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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Family-based study reveals decreased abundance of sputum *Granulicatella* in asthmatics

To the Editor,

The search for a bacterial signature in asthma is ongoing.^{1,2} One potential limitation of existing studies is the case-control

study design may not adequately control for baseline variation in the bacterial composition between individuals, which may lead to attenuated association results. Family members generally have relatively similar bacteria composition.^{3,4} It is, therefore, advantageous to adopt a family-based study design to reduce

Abbreviations: FEV1, forced expiratory volume in 1 second; rRNA, ribosomal RNA.

interfamilial variation and identify novel asthma-associated microbes.

Microbial exposure in early life determines bacteria colonization.^{5,6} As Firmicutes and Actinobacteria dominate in the indoor environment, a more similar pattern of airway bacteria from these two phyla is expected among family members, especially siblings, compared to nonfamily members.⁷ Compositional discrepancies between siblings with and without asthma may indicate a disease-specific microbe.

Here, we conducted a preliminary family-based study. The bacterial phyla Firmicutes and Actinobacteria from induced sputum were evaluated using 16S ribosomal RNA (rRNA) sequencing to compare the relative abundance of different bacteria genera between asthmatic and nonasthmatic sibling(s). Our study identified that the relative abundance of *Granulicatella* is decreased in asthmatic siblings compared to nonasthmatic siblings across all three families.

This study has been approved by Yale University Human Research Protection Program. Fourteen subjects from three two-generation pedigrees were recruited (Figure S1). Each participating family had both asthmatic and nonasthmatic offspring. Enrolled subjects were invited to the Yale-New Haven Hospital Research Unit for sputum induction and collection. The induced sputum sample was incubated with storage reagent and then extracted and subjected to 16S rRNA sequencing. Sequence data were trimmed, filtered for quality, and clustered at 97% identity into operational taxonomic units. Only bacterial sequencing from phyla Firmicutes and Actinobacteria (>98% in total sequencing reads) was retained for analysis. The bacteria profiling is shown in Figure S2. The α -diversity of each microbial community was calculated and compared by family and by

asthma status. UniFrac distance between the sputum bacterial communities of each subject pair was generated. The comparison of genus percentage between asthmatic and healthy siblings within each family was analyzed. The significance across the three families was then evaluated. Detailed methods are in Supporting information.

The characteristics of subjects are summarized in Table S1. Compared with nonasthmatics, asthmatics have higher BMI and lower percentage of predicted forced expiratory volume in 1 second (FEV1). As shown in Figure 1A,B, the α -diversity (Shannon's index) did not significantly differ by family or asthma status. However, the α -diversity tended to have higher variation and lower values, in general, for asthmatic compared to nonasthmatic subjects. This observation indicates that the sputum microbial community in asthmatics has been disturbed and is prone to harbor less diverse gram-positive bacteria. UniFrac distances between the bacterial communities of participating subjects were calculated. The mean distance of each individual with all subjects within the same family and the mean distance with all subjects outside that family are plotted in Figure 1C. In two of the three families (Family 1 and Family 3), family members have more similar sputum bacterial composition than nonfamily members. Asthmatics exhibit the same trend with other nonasthmatic family members.

Next, we focused on the discordant siblings in each household and conducted a genus-level analysis to identify bacteria that displayed differences in abundance between the asthmatic and nonasthmatic sibling(s). We identified eight genera whose average relative abundance is greater than 1% among offspring. As shown in Figure 2 and Table S2, two genera, *Granulicatella* and *Veillonella*, have concordant direction of association with

