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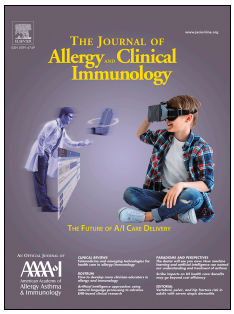

Developmental patterns in the nasopharyngeal microbiome during infancy are associated with asthma risk

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Developmental patterns in the nasopharyngeal microbiome during infancy are associated with asthma risk

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Abstract

Background: Studies indicate that the nasal microbiome may correlate strongly with the presence or future risk of childhood asthma.

Objectives: In this study, we tested whether developmental trajectories of the nasopharyngeal microbiome in early life and the composition of the microbiome during illnesses were related to risk of childhood asthma.

Methods: Children participating in the Childhood Origins of Asthma study (n=285) provided nasopharyngeal mucus samples in the first two years of life, during routine healthy study visits (2, 4, 6, 9, 12, 18 and 24 months of age) and episodes of respiratory illnesses, which were analyzed for respiratory viruses and bacteria. We identified developmental trajectories of early-life microbiome composition, as well as predominant bacteria during respiratory illnesses, and correlated these with presence of asthma at 6, 8, 11, 13 and 18 years of age.

Results: Of the four microbiome trajectories identified, a *Staphylococcus*-dominant microbiome in the first 6 months of life was associated with increased risk of recurrent wheezing by age 3 years and asthma that persisted throughout childhood. In addition, this trajectory was associated with the early onset of allergic sensitization. During wheezing illnesses, detection of rhinoviruses and predominance of *Moraxella* were associated with asthma that persisted throughout later childhood.

Conclusion: In infancy, the developmental composition of the microbiome during healthy periods and the predominant microbes during acute wheezing illnesses are both associated with the subsequent risk of developing persistent childhood asthma.

Clinical Implication: Identifying factors that promote early colonization with *S. aureus* may lead to future interventional studies to prevent childhood asthma.

Capsule summary: In a birth cohort study, early colonization of the upper airway with *Staphylococcus aureus* and detection of rhinoviruses and *Moraxella catarrhalis* during illnesses were associated with subsequent childhood asthma.

Key words: Microbiome, children, asthma, development, birth cohort

Abbreviations: RV, rhinovirus; RSV, respiratory syncytial virus; ASV, amplicon sequence variant; MPG, microbiome predominance group; COAST, Childhood Origins of Asthma birth cohort study

Introduction

Asthma is a chronic inflammatory disease that affects 6 million children in the United States alone.[1] While childhood asthma can be treated, the lack of a cure underscores the need to understand its early life developmental origins. Most cases of persistent childhood asthma begin with acute infectious wheezing illnesses in infancy. While these illnesses are initiated by respiratory viruses, there is strong evidence that bacterial pathogens also contribute,[2-9] and both types of microorganisms have also been related to the subsequent risk of developing asthma. Wheezing illnesses caused by respiratory syncytial virus (RSV) and rhinovirus (RV) are associated with asthma, especially in children who develop early allergic sensitization.[10, 11] In addition, detection of specific bacteria by culture (*S. pneumoniae*, *M. catarrhalis* or *H. influenzae*) or 16S sequencing (e.g. *Prevotella*, *Veillonella*) in oral or nasopharyngeal aspirates of babies have been related to asthma in early childhood.[2, 12, 13] In an Australian birth cohort (Childhood Asthma Study) using bacterial metagenomics based on 16S rRNA; predominance of *S. pneumoniae*, *M. catarrhalis* or *H. influenzae* was found to interact with early allergic sensitization to increase the risk of later asthma.[3, 7] Others have found co-association between eosinophil counts, severe RV bronchiolitis and a *Haemophilus* or *Moraxella*-dominated profile of nasopharyngeal microbiota in infants.[14] These studies suggest that infection by viral and bacterial pathogens promote acute wheezing illnesses and increase the risk of asthma, while possibly interacting with other host factors such as allergy.

There is also considerable interest in determining whether the dynamic transformation of the airway microbiota with time – its developmental pattern – is associated with acute or chronic respiratory illness. The composition of the airway microbiome typically undergoes marked changes in the first postnatal weeks and months, and this process can be influenced by mode of delivery,[15, 16] viral illnesses[17, 18] and exposure to other children.[17] Given the likewise rapid maturation of mucosal immunity in early life, host microbiome dynamics during early childhood may impact future health and disease through interactions with immune development.[8, 19]

Collectively, these findings suggest that both the developmental trajectory of the airway microbiome in early life, and episodic incursions with viral and bacterial pathogens during respiratory illnesses modify the risk of developing childhood asthma. To further test these hypotheses, we analyzed respiratory bacteria and viruses in nasopharyngeal mucus specimens collected from children enrolled in the Childhood Origins of Asthma (COAST) study under two set of conditions: 1) multiple scheduled visits mostly during periods of good health through 24 months of age, and 2) acute respiratory illnesses.[20] We derived developmental trajectories of airway microbiome assembly based on the routine samples, then tested these trajectories and microbial composition during respiratory illnesses for associations with asthma throughout childhood.

