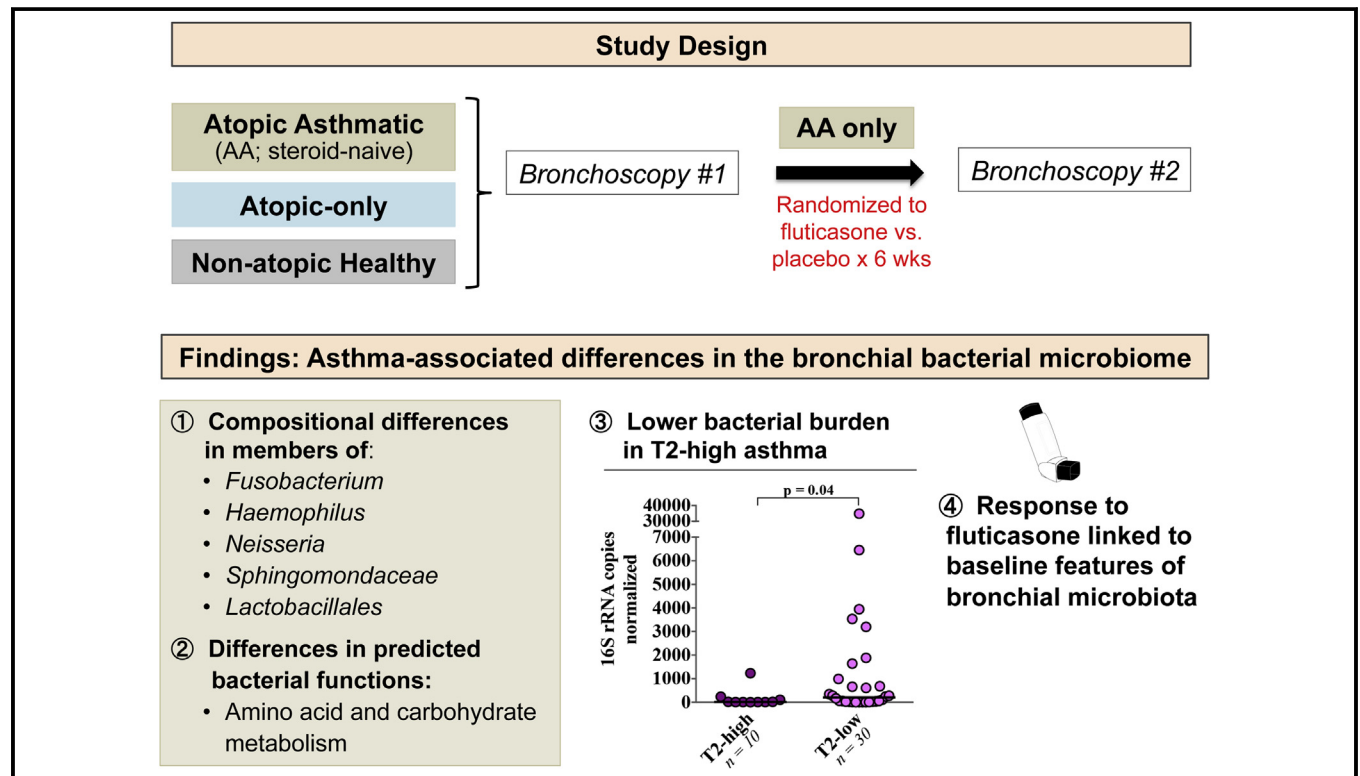


Features of the bronchial bacterial microbiome associated with atopy, asthma, and responsiveness to inhaled corticosteroid treatment

Juliana Durack, PhD,^a Susan V. Lynch, PhD,^a Snehal Nariya, BS,^b Nirav R. Bhakta, MD, PhD,^c Avraham Beigelman, MD,^d Mario Castro, MD,^{d,e} Anne-Marie Dyer, MS,^f Elliot Israel, MD,^g Monica Kraft, MD,^h Richard J. Martin, MD,ⁱ David T. Mauger, PhD,^f Sharon R. Rosenberg, MD, MS,^j Tonya Sharp-King, PhD,^f Steven R. White, MD,^k Prescott G. Woodruff, MD, MPH,^c Pedro C. Avila, MD, MPH,^j Loren C. Denlinger, MD, PhD,^l Fernando Holguin, MD,^m Stephen C. Lazarus, MD,^c Njira Lugogo, MD,ⁿ Wendy C. Moore, MD,^o Stephen P. Peters, MD,^o Loretta Que, MD,ⁿ Lewis J. Smith, MD,^j Christine A. Sorkness, PharmD,^l Michael E. Wechsler, MD, MMSc,ⁱ Sally E. Wenzel, MD,^m Homer A. Boushey, MD,^c and Yvonne J. Huang, MD,^b for the National Heart, Lung and Blood Institute's "AsthmaNet"
San Francisco, Calif, Ann Arbor, Mich, St Louis, Mo, Hershey, Pa, Boston, Mass, Tucson, Ariz, Denver, Colo, Chicago, Ill, Madison, Wis, Pittsburgh, Pa, and Durham and Winston-Salem, NC

GRAPHICAL ABSTRACT



From ^athe Department of Medicine, Division of Gastroenterology, University of California, San Francisco; ^bthe Division of Pulmonary & Critical Care Medicine, University of Michigan, Ann Arbor; ^cthe Department of Medicine, University of California, San Francisco; the Divisions of ^dPediatrics and ^ePulmonary and Critical Care Medicine, Washington University School of Medicine, St Louis; ^fthe Department of Public Health Sciences, Penn State University, Hershey; ^gthe Department of Medicine, Brigham & Women's Hospital, Boston; ^hHealth Sciences, University of Arizona, Tucson;

ⁱthe Department of Medicine, National Jewish Hospital, Denver; ^jthe Department of Medicine, Northwestern University, Chicago; ^kthe Department of Medicine, University of Chicago; ^lthe Department of Medicine, University of Wisconsin-Madison; ^mthe University of Pittsburgh Asthma Institute at UPMC/UPSOM, Pittsburgh; ⁿthe Duke Asthma, Allergy & Airway Center, Duke University School of Medicine, Durham; and ^oWake Forest School of Medicine, Winston-Salem.

Background: Compositional differences in the bronchial bacterial microbiota have been associated with asthma, but it remains unclear whether the findings are attributable to asthma, to aeroallergen sensitization, or to inhaled corticosteroid treatment.

Objectives: We sought to compare the bronchial bacterial microbiota in adults with steroid-naïve atopic asthma, subjects with atopy but no asthma, and nonatopic healthy control subjects and to determine relationships of the bronchial microbiota to phenotypic features of asthma.

Methods: Bacterial communities in protected bronchial brushings from 42 atopic asthmatic subjects, 21 subjects with atopy but no asthma, and 21 healthy control subjects were profiled by using 16S rRNA gene sequencing. Bacterial composition and community-level functions inferred from sequence profiles were analyzed for between-group differences. Associations with clinical and inflammatory variables were examined, including markers of type 2–related inflammation and change in airway hyperresponsiveness after 6 weeks of fluticasone treatment.

Results: The bronchial microbiome differed significantly among the 3 groups. Asthmatic subjects were uniquely enriched in members of the *Haemophilus*, *Neisseria*, *Fusobacterium*, and *Porphyromonas* species and the Sphingomonadaceae family and depleted in members of the Mogibacteriaceae family and Lactobacillales order. Asthma-associated differences in predicted bacterial functions included involvement of amino acid and short-chain fatty acid metabolism pathways. Subjects with type 2–high asthma harbored significantly lower bronchial bacterial burden. Distinct changes in specific microbiota members were seen after fluticasone treatment. Steroid responsiveness was linked to

differences in baseline compositional and functional features of the bacterial microbiome.

Conclusion: Even in subjects with mild steroid-naïve asthma, differences in the bronchial microbiome are associated with immunologic and clinical features of the disease. The specific differences identified suggest possible microbiome targets for future approaches to asthma treatment or prevention. (J Allergy Clin Immunol 2016;■■■:■■■–■■■.)