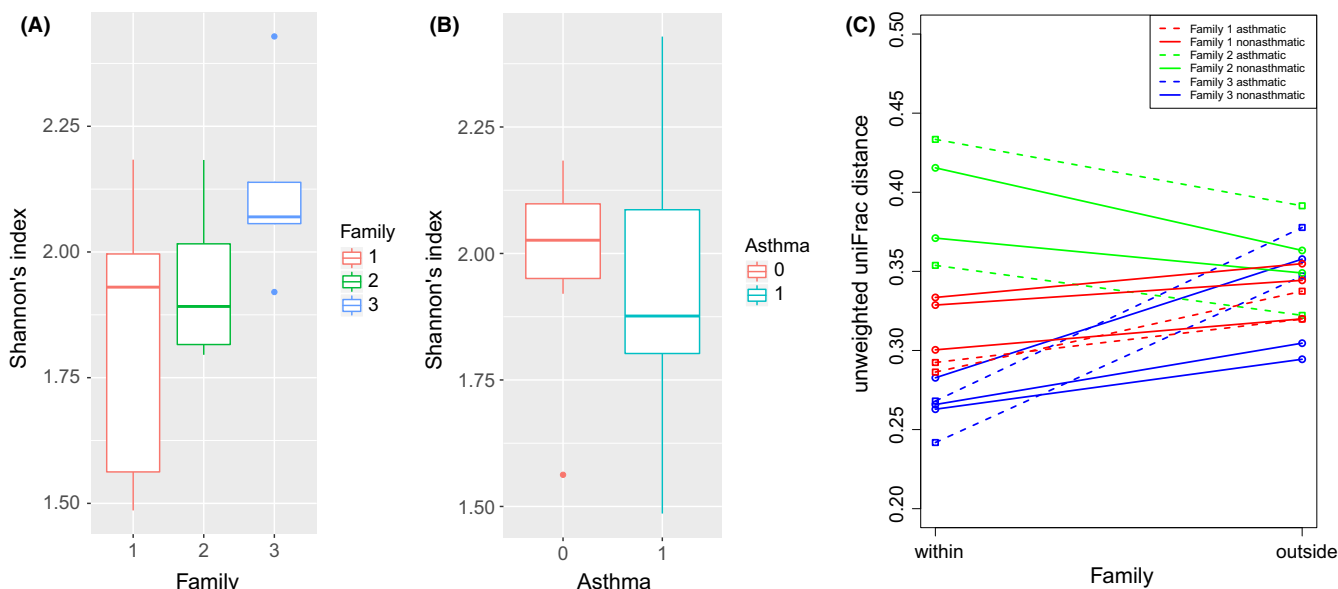


FIGURE 1 α -diversity (Shannon's index) among families (A), and between people with asthma (indicated as 1) and not with asthma (indicated as 0) (B). No significant differences were observed by Student's *t* test. Mean distance of an individual with subjects within his/her family and mean distance of the individual with subjects outside his/her family (unweighted UniFrac) (C). Family is indicated by colors; asthma status is indicated by line types

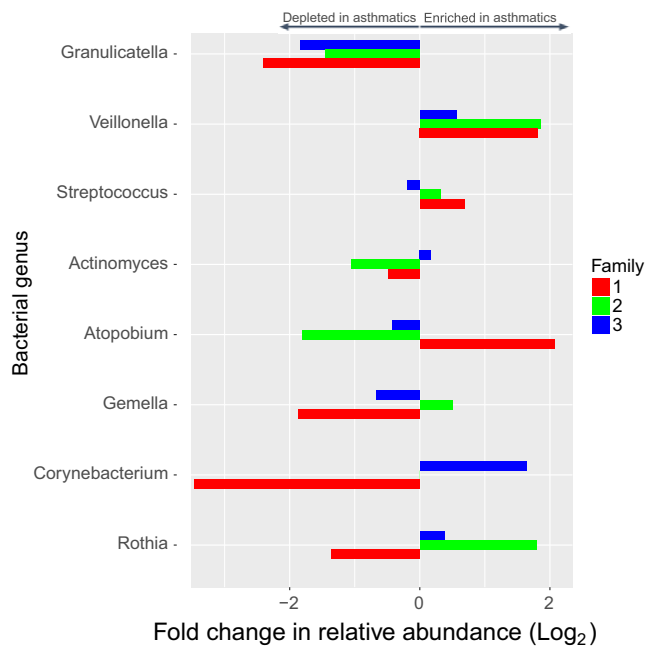


FIGURE 2 Bacterial genera that were enriched or depleted in relative abundance in asthmatic versus nonasthmatic sibling(s) across three families

asthma across the three families. The genus, *Granulicatella*, exhibited a significantly reduced abundance in asthmatics, compared to nonasthmatics (P -value = .043, paired t test) and genus *Veillonella* exhibited an increased abundance, however nonsignificant, in asthmatics compared to nonasthmatics (P -value = .078, paired t test).