Methods

Study design

Participants of the COAST birth cohort study (initial *N*=289)[20] were recruited in Madison, Wisconsin and surrounding areas from November 1998 to May 2000. The study was approved by the University of Wisconsin-Madison Human Subjects Committee, all families provided informed consent before enrollment, and children provided assent when they reached 7 years of age. All recruited children had at least one parent with an allergic disease or asthma. Routine scheduled nasopharyngeal sampling was performed at timepoints of 2, 4, 6, 9, 12, 18 and 24 months of age. Most routine samples were collected from children during periods of good health, though some coincided with symptoms of mild respiratory illness. From birth until age 3 years, additional samples were collected from children with upper respiratory illness of at least moderate severity, or any lower respiratory illness, as previously described.[21]

The children had yearly routine visits to the clinic where they underwent procedures including assessment of IgE sensitization to aeroallergens (cat, dog, *Dermatophagoides pteronyssinus*, *D. farinae*, and *Alternaria*), blood eosinophil counts, lung function and asthma diagnosis from ages 6-18 years.[22] Information on environmental exposures and allergic conditions was collected. Wheezing illnesses, rhinitis, asthma and atopic dermatitis latent classes were defined as previously described.[21, 23-25]

Detection of viruses and bacteria

We performed 16S rRNA amplicon sequencing of nasopharyngeal samples (swab or aspirate) and negative controls.[7] Microbiome data was processed using QIIME2 (v2017.10/12)[26] and DADA2[27] to produce relative abundance data for amplicon

147 sequence variants (ASVs), representing unique 16S rRNA V4 sequences. The
148 nasopharyngeal samples were clustered into microbiome profile groups (MPGs) using
149 hierarchical clustering methods.[3, 7] Nasal specimens were analyzed for common
150 respiratory viruses as previously described.[28, 29]
151
152 ***Statistical methods***
153 We used the relative abundances of common ASVs to determine clusters of individuals
154 who shared similar patterns ("trajectories") of changing microbiome during routine visits
155 (with healthy or mildly-ill samples). To generate these trajectories, we omitted all
156 samples obtained at 18 months of age because of a high rate of missing samples at this
157 timepoint. We then performed Multiple Factor Analysis (R package "FactoMineR"),[30]
158 followed by K-means clustering.
159
160 To estimate a longitudinal asthma phenotype, simple latent class models were fit using
161 asthma diagnoses at ages 6, 8, 11, 13 and 18 years as variables. Next, conditional
162 variable importance measures from random forest ensembles were used to identify
163 microbial and viral features (MPG wheezing burdens, viral wheezing episodes, and
164 routine visit microbiome trajectory) for additional analysis based on associations with
165 the 4-class asthma phenotype.
166
167 To compare MPGs and MFA-k-means trajectories, Fisher exact tests and Chi-square
168 tests were used for categorical variables; Kruskal-Wallis, t-tests and ANOVAs for
169 continuous variables. More complex associations were assessed using generalized

linear models (GLM) for subject-based analyses, or generalized estimating equations (GEE, using R package "gee" v4.13.20)[31, 32] for sample-based analyses, adjusting for gender, and age and season with repeated measures of multiple samples per child subject, and unstructured correlation. These analyses were conducted using R v3.5.0. Post-hoc comparisons with FDR correction were conducted where required.

Additional details on study and statistical methods are listed in the online data supplement.

Results

Composition of nasopharyngeal microbiome in COAST

A total of 3147 nasal samples were analyzed for bacteria, including 1654 collected during routine scheduled visits (2, 4, 6, 9, 12, 18 and 24 months of age) and 1493 additional specimens collected during respiratory illnesses. From these samples, 2922 passed quality controls (1488 routine, 1434 episodic), of which 1059 were routine and truly healthy, while 1863 were illness or mild illness samples collected during either routine or episodic visits. There were 414 distinct samples corresponding to wheezing illnesses. The most common ASVs belonged to six genera; *Dolosigranulum*.dd2e, *Corynebacterium*.cb50, *Haemophilus.*bc0d, *Haemophilus.*f579, *Moraxella*.d253, *Streptococcus*.4060, *Staphylococcus*.29eb, and *Streptococcus.*3575 (Figure 1A). These ASV sequences most closely match those of bacterial species *Dolosigranulum pigrum*, *Corynebacterium pseudodiphtheriticum*, two subtypes of *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, multiple *Staphylococcus* species

193 (incl. *S. aureus* and *S. epidermidis*), and *Streptococcus mitis*, respectively
194 (Supplementary Table 1).
195
196 ***Clustering into microbiome profile groups (MPGs)***
197 Consistent with previous similar studies,[3, 7] each nasopharyngeal sample had a simple
198 structure, being largely dominated (>50% of reads per sample) by a single ASV.
199 Hierarchical clustering identified 12 microbiome profile or predominance groups
200 (MPGs). Each MPG described a pattern with a single dominant ASV, and was named
201 according to this dominant taxon. Incidentally, the 12 MPGs corresponded to the most
202 abundant ASVs (Figure 1A; relative abundances for all features shown in
203 Supplementary Figure 1).
204
205 ***MPG association with acute respiratory illness***
206 Four specific MPGs were significantly overrepresented in respiratory illness samples
207 compared to samples from healthy children (p<0.05; Figure 1B and Supplementary
208 Table 2A). These MPGs were those of known respiratory pathogens *Moraxella*.d253 (*M.*
209 *catarrhalis*), *Streptococcus.*4060 (*S. pneumoniae*), *Haemophilus*.f579 and
210 *Haemophilus*.bc0d (both *H. influenzae*). Conversely, MPGs dominated by
211 *Corynebacterium*.cb50 (*C. pseudodiphtheriticum*), *Dolosigranulum*.dd2e
212 (*Dolosigranulum pigrum*), *Staphylococcus*.29eb (*Staphylococcus* spp.), and
213 *Streptococcus*.3575 (*S. mitis*) were more common in healthy rather than sick samples.
214 In a similar analysis testing for association of MPGs with acute wheezing illnesses
215 (compared to healthy samples), *Streptococcus*.4060 (*S. pneumoniae*) showed

