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Authors

Durack, Juliana
Lynch, Susan V
Nariya, Snehal
et al.

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

Juliana Durack, PhD[1], **Susan V. Lynch, PhD**[1], **Snehal Nariya, BS**[2], **Nirav R. Bhakta, MD, PhD**[3], **Avraham Beigelman, MD**[4], **Mario Castro, MD**[4,5], **Anne-Marie Dyer, MS**[6], **Elliot Israel, MD**[7], **Monica Kraft, MD**[8], **Richard J. Martin, MD**[9], **David T. Mauger, PhD**[6], **Sharon R. Rosenberg, MD, MS**[10], **Tonya Sharp-King, PhD**[6], **Steven R. White, MD**[11], **Prescott G. Woodruff, MD, MPH**[3], **Pedro C. Avila, MD, MPH**[10], **Loren C. Denlinger, MD, PhD**[12], **Fernando Holguin, MD**[13], **Stephen C. Lazarus, MD**[3], **Njira Lugogo, MD**[14], **Wendy C. Moore, MD**[15], **Stephen P. Peters, MD**[15], **Loretta Que, MD**[14], **Lewis J. Smith, MD**[10], **Christine A. Sorkness, PharmD**[12], **Michael Wechsler, MD, MMSc**[9], **Sally E. Wenzel, MD**[13], **Homer A. Boushey, MD**[3], and **Yvonne J. Huang, MD**[2] **for the National Heart, Lung and Blood Institute's "AsthmaNet"**

[1]University of California, Dept. of Medicine, Division of Gastroenterology, San Francisco, CA

[2]University of Michigan, Division of Pulmonary & Critical Care Medicine, Ann Arbor, MI

[3]University of California, Dept. of Medicine, San Francisco, CA

[4]Washington University School of Medicine, Division of Pediatrics, St Louis, MO

[5]Washington University School of Medicine, Division of Pulmonary and Critical Care Medicine, St Louis, MO

[6]Penn State University, Department of Public Health Sciences, Hershey, PA

[7]Brigham & Women's Hospital, Dept. of Medicine, Boston, MA

[8]University of Arizona, Health Sciences, Tucson, AZ

[9]National Jewish Hospital, Dept. of Medicine, Denver, CO

[10]Northwestern University, Dept. of Medicine, Chicago, IL

[11]University of Chicago, Dept. of Medicine, Chicago, IL

[12]University of Wisconsin-Madison, Dept. of Medicine, Madison, WI

[13]The University of Pittsburgh Asthma Institute at UPMC/UPSOM, Pittsburgh, PA

[14]Duke University School of Medicine, Duke Asthma, Allergy & Airway Center, Durham, NC

[15]Wake Forest School of Medicine, Winston-Salem, NC

Abstract

Corresponding author: Homer A. Boushey, Professor of Medicine, Div. of Pulmonary/Critical Care Medicine, University of California, San Francisco, 505 Parnassus Avenue, M1292, Box 0130, San Francisco, CA 94143, Phone: 415-476-8019, Fax: 415-502-6235 homer.boushev@ucsf.edu.

Background—Compositional differences in 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—To compare the bronchial bacterial microbiota in adults with steroid-naive atopic asthma (AA), with atopy but no asthma (ANA), and non-atopic healthy subjects (HC), and determine relationships of bronchial microbiota to phenotypic features of asthma.

Methods—Bacterial communities in protected bronchial brushings from 42 AA, 21 ANA, and 21 HC subjects were profiled by 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 following six weeks of fluticasone treatment.

Results—The bronchial microbiome differed significantly among the three groups. Asthmatic subjects were uniquely enriched in members of the *Haemophilus, Neisseria., Fusobacterium, Porphyromonas* and *Sphingomonodaceae,* and depleted in members of the *Mogibacteriaceae and Lactobacillales.* 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 following fluticasone treatment. Steroid-responsiveness was linked to differences in baseline compositional and functional features of the bacterial microbiome.

Conclusion—Even in mild steroid-naive asthma subjects, 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.

Keywords

Asthma; atopy; microbiome; corticosteroids; 16S ribosomal RNA; bacteria; Th2 inflammation; Three-gene mean; metabolic pathways; SCFAs

Introduction

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 hyper-responsiveness, asthma control, and transcriptional response to steroids, correlate with patterns of bronchial microbiota composition (4, 5). Though different studies have reported asthma-associated enrichment (higher relative abundance) of different taxa [i.e. bacterial-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 (ICS), 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 as to whether asthma- associated differences in respiratory

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

Accordingly, we compared the bronchial bacterial microbiome among adults with mild steroid-naive atopic asthma, with atopy without asthma, and healthy non-atopic nonasthmatic controls. 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 Th2 inflammation and responsiveness to ICS treatment.

Methods

Study Population and Sample Collection

This study was conducted at nine sites in the NHLBI AsthmaNet, using a standardized bronchoscopy protocol for sample collection. Of 186 adults screened, 84 subjects were enrolled (Figure S1A-B): 42 atopic asthmatics (AA), 21 atopic non-asthmatics (ANA) and 21 non-atopic healthy control subjects (HC). Atopy was defined by serologic evidence (>0.35 kU/l) of sensitivity to >1 of 12 aeroallergens (specific IgE by ImmunoCap; Thermo-Scientific; Table S1). Asthma was confirmed by airway hyperresponsiveness (methacholine PC_{20} ≤8 mg/mL or FEV-i improvement >12% postalbuterol). At enrollment, asthmatics had been clinically stable for three months, and had an Asthma Control Questionnaire (ACQ) score of <1.5 (11) without the use of a controller medication. Exclusion criteria included a history of smoking, respiratory infection within six weeks or antibiotic use within 3 months of enrollment (Supplementary Methods).

Samples processed for microbiota analysis included oral wash (OW) (12), a saline flush (10 mL) of the bronchoscope suction channel (“scope-flush”) and protected bronchial brushings (BB; Figure S1C; Supplemental Methods). AA were further randomized in a 2:1 ratio to treatment with inhaled fluticasone propionate (250 mcg, GlaxoSmithKline) or placebo twice daily for six weeks and re-assessed post-treatment. Each subject signed informed consent approved by their center’s IRB; an NHLBI-appointed Data Safety Monitoring Board (DSMB) oversaw the study conduct.