To our knowledge, this is the first attempt to apply a family-based design to study the sputum microbiome for asthma. Our results suggest that family members share more similar bacterial composition in the induced sputum, compared to nonfamily members. A family-based study design is, therefore, promising to diminish baseline variation caused by different household environmental exposures and better reveal the association between asthma and the sputum microbes.

We applied a two-step strategy that leveraged the family-based nature of our study: We first compared the differences in the relative abundance of bacteria between asthmatic sibling and nonasthmatic siblings within each household. Next, we horizontally compared the abundance of each bacterial genus across the three families to identify those with the same trend. For the first time, our study associates asthma with *Granulicatella* in the sputum microbiome. *Granulicatella* are commensal microbes in the respiratory tract.⁸ It shows a wide range of relative abundance in the sputum microbiome in our data (1%–14.3%), suggesting the abundance of *Granulicatella* is highly influenced by environmental exposure, which has been reported before in a home-based study.⁸ Individuals from different household harbor very different relative abundance of *Granulicatella* in the sputum microbiome, making it difficult for case-control studies to capture this association. However, in our family-based study we were able to

compare the relative abundance between case and control siblings to reveal this pattern. We also found the Genus *Veillonella* was enriched in asthmatic siblings in all three families, which supports previous finding that increased *Veillonella* is associated with enhanced lung inflammation.⁹ This is also concordant with the finding that *Veillonella* is enriched in the bronchial microbiome of asthmatic subjects.¹

Due to lack of information on diet, we made the assumption that siblings within the same household were exposed to similar diet and the eating habits were initiated and nurtured within the family settings. Future study should gather information on diet to define its role in influencing airway bacterial community within each household.

We have included a limited number of families for this study. A large-scale family-based study is necessary to further investigate the impact of shared indoor environment on the host microbiome and improve our understanding of asthma-associated microbes in the airway.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Cosensitization to profilin is associated with less severe reactions to foods in nsLTPs and storage proteins reactors and with less severe respiratory allergy

To the Editor,

Profilin was originally identified as an allergenic component in birch pollen and as a relevant pan-allergen in both plant and vegetable food.¹ 48 profilins are currently registered in the WHO/IUIS database. Despite their well-known relevance as allergens, profilin's precise role in eliciting allergic symptoms from a clinical point of view is still a matter of debate.² Previous studies showed that profilin sensitization may underlie severe food-induced allergic reactions in some patients³ but also exert a protective effect against systemic reactions associated with the sensitization to a stable allergen such as nsLTP.⁴ In view of these diverse findings, we used a micro-arrayed detection system to identify and characterize profilin IgE reactivity in a large cohort of unselected patients, with the aim to study the influence of profilin hypersensitivity on the clinical expression of allergic disease.

The study was carried out on 4753 unselected persons born in Central or Southern Italy, attending the outpatient Allergy Clinic of IDI-IRCCS, Rome, between September 2015 and December 2017. Inclusion criteria were as follows: signed written informed consent, history of allergic rhinitis, bronchial asthma, or adverse reactions to foods, including oral allergic syndrome and urticaria. A total of 626 individuals (291 women; age 29 ± 17 years, range 1-82 years) sensitized to at least one of Bet v 2, Hev b 8, Mer a 1, and Phl p 12 were identified. Subjects not sensitized to profilin (4127 patients), acted as allergic controls, to investigate the different behavior of both species-specific and pan-allergen (ie, nsLTP, seed-storage protein [SSP], or polcalcin) hyper-reactors in the presence or absence of profilin sensitization. Demographic details and clinical data (food-related reactions, respiratory, and dermatologic symptoms) were recorded using the TD-Synergy[®] Laboratory Information System (Siemens Healthcare Diagnostics, Muenchen, Germany) and a customized electronic database. IgE reactivity of sera collected after the first visit was analyzed using the 112 ImmunoCAP-ISAC platform (Thermo

Abbreviations: BA, bronchial asthma; ISAC, immuno solid-phase allergen chip; nsLTP, nonspecific lipid transfer protein; OAS, oral allergic syndrome; SR, systemic reaction to food; SSP, seed-storage proteins.