significant positive association (p=0.00035), and *Dolosigranulum*.dd23 (*Dolosigranulum pigrum*) negative association (p=2.6×10^{-5}; Supplementary Table 2A). Similar results were attained after adjusting for other asthma-related covariates including parental allergy, parental asthma, environmental smoke exposure, presence of pets, breastfeeding, and birth by Cesarean (Supplementary Table 2B).

As noted in our previous publications,[33, 34] the viruses most commonly detected in the specimens were RV, RSV, parainfluenza virus, coronavirus, and metapneumovirus. We observed that pathogen MPGs (*Moraxella*.d253, *Streptococcus.*4060, *Haemophilus*.f579, *Haemophilus*.bc0d) and certain respiratory viruses (RSV, influenza) often co-existed in the same sample, especially during illnesses in the winter months (Supplementary Figures 2 and 3). The distribution of detected MPGs was generally similar across all viruses, whether we examined all samples or only those samples from wheezing illnesses (Supplementary Figure 3). During illnesses (n=1863), pathogen-related MPGs and viruses were most often detected together (n=1224, 66%), followed by viruses alone (n=422, 23%). pathogen-related MPGs alone (n=145, 7.8%) and neither (n=72, 3.9%). The presence of any pathogen MPG and the presence of virus each remained independently associated with respiratory illnesses, even when adjusting for each other, age, gender and seasonality (GEE model, for any pathogen MPG: OR=3.4, p=7.3×10^{-8}; for any virus: OR=12, p<1×10^{-10}).

Trajectory analysis of the nasopharyngeal microbiome

Nasopharyngeal samples from routine study visits across the first two years of life were analyzed to identify temporal trajectories of microbiome assembly. We identified four clusters of children distinguished by distinct patterns of microbial composition over time (Figure 2A). Each trajectory appeared to be driven by a different MPG in the first six months of life: Trajectory A (N=79) by *Dolosigranulum*.dd2e and *Corynebacterium*.cb50; Trajectory B (N=43) by *Moraxella*.d253; Trajectory C (N=26) by *Staphylococcus*.29eb; and Trajectory D (N=135) by *Streptococcus*.3575 and other streptococci. Since V3-V4 primers do not reliably differentiate *S. aureus* and *S. epidermidis*, 20 Trajectory C nasal mucus samples obtained at 2 months of age were analyzed by qPCR and revealed a predominance of *S. aureus* (Supplementary Figure 4).

Notably, as the children grew older the trajectories became more similar, and by age two years had converged towards a generally-mixed composition (with many dominated by *Moraxella*.d253). At age 2 months, between-trajectories dissimilarity (Bray-Curtis) was greatest (0.86), while the dissimilarity within the same trajectory was smallest (0.55). These gradually shifted with age, until by age 2 years both between- and within-trajectories dissimilarities were roughly equal (0.71).

During wheezing illnesses, nasal bacteria were typically dominated by illness-associated taxa (e.g. *Moraxella*.d253, *Streptococcus*.4060, *Haemophilus* taxa) irrespective of trajectory (Figure 2B). There were no significant differences in the rate of detection of specific viruses between any of the four trajectories in routine samples or in wheezing illness samples (Supplementary Figure 5).

Demographic characteristics were similar among children in the four trajectories (Table 1). There were no significant differences among the microbiome trajectories in terms of other environmental variables including mode of delivery, presence of home pets (cat, dog), number of siblings at time of birth, exclusive breast feeding during the first 6 months of life, and antibiotic use in the first year of life (Table 1).

Association of microbiome trajectories with early wheezing illness and later asthma

Trajectory C, dominated by *Staphylococcus*.29eb, was associated with the greatest frequency of wheezing illness in the first three years of life; however, this association differed by age (Figure 3). The number of wheezing illnesses per trajectory was most similar in the first year of life, lowest for trajectories A (*Dolosigranulum*) and C (*Staphylococcus*), and highest for Trajectory D (*Streptococcus mitis*). However, Trajectory C was associated with a progressive increase in wheezing illnesses with time, overtaking the other trajectories to give the greatest frequency at year 3 (p = 0.0006 for Trajectory C).

In addition, Trajectory C was also associated with greater frequency of physician-diagnosed asthma from age 6 years (47%, p=0.053) to 18 years (58%, p=0.019) compared to the other trajectories (Figure 4A). Furthermore, we applied a latent class model to asthma diagnoses at age 6, 8, 11, 13 and 18 years to identify four longitudinal patterns of asthma (Supplementary Figure 6): none/intermittent (63% subjects),

persistent (19% subjects), remitting (10% subjects), and late onset (8% subjects). Compared to other microbiome trajectories, Trajectory C (*Staphylococcus*.29eb dominance) tended to be positively associated with a persistent asthma phenotype (p=0.08, Figure 4B).

