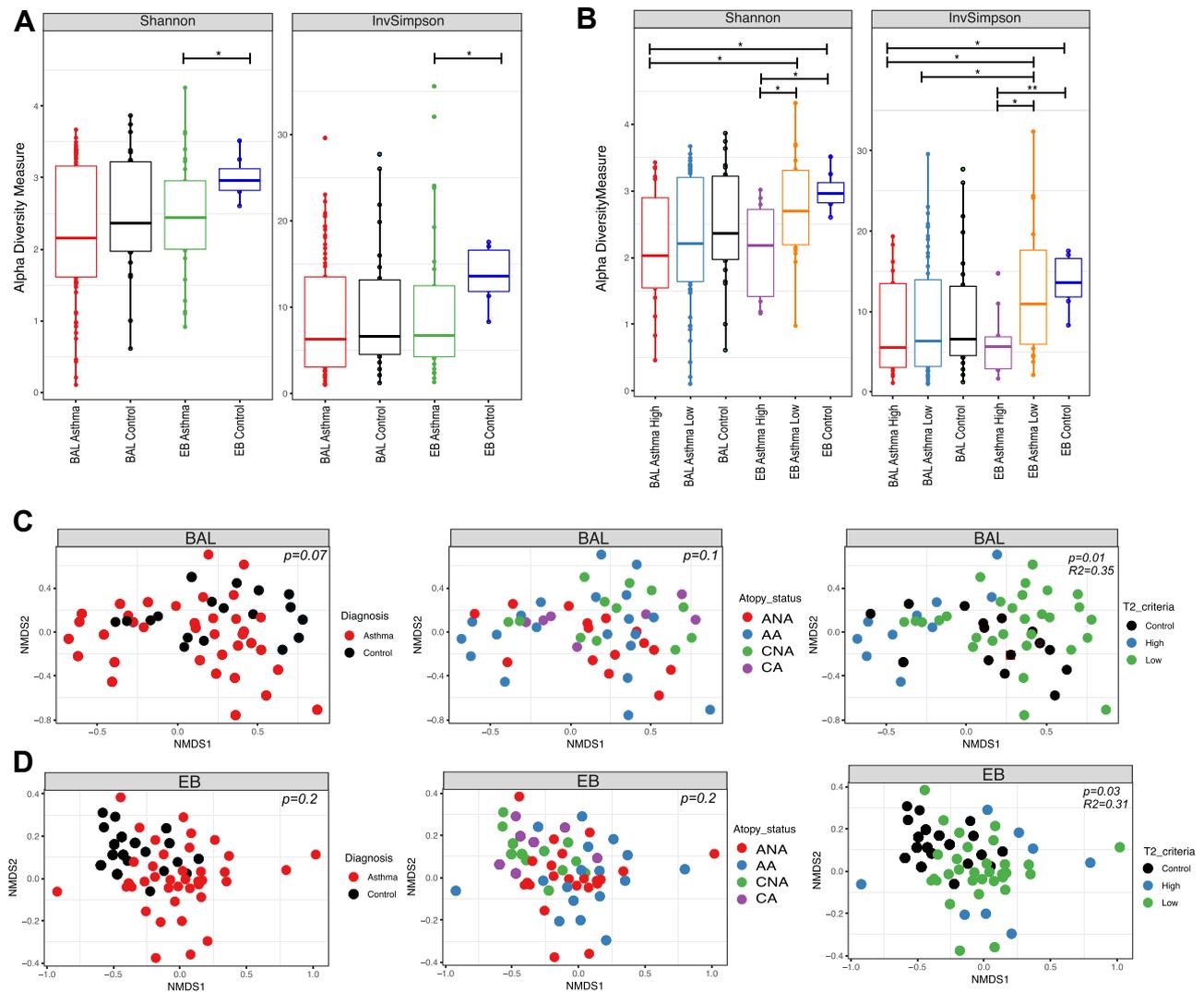


FIG 1. Fungal diversity comparison between various asthma-associated phenotypes. **A** and **B**, Box plots of Shannon and inverse Simpson diversity indices distinguishing asthmatic patients and healthy control subjects (Fig 1, **A**) and patients with T2-high asthma, patients with T2-low asthma, and healthy control subjects (Fig 1, **B**) based on T2 inflammation. Statistical analysis was performed with paired *t* tests. Plotted are inter-quartile ranges (*boxes*), and *dark lines in boxes* are medians. **P* ≤ .05 and ***P* ≤ .01. **C**, NMDS plots based on the Bray-Curtis index between the above-mentioned groups, including comparisons between AAs, ANAs, CAs, and CNAs for BAL samples. **D**, NMDS plots between the groups mentioned in Fig 1, **C**, for EB samples. Statistical analysis was performed with PERMANOVA, and *P* values are labeled in plots. *R*² values are noted for comparisons with significant *P* values and stand for percentage variance explained by the variable of interest. We observed a greater than 30% variance explained by T2 criteria in both BAL fluid and EB samples.

regard to BAL fluid samples, associated with atopic patients with T2-high asthma compared with healthy control subjects and asthmatic patients who were in the T2-low and no-atopy groups.

In addition, we performed differential abundance analyses between asthmatic patients with and without inhaled corticosteroid (ICS) use. Interestingly, we identified 2 different ESVs belonging to the genus *Penicillium* to be differentially abundant between the 2 groups in both BAL fluid and EB samples (see Fig E2 in this article's Online Repository at www.jacionline.org). ESV from *Penicillium chrysogenum* was significantly enriched in asthmatic patients using ICSs in BAL fluid samples, whereas 1 ESV from *Penicillium* species (unidentified species) was enriched in asthmatic subjects not using ICSs in EB samples (see Fig E2).

Association between the fungal bronchial microbiota and clinical parameters

GLMs were performed by using genera identified by means of ANCOM to determine their associations with select clinical parameters. Because of differential microbial enrichment between BAL fluid and EB samples, we expected to see different associations. For BAL fluid samples, FEV₁ was a significant contributor to the variance in key genera (*P* < .05); however, the direction of correlation differed (Fig 3). *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Mycosphaerella* species were positively associated with FEV₁, whereas *Alternaria* and *Cladosporium* species were negatively associated (Fig 3). *Trichoderma* species showed significantly positive association with BAL fluid lymphocytes and neutrophils, whereas there was a negative

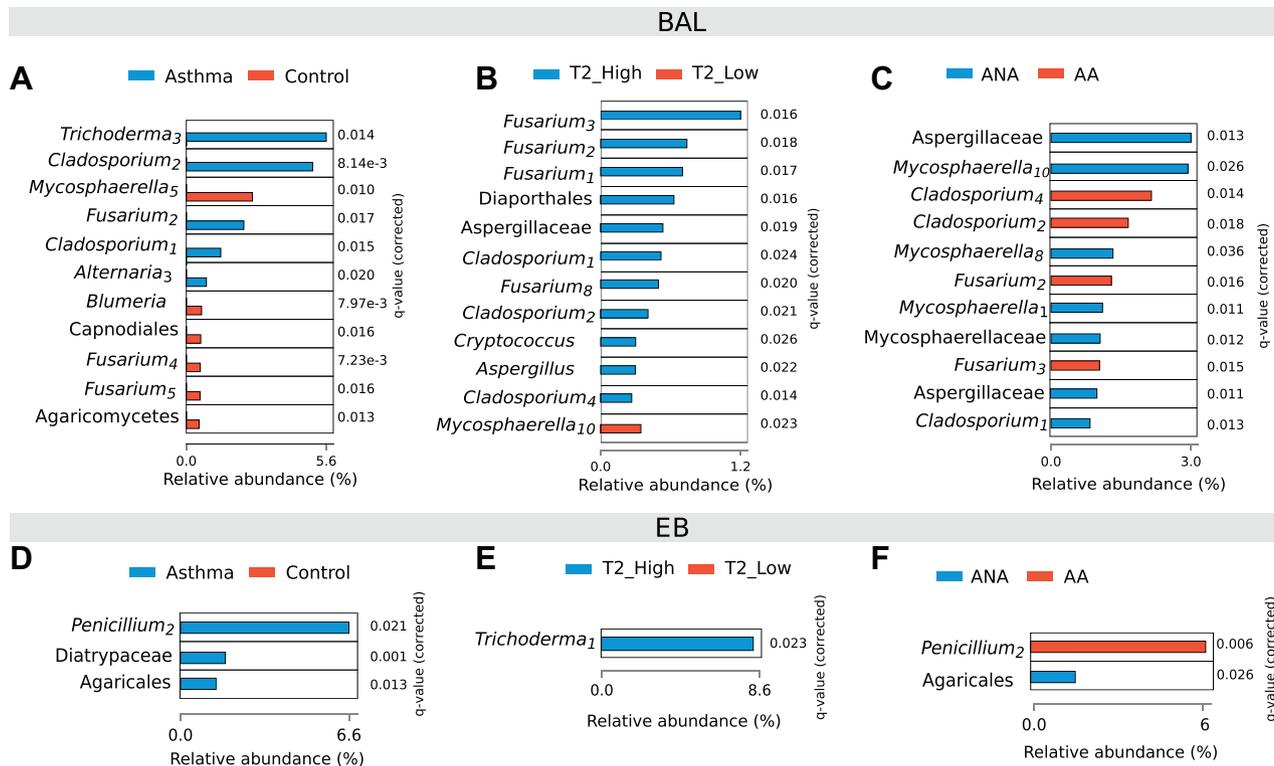


FIG 2. A-C, Differentially abundant ESVs between asthmatic patients and healthy control subjects (Fig 2, A), patients with T2-high or T2-low asthma (Fig 2, B), and AAs and ANAs by using ANCOM for BAL fluid samples (Fig 2, C). D-F, Differentially abundant ESVs between asthmatic patients and healthy control subjects (Fig 2, D), patients with T2-high and T2-low asthma (Fig 2, E), and ANAs and AAs (Fig 2, F) for EB samples are shown. BH-FDR-corrected P values are displayed for each ESV. For genera with more than 1 differentially abundant ESV, the ESV number is shown as subscripts.