Supporting information

Family-based study reveals decreased abundance of sputum *Granulicatella* in asthmatics

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Methods

Patient recruitment

Recruitment, questionnaire, sputum collection and 16S ribosomal RNA (rRNA) sequencing were approved by the Yale University Human Research Protection Program. The families with both asthmatic and non-asthmatic offspring were identified from an ongoing family-based asthma study being conducted at the Yale School of Public Health. Asthma was defined as a self-reported physician diagnosed asthma and two or more asthma symptoms in the last 12 months. We contacted and screened the members of 5 families. The questionnaire gathered information on symptoms and medications used for asthma or respiratory infection over the preceding 4 weeks. Exclusion criteria included: 1) presence of other respiratory diseases in the subject; 2) having had symptoms or treatment for a respiratory infection or asthma exacerbation in the past 4 weeks, with questions based on previously validated clinical tools (1); and 3) use of antibiotics, omalizumab or other unknown substances that could potentially impact results. Three families were eligible for this study. Figure E1 shows the pedigree of each family. Table E1 shows the characteristics of the 14 subjects. Atopy was defined by reported positive skin prick testing of aeroallergens (cat, dog, dust/dust mites, cockroach, pollens, trees, grass, mold/fungi, and ragweed).

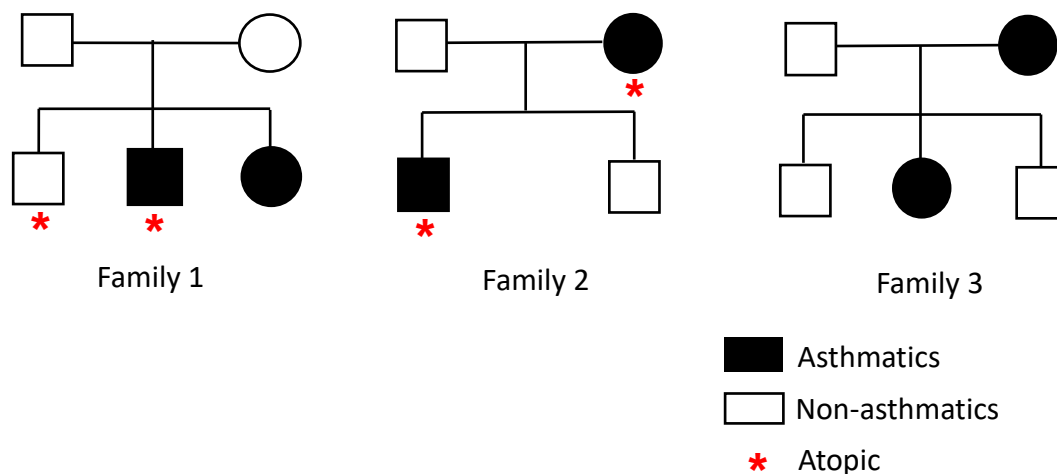


Figure E1. Pedigree drawings for each participating family. Each family has both asthmatic and non-asthmatic offspring.

Table E1. Study cohort characteristics

Variable	Asthmatics (n=6)	Non-asthmatics (n=8)	P value
Age, mean (range), y	28 (18-49)	36 (13-54)	
Sex, male, n	2	7	
White race, n	6	8	
BMI (kg/m ²)	31.0 ± 3.6	27.6 ± 2.2	0.42
Exposure to smoke, n	1	1	0.41
Atopic ¹ , n	3	1	0.17
FEV1 (% predicted) baseline	92 ± 6	95 ± 5	0.66
FEV1 (% predicted) minimum	82 ± 3	94 ± 5	0.09
FEV1 (% predicted) maximum	100 ± 4	101 ± 6	0.90

¹Atopy was defined by reported positive skin prick testing of aeroallergens (cat, dog, dust/dust mites, cockroach, pollens, trees, grass, mold/fungi, and ragweed).

Statistical significance was determined by t-test for the continuous variables and Chi-squared with Yates correction for the categorical variables.