We next evaluated microbial predictors of asthma phenotypes in a random forest model that included the routine visit microbiome trajectories together with MPG and virus detection during wheezing illnesses (Supplemental Figure 7). In the first year of life, microbiome trajectory C along with detection of illness-associated MPGs (*Moraxella*.d253, *Haemophilus*.bc0d) were most predictive of asthma class. When the predictors were evaluated over the first three years, the microbiome trajectory was no longer among the key predictors of asthma class. Instead, detection of RV during illnesses was an important predictor, and illness-associated MPG *Moraxella*.d253 remained an important asthma class predictor. These relationships were modified by the age of the child at the time of the wheezing illness (Figure 6). Both RV and *Moraxella*.d253 wheezing illnesses in the first year of life were modestly associated with the persistent asthma latent class, while wheezing illnesses associated with RV or *Moraxella*.d253 during years 2 and 3 were strongly related to persistent asthma.

Association of microbiome trajectories with allergic variables

Given the close association between early onset of atopy and persistent asthma, we next tested for associations between microbiome Trajectory C and indicators of type II inflammation and allergic outcomes. Trajectory C was associated with a greater

frequency of aeroallergen sensitization, especially during early childhood (Figure 5A). The difference of trajectory C from the others was significant through to age 5 ($p<0.05$ at each age) and also when all years were considered together (Trajectory C vs. others, $p=0.05$). There were similar nonsignificant trends for associations between Trajectory C and increases in both total IgE and absolute eosinophil counts (Figure 5, B and C). Trajectory C was associated with a nonsignificant trend for increased risk of allergic rhinitis at age 6 years (overall $p = 0.05$, Trajectory C vs. others $p = 0.12$), but not with early-onset atopic dermatitis (Supplementary Figure 8) or lung function (FEV_1 or FEV_1/FVC ratio, Supplementary Table 3). A panel of cytokines were analyzed in samples of nasal lavage fluid from a subset of 80 COAST children, with approximately equal representation from the four MPGs. In general, pro-inflammatory cytokine production was greatest in the *Moraxella* MPG, followed by *Staphylococcus*, *Streptococcus* and *Dolosigranulum* (Supplementary Figure 9).

We next tested whether the association between Trajectory C and asthma was mediated via viral wheezing illnesses or allergic sensitization in early life. To test this, all three variables (trajectory, early wheezing illness, aeroallergen sensitization) were included in multivariable models with asthma diagnosis at various timepoints as outcomes. The association between Trajectory C and asthma diagnoses at ages 6 to 13 was partially ablated when adjusting for both early aeroallergen sensitization (allergen-specific IgE>0.35 kU/L by age two) and number of early-life wheezing illnesses up to age 3 (Supplementary Table 4). However, Trajectory C remained a statistically-significant predictor for asthma diagnosis at ages 11 and 13, suggesting that the

microbiome trajectory may be acting via mechanisms not fully captured by wheezing illnesses or early-life aeroallergen sensitization.

The trajectories were robust to modifications in their derivation. We reproduced trajectories using (1) only routine samples within the first 6 months of life (Online Supplement), or (2) only healthy samples. Both analyses yielded trajectories that were very similar to the original ones (Supplementary Table 5), with similar associations with most asthma outcomes ($p < 0.05$ for all GLM associations of asthma age 8, 11 or 13 ~ Trajectory C).

Discussion

Developmental patterns of microbiome composition in the gut and skin can influence local immune function and the risk for developing allergic diseases.[35-37] Similarly, we hypothesized that the developmental trajectory of the airway microbiome influences the risk for developing wheezing illnesses and asthma. Children in the COAST study could be separated into four developmental trajectories of microbiome composition, each characterized by nasopharyngeal samples in the first 4-6 months of life being dominated by a distinct bacterial taxon. In particular, Trajectory C, which was characterized by early *Staphylococcus* colonization, was associated with higher frequency of wheezing illnesses during the second and third years of life. Furthermore, membership in the *Staphylococcus*-dominated Trajectory C was linked to increased allergen sensitization, allergic rhinitis, and increased risk for asthma diagnosis from age six years through adolescence. The association between Trajectory C and asthma was partially mediated by allergic sensitization, RV infections and early-life wheezing illnesses. Finally, in

addition to identifying a novel association between *Staphylococcus*-dominated nasal microbiome in early life and asthma, we confirmed previously reported relationships between detection of viral (RV)[33] and bacterial (e.g. *M. catarrhalis*)[7] pathogens during periods of illness and the risk of childhood asthma.

Previous observational studies have provided information on temporal changes in composition of the airway microbiome in early life, and both community composition and maturation of the microbiome have been related to more frequent respiratory illnesses. In a study of 60 healthy children sampled several times (1.5, 6, 12, and 24 mo) during the first two years of age, initial colonization with *Haemophilus*, *Streptococcus* or *Staphylococcus* communities were associated with more frequent respiratory illnesses, and were relatively unstable.[16] In contrast, microbial communities associated with *Moraxella* and *Corynebacterium*/*Dolosigranulum* in the first few months were more stable. Our findings were similar in that Trajectory B had the most stable composition with *Moraxella* MPG detected most often at all ages tested.