association with BAL fluid eosinophils (Fig 3). *Cladosporium* species was also demonstrated to be positively associated with ICSs, whereas *Mycosphaerella* species showed a negative association with ICSs. This observation is supportive of the prior ANCOM analyses results, whereby *Mycosphaerella* species were enriched in patients with milder asthma and healthy control subjects. For EB samples, *Alternaria* and *Aspergillus* species were positively correlated with BAL fluid macrophages and neutrophils, whereas *Cladosporium*, *Fusarium*, and *Trichoderma* species were negatively correlated (see Fig E2).

Bacterial-fungal co-occurrence patterns across asthma-associated phenotypes

Co-occurrence networks were calculated to determine differential associations caused by similar shifts in the proportion of different taxa between asthma-associated phenotypes and healthy control subjects. Modularity-based co-occurrence networks between fungal and bacterial taxa were parsed at a Spearman correlation cutoff of 0.6 and BH-FDR corrected to a P_{FDR} value of less than .001. Modules were identified that maintained highly connected sets of nodes (ESVs), with few connections to other modules. The healthy control network had fewer connections overall compared with the asthma networks, which had a greater density of connections between nodes (Fig 3). Because of the small sample sizes for the T2-high, T2-low, AA, and ANA subgroups, we were unable to construct modularity networks with

a P_{FDR} of less than .05. Interestingly, unlike the ANCOM and GLM analysis, we identified fungal “keystone” taxa (betweenness centrality >50), which were defined as organisms whose removal would have a disproportionate deleterious effect on community stability and function,⁴⁴ that were found in both BAL fluid and EB samples from asthmatic patients.

Overall, EB samples had a greater density of connections between nodes compared with BAL fluid samples. In the asthma BAL network we observed small co-occurring bacterial networks associated with fungal nodes, such as *Cladosporium*, *Trichoderma*, *Penicillium*, and *Alternaria* species. *Cladosporium* species ESVs correlated significantly with bacterial ESVs, including *Rickettsia* and *Lactobacillus* species, and with *Fusobacterium*, *Lactobacillus*, and *Actinomyces* species (Fig 4, A). *Alternaria* species were associated with *Mycosphaerella* species in 2 separate modules, suggesting a robust relationship (Fig 4, A). In the dark green network both fungal ESVs showed associations with bacterial ESVs from *Prevotella* and *Synechococcus* species (Fig 4, A). In the light green module the 2 fungal ESVs showed interactions with *Veillonella*, *Atopobium*, and *Actinomyces* species in addition to *Prevotella* species (Fig 4, A). Another major module (blue) demonstrated interactions between *Cladosporium* and *Streptococcus*, *Lactobacillus*, *Megasphaera*, *Gemella*, *Veillonella*, and *Haemophilus* species (Fig 4, A).

In the EB asthma network *Cladosporium* species were significantly associated with *Prevotella*, *Aggregatibacter*, *Leptotrichia*,

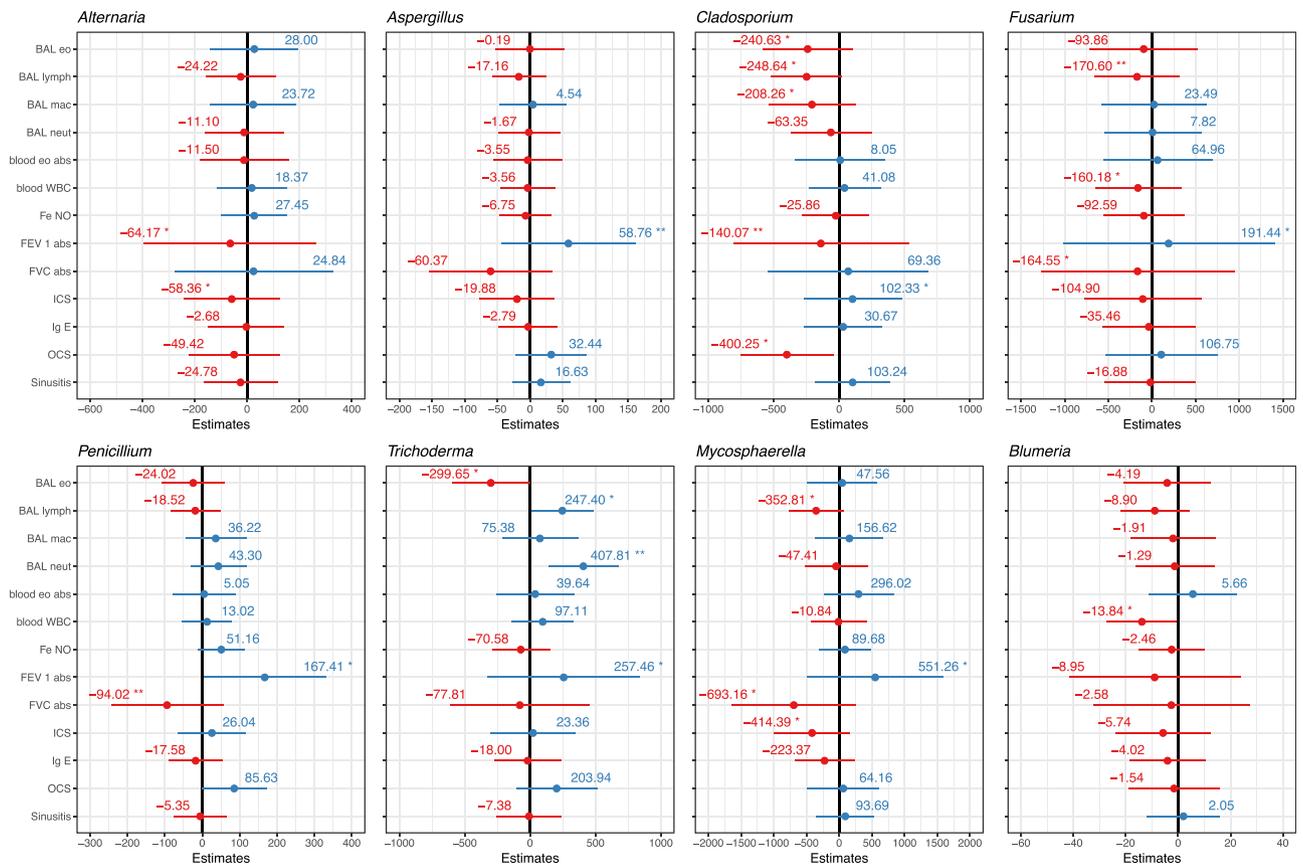


FIG 3. Associations between key fungal genera and clinical variables in BAL fluid samples using GLMs. *Asperisks* represent significant correlations (positive or negative) with BH-FDR–corrected P values as follows: * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .01$. The x -axis represents GLM estimates (ie, standardized β -coefficients of each model, which are rescaled as suggested by Gelman³⁹). Each horizontal bar provides a range of these estimates, with an average indicated by a node. Estimates represent the strength of the association. Estimates on the left-hand side (red line) of 0 (neutral line) demonstrate a negative association, and estimates on the right-hand side (blue line) of 0 demonstrate a positive association, with the variable on the y -axis.

Fusobacterium, *Porphyromonas*, and *Mogibacterium* species (green module; Fig 4, B), whereas *Trichoderma* species associated with *Synechococcus* and *Prevotella* species (pink module; Fig 4, B), and *Fusarium* species associated with *Nitrosopumilus* and *Stenotrophomonas* species (golden module; Fig 4, B). Although the keystone fungal taxa remained similar between BAL fluid and EB samples, co-occurring bacterial ESVs were distinct between the 2. The occurrence of different network modules in each network represents different coregulated groups of genera that in turn highlight distinct community partitions.