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

Nucleic acids from OW, and 3 BB were extracted as previously described (5, 12) using a modified bead-beating protocol and the AllPrep kit (Qiagen). 16S rRNA gene copy number was assessed by quantitative PCR using universal primers (Supplemental Methods).

16S rRNA-based sequencing and raw data processing

For BB and OW samples, variable region 4 (V4) of the 16S rRNA gene was amplified using the primer combination 515F/806R (13, 14) and sequenced on the Illumina MiSeq platform. Using chimera-checked and quality-filtered sequence reads (Supplemental Methods), a 97%

sequence homology cut-off was used to define bacterial taxa (also referred to as operational taxonomic units; OTUs) and classified using the Greengenes database (15). A phylogenetic tree was built using FastTree (16) and used to compute Faith's Phylogenetic Diversity (17) of samples using an OTU table multiply-rarefied to 52,317 sequences per sample (Supplemental Methods).

"Three-gene mean" bronchial epithelial signature of type 2 inflammation

Expression levels of three bronchial epithelial genes *(CLCA1, SERPINB2 and POSTN)* previously shown to be induced by IL-13 (18, 19), were measured using RNA extracted in parallel with DNA in this study to calculate the "three-gene mean" (TGM) score for each participant (20). Type 2 (T2) - high asthma was defined by TGM scores >1.117 (two standard deviations above the average TGM-score in HC), confirmed by unsupervised cluster and principal component analysis.

Statistical analyses

We applied principal coordinates analyses (PCoA) on an unweighted UniFrac distance matrix and PERMANOVA (21) to identify the determinants of variation in bacterial community beta-diversity. Linear mixed-effects model (LME) (22) was employed to examine relationships between paired OW and BB microbiota; negative binomial (NB) and zero-inflated negative binomial (ZINB) regression models (23-26) corrected for false discovery (Benjamini-Hochberg, q-value <0.1) 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 microbiota (27), and NB regression to compare inferred functional pathway predictions across groups. Procrustes analysis (13) was used to explore the strength of relationships between paired samples in the AA group (Supplemental Methods).

Results

Study group characteristics

AA had mild well-controlled disease, significantly higher serum total IgE and blood and sputum eosinophil cell counts (Table 1, Figure S2A-C) than HC. Compared to the ANA, the asthmatics had significantly higher serum IgE, were sensitized to more of the aeroallergens tested (Table 1), were more likely to be sensitive to cat, dog, and mouse (Table S1), and to report a history of allergic rhinitis and eczema (Table S2). However, they did not exhibit significant differences in environmental exposures as assessed by questionnaire (Table S3).

Bacterial microbiota in bronchial brushings are compositionally distinct from oral wash

To evaluate mucosa-associated bronchial microbiota, we focused on protected BB. Sequence-based bacterial community analysis could be performed in the same proportion (67%) of samples collected in each of the three groups (Figure S1D-E). Samples that could not be sequenced had lower bacterial burden as indicated from 16S rRNA ($p<0.0001$; Figure S2D), despite recovery of similar mammalian cell burden as assessed by quantifyingβ -actin copy number (Supplemental Methods). Subjects with insufficient 16S rRNA amplicon for

microbiota profiling were younger [median 28 (22-37) vs. 34 yrs. (25-44); p=0.03] but did not differ in any other characteristic measured.

Potential oral contamination of BB was evaluated by comparing a random subset of 30 BB-paired scope-flushes. Bacterial burden of scope-flushes for BB with sufficient 16S rRNA for sequencing was indistinguishable from those unable to be sequenced ($p>0.1$; Figure S2E). Additionally, PCoA analysis of bacterial community composition in paired OW and BB samples showed these two niches to be compositionally distinct (LME $\beta=0.31$, $p<0.0001$; Figure 1A), with BB samples exhibiting lower phylogenetic diversity (Faith's index; $p<0.0001$; Figure 1B).

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

Alpha-diversity indices, such as richness (number of observed taxa), Shannon's diversity, and evenness, as well as bacterial burden did not differ amongst the groups (Figure S2F-I). However, phylogenetic diversity (Faith's index) tended to be higher in AA compared with HC ($p=0.06$; Figure 1C). Since this index weights phylogenetic relatedness of the bacteria detected, this observation suggests that the bronchial airways of AA harbor more phylogenetically diverse bacterial communities. Although phylogenetic diversity in the ANA also appeared to be greater than in HC (Figure 1C), the difference fell short of significance. Overall, inter-subject bacterial community composition (beta-diversity) was highly heterogeneous across subjects (Figure S3A). This was significantly related to bacterial richness independent of the study group (Figure S3B-E), but was not associated with any of the clinical measures or environmental exposures evaluated in this study (Tables 1 and S1-3). Compositional variability in bronchial bacterial microbiota was significantly greater within the asthmatic group (weighted UniFrac distance F-test $p<0.001$; Figure S3F), indicating a greater degree of bacterial community heterogeneity in this group.

Following these initial assessments for differences in overall bacterial community composition, we conducted OTU-level analyses to determine if the relative abundance of specific taxa differed significantly across groups; 76 taxa were found to do so in the AA vs HC comparison (Table S4). Phylum-level differences in AA (Figures 2A and S4A) included significant enrichment for members of the *Bacteroidetes (Prevotella), Fusobacteria (Fusobacterium), Actinobacteria (Actinomyces)* and *Proteobacteria (Haemophilus* and *Neisseria),* 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), Proteobacteria (Actinobacillus),* and *Firmicutes (Lactobacillus*). ANA subjects also demonstrated significant differences in the relative abundance of 100 taxa compared to HC (Table S5B). The taxa most relatively enriched in ANA included members of the *Proteobacteria (Aggregatibacter, Haemophilus*), *Firmicutes (Granulicatella)* and *Actinobacteria (Corynebacterium)* phyla (Figures 2B and S4B); those depleted included members of the phylum *Bacteroidetes (Porphyromonas* and *Prevotella).*

We noted that the ANA shared 26% and 29% of taxa that were also relatively enriched or depleted in the AA, when compared to HC. Despite these similarities, OTU-level analysis identified 103 taxa that differed significantly between the AA and ANA groups (Table S6;