Key words: Asthma, atopy, microbiome, corticosteroids, 16S ribosomal RNA, bacteria, T_H2 inflammation, three-gene mean, metabolic pathways, short-chain fatty acids

Recent culture-independent studies have documented that the composition of commensal lower respiratory tract bacteria (microbiota) differs between asthmatic and healthy adults.^{1–6} Additionally, phenotypic features of asthma, such as measures of airway hyperresponsiveness, asthma control, and transcriptional response to steroids, correlate with patterns of bronchial microbiota composition.^{4,5} Although different studies have reported asthma-associated enrichment (higher relative abundance) of different taxa (ie, bacteria-derived 16S ribosomal RNA gene sequences that exhibit an operator-defined level of sequence homology [typically 97%]), enrichment in members of the phylum Proteobacteria is a repeating signature. Asthmatic subjects in most previous studies were treated with inhaled corticosteroids (ICSs), casting some uncertainty on whether the findings reflect the effects of ICS treatment or of asthma itself. Similarly, many asthmatic patients are atopic,^{7,8} raising the question of whether asthma-associated differences in respiratory microbiota are

Disclosure of potential conflict of interest: S. V. Lynch has received grants from the National Institutes of Health (NIH)/National Institute of Allergy and Infectious Disease (NIAID; AI113916, AI089473-01, and AI097172), Janssen, the Broad Foundation, the Sloan Foundation, Pfizer, the NIH/National Institute of Child Health and Human Development (HD082147-01), the NIH/National Heart, Lung, and Blood Institute (NHLBI; HL098107 and HL098964), and the NIH/National Institute of Diabetes and Digestive and Kidney Diseases (DK104664); has a patent Stan449-PRV REDUCTIVE PRODRUG CANCER CHEMOTHERA issued, a patent Combination antibiotic and antibody therapy for the treatment of *Pseudomonas aeruginosa* infection with royalties paid, a patent provisional filing for use of *Lactobacillus sakei* and other lactic acid bacteria as a therapeutic strategy for chronic rhinosinusitis issued, a patent provisional filing for claims associated with use of PhyloChip as a diagnostic and prognostic clinical tool issued, and a patent Bacterial Therapeutic Consortium for induction of Immune Tolerance pending. S. Nariya has received a grant from the NHLBI and the National Institute of Allergy and Infectious Disease (NIAID). N. R. Bhakta has received a grant from the NIH/NHLBI and has received personal fees from Roche/Genentech. M. Castro has received personal fees from Boston Scientific, Holaira, Genentech, Teva, GlaxoSmithKline, Boehringer Ingelheim, Elsevier, and Neostem; has received grants from Amgen, Teva, Novartis, GlaxoSmithKline, Sanofi-Aventis, Vectura, Boehringer Ingelheim, MedImmune, Invion, and Pfizer; and owns stock in Sparo. A.-M. Dyer has received a grant from the NHLBI. E. Israel has received personal fees from AstraZeneca, Novartis, Philips Respironics, Regeneron Pharmaceuticals, Campbell, Campbell, Edwards & Conroy, Crammer, Bishop & O'Brien, Fox Rothschild, Ryan Deluca LLP, TEVA Specialty Pharmaceuticals, UpToDate, Cowen & Co, Bird Rock Bio, Nuvelution Pharmaceuticals, and Vitaeris; has received a grant from Genentech and Sanofi; has received nonfinancial support from Boehringer Ingelheim, GlaxoSmithKline, Merck, Sunovion, and TEVA; and has received other compensation from Novartis, Research in Real Life, and TEVA Specialty Pharmaceuticals. R. J. Martin has received a grant from MedImmune; has received personal fees from Teva, AstraZeneca, and Genentech; and has received travel support from the Respiratory Effectiveness Group. D. T. Mauger has received a grant from the NHLBI and has received nonfinancial support from GlaxoSmithKline, Merck, Boehringer Ingelheim, Teva, and Sunovion. S. R. Rosenberg has received grants from AstraZeneca and Boehringer Ingelheim. T. Sharp-King has received a grant from the NHLBI and has received personal fees from Pearl Therapeutics and Insmid. S. R. White has received grants

from the NIAID (U19-AI-095230) and AstraZeneca and has received personal fees from Marathon Pharmaceuticals. P. G. Woodruff has received personal fees from Genentech, Johnson & Johnson, Roche, and Neostem and has a patent pending related to asthma biomarkers with Genentech. P. C. Avila has received grants from the NIH/NHLBI, Genentech, AstraZeneca, and Novartis. L. C. Denlinger has received a grant from the NIH/NHLBI and has received personal fees from Novartis and GlaxoSmithKline. S. C. Lazarus has received a grant from the NIH/NHLBI and has received personal fees from Respiratory Disease Young Investigators' Research Forum. N. Lugogo has received a grant from the NIH. W. C. Moore has received a grant from the NHLBI. S. P. Peters has received a grant from the NIH/NHLBI. C. A. Sorkness has received grants from the NHLBI and the NIAID. M. E. Wechsler has received personal fees from Sepracor/Sunovion, Asthmatx/BSI, Merck, Regeneron, MedImmune, Ambitbio, Vectura, Sanofi, Teva, Mylan, AstraZeneca, Genentech, Meda, Theravance, Novartis, Boehringer Ingelheim, GlaxoSmithKline, Tunitas, and Gliacure. S. E. Wenzel has received grants from Sanofi, Genentech, AstraZeneca, GlaxoSmithKline, and Boehringer Ingelheim and has received personal fees from AstraZeneca, Aerocrine, GlaxoSmithKline, Actelion, and Boehringer Ingelheim. H. A. Boushey has received grants from the NIH/NHLBI and the NIH/NIAID and has received personal fees from the McGraw-Hill Companies. Y. J. Huang has received grants from the NHLBI (K23HL105572) and the Michigan Institute of Health and Clinical Research, has received travel and lodging compensation from the NIH, and has received payment for lectures from the American Academy of Allergy, Asthma & Immunology; the Massachusetts Institute of Technology; the European Respiratory Society; the Microbiome R&D Business Forum; and the European Academy of Allergy, Asthma, and Clinical Immunology. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication March 21, 2016; revised August 2, 2016; accepted for publication August 12, 2016.

Corresponding author: Homer A. Boushey, MD, Division of Pulmonary/Critical Care Medicine, University of California, San Francisco, 505 Parnassus Ave, M1292, Box 0130, San Francisco, CA 94143. E-mail: homer.boushey@ucsf.edu.

0091-6749/\$36.00

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2016.08.055>

Abbreviations used

AA:	Atopic asthmatic subject
ANA:	Atopic nonasthmatic subject
BB:	Bronchial brushing
HC:	Healthy control subject
ICS:	Inhaled corticosteroid
OTU:	Operational taxonomic unit
OW:	Oral wash
PICRUSt:	Phylogenetic Reconstruction of Unobserved States
SCFA:	Short-chain fatty acid
T2:	Type 2
TGM:	Three-gene mean

related to underlying atopy, which is itself associated with altered mucosal immune function.^{9,10} Collectively, these considerations indicate a need to elucidate differences in the bronchial microbiota associated with asthma versus atopy and with important phenotypic features of this disease, such as the level of type 2 (T2) inflammation and responsiveness to ICS treatment.

Accordingly, we compared the bronchial bacterial microbiome among adults with mild steroid-naïve atopic asthma, subjects with atopy without asthma, and healthy nonatopic nonasthmatic control subjects. We hypothesized that specific compositional and functional differences in bronchial microbiota are associated with asthma and with distinguishing phenotypic features of the disease, including evidence of T_H2 inflammation and responsiveness to ICS treatment.

METHODS**Study population and sample collection**

This study was conducted at 9 sites in the National Heart, Lung, and Blood Institute AsthmaNet network by using a standardized bronchoscopy protocol for sample collection. Of 186 adults screened, 84 subjects were enrolled (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org): 42 atopic asthmatic subjects (AAs), 21 atopic nonasthmatic subjects (ANAs), and 21 nonatopic healthy control subjects (HCs). Atopy was defined based on serologic evidence (>0.35 kU/L) of sensitivity to 1 or more of 12 aeroallergens (specific IgE measured by means of ImmunoCAP, Thermo Scientific, Waltham, Mass; see Table E1 in this article's Online Repository at www.jacionline.org). Asthma was confirmed based on measurement of airway hyperresponsiveness (methacholine PC₂₀ ≤8 mg/mL or FEV₁ improvement ≥12% after albuterol). At enrollment, asthmatic subjects had been clinically stable for 3 months and had an Asthma Control Questionnaire score of less than 1.5¹¹ without use of a controller medication. Exclusion criteria included a history of smoking, respiratory tract infection within 6 weeks, or antibiotic use within 3 months of enrollment (see the Methods section in this article's Online Repository at www.jacionline.org).

Samples processed for microbiota analysis included oral wash (OW) samples,¹² a saline flush (10 mL) of the bronchoscope suction channel (scope-flush), and protected bronchial brushings (BBs; see Fig E1, C, and see the Methods section in this article's Online Repository). AAs were further randomized in a 2:1 ratio to treatment with inhaled fluticasone propionate (250 µg; GlaxoSmithKline, London, United Kingdom) or placebo twice daily for 6 weeks and reassessed after treatment. Each subject signed informed consent forms approved by their center's institutional review board; a National Heart, Lung, and Blood Institute-appointed Data Safety Monitoring Board oversaw the study conduct.

Nucleic acid extraction and quantitation of 16S rRNA gene copy number

Nucleic acids from OW samples and 3 BB samples were extracted, as previously described,^{5,12} by using a modified bead-beating protocol and the AllPrep kit (Qiagen, Hilden, Germany). 16S rRNA gene copy number was

assessed by means of quantitative PCR with universal primers (see the Methods section in this article's Online Repository).

16S rRNA-based sequencing and raw data processing

For BB and OW samples, variable region 4 (V4) of the 16S rRNA gene was amplified by using the primer combination 515F/806R^{13,14} and sequenced on the Illumina MiSeq platform (Illumina, San Diego, Calif). By using chimera-checked and quality-filtered sequence reads (see the Methods section in this article's Online Repository), a 97% sequence homology cutoff was used to define bacterial taxa (also referred to as operational taxonomic units [OTUs]) and classified by using the Greengenes database.¹⁵ A phylogenetic tree was built with FastTree¹⁶ and used to compute the Faith phylogenetic diversity¹⁷ of samples by using an OTU table multiply rarefied to 52,317 sequences per sample (see the Methods section in this article's Online Repository). All sequence data related to this study are available from the European Nucleotide Archive (ENA) under accession number PRJEB15534.