In healthy control samples the keystone fungal taxa included *Mycosphaerella*, Pleosporaceae, and *Agaricomycetes* species, which showed associations between different bacteria compared with the asthma networks, including *Corynebacterium*, *Neisseria*, *Oribacterium*, *Rothia*, *Delftia*, *Bulleidia*, *Prochlorococcus*, and *Pseudomonas* species (in the asthma network these fungi associated with *Lactobacillus*, *Prevotella*, *Streptococcus*, and *Actinomyces* species and others; see Fig E3 in this article's Online Repository at www.jacionline.org). Overall, the asthmatic patient- and healthy control subject-associated networks both elucidated the roles of different genera in different asthma-

associated endotypes and also highlighted the distinct community partitions that exist in each phenotype.

Bacterial community structure and predicted functional associations with atopy and T2 inflammation

Across our cohort of 39 asthmatic patients, the 16S rRNA data revealed significant variations in α - and β -diversity between the different groups (see Fig E4 in this article's Online Repository at www.jacionline.org). Adding to the observations made in the study by Denner et al⁹ and confirming the results of the Asthma-Net microbiome study,⁸ bacterial diversity was significantly lower in the T2-high group compared with the T2-low group in EB samples (BH-FDR–corrected $P_{FDR} < .05$; see Fig E4). In the study by Denner et al, we demonstrated that the bacterial microbiota of the EB samples correlated with airflow obstruction, and hence we used the EB samples for bacterial microbiota analyses in the present study. Multigroup ANCOM analysis revealed a differential abundance pattern ($P_{FDR} < .05$) between the AAs, ANAs, and control subjects (ie, both CNAs and CAs as separate

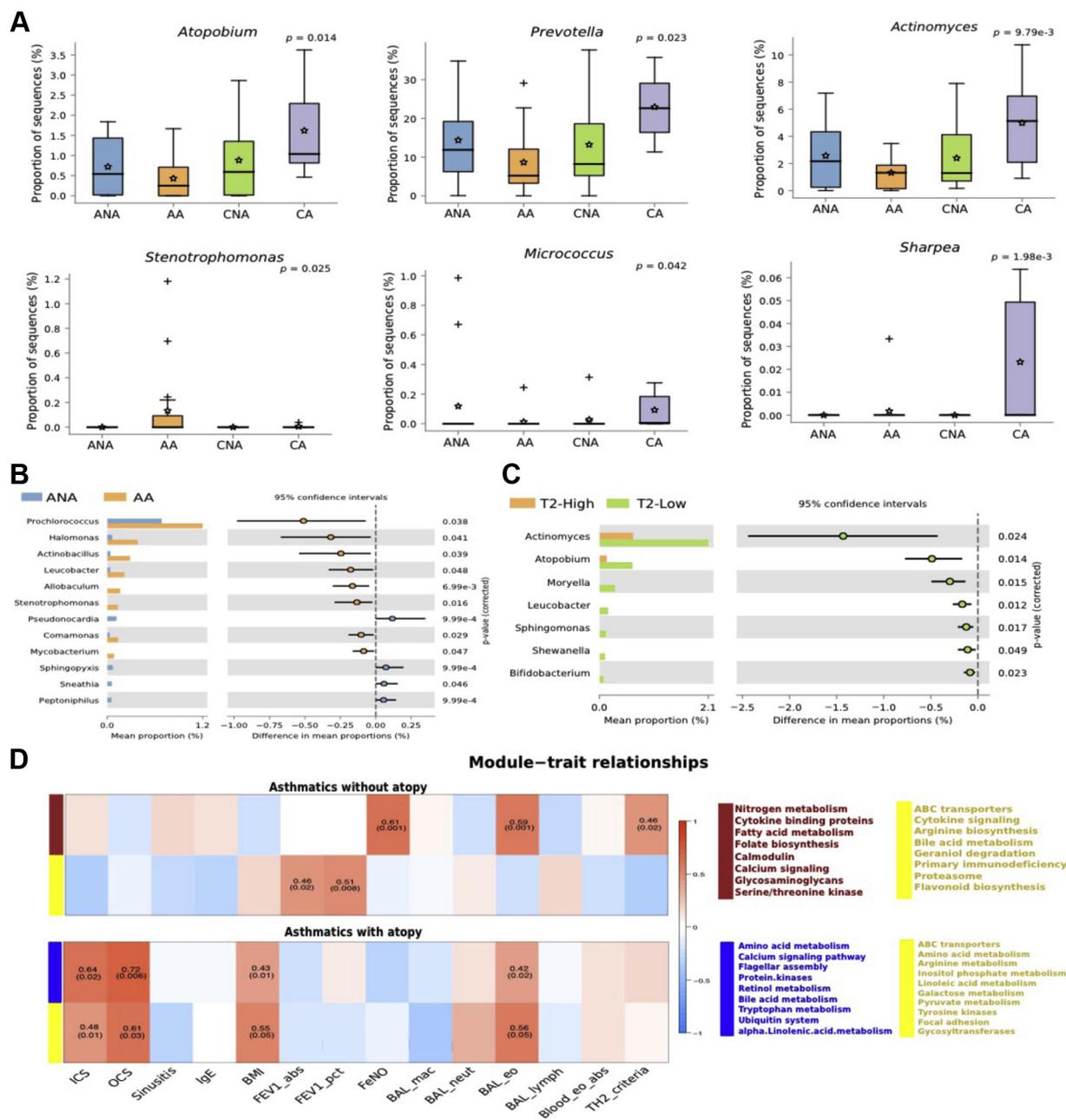


FIG 5. Bacterial community and functional associations with asthma-associated atopy and T2 inflammation. **A**, Multigroup nonparametric ANCOM test showing results for statistically significant (BH-FDR-corrected $P < .05$) genera differentiating 4 groups: AAs, ANAs, CAs, and CNAs. **B** and **C**, Two-group nonparametric test with BH-FDR correction displaying statistically significant genera between ANAs and AAs (Fig 5, **B**) and patients with T2-high and T2-low asthma (Fig 5, **C**). **D**, WGCNA to assess significant associations between functional modules and specific clinical traits. Each row corresponds to a module eigengene, and each column corresponds to a trait. Each cell contains the corresponding correlation and P value (BH-FDR corrected). The table is color coded by correlation according to the color legend.

groups). *Stenotrophomonas* species were significantly ($P_{FDR} < .05$) enriched in the AA group, whereas *Atopobium*, *Prevotella*, and *Actinomyces* species were enriched ($P_{FDR} < .05$) in the ANA group and in healthy control subjects (ie, both CNAs and CAs as separate groups; Fig 5, **A**). *Sharpea* and *Micrococcus* species were significantly ($P_{FDR} < .05$) enriched only in the CA group (Fig 5, **A**). However, using a 2-group test (Mann-Whitney) within asthmatic patients (ie, ANAs vs AAs), additional genera

had significantly ($P_{FDR} < .05$) greater abundance, such as *Actinobacillus*, *Halomonas*, and *Leucobacter* species ($P_{FDR} < .05$), in the AA compared with the ANA groups (Fig 5, **B**). Additionally, 2-group analyses between the T2-high and T2-low groups revealed genera, such as *Actinomyces*, *Atopobium*, and *Moryella* species, to be relatively more enriched ($P_{FDR} < .05$) in the T2-low group (Fig 5, **C**). Additionally, comparing asthmatic patients with and without ICS use, we identified ESVs belonging to the