Figures 2C and S4C). To pinpoint bacteria discretely associated with asthma, we evaluated which taxa among those that distinguished AA from HC, also distinguished AA from ANA (Figure 3). By this approach, asthma-associated taxa included members of the *Haemophilus* (OTU4406393), *Fusobacterium* (OTU4405869; OTU2438396), *Neisseria* (OTU1304), *Porphyromonas* (OTU495451) and *Sphingomonodaceae* (OTU8331815); while taxa negatively associated with asthma included members of the *Mogibacteriaceae* (OTU4335578) *and Lactobacillales* (OTU4480189; OTU4469032). The same approach also identified taxa specific to atopy- alone, which included enrichment in *Aggregatibacter* (OTU4432431), *Corynebacterium* (OTU1015518; OTU495067) and *Prevotella* (OTU4372058; OTU134265) (Figure 3). Of the taxa identified as specifically associated with either asthma or atopy-alone, a subset exhibited strong, significant correlations with features of atopy (IgE, blood and sputum eosinophil counts); these associations were distinct in the two atopic groups. For example, specific taxa uniquely enriched in AA, belonging to *Sphingomonodaceae* and *Fusobacteria,* were positively associated with all three markers of atopy, while of those specifically enriched in ANA, *Sharpea* and *Prevotella* (OTU1052181; OTU134265) correlated with IgE and blood eosinophil counts, respectively (Figure 3). These data indicate that while taxonomic overlap exists between AA and ANA subjects, 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 three groups using PICRUSt (27), an algorithm that predicts bacterial metagenomes *in silico* from 16S rRNA sequence. This analysis revealed significant differences across groups (Figure 4). Predicted bacterial gene functions enriched in AA 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 lipopolysaccharide biosynthesis among asthma-associated bacterial communities.

Type 2-high asthma is associated with reduced bronchial bacterial burden

TGM scores were higher in AA (Figure 5A) and, consistent with prior reports (20, 29), correlated positively with serum IgE ($r_{spearman}$=0.36, p<0.01), blood and sputum eosinophil counts (r_S=0.32 and r_S=0.37, respectively, p<0.01), and negatively with FEV-i (r_S= -0.45, p<0.005) and PC_{20} (r_S= -0.41, p<0.005). Ten of 40 AA had T2-high asthma and exhibited significantly higher ACQ scores, serum IgE, blood and sputum eosinophil counts, and younger age, than T2-low asthmatics (Table S7). T2-high asthmatics demonstrated significantly lower bronchial bacterial burden than T2-low asthmatics (Figure 5C); bacterial burden also was negatively correlated with TGM scores across all asthmatic subjects (r_S= -0.43, p<0.01) independent of age (GLM p=0.03). Because of their low bacterial burden BBs from only four T2-high asthmatics, could be sequenced for profiling, an insufficient sample size to allow meaningful assessment of differences in bacterial microbiota composition between T2-high and T2-low subjects.

Inhaled corticosteroid-responsiveness is associated with distinct features of the bronchial bacterial microbiota present before treatment

We hypothesized that differences in baseline airway microbiota characteristics may be associated with ICS-responsiveness, defined as ≥2-fold increase in PC_{20}Mch after ICS-treatment. Of the asthmatics included, 15 were classified as ICS-responders, 10 as non-responders, and three were excluded as PC_{20} was not performed (Figure S5A-B). Compared to non-responders, ICS-responders did not differ in PC_{20}, serum IgE, blood and sputum eosinophil counts, or bronchial burden at baseline but did have lower FEV-i and a trend toward a higher TGM score ($p<0.08$) (Table S8; Figure S5C), which decreased following treatment ($p<0.05$; Figure S4D).

The baseline composition of profiled bronchial bacterial microbiota in ICS-responders (n=10) differed from that in the non-responders (n=5; unweighted UniFrac PERMANOVA $R^2=0.13$, $p=0.01$), and was significantly more similar to that of HC (Figure 6A). Bacterial families enriched at baseline in ICS non-responders included *Microbacteriaceae, Pasteurellaceae* (e.g. asthma-associated *Haemophilus* OTU4406393) and several others (Figure 6C and Table S9). Conversely, bacterial families enriched at baseline in ICS-responders included *Streptococcaceae, Fusobacteriaceae and Sphingomonodaceae.* Compared with ICS-responders, the predicted functions of bacterial communities of non-responders were enriched in xenobiotic biodegradation pathways (Figure 6D and Table S10), implicating potential enhanced capacity for synthetic chemical degradation.

Following six weeks of ICS vs. placebo treatment, we found no significant changes in bacterial burden or phylogenetic diversity in the AA group (Figure S6A-B). Bacterial community analysis in paired samples before and after treatment was limited, as 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 non-responders and ICS-responders (2) and explored ICS-induced compositional changes in the latter, more predominant group. No significant differences were seen in the magnitude of changes (beta-diversity assessed by unweighted Unifrac distance) between paired samples (n=8 each in placebo or ICS-treated responders; Figure S6C-E). At the taxon level, however, ICS treatment resulted in increased relative abundance of *Microbacteriaceae, Neisseria* and *Moraxella* and depletion of a specific *Fusobacterium,* which was not observed with the placebo treatment (Figure 6D, Tables S11-12). We unfortunately could not analyze compositional changes in ICS-non- responders, as only 2 subjects in this subgroup had sufficient 16S rRNA in both pre- and post- treatment samples.

Discussion

Our findings show compositional and predicted functional differences in the bronchial bacterial microbiomes of atopic asthmatic, atopic non-asthmatic, and healthy individuals. 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 were discretely associated with asthma. Analyses based on metagenomic inference further suggest

that genes for pathways involved in the metabolism of short- chain fatty acids and amino acids are enriched in the asthmatic bronchial microbiome. We additionally observed that the bronchial microbiome among asthmatics at baseline differed compositionally and functionally according to their responsiveness to treatment with inhaled corticosteroids.

Commonly reported microbial community metrics (e.g., richness, evenness, and burden) did not differ significantly among our three groups. This is not unexpected due to the broad characterization of microbial composition provided by such measures, the inter-individual heterogeneity of microbiota composition found in all human niches, including the lung (30), 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 T2-low asthma subjects, in whom bronchial bacterial burden was significantly greater than in T2-high subjects. The trend towards higher bacterial phylogenetic diversity in our mild asthmatic subjects 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, in clearance of microorganisms, or in other immune function parameters not identified here.