"Three-gene mean" bronchial epithelial signature of T2 inflammation

Expression levels of 3 bronchial epithelial genes (*CLCA1*, *SERPINB2*, and *POSTN*) previously shown to be induced by IL-13^{18,19} were measured by using RNA extracted in parallel with DNA in this study to calculate the "three-gene mean" (TGM) score for each participant.²⁰ T2-high asthma was defined by TGM scores of 1.117 or greater (2 SDs greater than the average TGM score in HCs), as confirmed by using unsupervised cluster and principal component analysis.

Statistical analyses

We applied principal coordinates analyses on an unweighted UniFrac distance matrix and PERMANOVA²¹ to identify the determinants of variation in bacterial community β-diversity. A linear mixed-effects model²² was used to examine relationships between paired OW and BB microbiota; negative binomial and zero-inflated negative binomial regression models²³⁻²⁶ corrected for false discovery ($q < 0.1$, Benjamini-Hochberg) were used to identify OTUs differentially abundant between subject groups and paired samples, respectively. We applied Phylogenetic Reconstruction of Unobserved States (PICRUSt) to predict functional capacities of the microbiota²⁷ and negative binomial regression to compare inferred functional pathway predictions across groups. Procrustes analysis¹³ was used to explore the strength of relationships between paired samples in the AA group (see the Methods section in this article's Online Repository).

RESULTS**Study group characteristics**

AAs had mild well-controlled disease and significantly higher serum total IgE levels and blood and sputum eosinophil counts (Table I and see Fig E2, A-C, in this article's Online Repository at www.jacionline.org) than HCs. Compared with the ANAs, asthmatic subjects had significantly higher serum IgE levels; were sensitized to more of the aeroallergens tested (Table I); were more likely to be sensitive to cat, dog, and mouse (see Table E1); and were more likely to report a history of allergic rhinitis and eczema (see Table E2 in this article's Online Repository at www.jacionline.org). However, they did not exhibit significant differences in environmental exposures, as assessed by questionnaire (see Table E3 in this article's Online Repository at www.jacionline.org).

Bacterial microbiota in BB samples is compositionally distinct from that in OW samples

To evaluate mucosa-associated bronchial microbiota, we focused on protected BB samples. Sequence-based bacterial

TABLE I. Study cohort characteristics

Variable	AAs (n = 42)*	ANAs (n = 21)	HCS (n = 21)	P value
Age (y)	33 (25 to 41)	28 (24 to 44)	28 (23 to 46)	NS
Age of asthma diagnosis (y)	9 (5 to 22)	—	—	—
Duration of asthma (y)	21 (14 to 27)	—	—	—
ACQ score	0.3 (0 to 1.3)	—	—	—
Male sex (%)	45	48	43	NS¶
White race (%)	60	52	71	NS¶
BMI (kg/m ²)	26 (23 to 29)	25 (21 to 28)	26 (22 to 28)	NS
FEV ₁ (% predicted) before albuterol	89 (76 to 97)	98 (89 to 109)	100 (93 to 108)	<.01#
FEV ₁ (% predicted) after albuterol	100 (84 to 106)	104 (97 to 109)	102 (98 to 116)	NS#
Change in FEV ₁ (%)	7.5 (5.0 to 14.3)	3.0 (−0.5 to 5.5)	5.0 (2.5 to 5.0)	<.0001#
Methacholine PC ₂₀	1.2 (0.3 to 3.2)	>32†	>32†	—
Blood eosinophils (absolute)	200 (100 to 393)	100 (87 to 200)	100 (60 to 200)	<.01
Blood eosinophils (%)	3.3 (1.5 to 5.6)	2.0 (1.4 to 3.0)	1.4 (1.0 to 3.0)	<.01‡
Blood neutrophils (%)	55.5 (49.3 to 62.0)	56.9 (51.0 to 63.8)	58.4 (52.7 to 63.5)	NS
Sputum neutrophils (%)	54.6 (31.8 to 64.9)	39.5 (26.5 to 50.0)	41.8 (29.2 to 76.5)	NS
Sputum eosinophils (%)	0.4 (0.0 to 1.1)	0.0 (0.0 to 0.6)	0.0 (0.0 to 0.4)	NS‡
Serum IgE (IU/mL)	169.5 (56.3 to 321.3)	64.0 (22.0 to 164.5)	15.0 (5.0 to 31.0)	<.01‡#
No. of positive sIgE§	6 (2 to 7)	3 (2 to 5)	—	<.05#

All values are medians (interquartile ranges).

Statistical significance was determined by using the following tests: ||Kruskal-Wallis, ¶ χ^2 , or #Mann-Whitney for AA versus ANA comparison.

ACQ, Asthma Control Questionnaire; NS, not significant.

*Number of exacerbations requiring oral steroids in the past 5 years: 0 exacerbations, 38 subjects; 1 exacerbation, 3 subjects; and 2 exacerbations, 1 subject.

†Methacholine challenge was stopped at 32 mg/dL, and PC₂₀ values for these subjects were censored.

‡For between-group statistical comparisons, see Fig E2, A-C.

§Number of positive specific IgE measurements (sIgE >0.35 kU/L) from a total of 12 aeroallergens tested by using the ImmunoCAP assay (for breakdown of specific aeroallergens, see Table E1).

community analysis could be performed in the same proportion (67%) of samples collected in each of the 3 groups (see Fig E1, D and E). Samples that could not be sequenced had lower bacterial burden, as indicated from 16S rRNA ($P < .0001$; see Fig E2, D), despite recovery of similar mammalian cell burden, as assessed by quantifying β -actin copy number (see the Methods section in this article's Online Repository). Subjects with insufficient 16S rRNA amplicons for microbiota profiling were younger (median, 28 [22–37] vs 34 [25–44] years; $P = .03$) but did not differ in any other characteristics measured.

Potential oral contamination of BB samples was evaluated by comparing a random subset of 30 BB-paired scope-flush samples. Bacterial burden of scope-flushes for BB samples with sufficient 16S rRNA for sequencing was indistinguishable from those unable to be sequenced ($P > .1$; see Fig E2, E). Additionally, principal coordinates analysis of bacterial community composition in paired OW and BB samples showed these 2 niches to be compositionally distinct ($\beta = 0.31$ and $P < .0001$, linear mixed-effects model; Fig 1, A), with BB samples exhibiting lower phylogenetic diversity (Faith index: $P < .0001$; Fig 1, B).

Features of the bronchial bacterial microbiota and distinct combinations of specific bacterial taxa are associated with atopic asthma or atopy alone

α -Diversity indices, such as richness (number of observed taxa), Shannon diversity, and evenness, as well as bacterial burden, did not differ among the groups (see Fig E2, F-I). However, phylogenetic diversity (Faith index) tended to be higher in AAs compared with that in HCs ($P = .06$; Fig 1, C). Because this index weights the phylogenetic relatedness of the bacteria detected, this observation suggests that the bronchial airways of AAs harbor more phylogenetically diverse bacterial

communities. Although phylogenetic diversity in ANAs also appeared to be greater than that in HCs (Fig 1, C), the difference fell short of significance. Overall, intersubject bacterial community composition (β -diversity) was highly heterogeneous across subjects (see Fig E3, A, in this article's Online Repository at www.jacionline.org). This was significantly related to bacterial richness independent of study group (see Fig E3, B-E) but was not associated with any of the clinical measures or environmental exposures evaluated in this study (Table I and see Tables E1–E3). Compositional variability in bronchial bacterial microbiota was significantly greater within the asthmatic group ($P < .001$, weighted UniFrac distance F-test; see Fig E3, F), indicating a greater degree of bacterial community heterogeneity in this group.

After these initial assessments for differences in overall bacterial community composition, we conducted OTU-level analyses to determine whether the relative abundance of specific taxa differed significantly across groups; 76 taxa were found to do so in the AA versus HC comparison (see Table E4 in this article's Online Repository at www.jacionline.org). Phylum-level differences in AAs (Fig 2, A, and see Fig E4, A, in this article's Online Repository at www.jacionline.org) included significant enrichment for members of the Bacteroidetes (*Prevotella* species), Fusobacteria (*Fusobacterium* species), Actinobacteria (*Actinomyces* species), and Proteobacteria (*Haemophilus* and *Neisseria* species), the latter previously associated with more severe, ICS-requiring asthma.^{2–6,28} Conversely, asthmatic subjects showed reduced relative abundance of members of the Fusobacteria (*Leptotrichia* species), Proteobacteria (*Actinobacillus* species), and Firmicutes (*Lactobacillus* species). ANAs also demonstrated significant differences in the relative abundance of 100 taxa compared with HCs (see Table E5, B, in this article's Online Repository at www.jacionline.org). The