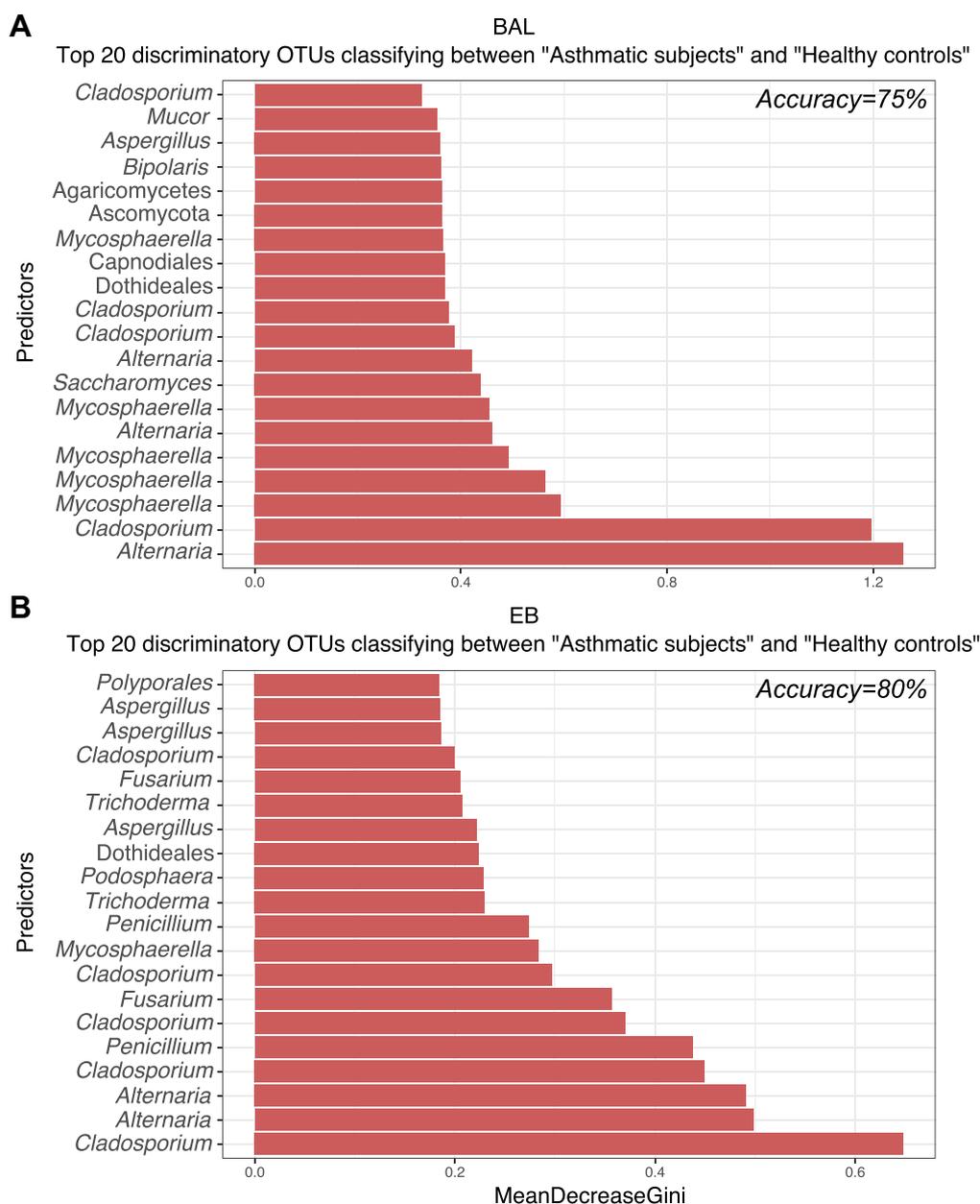


FIG 6. The 20 most predictive fungal ESVs for classification of samples into asthmatic patients versus healthy control subjects. Data are shown for both BAL fluid (**A**) and EB (**B**) samples. The *x-axis* is the mean decrease in Gini score (a variable importance score that measures the importance of the predictor ESVs). Gini is defined as inequity or measure of "node impurity" in the random forest decision tree. A low Gini (ie, greater mean decrease in Gini score) demonstrates greater significance or importance of that ESV in classification of the data set and that the removal of that particular ESV will lead to decreased accuracy of the model and suggests that a particular predictor ESV has a greater role in partitioning the data into the defined classes.

Acinetobacter genes, the Rickettsiales order, and the Rhodobacteraceae family enriched ($P_{FDR} < .05$) in those using ICSs (see Fig E2).

We further tested the significance of association between the key bacterial signature genera and clinical parameters using GLMs (see Fig E5 in this article's Online Repository at www.jacionline.org). We identified genera, such as *Prevotella*, *Actinomyces*, *Sharpea*, *Atopobium*, *Stenotrophomonas*, *Micrococcus*,

and *Leucobacter* species, to be associated ($P_{FDR} < .05$) with atopy (see Fig E4). The composition and proportion of bacterial and fungal taxa in each sample significantly ($P_{FDR} < .05$) differentiated the T2-high and T2-low groups. Statistically significant correlations ($P_{FDR} < .05$) were seen with ICS use, OCS use, blood eosinophil counts, FEV₁, and FENO values (see Fig E6 in this article's Online Repository at www.jacionline.org). These data extend our previous observations⁹ to show that there are

significant differences in bacterial community composition in the lower airways and further confirmed the observations of Durack et al⁸ concerning differences in bacterial burden and community structure based on atopy and T2-associated inflammation.

We examined the relationship between predicted bacterial function and clinical parameters of airway inflammation, such as corticosteroid use, FEV₁, and FENO value. To do this, we performed additional WGCNAs based on functional annotations of 16S data, as predicted by using PICRUSt.⁴⁵ This analysis revealed significant correlation between functional modules and clinical traits (Fig 5, D). Two significant modules (Spearman $R^2 > 0.5$ and BH-FDR-corrected $P_{FDR} < .05$) were found each in the ANA and AA groups. However, no significant associations were seen for the bacteria-associated functions for the T2-high/low groups. Across the ANA group, the red module comprising bacterial functions, such as nitrogen metabolism, cytokine-binding proteins, fatty acid metabolism, calmodulin-like factors, and folate biosynthesis, correlated significantly ($P_{FDR} < .05$) with FENO values, BAL fluid eosinophil counts, and T2 status (Fig 5, D). Bacterial calmodulin-like factors are known to play a significant role in calcium signaling in the lungs, which has been implicated in regulating airway inflammatory responses.⁴⁶⁻⁴⁸ Likewise, bacterial cytokine-binding proteins are also known to initiate and amplify inflammatory responses in the lungs.⁴⁹ The yellow module comprised of ATP-binding cassette (ABC) transporters, mitogen-activated protein kinase signaling, arginine biosynthesis, flavonoid biosynthesis, and bile-acid metabolism correlated significantly ($P_{FDR} < .05$) with FEV₁ and has been associated with asthma in several reports (Fig 5, D).⁵⁰⁻⁵⁴ Likewise, across the AA group, the yellow and blue modules correlated with clinical observations. The yellow module, including ABC transporters, amino acid metabolism, inositol phosphate metabolism, tyrosine kinases, and focal adhesion, significantly ($P_{FDR} < .05$) associated with OCS use, BMI, and BAL fluid eosinophil counts (Fig 5, D). The blue module demonstrated association with ICS and OCS use ($P_{FDR} < .05$) and included pathways for mitogen-activated protein kinase signaling, flagellar assembly, tryptophan metabolism, and retinoid metabolism (Fig 5, D).⁵⁵⁻⁵⁸ Therefore each module contained bacteria-predicted functions that were associated with asthma and airway inflammation.

Finally, to supplement the microbial signatures (both fungal and bacterial) associated with asthmatic patients, we built random forest models against a 10-fold cross-validation. These models identified discriminatory ESVs that predicted whether the subjects had asthma. By using the random forest classifier trained with fungal ESVs, the diagnostic accuracy increased up to 75% (Out-Of-Bag [OOB] error = 0.25) in BAL fluid samples and 80% (OOB error = 0.21) in EB samples (Fig 6). These models based on machine learning revealed a consistent group of ESVs that were important for discrimination in both BAL fluid and EB samples. These discriminatory ESVs were *Alternaria*, *Cladosporium*, *Mycosphaerella*, and *Aspergillus* species (Fig 6). Thus these taxa could be biomarkers for different clinical phenotypes associated with asthma. The random forest bacterial classifier predicted the 2 states (ie, asthma and no asthma) with an accuracy of 72% (OOB error = 0.28) based on the discriminatory ESVs from *Prevotella*, *Actinomyces*, *Streptococcus*, and *Atopobium* species in the EB samples. Using BAL fluid samples, we observed an accuracy of 70% (OOB

error = 0.32) with discriminatory ESVs from *Lactobacillus*, *Pseudomonas*, and *Rickettsia* species.

DISCUSSION

Our findings demonstrate fungal and bacterial compositional differences between different asthma-associated phenotypes based on atopy and T2-mediated inflammation from a total cohort of 39 asthmatic patients and 19 healthy control subjects.⁹ As with the bacterial analysis, we identified a significantly lower fungal diversity in patients with T2-high inflammation when compared with patients with T2-low inflammation in EB samples. Previously, it was suggested that lower bacterial diversity in patients with T2-high inflammation might correlate with greater fungal diversity in this subtype.⁸ However, we provide the first evidence that both fungal and bacterial diversity remain lower in the central airways of patients with T2-high inflammation. It was also interesting to note there were no significant differences observed in the fungal microbiota between AAs and ANAs based on both α - and β -diversity for both BAL fluid and EB samples.