Reasons for the low bronchial bacterial burden in T2-high asthma are unclear, but the finding echoes a recent observation in 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 T2-high airway inflammation. Given prior reports of inverse relationships between bacterial and fungal richness (33, 34), the low bacterial burden in T2-high asthma could also reflect increased fungal or bacteriophage burden. Indeed microbial interactions are not limited to bacteria, as bacterial-viral interactions have been associated with asthma exacerbations and risk for asthma development (35, 36). Such inter-kingdom interactions (37) 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 (e.g. *Haemophilus and Neisseria)* among our ICS-naïve asthmatics resembles prior findings in ICS-using patients (2-6, 28). However, we found other taxa discretely associated with asthma including *Fusobacterium* and *Porphyromonas,* two oral-associated anaerobes capable of augmenting pathogenic behavior of opportunistic respiratory pathogens, such as *Pseudomonas aeruginosa* (38). Additionally, both *Fusobacterium* (39) and *Haemophilus* (40) induce MUC5AC expression in bronchial epithelial cells. *Sphingomonodaceae* represented another asthma-specific taxon whose abundance, like that of *Fusobacterium*, correlated with sputum eosinophilia. This finding echoed the reported activity of glycosphingolipids (present in the cell membrane of *Sphingomonodaceae)* in activating NKT-cells to produce T2-cytokines (41-43) 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* strains against atopy by various mechanisms including alteration of gastrointestinal (44) and respiratory tract permeability (45, 46). Our finding that the predicted functions of asthma-associated microbiota were relatively depleted in machinery for LPS biosynthesis suggests another possibility, for continuous stimulation of airways with LPS has been shown to suppress T2 immune activation (47). We propose, though, that any "pro-asthmatic" 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 asthma 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 utilization of anti-inflammatory SCFAs by members of the asthmatic airway microbiota may contribute to atopic asthma by reducing their bioavailability and the consequent capacity to down-regulate host inflammatory responses to aeroallergens and pathogens.

Microbiome-related functions might also affect responsiveness to corticosteroid treatment. We found that pre-treatment enrichment in an asthma-associated *Haemophilus,* a genus with species previously shown to reduce response of BAL macrophages to corticosteroids (2), was associated with diminished response to six weeks of treatment with inhaled fluticasone. Analysis of the predicted metagenome of pre-treatment bronchial microbiota present in ICS-non-responders also indicated enhancement of xenobiotic degradation capacity, which we hypothesize may contribute to their diminished response. In contrast, ICS-induced changes to the bronchial microbiota in ICS-responsive asthmatics showed enrichment of previously reported asthma-associated taxa such as *Neisseria* and *Moraxella* (2, 3, 6). Community shift detected in response to lactose-containing placebo inhaler was not surprising, as 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 utilize 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 is also a novel finding of this study. ANA subjects were less sensitized than the AA subjects and showed primarily low T2 inflammation of bronchial epithelium. While the greater severity of allergy in the AA group cannot be ruled out as contributing to the difference in bacterial signature observed between AA and ANA subjects, 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 two patient groups. These observations in ANA subjects 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).* Members of *Aggregatibacter* are associated with periodontal disease driven by high pro-inflammatory cytokines (e.g., TNF-a, IL1B, IL-6 and IL-8;) and reduced levels of IL-10 (51, 52). It is tempting to speculate that airway enrichment of this genus in ANA subjects 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 (e.g., *Prevotella* and *Haemophilus)*

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 non-atopic 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 vs. 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 non-bronchial sources of bacterial DNA to the dataset.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	Atopic asthmatic subjects
ANA	Atopic non-asthmatic subjects
HC	Healthy controls
ICS	Inhaled corticosteroid
ACQ	Asthma Control Questionnaire
OW	Oral wash
BB	Bronchial brush
OUT	Operational taxonomic unit
SCFAs	Short chain fatty acids
TGM	Three-gene mean
PICRUSt	Phylogenetic Reconstruction of Unobserved States
KEGG	Kyoto Encyclopedia of Genes and Genomes

Key messages

- The bronchial bacterial microbiota of both mild atopic-asthma (steroid-naïve) and atopy-alone differ from that of healthy controls and also differ from each other.
- Asthma is associated with enrichment in members of the Haemophilus, Neisseria, Fusobacterium, Porphyromonas and Sphingomonodaceae and with depletion of Lactobacillus.
- 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 prior to steroid exposure.