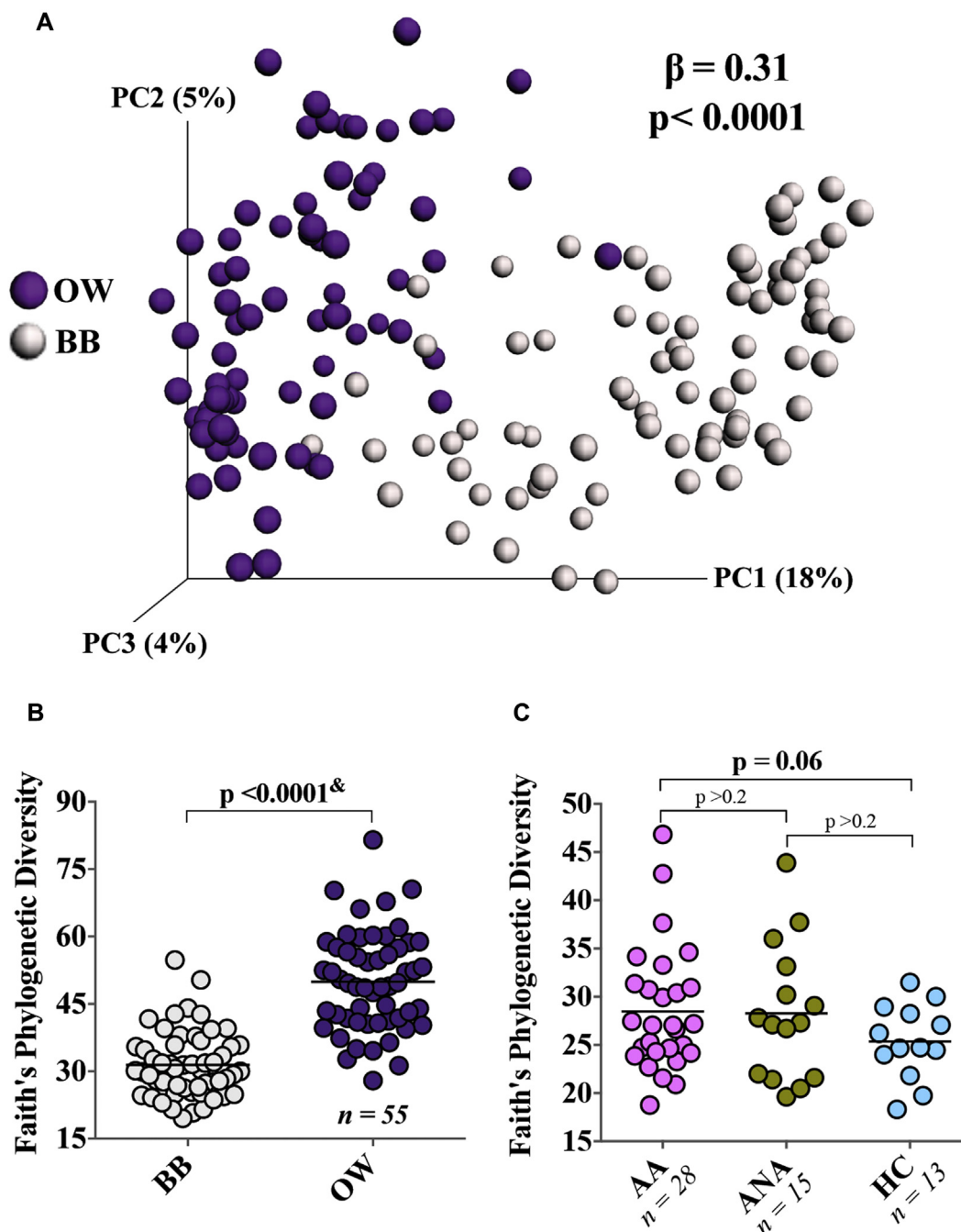


FIG 1. **A**, Principal coordinate analysis (unweighted UniFrac) shows compositional dissimilarity between paired BB and OW samples ($P < .0001$, linear mixed-effects model). **B**, Phylogenetic diversity (Faith index) in BB and paired OW samples. $\&$ Wilcoxon matched-pairs signed-rank test. **C**, Phylogenetic diversity (Faith index) in BB samples for the 3 subject groups (Welch corrected t test).

taxa most relatively enriched in ANAs included members of the Proteobacteria (*Aggregatibacter* and *Haemophilus* species), Firmicutes (*Granulicatella* species), and Actinobacteria (*Corynebacterium* species) phyla (Fig 2, B, and see Fig E4, B); those depleted included members of the phylum Bacteroidetes (*Porphyromonas* and *Prevotella* species).

We noted that the ANAs shared 26% and 29% of taxa that were also relatively enriched or depleted in AAs when compared with HCs. Despite these similarities, OTU-level analysis identified 103

taxa that differed significantly between the AA and ANA groups (Fig 2, C, and see Fig E4, C, Table E6 in this article's Online Repository at www.jacionline.org). To pinpoint bacteria discretely associated with asthma, we evaluated which taxa among those that distinguished AAs from HCs also distinguished AAs from ANAs (Fig 3). By using this approach, asthma-associated taxa included members of the *Haemophilus* (OTU4406393), *Fusobacterium* (OTU4405869; OTU2438396), *Neisseria* (OTU1304), and *Porphyromonas*

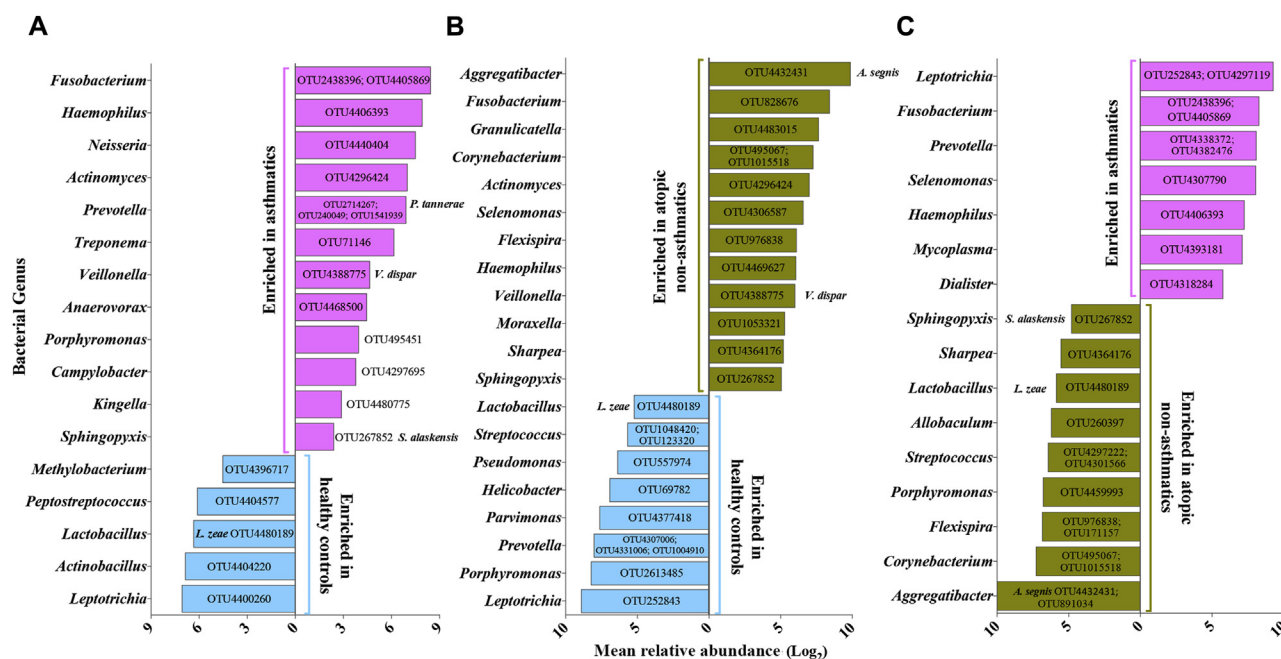


FIG 2. Bacterial taxa that were significantly enriched or depleted in relative abundance (at least 2-fold; $q < 0.1$, negative binomial regression) in AAs ($n = 28$) compared with HCs ($n = 13$; **A**); ANAs ($n = 15$) compared with HCs (**B**); and AAs compared with ANAs (**C**). OTUs represent the most abundant representatives within the indicated genus.

(OTU495451) species and the Sphingomonadaceae family (OTU8331815), whereas taxa negatively associated with asthma included members of the Mogibacteriaceae family (OTU4335578) and Lactobacillales order (OTU4480189; OTU4469032). The same approach also identified taxa specific to atopy alone, which included enrichment in *Aggregatibacter* (OTU4432431), *Corynebacterium* (OTU1015518; OTU 495067), and *Prevotella* (OTU4372058; OTU134265) species (Fig 3). Of the taxa identified as specifically associated with either asthma or atopy alone, a subset exhibited strong and significant correlations with features of atopy (IgE levels and blood and sputum eosinophil counts); these associations were distinct in the 2 atopic groups. For example, specific taxa uniquely enriched in AAs, belonging to the Sphingomonadaceae and Fusobacteria families, were positively associated with all 3 markers of atopy, whereas of those specifically enriched in ANAs, *Sharpea* and *Prevotella* species (OTU1052181; OTU134265) correlated with IgE levels and blood eosinophil counts, respectively (Fig 3). These data indicate that although taxonomic overlap exists between AAs and ANAs, discrete bacterial enrichments characterize these groups, a subset of which are associated with biomarkers of atopic disease.

We further explored whether predicted functions of the bronchial bacterial microbiome differed among the 3 groups by using PICRUST,²⁷ an algorithm that predicts bacterial metagenomes *in silico* from 16S rRNA sequence. This analysis revealed significant differences across groups (Fig 4). Predicted bacterial gene functions enriched in AAs included those involved in metabolism of amino acids and carbohydrates, especially of short-chain fatty acids (SCFAs), such as butanoate and propanoate (more commonly known as butyrate and propionate, respectively). Also noteworthy was a relative depletion in predicted bacterial functions involved in LPS biosynthesis among asthma-associated bacterial communities.