We identified 7 key fungal genera, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Mycosphaerella*, that were significantly associated with asthma and its associated T2 inflammation and atopy. A number of these genera have previously been associated with asthma and atopy. For example, sensitivity to *Alternaria* species, an environmental fungus commonly found indoors, has been suggested as a risk factor for the development and persistence of asthma.⁵⁹ *Alternaria* species is known to possess type 2 inflammatory adjuvant effects *in vivo* and can induce airway sensitization to allergens.⁶⁰ Another genus, *Aspergillus* species, is known for its different allergic presentations, particularly in atopic subjects, including asthma and allergic bronchopulmonary aspergillosis, and is known to be a T2 inflammation adjuvant.^{61,62} A recent meta-analysis suggests that *Cladosporium* species, along with *Penicillium* and *Aspergillus* species, might also increase the risk of asthma.⁶³ *Trichoderma* and *Fusarium* species sensitization has also been demonstrated in patients with asthma, atopy, and allergic rhinitis.^{64,65} The presence of these fungal genera in the airway microbiota in select asthma phenotypes, coupled with these associations, suggests either a colonizing or a potential pathogenic role. Their presence in select phenotypes offers the possibility that they might be useful, either alone or as part of a cluster of other biological or clinical markers, to delineate clinical asthma endotypes.

We identified potential associations between key fungal genera and clinical variables of interest by using GLM in both BAL fluid and EB samples. Interestingly, different fungal ESVs demonstrated different directions of association with the same clinical variable. For instance, in BAL fluid samples 2 groups were evident: one with positive associations, including *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Mycosphaerella* species, and the second with negative associations, which included *Alternaria* and *Cladosporium* species. *Cladosporium* species was also seen to be positively associated with ICSs, whereas *Mycosphaerella* species showed a negative association with ICSs. This observation again supports our finding that *Mycosphaerella* species were associated with mild asthma or a healthy phenotype. In EB samples we identified potential relationships between fungal genera, such as *Alternaria*, *Aspergillus*, and *Fusarium* species, with the presence of macrophages and neutrophils in BAL fluid. Using co-occurrence networks, we were able to identify small coregulated subnetworks

in asthmatic patients for both BAL fluid and EB samples, with fungal and bacterial ESVs interacting with each other. There was a significant overlap of keystone asthma-associated fungal taxa between both BAL fluid and EB samples; however, the interacting bacterial ESVs for each of these subnetworks were quite different. Our data highlight that there is a consistent fungal signature in both the peripheral and central airways that might be useful in identifying clinical asthma endotypes.

Recent studies have shown compositional differences in bacterial communities in asthmatic and normal airways, but our understanding of microbial differences between the T2-high and T2-low phenotypes remains incomplete.^{3,6,8,9,66,67} Therefore in a re-analysis of our previous study,⁹ we clustered 16S rRNA data at ESVs and used ANCOM to delineate taxa that differentiate between T2-low and T2-high phenotypes. The T2-low group was significantly enriched in *Leucobacter*, *Moryella*, *Actinomyces*, and *Atopobium* species compared with T2-high samples or healthy control subjects. A similar analysis differentiated between atopic and nonatopic asthmatic patients demonstrated that *Allobaculum*, *Leucobacter*, *Stenotrophomonas*, and *Pseudonocardia* species were significantly enriched in the AA group compared with the ANA group. Genera, such as *Prevotella*, *Actinomyces*, and *Sharpea*, were previously shown by Durack et al⁸ to be associated with atopy; in the present study we identified additional genera, such as *Atopobium*, *Stenotrophomonas*, *Micrococcus*, *Leucobacter*, *Peptoniphilus*, and *Pseudonocardia*, that were also associated with atopy. These differential bacterial enrichment patterns between the 2 studies can be attributed to the different demographics of the cohorts: our cohort was recruited at single site (University of Chicago), whereas the Durack et al⁸ study was conducted at 9 different sites in the National Heart, Lung, and Blood Institute AsthmaNet network.

This study refines the list of bacterial groups previously associated with asthma to a more precise set of bacterial candidates associated with symptoms, such as atopy and T2 inflammation.^{8,9} Our findings further highlight the significant relationship between the central airway microbiota and the state of asthma and underlying atopy and again suggest the possibility of more targeted therapies that can influence clinical control of asthma by modulating select bacterial genera.

We also investigated correlations between bacteria-associated functions and clinical features. Durack et al⁸ demonstrated a significant enrichment of amino acid and carbohydrate metabolism in AAs. In our data set using predicted metabolic function (PIC-RUS_t) and an analytic workflow that included WGCNA, we were able to predict significant relationships between bacteria-encoded functions and corticosteroid therapy (ICSs and OCSs), BMI, and BAL eosinophil counts among the AAs. We identified significant associations between pathways, including calcium signaling, amino acid metabolism (eg, tryptophan and arginine metabolism), pyruvate metabolism, and corticosteroid therapy. These bacteria-associated pathways are known to be involved in cell migration, regulation of epithelial and endothelial barriers, and inflammatory pathways.⁶⁸ Interestingly, although we were not able to identify any significant association between BMI and taxa enriched in our data, we observed significant positive relationships between functional pathways (eg, linoleic acid metabolism and galactose metabolism) and BMI. Many cross-sectional studies have shown a relationship between increasing BMI and risk of asthma,⁶⁹ emphasizing obesity-altered functional pathways, such as fatty acid and lipid metabolism.⁷⁰

Similarly, we identified significant associations of pathways, such as bile-acid metabolism, ABC transporters, protein kinases, and the ubiquitin proteasome system, with both ICSs and OCSs. Bile-acid reflux into the airways can exacerbate asthma and chronic cough.⁷¹ The ABC transporters have a significant role in transportation of various substrates in the lungs, and their disruption can increase lipid accumulation and inflammatory cytokine levels in lung tissue.⁵⁴ Bacterial protein kinases and their receptors are known to modulate immune response by regulating proinflammatory cytokines, such as IL-6, interferons, and TNF- α .⁷² These results are in agreement with previous studies that demonstrate a relationship between these pathways with atopy.^{50,51,53} The ubiquitin proteasome system is also known to be involved in pathobiology of multiple pulmonary diseases, including asthma.⁷³ Hence association of this set of pathways with corticosteroid therapy suggests that perturbations of these bacterial metabolic pathways by concomitant corticosteroid therapy could be associated with greater manifestations of atopy. This is counterintuitive to the current use of this therapy in asthma and atopy and will require exploration in future studies.

Our study, the largest single-center asthma cohort investigation of the microbiota, still remains modest in sample size compared with microbiome-wide association studies in other disease models, such as ulcerative colitis and atopic dermatitis. A major limiting factor in the size of asthma microbiota studies has been the need for sampling of the lower airways; future studies that rely on induced or expectorated sputum as a surrogate marker for the lower airway microbiota have promise.⁷⁴ One major focus of this study has been understanding the predictive fungal microbiota in the T2-high, T2-low, and atopy-associated phenotypes, and grouping our cohort in this manner reduced the sample size, which in turn made it difficult to run successful predictive random forest models caused by an insufficient validation data set for testing credibility of the models. We were also limited in generating statistically significant co-occurrence networks for the asthma subtypes caused by the small sample size.

A further limitation is the variations in medication use in patients with asthma, a natural consequence of recruiting a heterogeneous population of differing phenotypes and disease severity. Answering whether medication use affects the microbiome will require either very large studies to have sufficient power in subgroup analyses or, alternatively, mechanistic studies in which asthmatic patients are treated longitudinally with specific medications with a proper wash-in and wash-out phase. Both types of studies will be challenging. A third option is the use of gnotobiotic mouse models informed by the findings to date in human asthma.

In conclusion, our study demonstrates (1) a potential association between the fungal and bacterial microbiota and asthma-associated phenotypes and endotypes, (2) significant coregulation between bacterial and fungal genera in co-occurrence networks, and (3) correlation between select predicted bacterial functional pathways and clinical parameters. Our findings provide potential new biomarkers that might be useful, particularly when combined with other clinical and biological markers, to distinguish asthma endotypes.

ITS fungal data and 16S data are available in EMBL-ENA under project accession numbers PRJEB26930 and PRJEB20527, respectively.

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Key messages

- Bacterial and fungal microbiota differ between asthmatic and normal airways.
- Patients with T2-high asthma have lower diversity of bacterial and fungal microbiota.
- Coassociations between key fungal and bacterial genera differ with asthma and atopy status.

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METHODS

Sample collection

Bronchoscopy was done by using standard methods and conscious sedation. All subjects tolerated bronchoscopy without complications. After introduction of the bronchoscope into the airways, 4 EB samples were collected from the proximal portion of the right lower lobe bronchus by using an epithelial cell cytology brush (Medical Engineering Laboratory, Shelby, NC). Brushes were initially stored in 1 mL of QIAzol reagent (Qiagen, Hilden, Germany) and vortexed for 3 minutes. Samples then were stored at -80°C until processing for DNA extraction. At that time, samples were transferred to 2-mL bead tubes containing sterile 0.1-mm glass beads (MoBio Laboratories, Carlsbad, Calif).