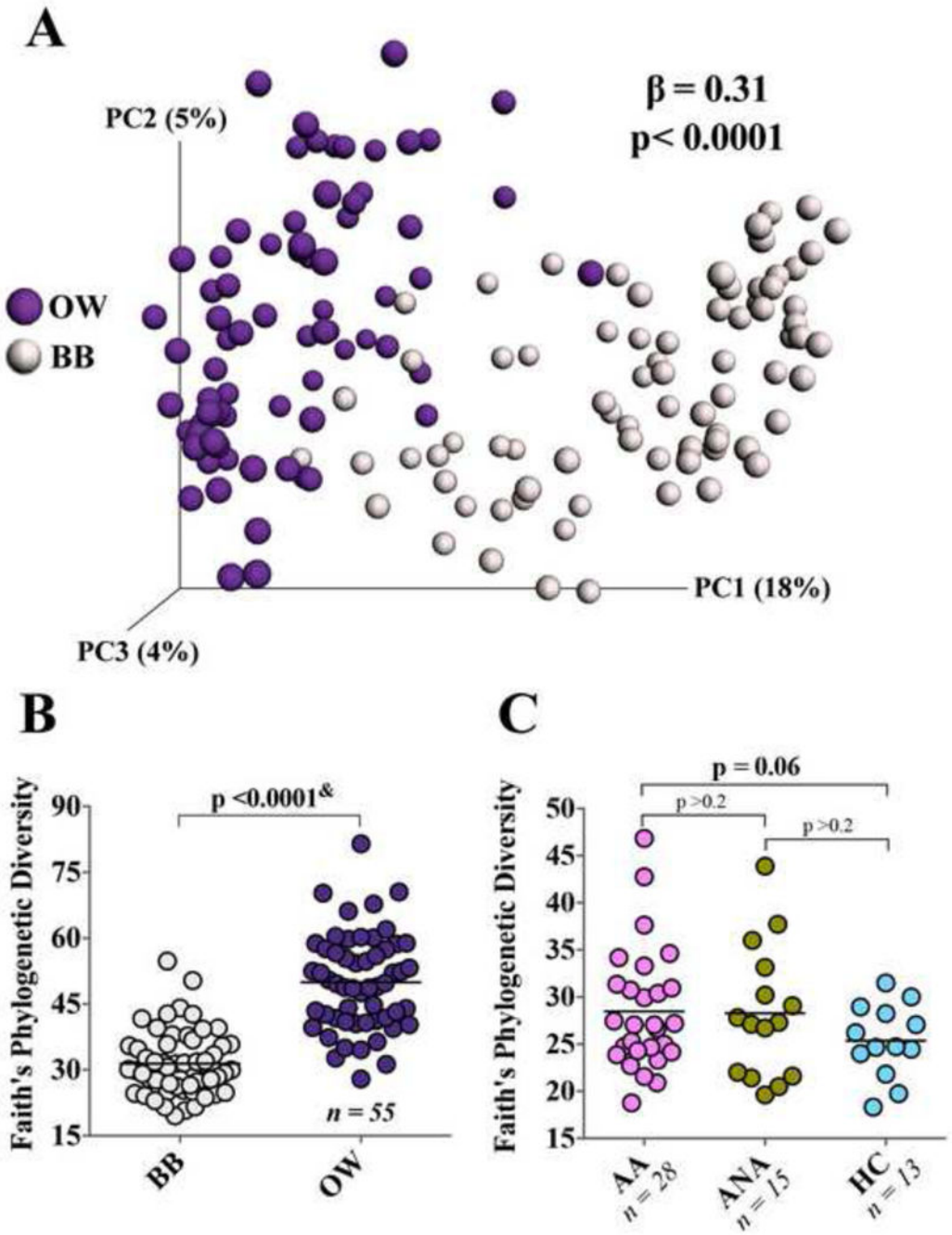

Figure 1.

(A) Principal coordinate analysis (unweighted UniFrac) shows compositional dissimilarity between paired BB and OW samples (LME p<0.0001). **(B)** Phylogenetic diversity (Faith's index) in BB and paired OW samples (& Wilcoxon matched-pairs signed rank test). **(C)** Phylogenetic diversity (Faith's index) in BB samples for the three subject groups (Welch's corrected t-test).

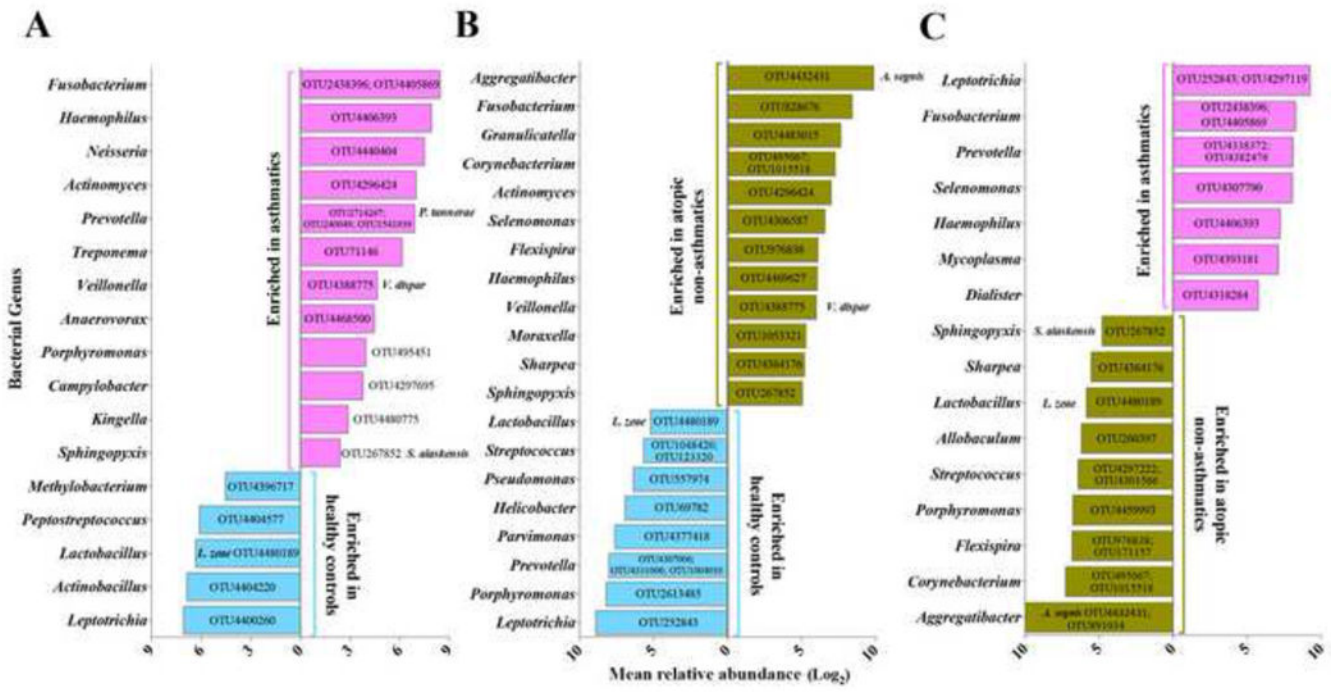

Figure 2.

Bacterial taxa, significantly enriched or depleted in relative abundance (at least 2 fold; NB regression, q<0.1) in **(A)** AA (n=28) compared to HC (n=13); **(B)** ANA (n=15) compared to HC; **(C)** AA compared to ANA. The OTUs indicated represent the most abundant representatives within the indicated genus.