T2-high asthma is associated with reduced bronchial bacterial burden

TGM scores were higher in AAs (Fig 5, A) and, consistent with prior reports,^{20,29} correlated positively with serum IgE levels ($r_s = 0.36$, $P < .01$) and blood and sputum eosinophil counts ($r_s = 0.32$ and $r_s = .37$, respectively; $P \leq .01$) and correlated negatively with FEV₁ ($r_s = -0.45$, $P < .005$) and PC₂₀ ($r_s = -0.41$, $P < .005$) values. Ten of 40 AAs had T2-high asthma and exhibited significantly higher Asthma Control Questionnaire scores, serum IgE levels, and blood and sputum eosinophil counts and younger age than subjects with T2-low asthma (see Table E7 in this article's Online Repository at www.jacionline.org). Subjects with T2-high asthma demonstrated significantly lower bronchial bacterial burden than subjects with T2-low asthma (Fig 5, C); bacterial burden also was negatively correlated with TGM scores across all asthmatic subjects ($r_s = -0.43$, $P < .01$) independent of age (general linear model: $P = .03$). Because of their low bacterial burden, BB samples from only 4 subjects with T2-high asthma could be sequenced for profiling, an insufficient sample size to allow meaningful assessment of differences in bacterial microbiota composition between those with T2-high asthma and those with T2-low asthma.

ICS responsiveness is associated with distinct features of the bronchial bacterial microbiota present before treatment

We hypothesized that differences in baseline airway microbiota characteristics might be associated with ICS responsiveness, which was defined as a 2-fold or greater increase in methacholine PC₂₀ after ICS treatment. Of the asthmatic subjects included, 15 were classified as ICS responders and 10 as nonresponders, and 3 were excluded because PC₂₀ measurement was not performed (see Fig E5, A and B). Compared

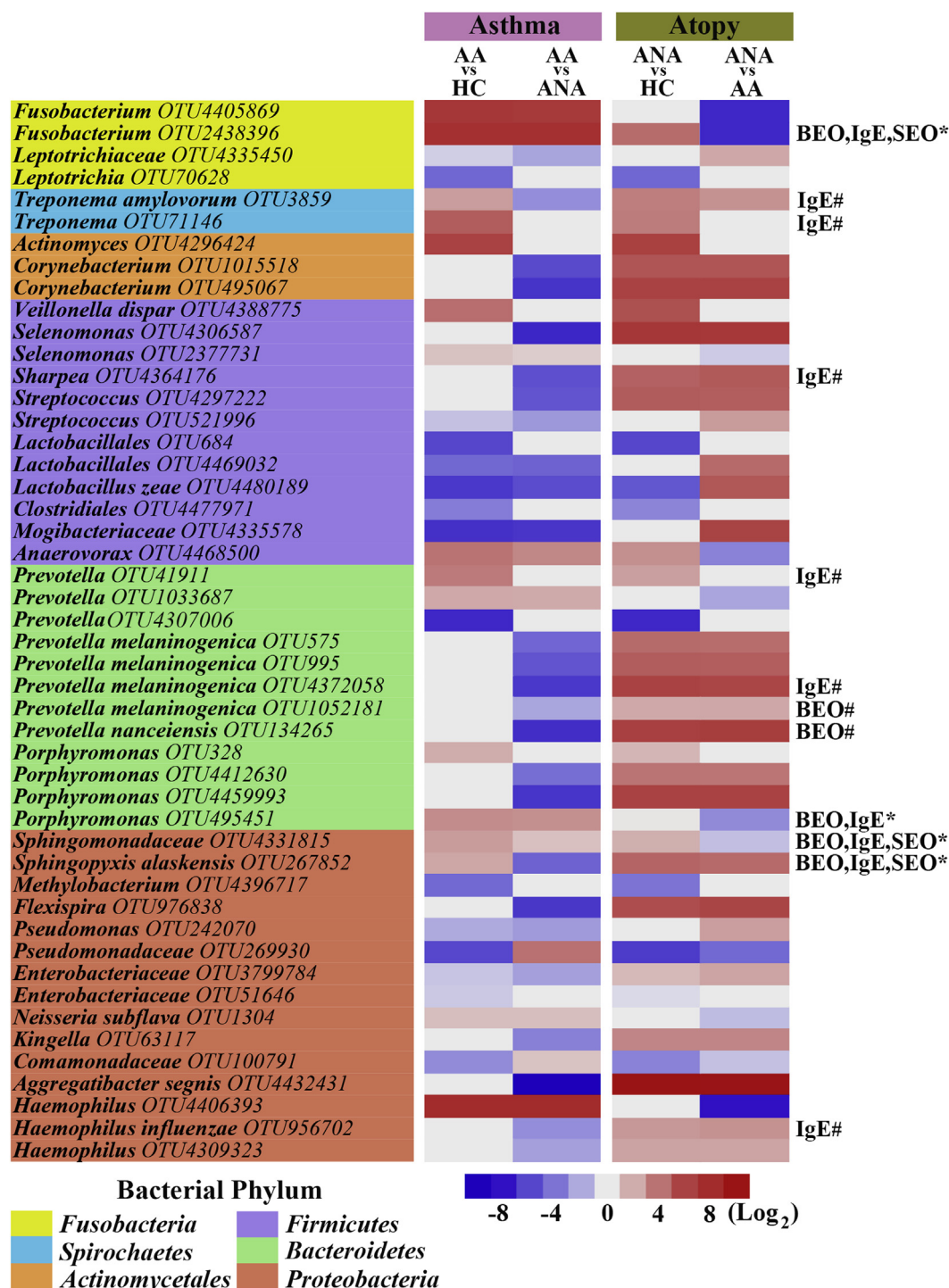


FIG 3. Mean difference in specific bacterial taxa between groups. Asthma-specific taxa are similarly abundant in AAs compared with HCs and ANAs ($q < 0.1$, negative binomial regression). Atopy-only taxa are similarly abundant in ANAs versus both HCs and AAs. Taxa positively correlated ($r_p \geq 0.5$, $q < 0.1$) with blood eosinophil counts (BEO), serum IgE levels (IgE), or sputum eosinophil counts (SEO) in AAs (*) or ANAs (#).

with nonresponders, ICS responders did not differ in PC₂₀ values, serum IgE levels, and blood and sputum eosinophil counts or bronchial burden at baseline but did have lower FEV₁ values and a trend toward a higher TGM score ($P < .08$; see Fig E5, C, and Table E8 in this article's Online Repository at

www.jacionline.org), which decreased after treatment ($P < .05$; see Fig E4, D).

The baseline composition of profiled bronchial bacterial microbiota in ICS responders ($n = 10$) differed from that in nonresponders ($n = 5$; unweighted UniFrac

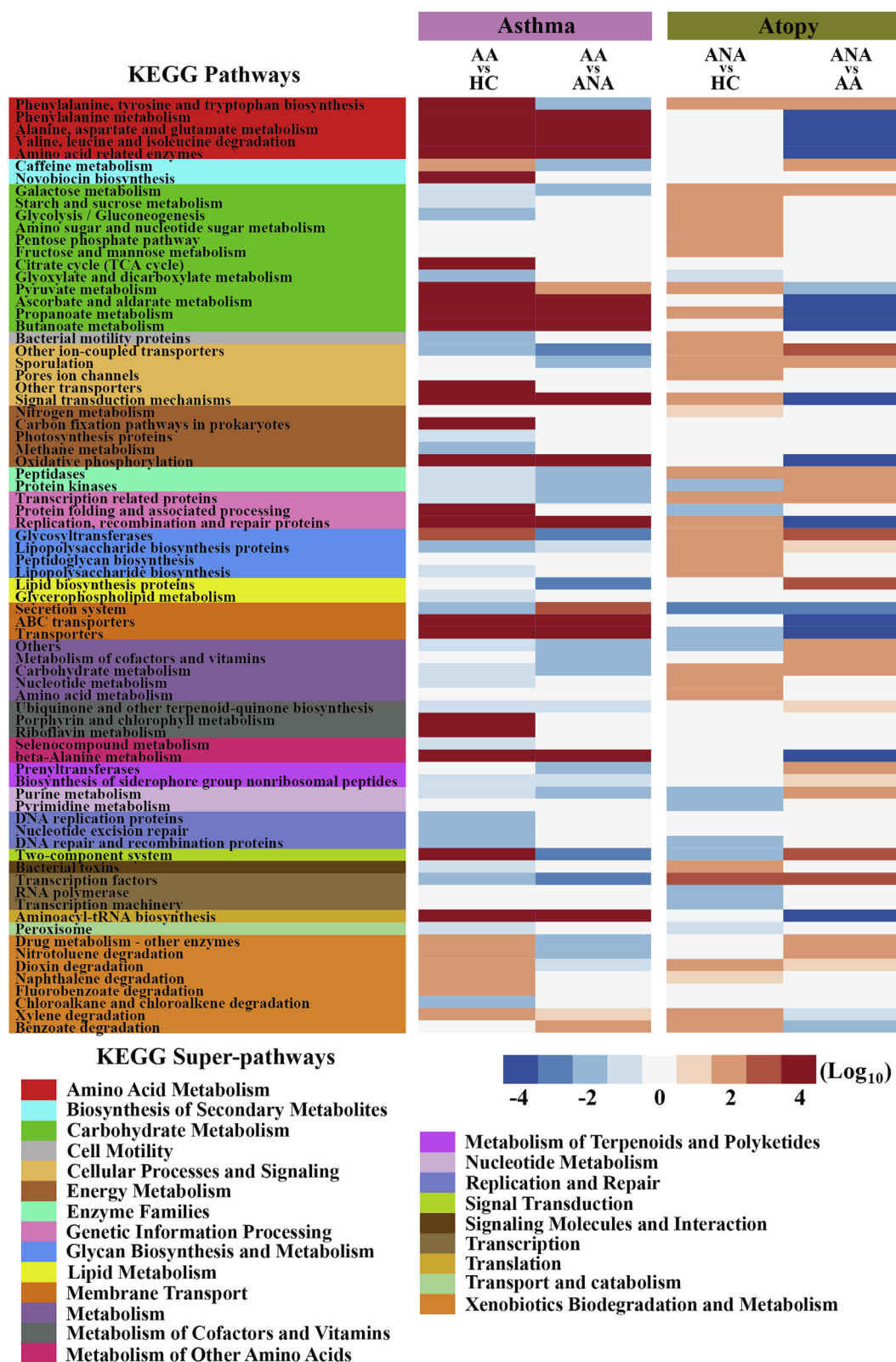


FIG 4. Mean difference in predicted Kyoto Encyclopedia of Genes and Genomes orthologs (KOs) between groups. Asthma-specific pathways are similarly abundant in AAs compared with HCs and ANAs. Atopy-only pathways are similarly abundant in ANAs compared with HCs and AAs. Statistical significance was determined by using a negative binomial regression model corrected for false discovery rate ($q < 0.1$).