The relationship between wheezing illnesses and *Staphylococcus* appears to be age-dependent. Our study and others[2, 7] found that *Staphylococcus* was more prevalent in secretions from healthy young infants and was less likely to be detected in the first year of life during periods of illness. On the other hand, Trajectory C, characterized by *Staphylococcus* MPG, was associated with increased wheezing by age 3 years. To reconcile these findings, it is important to consider that the *Staphylococcus* MPG was only predominant in Trajectory C for the first 6 months of life in COAST, and thereafter

377 *Moraxella* was the most common MPG. Accordingly, Trajectory C was associated with
378 fewer illnesses during the first year, followed by the highest frequency of illnesses
379 during years 2 and 3. Teo *et al*[3] had also found that the negative association between
380 *Staphylococcus* MPG and respiratory illness attenuated over time. Similarly, Bosch and
381 colleagues[17] reported that early predominance of *Staphylococcus* transitioning to
382 *Moraxella* was related to increased frequency of respiratory illnesses in a birth cohort
383 study. Notably, nasal *S. aureus* has also been related to asthma and bronchial
384 hyperresponsiveness in children[38] and wheeze in children and adults.[39]
385
386 There are several potential mechanisms that could link *S. aureus* colonization to
387 childhood asthma. First, *S. aureus* can produce superantigens that are potent
388 stimulators of proinflammatory T cell responses,[40] and can promote type 2 inflammation
389 by directly activating mast cells,[41] and by inducing thymic stromal lymphopoetin
390 (TSLP),[42] However, analysis of nasal cytokines did not indicate that the S. aureus MPG
391 was associated with increased TSLP or a greater inflammatory milieu in well infants.
392 Alternatively, Staphylococci can produce toxins that can enhance viral replication,[43] and
393 this effect could lead to increased viral wheezing illnesses. Furthermore, *S. aureus*
394 quorum sensing systems (*agr*) sense self-produced peptides and upregulate the
395 production of toxins, providing a mechanism for enhanced virulence when *S. aureus* is
396 present in higher quantities.[44, 45] On the skin, *S. aureus* colonization is closely linked to
397 epithelial barrier dysfunction and disease activity in atopic dermatitis, and furthermore is
398 associated with a greater risk of sensitization and allergy to foods.[46] Accordingly,
399 Trajectory C was linked to early aeroallergen sensitization and allergic rhinitis in

COAST. Alternatively, considering that *Staphylococcus* is a predominant organism in the neonatal airway,[15, 47] Trajectory C could indicate delayed maturation of the nasal microbiome. Delayed maturation of the microbiome could in turn delay development of airway mucosal immunity, and hence lead to more frequent infections.

Detection of pathogen-dominated microbial communities (*Moraxella*, *Streptococcus*, *Haemophilus,* viruses) have previously been related to acute wheezing illnesses[7, 14, 17, 18, 48] and to childhood asthma at age 5 years.[3, 7, 49] Similarly, in COAST we found that both RV-associated illnesses and the presence of illness-associated bacteria (esp. *Moraxella*) in nasopharyngeal samples, especially in the second and third years of life, were predictive for persistent childhood asthma. Dumas et al found that a severe bronchiolitis profile characterized by eosinophilia and RV infections is associated with a *Moraxella* or *Haemophilus*-dominated nasopharyngeal microbiota.[14] Conversely, Rosas-Salazar and colleagues found that co-presence of *Lactobacillus* during RSV infections may be protective against childhood wheeze.[50] These associations suggest that bacterial microbiota during health and disease may influence susceptibility to frequent early-life respiratory infectious illnesses, leading to inflammatory and/or structural changes and entrenchment of asthma. Furthermore, it is possible that there are two distinct mechanisms that link the early life microbiome to asthma – a developmental trajectory that is related to early colonization with *Staphylococcus*, and a second mechanism related to respiratory pathogens (*Moraxella*, *Streptococcus*, *Haemophilus, RV*) during periods of illness.

Strengths of this study included intensive sampling of the nasal microbiome and virome in the first two years of life, which enabled analyses both of microbiome assembly during routinely-observed states and during illness-related perturbations. In addition, COAST participants have been evaluated for asthma at regular intervals to the age of 18 years, which enabled identification of children with various patterns of asthma persistence, and association of these with microbial traits. One limitation is that COAST participants were specifically selected for family history of asthma and allergy;[20] this may limit the generalizability of our findings. In addition, the COAST sample size had limited power to detect associations between environmental factors, microbiome trajectories and clinical outcomes. The COAST cohort was already assembled prior to the introduction of conjugated pneumococcal vaccines in 2000, which could have changed patterns of microbial colonization in the upper airways. Finally, the associations in this study link the upper airway microbiome to lower airway outcomes (asthma). While the upper and lower airway microbiomes have distinct features, close relationships between upper airway microbiome, wheezing and asthma[2-7, 51] provide evidence of functional linkages. It is notable that bacteria overexpressed in the lower airways of asthmatic children and adults are also commonly present in upper airway samples.[52]

In summary, these findings suggest that both the initial development of the upper airway microbiome during health, and the incursion of specific viral or bacterial pathogens during respiratory illnesses, modify the risk of developing persistent childhood asthma. Identifying lifestyle and environmental exposures that promote early colonization with *S.*

446 *aureus* may lead to future interventional studies to test whether preventing this process
447 can reduce the risk for developing childhood asthma. Another possible opportunity to
448 reduce asthma risk may exist in the form of treatments to prevent infection or
449 proliferation of those major pathogens (e.g. RV, *M. catarrhalis*) closely associated with
450 acute wheezing illnesses in early life.