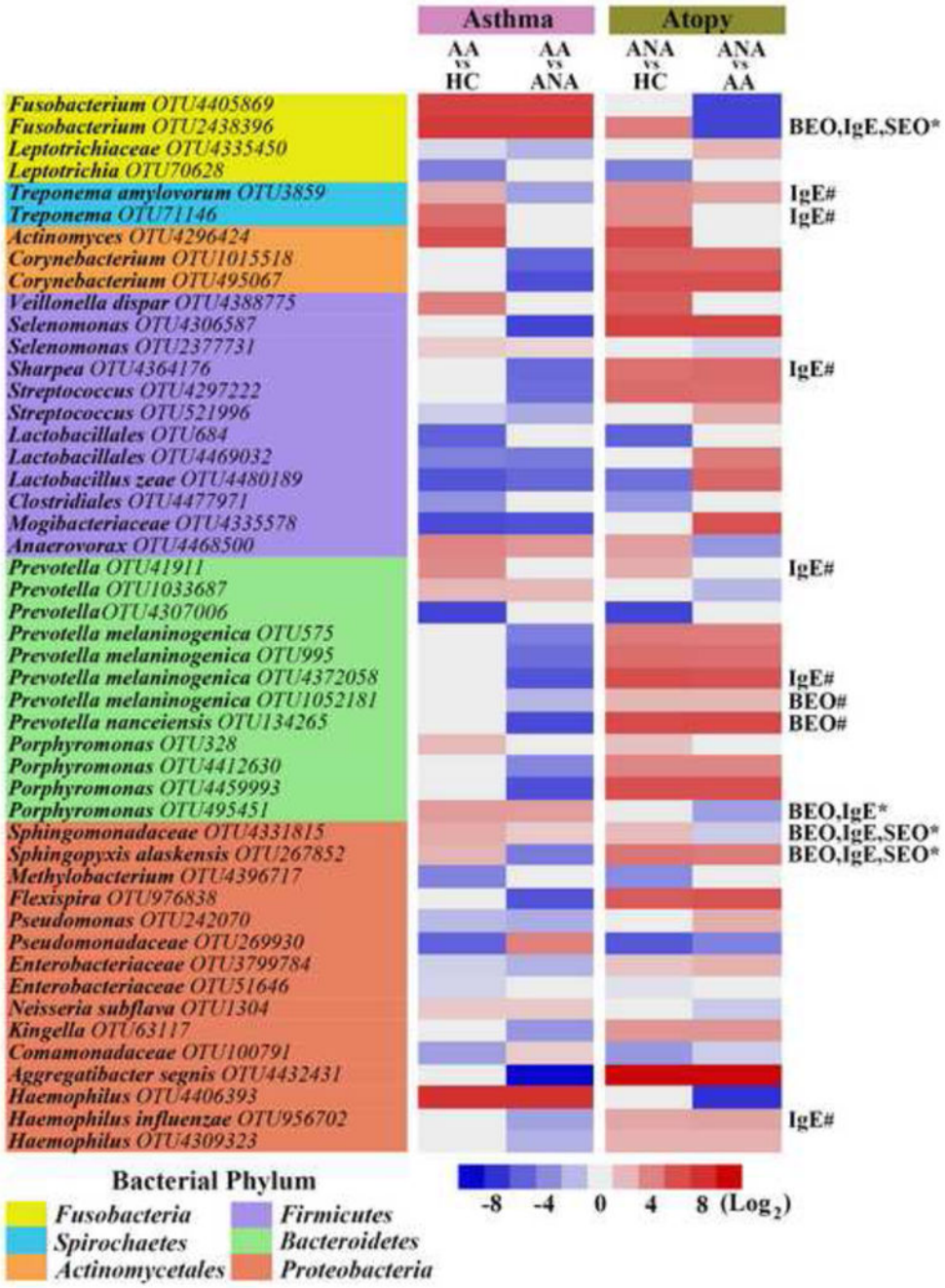

Figure 3.

Mean difference in specific bacterial taxa between groups. Asthma-specific taxa are similarly abundant in AA compared to HC and ANA (NB regression, $q<0.1$). Atopy-only taxa are similarly abundant in ANA vs. both HC and AA. Taxa positively correlated ($r_{person} \geq 0.5$, $q<0.1$) with blood eosinophil counts (BEO), serum IgE (IgE) or sputum eosinophil counts (SEO) in AA (*) or ANA (#) subjects.