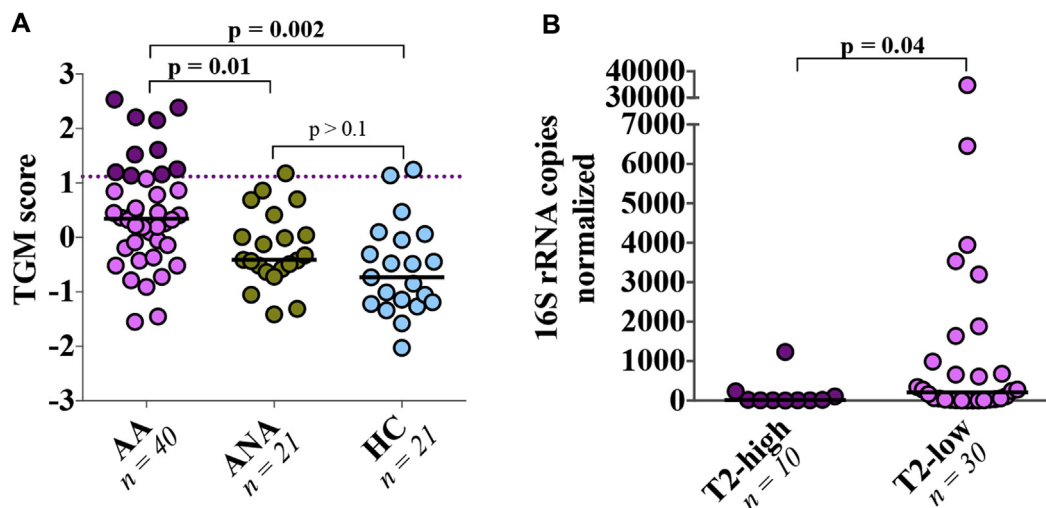


FIG 5. **A**, AAs displayed greater expression of epithelial genes induced by T2 cytokines compared with nonasthmatic subjects. T2-high AAs with a TGM of 1.117 or greater (cutoff value indicated by a dashed line) are colored in maroon red. **B**, Significantly lower bacterial burden was observed among subjects with T2-high asthma. Statistical significance was determined by using the Wilcoxon rank sum test.

PERMANOVA: $R^2 = 0.13$, $P = .01$) and was significantly more similar to that of HCs (Fig 6, A). Bacterial families enriched at baseline in ICS nonresponders included Microbacteriaceae, Pasteurellaceae (eg, asthma-associated *Haemophilus* OTU4406393), and several others (Fig 6, C, and see Table E9 in this article's Online Repository at www.jacionline.org). Conversely, bacterial families enriched at baseline in ICS responders included Streptococcaceae, Fusobacteriaceae, and Sphingomonadaceae. Compared with ICS responders, the predicted functions of bacterial communities of nonresponders were enriched in xenobiotic biodegradation pathways (Fig 6, D, and see Table E10 in this article's Online Repository at www.jacionline.org), implicating potential enhanced capacity for synthetic chemical degradation.

After 6 weeks of ICS versus placebo treatment, we found no significant changes in bacterial burden or phylogenetic diversity in the AA group (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org). Bacterial community analysis in paired samples before and after treatment was limited because several subjects did not have sufficient 16S rRNA to obtain sequence data at both time points. Nonetheless, we reasoned that ICS exposure might have distinct effects on the microbiotas of nonresponders and ICS responders² and explored ICS-induced compositional changes in the latter more predominant group. No significant differences were seen in the magnitude of changes (β -diversity assessed based on unweighted UniFrac distance) between paired samples ($n = 8$ each in placebo or ICS-treated responders; see Fig E6, C-E). At the taxon level, however, ICS treatment resulted in increased relative abundance of Microbacteriaceae and *Neisseria* and *Moraxella* species and depletion of a specific *Fusobacterium*, which was not observed with the placebo treatment (Fig 6, D, and see Tables E11 and E12 in this article's Online Repository at www.jacionline.org). Unfortunately, we could not analyze compositional changes in ICS nonresponders because only 2 subjects in this subgroup had sufficient 16S rRNA in both pretreatment and posttreatment samples.

DISCUSSION

Our findings show compositional and predicted functional differences in the bronchial bacterial microbiomes of AAs, ANAs, and HCs. An important implication of these findings is that control for allergic sensitization is necessary in studies aimed at understanding differences in the respiratory microbiome associated with asthma. Despite overlap in bacterial genera significantly associated with both atopic groups, our study identified specific bacterial taxa whose relative enrichment or depletion was discretely associated with asthma. Analyses based on metagenomic inference further suggest that genes for pathways involved in the metabolism of SCFAs and amino acids are enriched in the asthmatic bronchial microbiome. Additionally, we observed that the bronchial microbiome among asthmatic subjects at baseline differed compositionally and functionally according to their responsiveness to treatment with ICSs.

Commonly reported microbial community metrics (eg, richness, evenness, and burden) did not differ significantly among our 3 groups. This is not unexpected due to the broad characterization of microbial composition provided by such measures; the interindividual heterogeneity of microbiota composition found in all human niches, including the lung³⁰; and the mild disease severity in our subjects. Considering the clinical homogeneity of our asthmatic group, their heterogeneity in bronchial bacterial composition is striking. Moreover, this heterogeneity was predominantly observed among subjects with T2-low asthma, in whom bronchial bacterial burden was significantly greater than in subjects with T2-high asthma. The trend toward higher bacterial phylogenetic diversity in our subjects with mild asthma suggests their lower airways are receptive to colonization by a wider variety of bacteria. Although we could not pinpoint any specific clinical features associated with this finding, additional contributing factors might include differences in previous environmental exposures, clearance of microorganisms, or other immune function parameters not identified here.

Reasons for the low bronchial bacterial burden in subjects with T2-high asthma are unclear, but the finding echoes a recent