References

1. Zahran HS, Bailey CM, Damon SA, Garbe PL, Breysse PN. Vital Signs: Asthma in Children - United States, 2001-2016. MMWR Morb Mortal Wkly Rep 2018; 67:149-55.
2. Bisgaard H, Hermansen MN, Bonnelykke K, Stokholm J, Baty F, Skytt NL, et al. Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. BMJ 2010; 341:c4978.
3. Teo SM, Tang HHF, Mok D, Judd LM, Watts SC, Pham K, et al. Airway Microbiota Dynamics Uncover a Critical Window for Interplay of Pathogenic Bacteria and Allergy in Childhood Respiratory Disease. Cell Host Microbe 2018; 24:341-52 e5.
4. 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 e3.
5. Bashir H, Grindle K, Vrtis R, Vang F, Kang T, Salazar L, et al. Association of rhinovirus species with common cold and asthma symptoms and bacterial pathogens. J Allergy Clin Immunol 2018; 141:822-4 e9.
6. Kloepfer KM, Sarsani VK, Poroyko V, Lee WM, Pappas TE, Kang T, et al. Community Acquired Rhinovirus Infection Is Associated With Changes in the Airway Microbiome. J Allergy Clin Immunol 2017.
7. 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.

8. de Steenhuijsen Piters WA, Heinonen S, Hasrat R, Bunsow E, Smith B, Suarez-Arrabal MC, et al. Nasopharyngeal Microbiota, Host Transcriptome, and Disease Severity in Children with Respiratory Syncytial Virus Infection. Am J Respir Crit Care Med 2016; 194:1104-15.
9. Rosas-Salazar C, Shilts MH, Tovchigrechko A, Schobel S, Chappell JD, Larkin EK, et al. Differences in the Nasopharyngeal Microbiome During Acute Respiratory Tract Infection With Human Rhinovirus and Respiratory Syncytial Virus in Infancy. J Infect Dis 2016; 214:1924-8.
10. Kusel MM, de Klerk NH, Kebadze T, Vohma V, Holt PG, Johnston SL, et al. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J Allergy Clin Immunol 2007; 119:1105-10.
11. Jackson DJ, Evans MD, Gangnon RE, Tisler CJ, Pappas TE, Lee WM, et al. Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life. Am J Respir Crit Care Med 2012; 185:281-5.
12. Thorsen J, Rasmussen MA, Waage J, Mortensen M, Brejnrod A, Bonnelykke K, et al. Infant airway microbiota and topical immune perturbations in the origins of childhood asthma. Nat Commun 2019; 10:5001.
13. Powell EA, Fontanella S, Boakes E, Belgrave D, Shaw AG, Cornwell E, et al. Temporal association of the development of oropharyngeal microbiota with early life wheeze in a population-based birth cohort. EBioMedicine 2019; 46:486-98.
14. Dumas O, Hasegawa K, Mansbach JM, Sullivan AF, Piedra PA, Camargo CA, Jr. Severe bronchiolitis profiles and risk of recurrent wheeze by age 3 years. J Allergy Clin Immunol 2019; 143:1371-9 e7.

15. Shilts MH, Rosas-Salazar C, Tovchigrechko A, Larkin EK, Torralba M, Akopov A, et al. Minimally Invasive Sampling Method Identifies Differences in Taxonomic Richness of Nasal Microbiomes in Young Infants Associated with Mode of Delivery. Microb Ecol 2016; 71:233-42.
16. Biesbroek G, Tsivtsivadze E, Sanders EA, Montijn R, Veenhoven RH, Keijser BJ, et al. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. Am J Respir Crit Care Med 2014; 190:1283-92.
17. Bosch A, de Steenhuijsen Piters WAA, van Houten MA, Chu M, Biesbroek G, Kool J, et al. Maturation of the Infant Respiratory Microbiota, Environmental Drivers, and Health Consequences. A Prospective Cohort Study. Am J Respir Crit Care Med 2017; 196:1582-90.
18. Mansbach JM, Hasegawa K, Henke DM, Ajami NJ, Petrosino JF, Shaw CA, et al. Respiratory syncytial virus and rhinovirus severe bronchiolitis are associated with distinct nasopharyngeal microbiota. J Allergy Clin Immunol 2016; 137:1909-13 e4.
19. Folsgaard NV, Schjorring S, Chawes BL, Rasmussen MA, Krogfelt KA, Brix S, et al. Pathogenic bacteria colonizing the airways in asymptomatic neonates stimulates topical inflammatory mediator release. Am J Respir Crit Care Med 2013; 187:589-95.
20. Lemanske RF, Jr. The childhood origins of asthma (COAST) study. Pediatr. Allergy Immunol. 2002; 13 Suppl 15:38-43.