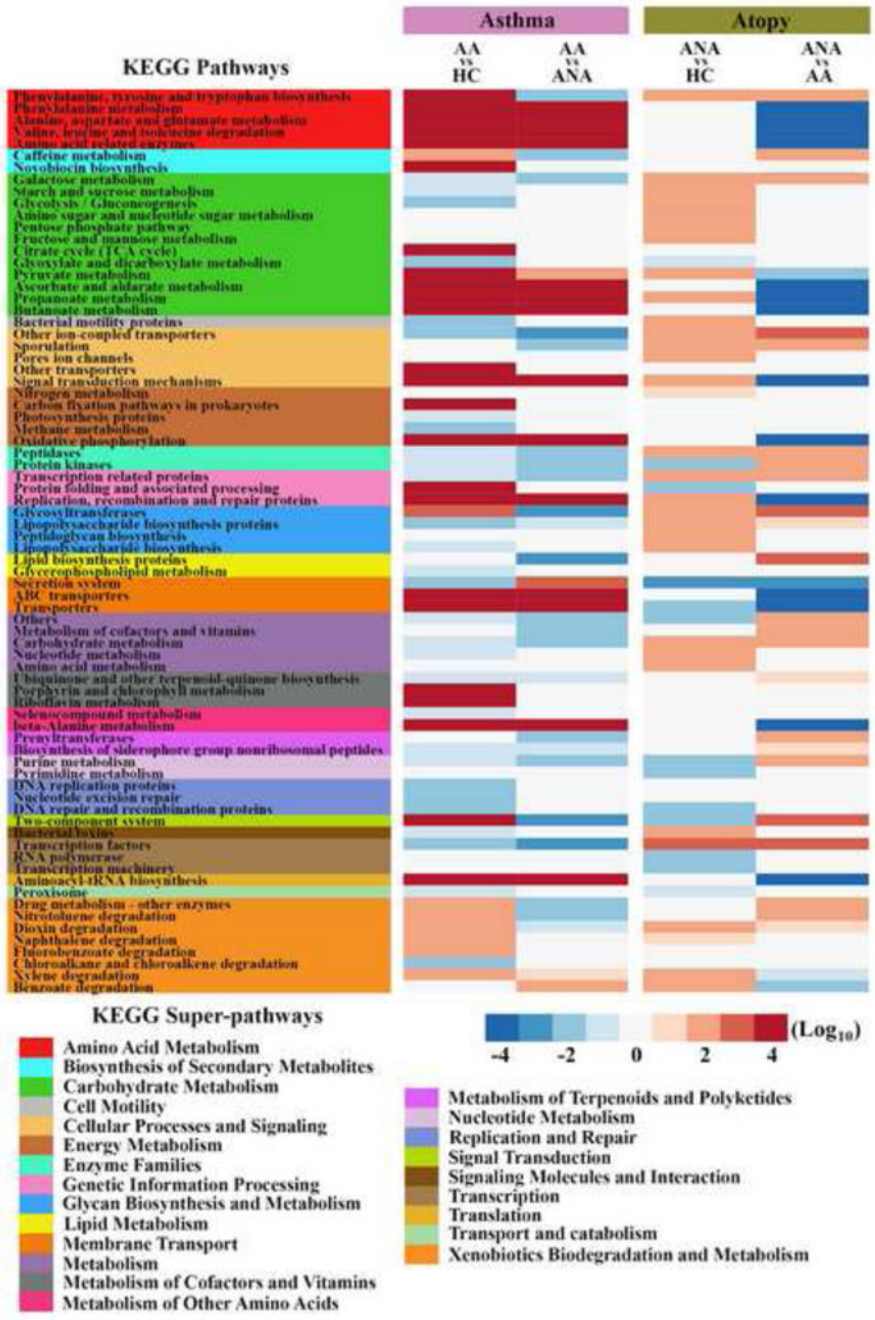

Figure 4.

Mean difference in predicted KEGG orthologs (KOs) between groups. Asthma- specific pathways are similarly abundant in AA compared to HC and ANA. Atopy-only pathways are similarly abundant in ANA compared to HC and AA. Statistical significance was determined using NB regression model corrected for false discovery rate ($q<0.1$).

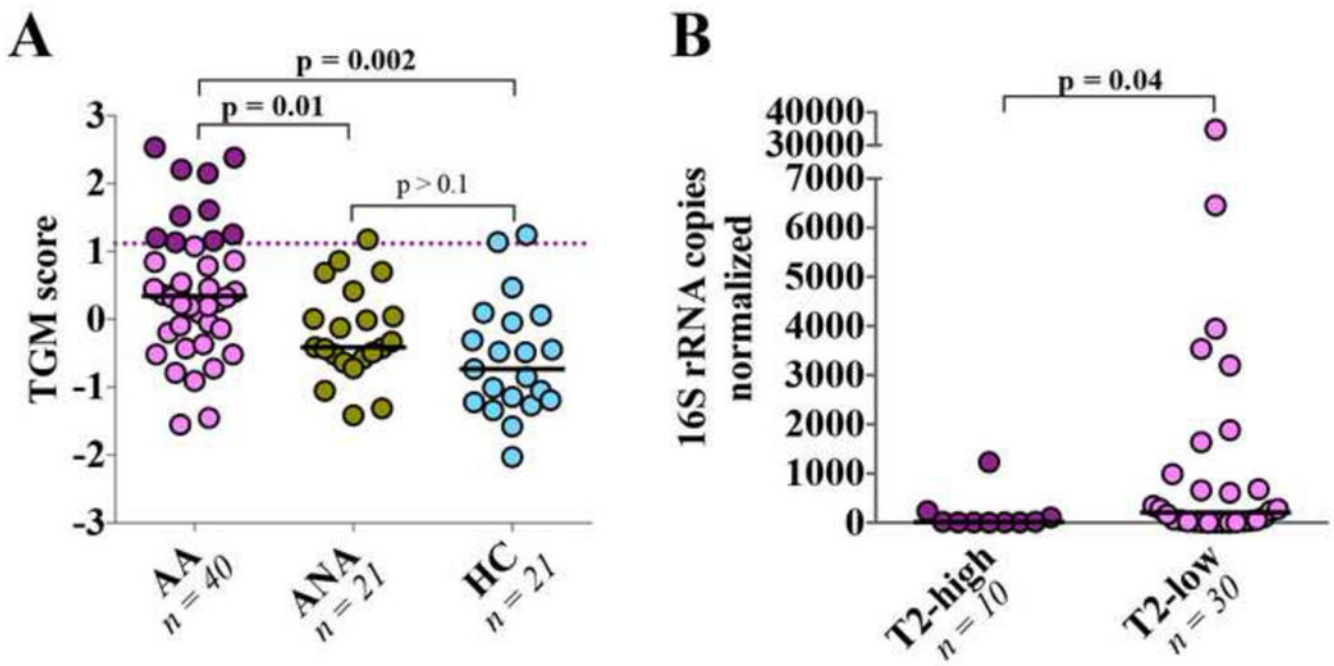

Figure 5.

(A) AA displayed greater expression of epithelial genes induced by type 2 cytokines compared to non-asthmatic subjects. T2-high AA, with a TGM >1.117 (cut-off value indicated by a dashed line) are colored in maroon red. **(B)** Significantly lower bacterial burden was observed among T2-high asthma subjects. Statistical significance was determined using Wilcoxon rank sum test.