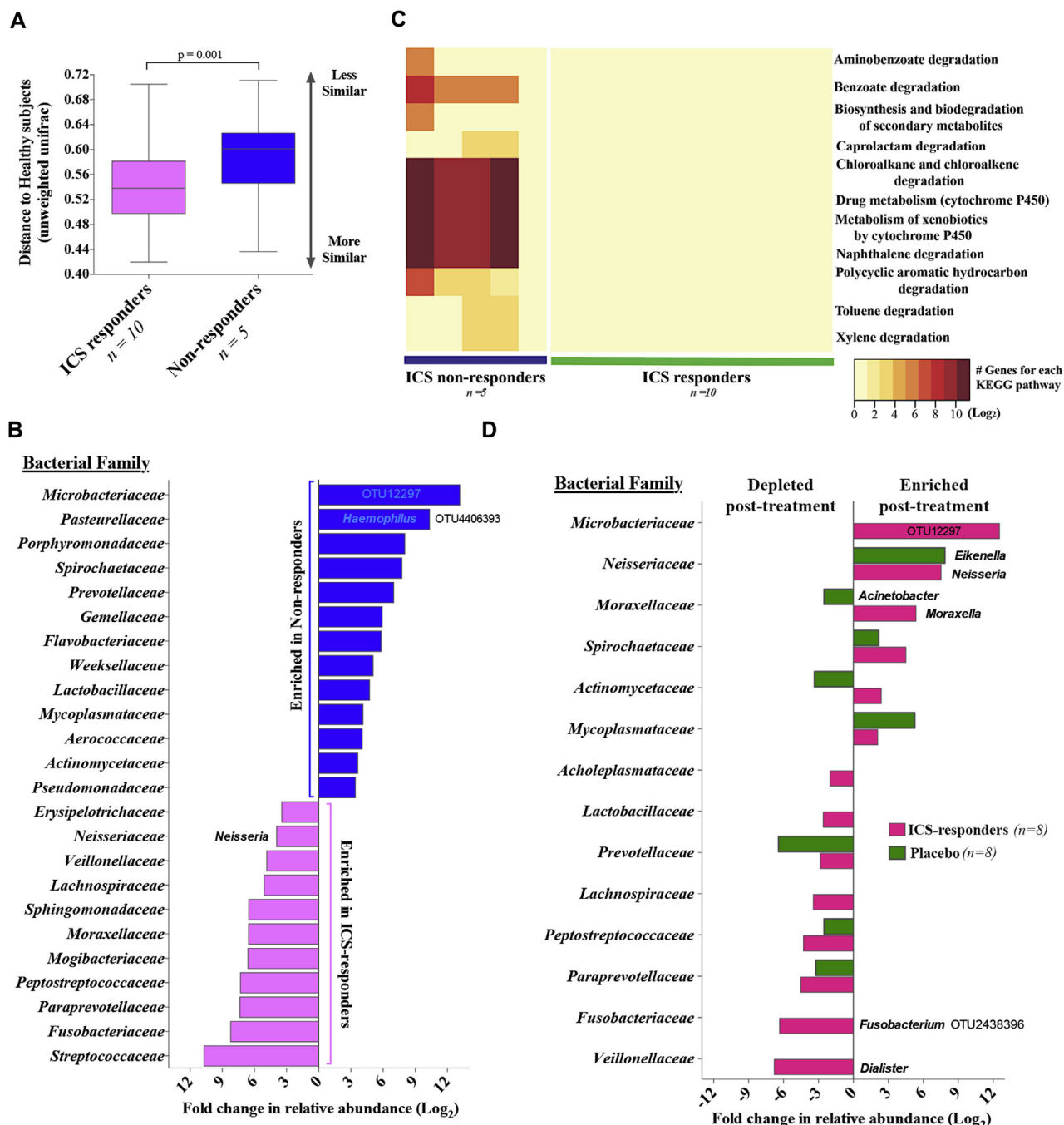


FIG 6. A, Mean distance to HCs (unweighted UniFrac; Bonferroni-corrected *t* test). B, Taxa significantly enriched or depleted in relative abundance (at least 2-fold; $q < 0.1$, negative binomial regression) in ICS responders versus nonresponders. C, Predicted Kyoto Encyclopedia of Genes and Genomes pathways associated with xenobiotic biodegradation and metabolism in ICS responders and nonresponders. D, Taxa, differentially expressed (at least 2-fold; $q < 0.1$, zero-inflated negative binomial regression) in asthmatic subjects after ICS or placebo treatment.

observation in subjects with severe asthma of an inverse relationship between bronchial bacterial burden and numbers of bronchial biopsy eosinophils.⁴ A possible explanation is the bactericidal activity of more numerous airway eosinophils^{31,32} in subjects with T2-high airway inflammation. Given prior reports of inverse relationships between bacterial and fungal richness,^{33,34} the low bacterial burden in subjects with T2-high asthma could also reflect

increased fungal or bacteriophage burden. Indeed, microbial interactions are not limited to bacteria because bacterial-viral interactions have been associated with asthma exacerbations and risk for asthma development.^{35,36} Such interkingdom interactions³⁷ should be considered in future studies.

This study expands the list of bacterial groups previously associated with asthma, further supporting the idea that

alterations in microbial composition from a healthy state is a characteristic of asthma. Enrichment in certain Proteobacteria members (eg, *Haemophilus* and *Neisseria* species) among our ICS-naïve asthmatic subjects resembles prior findings in ICS-using patients.^{2-6,28} However, we found other taxa discretely associated with asthma, including *Fusobacterium* and *Porphyromonas* species, 2 oral-associated anaerobes capable of augmenting pathogenic behavior of opportunistic respiratory pathogens, such as *Pseudomonas aeruginosa*.³⁸ Additionally, both *Fusobacterium* species³⁹ and *Haemophilus* species⁴⁰ induce MUC5AC expression in bronchial epithelial cells. Sphingomonadaceae represented another asthma-specific taxon the abundance of which, like that of *Fusobacterium* species, correlated with sputum eosinophilia. This finding echoed the reported activity of glycosphingolipids (present in the cell membrane of Sphingomonadaceae) in activating natural killer T cells to produce T2 cytokines⁴¹⁻⁴³ and the reported association of this bacterial family with bronchial reactivity.⁵

Asthma-related alterations in bronchial microbiota composition also involve relative depletion in certain taxa, including members of the Lactobacillales. Numerous studies have demonstrated protective effects of certain *Lactobacillus* species strains against atopy through various mechanisms, including alteration of gastrointestinal⁴⁴ and respiratory tract^{45,46} permeability. Our finding that the predicted functions of the asthma-associated microbiota were relatively depleted in machinery for LPS biosynthesis suggests another possibility, since continuous stimulation of the airways with LPS has been shown to suppress T2 immune activation.⁴⁷ However, we propose that any “proasthmatic” activity of a bronchial microbiome is likely dependent on functional effects consequent to interactions among many microbiota members in the airway microenvironment rather than the activity of any one species. Microbiome-related functions indicated by our analyses as potentially enhanced in asthmatic subjects include increased capacity for metabolism of butyrate and propionate, SCFAs that maintain epithelial barrier function, and immune tolerance in the gut.⁴⁸⁻⁵⁰ We speculate that use of anti-inflammatory SCFAs by members of the asthmatic airway microbiota might contribute to atopic asthma by reducing their bioavailability and the consequent capacity to downregulate host inflammatory responses to aeroallergens and pathogens.

Microbiome-related functions might also affect responsiveness to corticosteroid treatment. We found that pretreatment enrichment in asthma-associated *Haemophilus*, a genus with species previously shown to reduce response of BAL macrophages to corticosteroids,² was associated with diminished response to 6 weeks of treatment with inhaled fluticasone. Analysis of the predicted metagenome of the pretreatment bronchial microbiota present in ICS nonresponders also indicated enhancement of xenobiotic degradation capacity, which we hypothesize could contribute to their diminished response. In contrast, ICS-induced changes to the bronchial microbiota in ICS-responsive asthmatic subjects showed enrichment of previously reported asthma-associated taxa, such as *Neisseria* and *Moraxella* species.^{2,3,6} Community shift detected in response to a lactose-containing placebo inhaler was not surprising because an influx of an additional sugar would be expected to alter the composition of microbial communities in the airways, favoring those species with the metabolic capacity to use such carbon sources. As importantly, these results emphasize the need to

consider the effects of repeated inhalation of particles, especially of an ICS, as a selective pressure and nutritional source for airway microbiome members with the catalytic capacity to degrade such xenobiotics.

Perturbations to the bronchial microbiota associated with atopy alone are also a novel finding of this study. ANAs were less sensitized than AAs and showed primarily low T2 inflammation of bronchial epithelium. Although the greater severity of allergy in the AA group cannot be ruled out as contributing to the difference in bacterial signature observed between AAs and ANAs, distinct taxa associations with different markers of atopy and allergic inflammation were observed, suggesting that distinct microbial interactions with the host immune system occur in these 2 patient groups. These observations in ANAs support an association between airway colonization and atopy-related altered mucosal immune functions.^{9,10} Atopy-specific taxa included members of the Pasteurellaceae (specifically *Aggregatibacter* and *Haemophilus* species). Members of *Aggregatibacter* are associated with periodontal disease driven by high proinflammatory cytokine (eg, TNF- α , IL-1 β , IL-6, and IL-8) levels and reduced levels of IL-10.^{51,52} It is tempting to speculate that airway enrichment of this genus in ANAs could contribute to asthma protection through activation of the T1 arm of the immune system. We also highlight that specific taxa belonging to certain genera (eg, *Prevotella* and *Haemophilus* species) were associated with atopy only or asthma. This underscores the likely importance of species- or strain-level functional differences in microbial interactions related to disease status.

The limitations of our study include exclusive analysis of bacterial communities, the absence of nonatopic asthmatic subjects, and the relatively narrow breadth of asthma phenotype captured in this cohort. Moreover, our sample size was small in some comparisons, particularly in assessing changes in the bronchial microbiota in paired samples before and after ICS versus placebo treatment. However, strengths include the large number of subjects who were characterized and underwent invasive bronchoscopy to collect samples for analysis. Our study protocol also attended carefully to procedural and sampling methods to reduce contamination and analyze for possible contribution of nonbronchial sources of bacterial DNA to the data set.

In conclusion, our findings highlight the complexity of bacterial relationships to asthma in a background of atopy, conditions that are associated with distinct alterations in the airway microbiota. To achieve a comprehensive understanding of microbial factors involved in the induction of, management of, or protection against asthma, there is an important need to better understand functions collectively expressed by consortia of airway microbes, which could have a profound influence on asthma.