21. Lemanske RF, Jr., Jackson DJ, Gangnon RE, Evans MD, Li Z, Shult PA, et al. Rhinovirus illnesses during infancy predict subsequent childhood wheezing. J Allergy Clin Immunol 2005; 116:571-7.
22. Rubner FJ, Jackson DJ, Evans MD, Gangnon RE, Tisler CJ, Pappas TE, et al. Early life rhinovirus wheezing, allergic sensitization, and asthma risk at adolescence. J Allergy Clin Immunol 2017; 139:501-7.
23. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am J Respir Crit Care Med 2008; 178:667-72.
24. Jackson DJ, Virnig CM, Gangnon RE, Evans MD, Roberg KA, Anderson EL, et al. Fractional exhaled nitric oxide measurements are most closely associated with allergic sensitization in school-age children. J Allergy Clin Immunol 2009; 124:949-53.
25. Singh AM, Evans MD, Gangnon R, Roberg KA, Tisler C, DaSilva D, et al. Expression patterns of atopic eczema and respiratory illnesses in a high-risk birth cohort. Journal of Allergy & Clinical Immunology 2010; 125:491-3.
26. QIIME 2. 2018.] Available from https://qiime2.org/.
27. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 2016; 13:581-3.
28. Anderson HM, Lemanske RF, Jr., Evans MD, Gangnon RE, Pappas T, Grindle K, et al. Assessment of wheezing frequency and viral etiology on childhood and adolescent asthma risk. J Allergy Clin Immunol 2017; 139:692-4.

29. Bochkov YA, Grindle K, Vang F, Evans MD, Gern JE. Improved molecular typing assay for rhinovirus species A, B, and C. J Clin Microbiol 2014; 52:2461-71.
30. Lê S, Josse J, Husson F. FactoMineR: An R Package for Multivariate Analysis. 2008 2008; 25:18.
31. Liang K-Y, Zeger SL. Longitudinal data analysis using generalized linear models. Biometrika 1986; 73:13-22.
32. Generalized Estimation Equation Solver. 2015.] Available from https://cran.r-project.org/web/packages/gee/index.html.
33. Lee WM, Lemanske RF, Jr., Evans MD, Vang F, Pappas T, Gangnon R, et al. Human rhinovirus species and season of infection determine illness severity. Am J Respir Crit Care Med 2012; 186:886-91.
34. Gern JE, Pappas T, Visness CM, Jaffee KF, Lemanske RF, Togias A, et al. Comparison of the etiology of viral respiratory illnesses in inner-city and suburban infants. J Infect Dis 2012; 206:1342-9.
35. Fujimura KE, Sitarik AR, Havstad S, Lin DL, Levan S, Fadrosh D, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med 2016; 22:1187-91.
36. 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.
37. Kennedy EA, Connolly J, Hourihane JO, Fallon PG, McLean WHI, Murray D, et al. Skin microbiome before development of atopic dermatitis: Early colonization with

commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. J Allergy Clin Immunol 2017; 139:166-72.

38. Kim BS, Lee E, Lee MJ, Kang MJ, Yoon J, Cho HJ, et al. Different functional genes of upper airway microbiome associated with natural course of childhood asthma. Allergy 2018; 73:644-52.

39. Davis MF, Peng RD, McCormack MC, Matsui EC. Staphylococcus aureus colonization is associated with wheeze and asthma among US children and young adults. J Allergy Clin Immunol 2015; 135:811-3 e5.

40. Spaulding AR, Salgado-Pabon W, Kohler PL, Horswill AR, Leung DY, Schlievert PM. Staphylococcal and streptococcal superantigen exotoxins. Clin Microbiol Rev 2013; 26:422-47.

41. Nakamura Y, Oscherwitz J, Cease KB, Chan SM, Munoz-Planillo R, Hasegawa M, et al. Staphylococcus delta-toxin induces allergic skin disease by activating mast cells. Nature 2013; 503:397-401.

42. Vu AT, Baba T, Chen X, Le TA, Kinoshita H, Xie Y, et al. Staphylococcus aureus membrane and diacylated lipopeptide induce thymic stromal lymphopoietin in keratinocytes through the Toll-like receptor 2-Toll-like receptor 6 pathway. J Allergy Clin Immunol 2010; 126:985-93, 93 e1-3.

43. Bin L, Kim BE, Brauweiler A, Goleva E, Streib J, Ji Y, et al. Staphylococcus aureus alpha-toxin modulates skin host response to viral infection. J Allergy Clin Immunol 2012; 130:683-91 e2.

44. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 2007; 13:1510-4.
45. Baldry M, Nakamura Y, Nakagawa S, Frees D, Matsue H, Nunez G, et al. Application of an agr-Specific Antivirulence Compound as Therapy for Staphylococcus aureus-Induced Inflammatory Skin Disease. J Infect Dis 2018; 218:1009-13.
46. Jones AL, Curran-Everett D, Leung DYM. Food allergy is associated with Staphylococcus aureus colonization in children with atopic dermatitis. J Allergy Clin Immunol 2016; 137:1247-8 e3.
47. Pattaroni C, Watzenboeck ML, Schneidegger S, Kieser S, Wong NC, Bernasconi E, et al. Early-Life Formation of the Microbial and Immunological Environment of the Human Airways. Cell Host Microbe 2018; 24:857-65 e4.
48. Hasegawa K, Mansbach JM, Bochkov YA, Gern JE, Piedra PA, Bauer CS, et al. Association of Rhinovirus C Bronchiolitis and Immunoglobulin E Sensitization During Infancy With Development of Recurrent Wheeze. JAMA Pediatr 2019; 173:544-52.
49. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. N.Engl.J.Med. 2007; 357:1487-95.
50. Rosas-Salazar C, Shilts MH, Tovchigrechko A, Schobel S, Chappell JD, Larkin EK, et al. Nasopharyngeal Lactobacillus is associated with a reduced risk of childhood wheezing illnesses following acute respiratory syncytial virus infection in infancy. J Allergy Clin Immunol 2018; 142:1447-56 e9.