Samples were homogenized for 3 minutes in a BioSpec Mini-Beadbeater-16 cell disrupter (BioSpec Products, Bartlesville, Okla) and then allowed to incubate at room temperature for 5 minutes. Brush samples were centrifuged at 10,000g for 1 minute and then agitated with chloroform for 15 seconds, held at room temperature for 3 minutes, and then centrifuged at 12,000g for 15 minutes. Samples then were stored at 28°C until processed for DNA extraction.

After this, BAL was performed by means of instillation of 120 mL of saline into the anterior segment of the right middle lobe, followed by suction recovery. All subjects tolerated bronchoscopy without complications. Five negative specimens for microbiome analysis were generated from cytology brushes that were put through a bronchoscope that was precleared with sterile saline but were not introduced into a patient. As an additional control to identify reagent-derived contaminants, 7 wells with DNA extracted from reagents were used for processing of specimens on each day that batch processing was done. BAL fluid samples were handled at 4°C throughout initial processing. Samples were centrifuged at 1000g for 10 minutes, and then cytospin preparations of cells were made for differential counts. Supernatants were filtered through a 1.2-mm filter, pellets were collected after centrifugation at 10,000g for 10 minutes, and samples were frozen at 28°C until processing for DNA extraction. Epithelial cytology brushes (Medical Engineering Laboratory, Shelbyville, NC) used in bronchoscopy were initially stored in 1 mL of QIAzol reagent (Qiagen) and vortexed for 3 minutes.

After removal of residual aqueous phase, ethanol was added; samples were incubated at room temperature for 2 minutes and then centrifuged at 8,000g for 2 minutes to sediment DNA. The phenol/ethanol supernatant was discarded, after which DNA was washed in sodium citrate 3 times. The DNA pellet was washed in 75% ethanol for 20 minutes and centrifuged at 8,000g for 5 minutes, after which the pellet was air-dried and resuspended in 8 mmol/L NaOH. After resuspension, DNA was centrifuged at 14,000g for 10 minutes to remove insoluble material, and the DNA was placed in TE buffer solution. Final DNA purification with the Qiagen DNeasy Blood & Tissue Kit was done according to the kit's instructions. RNase activity was eliminated in this step. Isolated DNA then was dissolved in Qiagen AE buffer and stored at -80°C until analysis. DNA concentration and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher, Waltham, Mass).

Microbiome library preparation

Each 25- μL PCR reaction contained the following mixture: 12 μL of MoBio PCR Water (Certified DNA-Free), 10 μL of 5-Prime HotMasterMix (1 \times), 1 μL of forward primer (5 $\mu\text{mol/L}$ concentration and 200 pmol/L final), 1 μL of Golay Barcode Tagged Reverse Primer (5 $\mu\text{mol/L}$ concentration, 200 pmol/L final), and 1 μL of template DNA.^{E1} Conditions for PCR were as follows: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94°C for 45 seconds, 50°C for 60 seconds, and 72°C for 90 seconds and a final extension of 10 minutes at 72°C to ensure complete amplification.^{E1} Amplicons were quantified with PicoGreen (Invitrogen, Carlsbad, Calif) assays and a plate reader, followed by cleanup with the UltraClean PCR Clean-Up Kit (MoBio) and then quantification with Qubit readings (Invitrogen, Grand Island, NY).

For the ITS analysis, multiplexed primers designed to target the ITS1 region were used, as described in Fujita et al.^{E2} The PCR protocol for ITS is identical to the 16S rRNA gene protocol. Both the 16S and ITS samples were sequenced on an Illumina MiSeq platform at Argonne National Laboratory core sequencing facility according to Earth Microbiome Project standard protocols (<http://www.earthmicrobiome.org/emp-standard-protocols/its/>).^{E1}

Random forest supervised learning

Supervised learning was performed by using 2 different methodologies: (1) cross-validation sample sets in the caret package^{E3} and (2) OOB sample sets in the RandomForest package in R software. The training set (70% of the total samples) as per the sample type was used for learning models. Based on each condition (AAs, ANAs, CAs, and CNAs), the cross-validation set (30%) was created from the original data set available. Training was accomplished in RandomForest with bootstrapping at 1000 trees, and prediction accuracy (1-OOB) was estimated. We also predicted and annotated the top 10 important bacterial and fungal ESVs for each of the subset using RandomForest in R software.^{E4}

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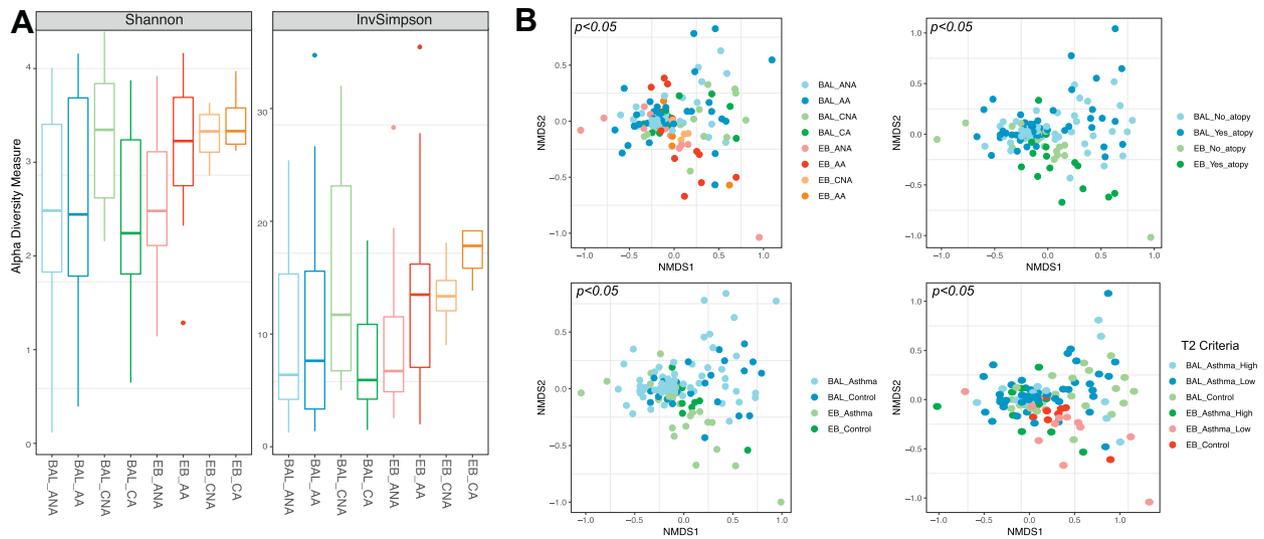


FIG E1. Fungal diversity comparison between various asthma-associated phenotypes. **A**, Box plots of Shannon and inverse Simpson diversity indices distinguishing AAs, ANAs, CAs, and CNAs for both EB and BAL fluid samples. **B**, NMDS plots based on the Bray-Curtis index between the above-mentioned groups. Statistical analysis was performed with PERMANOVA.

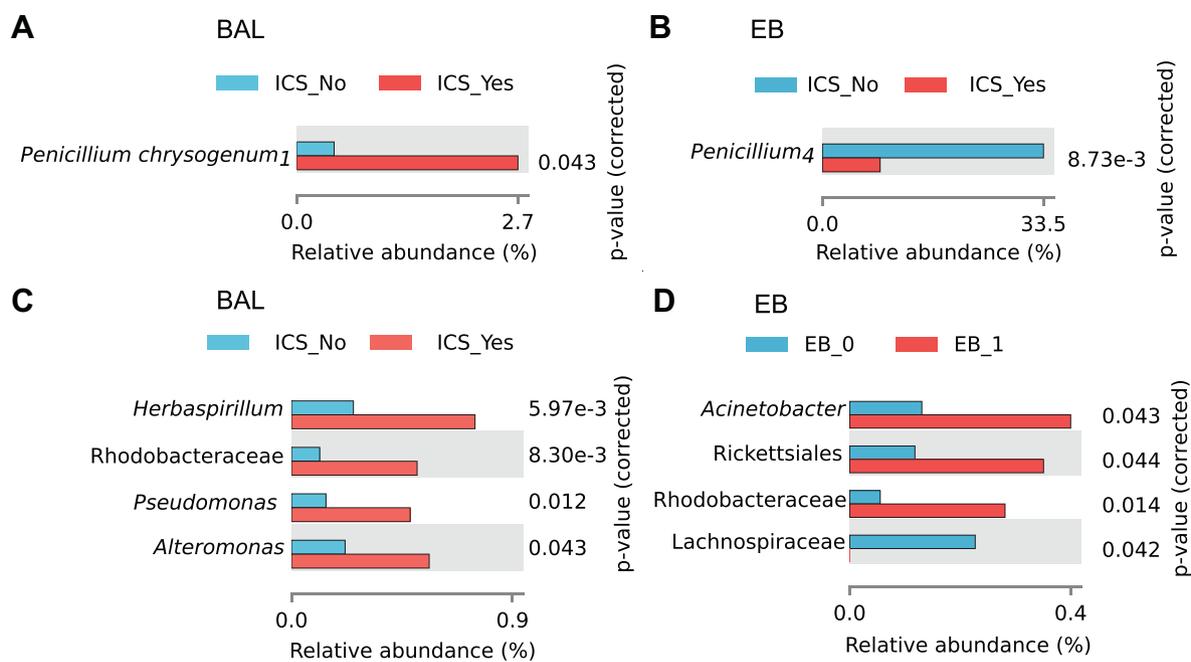


FIG E2. **A** and **B**, Differentially abundant fungal ESVs between BAL fluid (Fig E2, **A**) samples and EB samples (Fig E2, **B**) from asthmatic patients with and without ICS use. **C** and **D**, Differentially abundant bacterial ESVs between BAL fluid samples (Fig E2, **C**) and EB samples (Fig E2, **B**) from asthmatic patients with and without ICS use. For genera with more than 1 differentially abundant ESV, the ESV number is shown as a subscript.