We would like to thank the following coordinators and staff and to identify the grants supporting the study (further information can be found in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org): Robert Pedicini, BS¹; Kathy Zheng, MPH¹; Duanny Alva, MPH²; Assel Biyasheva, PhD²; Jenny Hixon, BS, CCRC²; Lucius Robinson III, BS, CMA, CCRC²; Mary Gill, RN, BSN³; James T. Good, MD³; Christena Kolakowski, MS³; Allen Stevens, CCRC, NREMT³; E. Rand Sutherland, MD³; Julia Bach, RN⁴; Rich Cornwell, MD⁴; Holly Eversoll, RN⁴; Tiffany Huard⁴; Keith Meyer, MD⁴; Barbara Miller, RN⁴; Ann Sexton, MPH⁴; Michele Wolff, RN⁴; Merritt Fajt, MD⁵; Sherri Hill, BS⁵; Lisa Lane, BS⁵; Russell Traister,

MD⁵; Cathy Vitari, RN, BSN AE-C⁵; Vanessa Curtis, RRT⁶; Brenda Patterson, RN, APN⁶; Cheryl Shelton, RN, BSN⁶; Kelly Norsworthy, BA, CPT⁷; Kelsey Wollen, BA⁷; Eugene Bleeker, MD⁸; Christopher Barrios, MD⁸; Suzan Farris, CCRP⁸; Jeffrey Krings, FNP⁸; Victor Ortega, MD⁸; Cheryl Wilmoth, CCRP⁸; Matthew Bowman, BS⁹; Linda Engle, BS⁹; Jennifer Lucier, BS⁹; Aimee J. Merchlinski, MS⁹; Kathryn Trasatt, BS⁹; Angela Updegrave⁹; Rachel Weber, BS⁹; and Ronald R. Zimmerman, Jr, MPA⁹. We also thank Elizabeth F. Juniper of McMaster University, Canada, for permitting use of her Asthma Control Questionnaire and Andrew Manies of UCSF for assistance with manuscript preparation.

Key messages

- The bronchial bacterial microbiota of both subjects with mild atopic asthma (steroid naive) and those with atopy alone differs from that of HCs and also differ from each other.
- Asthma is associated with enrichment in members of the *Haemophilus*, *Neisseria*, *Fusobacterium*, and *Porphyromonas* species and Sphingomonadaceae and with depletion of *Lactobacillus* species.
- The T2-high asthma phenotype is associated with low bronchial bacterial burden.
- ICS response is linked to a distinct bacterial community composition and functional profile before steroid exposure.

REFERENCES

- Denner DR, Sangwan N, Becker JB, Hogarth DK, Oldham J, Castillo J, et al. Corticosteroid therapy and airflow obstruction influence the bronchial microbiome, which is distinct from that of bronchoalveolar lavage in asthmatic airways. *J Allergy Clin Immunol* 2016;137:1398-405.e3.
- Goleva E, Jackson LP, Kirk Harris J, Robertson CE, Sutherland ER, Hall CF, et al. The effects of airway microbiome on corticosteroid responsiveness in asthma. *Am J Respir Crit Care Med* 2013;188:1193-201.
- Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. *PLoS One* 2010;5:e8578.
- Huang YJ, Nariya S, Harris JM, Lynch SV, Choy DF, Arron JR, et al. The airway microbiome in patients with severe asthma: associations with disease features and severity. *J Allergy Clin Immunol* 2015;136:874-84.
- Huang YJ, Nelson CE, Brodie EL, Desantis TZ, Baek MS, Liu J, et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol* 2011;127:372-81, e1-3.
- Marri PR, Stern DA, Wright AL, Billheimer D, Martinez FD. Asthma-associated differences in microbial composition of induced sputum. *J Allergy Clin Immunol* 2013;131:346-52, e1-3.
- Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Associations of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989;320:271-7.
- Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? *Thorax* 1999;54:268-72.
- Kariyawasam HH, Rotiroti G. Allergic rhinitis, chronic rhinosinusitis and asthma: unravelling a complex relationship. *Curr Opin Otolaryngol Head Neck Surg* 2013;21:79-86.
- Tomazic PV, Birner-Gruenberger R, Leitner A, Obrist B, Spoerke S, Lang-Loidolt D. Nasal mucus proteomic changes reflect altered immune responses and epithelial permeability in patients with allergic rhinitis. *J Allergy Clin Immunol* 2014;133:741-50.
- Juniper EF, O'Byrne PM, Guyatt GH, Ferrier PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999;14:902-7.
- Iwai S, Fei M, Huang D, Fong S, Subramanian A, Grieco K, et al. Oral and airway microbiota in HIV-infected pneumonia patients. *J Clin Microbiol* 2012;50:2995-3002.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 2012;6:1621-4.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 2011;108(suppl 1):4516-22.
- DeSantis TZ, Hugenholtz P, Larsen N, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;72:5069-72.
- Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 2009;26:1641-50.
- Faith DP. Conservation evaluation and phylogenetic diversity. *Biol Conserv* 1992;61:1-10.
- Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A* 2007;104:15858-68.
- Woodruff PG, Modrek B, Choy DF, Jia J, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 2009;180:388-95.
- Bhakta NR, Solberg OD, Nguyen CP, Nguyen CN, Arron JR, Fahy JV, et al. A qPCR-based metric of Th2 airway inflammation in asthma. *Clin Transl Allergy* 2013;3:24.
- Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001;26:32-46.
- Pinheiro JC. Linear mixed effects models for longitudinal data, encyclopedia of biostatistics. Hoboken (NJ): John Wiley & Sons; 2005.
- Gerber GK, Onderdonk AB, Bry L. Inferring dynamic signatures of microbes in complex host ecosystems. *PLoS Comput Biol* 2012;8:e1002624.
- McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput Biol* 2014;10:e1003531.
- Kassahun W, Neyens T, Molenberghs G, Faes C, Verbeke G. Marginalized multilevel hurdle and zero-inflated models for overdispersed and correlated count data with excess zeros. *Stat Med* 2014;33:4402-19.
- Min Y, Agresti A. Random effect models for repeated measures of zero-inflated count data. *Stat Model* 2005;5:1-19.
- Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotech* 2013;31:814-21.
- Simpson JL, Daly J, Baines KJ, Yang IA, Upham JW, Reynolds PN, et al. Airway dysbiosis: *Haemophilus influenzae* and *Tropheryma* in poorly controlled asthma. *Eur Respir J* 2016;47:792-800.
- Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. *J Allergy Clin Immunol* 2014;133:388-94.
- Ursell LK, Clemente JC, Rideout JR, Gevers D, Caporaso JG, Knight R. The interpersonal and intrapersonal diversity of human-associated microbiota in key body sites. *J Allergy Clin Immunol* 2012;129:1204-8.
- Hogan SP, Waddell A, Fulkerson PC. Eosinophils in infection and intestinal immunity. *Curr Opin Gastroenterol* 2013;29:7-14.
- Travers J, Rothenberg ME. Eosinophils in mucosal immune responses. *Mucosal Immunol* 2015;8:464-75.
- Dollive S, Chen Y-Y, Grunberg S, Bittinger K, Hoffmann C, Vandivier L, et al. Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. *PLoS One* 2013;8:e71806.
- Peleg AY, Hogan DA, Mylonakis E. Medically important bacterial-fungal interactions. *Nat Rev Micro* 2010;8:340-9.
- Kloepfer KM, Lee WM, Pappas TE, Kang TJ, Vrtis RF, Evans MD, et al. Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. *J Allergy Clin Immunol* 2014;133:1301-7, e1-3.
- Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 2015;17:704-15.
- Tarkka MT, Sarniguet A, Frey-Klett P. Inter-kingdom encounters: recent advances in molecular bacterium-fungus interactions. *Curr Genet* 2009;55:233-43.
- Pana Y, Tenga D, Burke AC, Haase EM, Scannapieco FA. Oral bacteria modulate invasion and induction of apoptosis in HEP-2 cells by *Pseudomonas aeruginosa*. *Microb Pathog* 2008;46:73-9.
- Nagaoka K, Yanagihara K, Harada Y, Yamada K, Migiyama Y, Morinaga Y, et al. Macrolides inhibit *Fusobacterium nucleatum*-induced MUC5AC production in human airway epithelial cells. *Antimicrob Agents Chemother* 2013;57:1844-9.
- Araki N, Yanagihara K, Morinaga Y, Yamada K, Nakamura S, Yamada Y, et al. Azithromycin inhibits nontypeable *Haemophilus influenzae*-induced MUC5AC expression and secretion via inhibition of activator protein-1 in human airway epithelial cells. *Eur J Pharmacol* 2010;644:209-14.

41. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003;9:582-8.
42. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 2005;434:520-5.
43. Zajonc DM, Girardi E. Recognition of microbial glycolipids by natural killer T cells. *Front Immunol* 2015;6:400.
44. Özdemir O. Various effects of different probiotic strains in allergic disorders: an update from laboratory and clinical data. *Clin Exp Immunol* 2010;160: 295-304.
45. Abreu NA, Nagalingam NA, Song Y, Roediger FC, Pletcher SD, Goldberg AN, et al. Sinus microbiome diversity depletion and *Corynebacterium tuberculoostearicum* enrichment mediates rhinosinusitis. *Sci Transl Med* 2012; 12:151ra124.
46. Fujimura KE, Demoor T, Rauch M, Faruqi AA, Jang S, Johnson CC, et al. House dust exposure mediates gut microbiome *Lactobacillus* enrichment and airway immune defense against allergens and virus infection. *Proc Natl Acad Sci U S A* 2014;111:805-10.
47. Schuijs MJ, Willart MA, Vergote K, Gras D, Deswarte K, Ege MJ, et al. Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. *Science* 2015;349:1106-10.
48. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med* 2015;7:307ra152.
49. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Cross-talk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* 2015;17:662-71.
50. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* 2014;20:159-66.
51. Bostanci N, Akgül B, Tsakanika V, Allaker RP, Hughes FJ, McKay IJ. Effects of low-dose doxycycline on cytokine secretion in human monocytes stimulated with *Aggregatibacter actinomycetemcomitans*. *Cytokine* 2011;56:656-61.
52. Hirose M, Ishihara K, Saito A, Nakagawa T, Yamada S, Okuda K. Expression of cytokines and inducible nitric oxide synthase in inflamed gingival tissue. *J Periodontol* 2001;72:590-7.