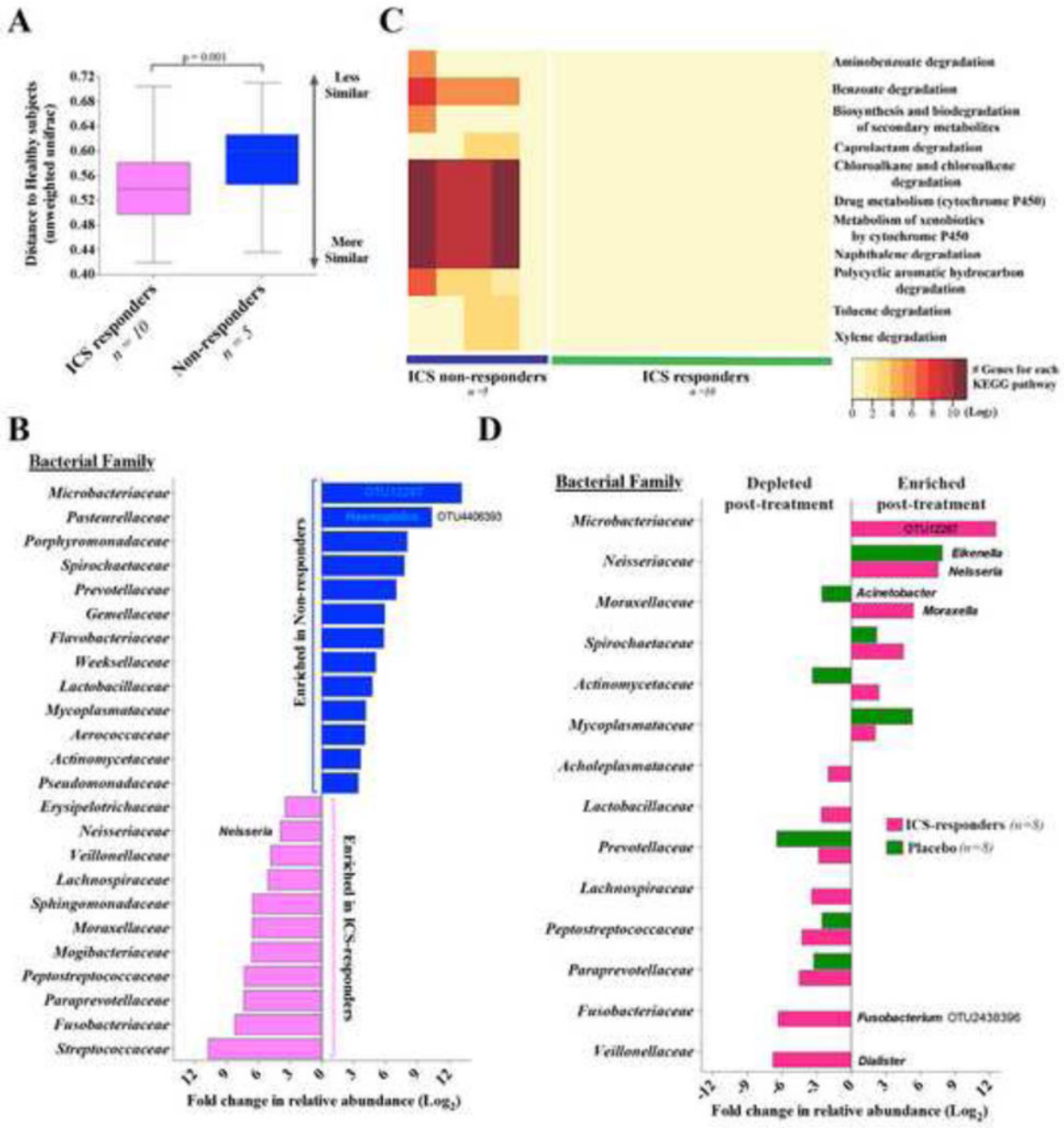

Figure 6.

(A) Mean distance to HC (unweighted UniFrac; Bonferroni-corrected t-test). **(B)** Taxa significantly enriched or depleted in relative abundance (at least 2 fold; NB regression, q<0.1) in ICS-responders vs. non-responders. **(C)** Predicted KEGG pathways associated with xenobiotic biodegradation and metabolism in ICS-responders and non-responders. (D) Taxa, differentially expressed (at least 2-fold; ZINB regression, q<0.1) in asthmatics following ICS or placebo treatment.

Table 1

Study cohort characteristics

Variable	Atopic asthmatic (AA) (n = 42)**	Atopic, non-asthmatic (ANA) (n = 21)	Healthy control (HC) (n = 21)	p-value#
Age (yrs)	33 (25 - 41)	28 (24 - 45)	28 (23 - 47)	NS
Age of asthma diagnosis (yrs)	9 (5 - 22)	-	-	-
Duration of Asthma (yrs)	21 (14 - 27)	-	-	-
ACQ Score*	0.3 (0 - 1.3)	-	-	-
% Male	45%	48%	43%	NS€
% White	60%	52%	71%	NS€
BMI (kg/m^2)	26 (23 - 29)	25 (21 - 28)	26 (22 - 28)	NS
FEV_1 % predicted (pre-albuterol)	89 (76 - 97)	98 (89 - 109)	100 (93 - 108)	**<0.01**&
FEV_1 % predicted (post-albuterol)	100 (84 - 106)	104 (97 - 109)	102 (98 - 116)	NS&
Change in FEV_1%	7.5 (5.0 - 14.3)	3.0 (-0.5 - 5.5)	5.0 (2.5 - 5.0)	**<0.0001**&
Methacholine PC_{20}	1.2 (0.3 - 3.2)	>32$	>32$	-
Blood eosinophils (absolute)	200 (100 - 393)	100 (87 - 200)	100 (60 - 200)	**<0.01**
Blood eosinophils (%)	3.3 (1.5 - 5.6)	2.0 (1.4 - 3.0)	1.4 (1.0 - 3.0)	**<0.01**¥
Blood neutrophils (%)	55.5 (49.3 - 62.0)	56.9 (51.0 - 63.8)	58.4 (52.7 -63.5)	NS
Sputum neutrophils (%)	54.6 (31.8 - 64.9)	39.5 (26.5 - 50.0)	41.8 (29.2 -76.5)	NS
Sputum eosinophils (%)	0.4 (0.0 - 1.1)	0.0 (0.0 - 0.6)	0.0 (0.0 - 0.4)	NS¥
Serum IgE (IU/mL)	169.5 (56.3 - 321.3)	64.0 (22.0 - 164.5)	15.0 (5.0 -31.0)	**<0.01**&¥
Number of positive sIgE¢	6 (2-7)	3 (2-5)	-	**<0.05**&

All values are medians (IQR).

*ACQ, Asthma Control Questionnaire

**Number of exacerbations requiring oral steroids in the past 5 years: zero exacerbations (38 subjects); one exacerbation (3 subjects); two exacerbations (one subject).

$Methacholine challenge was stopped at 32 mg/dL and PC_{20} for these subjects was censored.

¥For between group statistical comparisons see Supplementary Figure S2A-C.

¢Number of positive specific IgE (sIgE >0.35 kU/l) from a total of 12 aeroallergens tested by ImmunoCap assay (for breakdown of specific aeroallergens see Supplementary Table S1). Statistical significance was determined using

#Kruskal-Wallis or

€Chi-square or

&Mann-Whitney test for AA vs. ANA comparison.