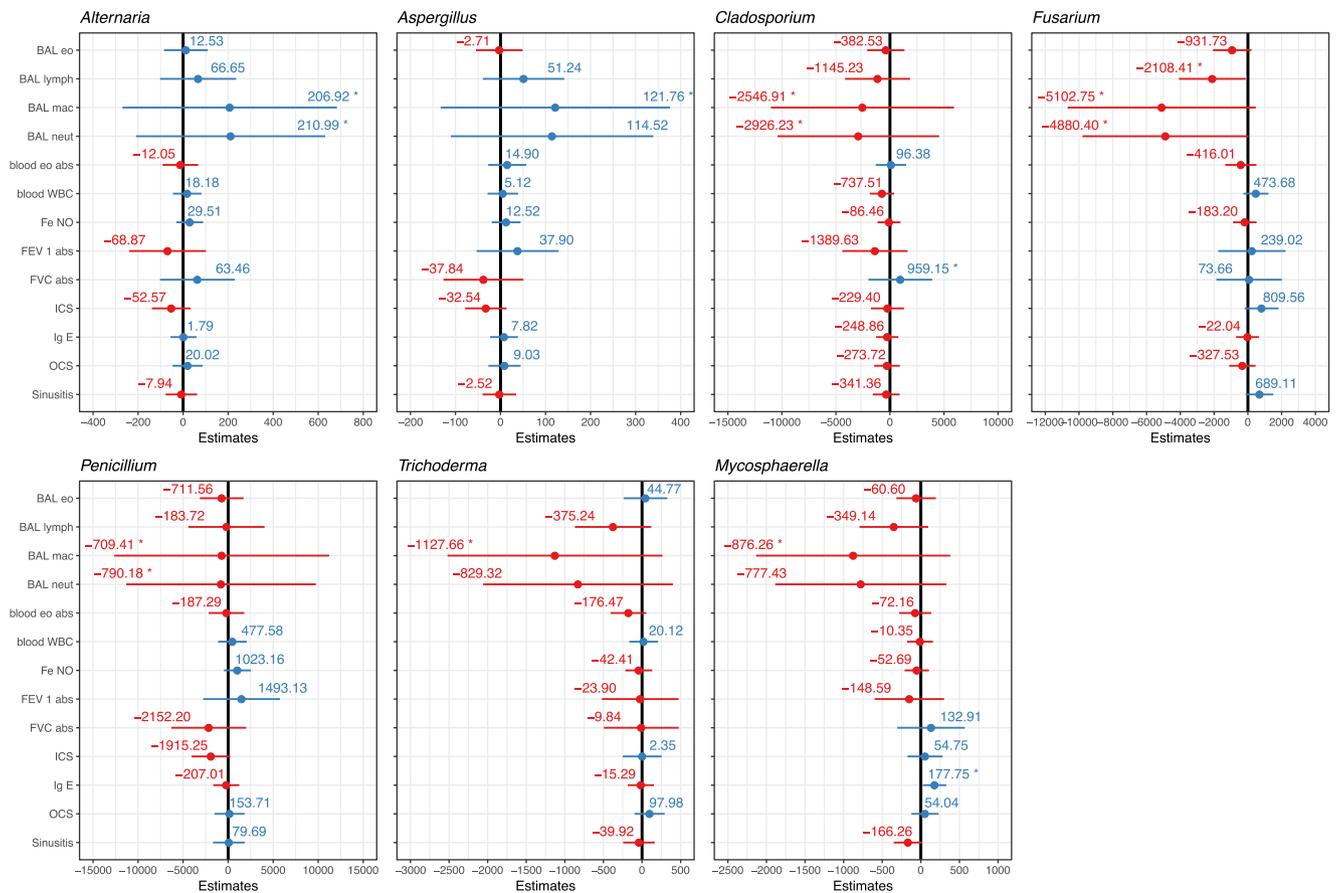


FIG E3. Associations between key fungal genera and clinical variables in EB samples examined by using GLMs. Significant correlations (positive or negative) with BH-FDR-corrected P values are shown as follows: * $P \leq .05$.

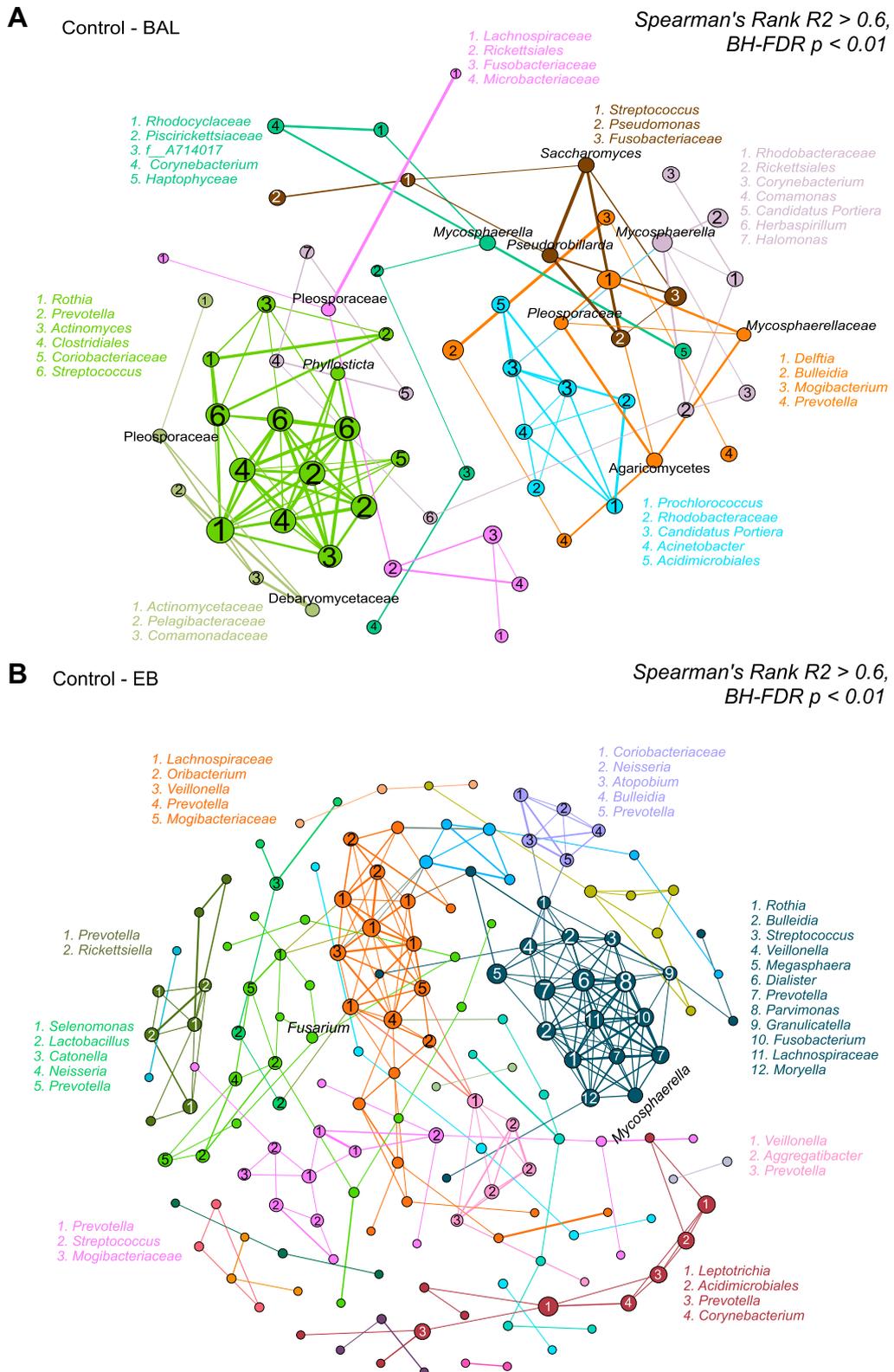


FIG E4. Significant co-occurrence relationships between different modules in healthy control subjects for BAL fluid (A) and EB (B) samples. All networks represent statistically significant correlations after a Spearman correlation cutoff of 0.6 and a BH-FDR-corrected P value cutoff of .01. A connection stands for a strong (Spearman $\rho > 0.6$) and significant ($P < .01$) correlation. Fungal nodes are labeled in black inside the network, and bacterial nodes are labeled outside the network in different colors depending on the module to which they belong. Nodes are colored by modules or communities (group of taxa) at a resolution of 0.9 using the "Modularity" function based on Louvain community detection algorithm implemented in Gephi software. The size of node is controlled by the degree (number of connections). The edge width is proportional to the weight of correlation. The level of modularity ranges between 8 and 16 for all networks.