51. Altman MC, Gill MA, Whalen E, Babineau DC, Shao B, Liu AH, et al. Transcriptome networks identify mechanisms of viral and nonviral asthma exacerbations in children. Nat Immunol 2019; 20:637-51.

52. Huang YJ, Nariya S, Lynch SV, Harris J, Choy D, Arron JR, et al. The airway microbiome in severe asthma. Ann Am Thorac Soc 2014; 11 Suppl 1:S78.

Data and materials availability: The microbial sequences have been uploaded to GenBank (accession number pending).

Figure Legends

Figure 1. Composition of nasopharyngeal microbiome in COAST subjects, and relationship to acute respiratory illness. **A.** Clustering of microbiomes into microbiome profile groups (MPGs), by relative abundances of amplicon sequence variants (ASVs) within each sample, as described in **Methods**. The heat in the heatmap represents relative abundance of each ASV (rows, color-coded on the right), arranged by samples (columns) clustered into MPGs (top bar separated by colors of dominant ASV). **B.** MPG association with respiratory illness, calculated from GEE models with gender, age, and season as covariates. Points (color-coded as per **Figure 1A**) represent the estimates as natural logarithms of odds ratios (OR) for association of each MPG with illness samples vs. healthy samples, while error bars represent 95% confidence intervals (CI) for estimates. Numeric results are given in **Supplementary Table 2A**.

Figure 2. Longitudinal trajectories of nasopharyngeal microbiome. Multiple factor analysis and k-means cluster analysis separated children into trajectories (vertical facets) based on similar patterns of "baseline" microbiome from routine samples, healthy or ill, in the first 2 years of life. **A.** Distribution of MPGs as proportions of routine samples (vertical axis) across each trajectory with timepoint of sampling (horizontal axis). Timepoints labelled by approximate time of routine visit (e.g. 2 mo refers to time period spanning 0 to 3 mo; 4 mo refers to 3 to 5 mo; etc.). Note the distinctive patterns observed for MPGs in each trajectory, especially in the first 6 months of life. **B.** Proportion of samples with MPG present during acute wheezing illness in the first 3

639 years of life, among individuals assigned to each “baseline”, routine sample-based
640 microbiome trajectory as in panel **A**.
641
642 **Figure 3.** Association of nasal microbiome trajectories with the frequency of wheezing
643 illnesses. Number of wheezing illnesses in year 1, 2 and 3 of life was determined for
644 individuals in each of the four nasal microbiome trajectories (A, B, C and D).
645 Microbiome trajectory C dominated by early Staphylococcus.29eb was associated with
646 increase in number of wheezing illnesses over time (Kruskal test, p = 0.0006 for
647 Trajectory C).
648
649 **Figure 4.** Association of nasal microbiome trajectories with asthma. Nasal microbiome
650 Trajectory C dominated by early Staphylococcus.29eb is associated with higher
651 frequency of asthma at each scheduled assessment (A). P-values were obtained using
652 Chi square test across all trajectories (top, in black) or post-hoc Bonferroni-corrected
653 comparisons for Trajectory C vs. all other Trajectories (A+B+D; bottom, in purple).
654 Nasal microbiome Trajectory C had higher proportion of children with a persistent
655 asthma phenotype compared to the other trajectories (B, Trajectory C vs. other
656 trajectories, p=0.08).
657
658 **Figure 5.** Associations between nasal microbiome trajectories and indicators of atopy.
659 Nasal microbiome Trajectory C had consistently higher proportion of children who were
660 sensitized to at least one aeroallergen (A), with similar nonsignificant trends for total IgE
661 (B) and blood eosinophils (C). *P <0.05 for Trajectory C vs. other all trajectories.

662

663 **Figure 6.** Association of microbial pathogen detection during illnesses with asthma.
664 Detection of rhinovirus during wheezing illnesses was associated with increased risks
665 for developing asthma at multiple ages (A). Wheezing illnesses during the second and
666 third years of life were most strongly related to persistent asthma (B). Similar patterns
667 were noted for Moraxella d253 (panels C and D). P<0.001 for all comparisons, Fisher's
668 Exact Test.

669

670 **Table 1.** Demographic characteristics of children in the four nasal microbiome
671 trajectories.*

672

Variable	Trajectory				
	A	**B**	**C**	**D**	**p-value**
	n=79	n=43	n=26	n=135	
Sex (% male)	51%	51%	69%	59%	0.30
Exclusive breastfeeding 6 mo	32%	30%	46%	30%	0.43
Dog in home at birth	42%	37%	23%	34%	0.36
Cat at home at birth	35%	21%	35%	28%	0.34
Cesarean delivery	16%	12%	12%	13%	0.91
Maternal asthma ever	46%	33%	35%	44%	0.40
Paternal asthma ever	36%	23%	27%	29%	0.51
Day care in first year	41%	53%	50%	47%	0.54
Non-Caucasian Race	14%	9%	8%	15%	0.75
Mother education (at least 3 years college)	73%	76%	73%	70%	0.91
Household income ≥ $50,000	57%	68%	62%	54%	0.43
Older siblings	52%	67%	46%	55%	0.28

673

674 * Association analyses were conducted using Fisher exact tests for categorical
675 variables, and Kruskal tests for continuous variables, across all trajectories.