Sputum induction and DNA extraction

Enrolled subjects attended the Yale-New Haven Hospital Research Unit, where they signed informed consent/assent. Subjects were required to rinse their mouth with water to minimize oral contamination. Prior to beginning, a baseline FEV1 was measured; subject's respiratory status was also monitored by a physician during the procedure. If the FEV1 was $\geq 80\%$ of predicted, procedure was started by administering nebulized 7% hypertonic saline for increasing time periods (minimum 30 s, maximum 4 min) with FEV1 measured each time based on previously described procedures (2). Predicted values for FEV1 were based on the variables and set values from NHANES III (3, 4).

Subjects were asked to expectorate sputum into storage reagent prototype provided by DNA Genotek (Ottawa, ON, Canada). The reagent keeps bacteria from phylum Firmicutes and Actinobacteria intact, but lyses most gram-negative bacteria from phylum Proteobacteria, Bacteroidetes and Fusobacteria. QIAamp DNA microbiome kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA. This kit first depletes all released free DNA (including the genomic material from human cells and broken bacteria) and then applies a combination of chemical and mechanical lysis to break and extract DNA from Firmicutes and Actinobacteria.

16S rRNA sequencing and analysis

The 16S rRNA coding gene fragments were sequenced as previously described (5). Briefly the V4 region of 16S ribosomal RNA coding gene was PCR amplified (34 cycles; primer pair F515/R806) with barcodes (Phusion polymerase, New England Bioscience). After amplification, PCR products were purified (SequalPrep™, Invitrogen, Waltham, MA), resuspended in 20 μ l of water and pooled. The concentration of sequencing library was assessed with qRT-PCR and final concentration of 11 pM was loaded on a MiSeq sequencer (Illumina, San Diego, CA), with sequencing conditions of 2x250 bp paired-end reads. Downstream analysis of raw data, paired end reads assembly, and UniFrac distance was analyzed with the Quantitative Insights Into Microbial Ecology (QIIME version 1.7) analysis suite (6). Reads were demultiplexed and quality filtered with a Q-score cutoff of 29. The open-reference OTU picking workflow in QIIME and the Greengenes reference database were used to cluster the reads into 97% identity Operational Taxonomic Units (OTUs). The Ribosomal Database Project classifier (RDP) and the May 2013 Greengenes taxonomy were used to assign taxonomy to representative OTUs. OTUs of less than 0.01% relative abundance, and contaminating OTUs that were also found after sequencing of 16S amplicons from PCR samples without template DNA, were filtered from OTU tables. Filtered OTU tables were rarefied to a depth of 3,000 sequences per sample for all analyses. Only the sequencing reads from Phylum Firmicutes or Actinobacteria (>98% of the total reads) were preserved for analysis. Figure E2 shows the bacterial profiling at Phylum and Genus level respectively.

abundance between the asthmatic and non-asthmatic sibling(s). The relative abundance of each genus in case and control of each family is shown in Table E2. Red color indicates the specific genus is depleted in case compared to control within the specific family. Green color indicates the specific genus is enriched in case compared to control within the specific family.

Table E2. Relative abundance of bacterial genera

Genus	Family 1		Family 2		Family 3		P-value
	Case	Control	Case	Control	Case	Control	
Granulicatella	0.010	0.053	0.052	0.143	0.036	0.128	0.043
Veillonella	0.215	0.061	0.130	0.036	0.161	0.109	0.078
Streptococcus	0.528	0.328	0.440	0.353	0.374	0.425	0.392
Actinomyces	0.050	0.070	0.012	0.025	0.070	0.062	0.426
Atopobium	0.021	0.005	0.004	0.014	0.006	0.008	0.457
Gemella	0.018	0.066	0.082	0.058	0.037	0.059	0.542
Corynebacterium	0.002	0.022	0.011	0.011	0.100	0.032	0.609
Rothia	0.036	0.092	0.066	0.019	0.030	0.023	0.984

Relative abundance of the top abundant bacterial genera between discordant sibling(s) within each family. Mean value was used if a family has more than one case or control sibling. P-value was calculated using paired t-test.

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