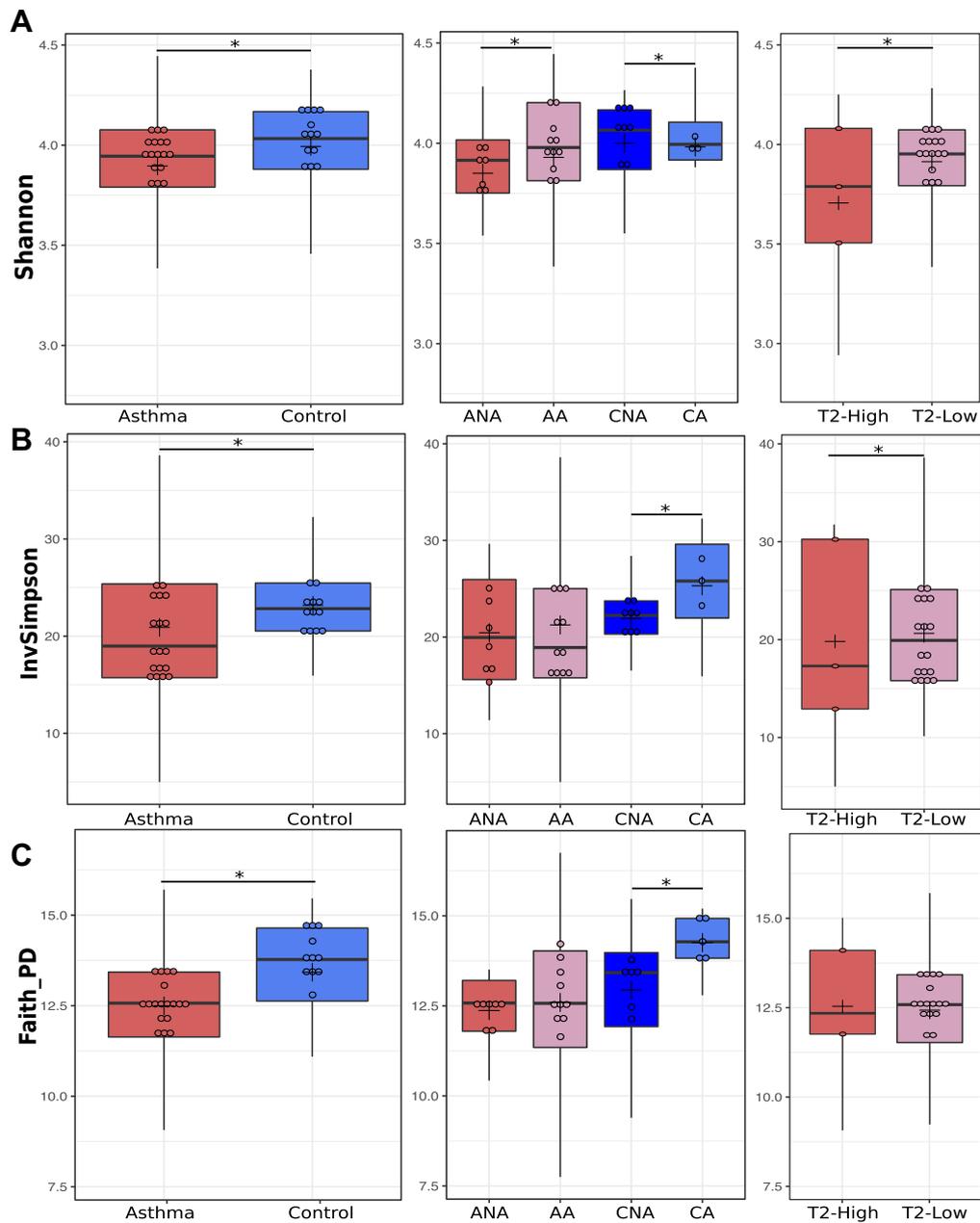
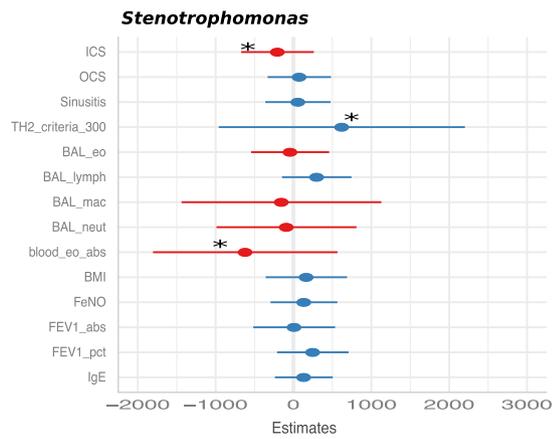
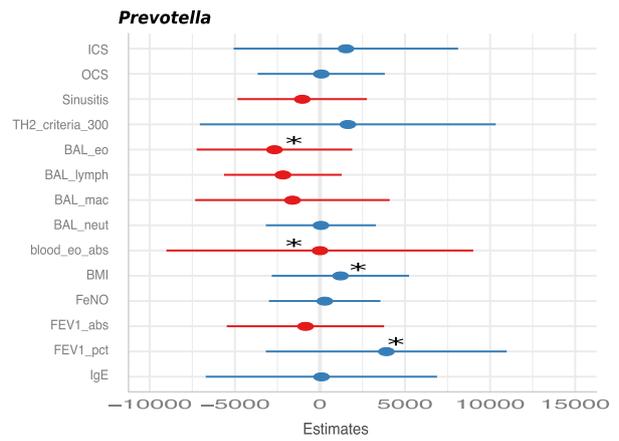


FIG E5. Bacterial diversity comparisons between select asthma-associated phenotypes. Distribution of Shannon (A), inverse Simpson (B), and Faith phylogenetic (C) α -diversity indices between the groups of ANAs, AAs, CAs, and CNAs and asthmatic patients grouped as having T2-high or T2-low asthma. Asterisks indicate comparisons with significant P values: $*P < .05$.

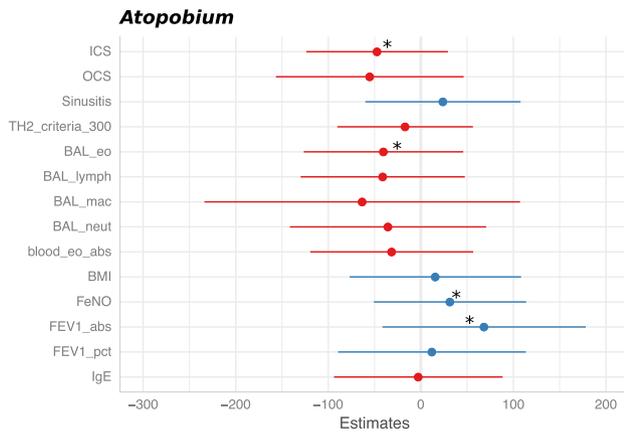
A Asthmatics with atopy (AA)



B Asthmatics without atopy (ANA)



C Controls with atopy (CA)



D TH2 low asthmatics

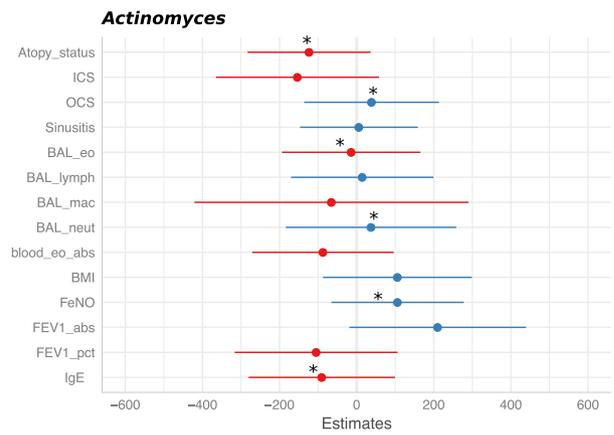


FIG E6. Generalized linear models generated between specific genera and clinical parameters among the groups shown (A-D). The *asterisks* represent the significant correlations (positive or negative) with BH-FDR corrected $*P < .05$.