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Seasonal airway microbiome and transcriptome interactions promote childhood asthma exacerbations

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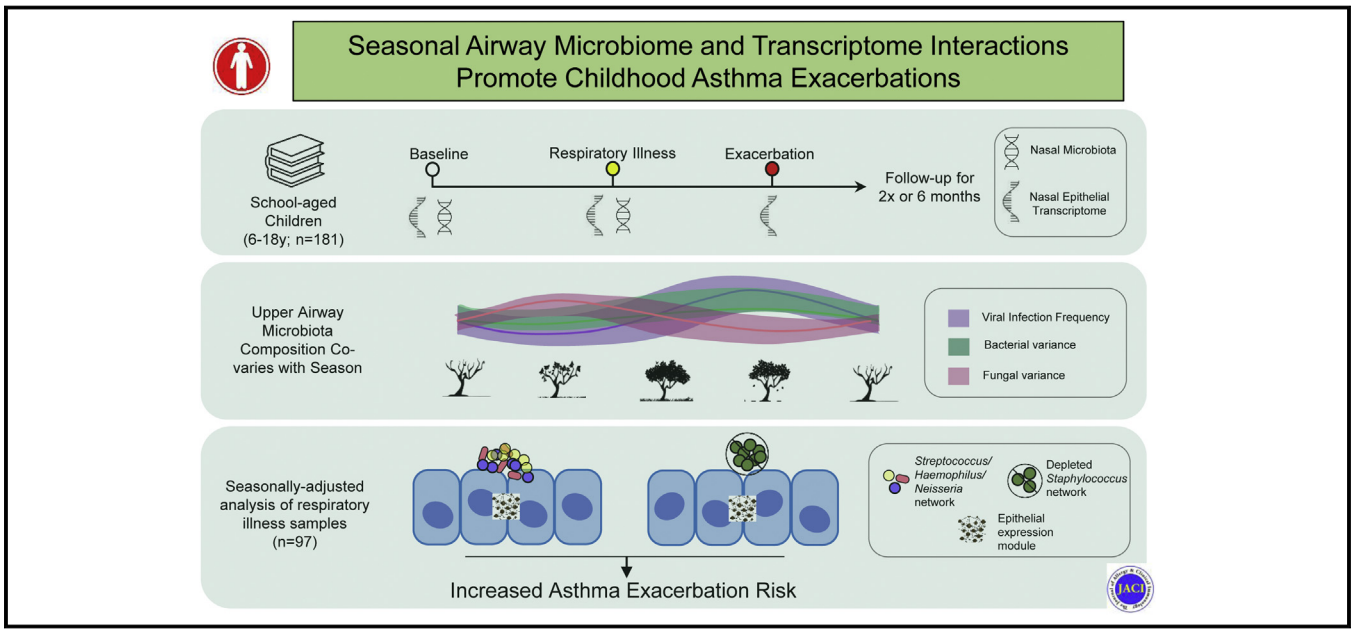
GRAPHICAL ABSTRACT



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Background: Seasonal variation in respiratory illnesses and exacerbations in pediatric populations with asthma is well described, though whether upper airway microbes play season-specific roles in these events is unknown.
Objective: We hypothesized that nasal microbiota composition is seasonally dynamic and that discrete microbe–host interactions modify risk of asthma exacerbation in a season-specific manner.
Methods: Repeated nasal samples from children with exacerbation-prone asthma collected during periods of respiratory health (baseline; n = 181 samples) or first captured respiratory illness (n = 97) across all seasons, underwent bacterial (16S ribosomal RNA gene) and fungal (internal transcribed spacer region 2) biomarker sequencing. Virus detection was performed by multiplex PCR. Paired nasal transcriptome data were examined for seasonal dynamics and integrative analyses.
Results: Upper airway bacterial and fungal microbiota and rhinovirus detection exhibited significant seasonal dynamics. In seasonally adjusted analysis, variation in both baseline and respiratory illness microbiota related to subsequent exacerbation. Specifically, in the fall, when respiratory illness and exacerbation events were most frequent, several *Moraxella* and *Haemophilus* members were enriched both in virus-positive respiratory illnesses and those that progressed to exacerbations. The abundance of 2 discrete bacterial networks, characteristically comprising either *Streptococcus* or *Staphylococcus,* exhibited opposing interactions with an exacerbation-associated *SMAD3* nasal epithelial transcriptional module to significantly increase the odds of subsequent exacerbation (odds ratio = 14.7, 95% confidence interval = 1.50-144, *P* = .02; odds ratio = 39.17, 95% confidence interval = 2.44-626, *P* = .008, respectively).
Conclusions: Upper airway microbiomes covary with season and with seasonal trends in respiratory illnesses and asthma exacerbations. Seasonally adjusted analyses reveal specific bacteria–host interactions that significantly increase risk of asthma exacerbation in these children. (J Allergy Clin Immunol 2022;■■■:■■■-■■■.)

Key words: *Microbiome, respiratory illness, transcriptomics, pediatric asthma, exacerbations, virus infection*

Abbreviations used
ITS2: Internal transcribed spacer region 2
OR: Odds ratio
OTU: Operational taxonomic unit
PERMANOVA: Permutational analysis of variance
RNA-Seq: RNA sequencing
rRNA: Ribosomal RNA
RV: Rhinovirus
SMAD3: Mothers against decapentaplegic homolog 3

Incidence of asthma exacerbation is seasonal and a leading cause of pediatric morbidity.[1] The annual peak in the United States is typically observed in the fall (September through November), with some variation by geographical region.[2] Seasonal variability in acute asthma exacerbations requiring hospitalization is also strongly associated with age and is more likely to vary by season in younger children.[3] Asthma exacerbations track with seasonal trajectories of viral upper respiratory infections.[4] Approximately 80% of acute asthma exacerbations in children present with a cooccurring respiratory virus, with rhinovirus (RV) accounting for 60% to 70% of virus infection–associated exacerbations.[5] Thus, understanding upper airway factors that covary with season and potentiate these clinical respiratory events may offer opportunities to prevent or modify their incidence.

Recent studies have revealed relationships among distinct upper airway microbiota, virus infections, and risk of asthma exacerbations, suggesting that interactions between the upper airway microbiome and airway mucosal responses may influence susceptibility to these respiratory events.[6,7] Specific bacterial genera dominate distinct upper airway microbiota structures, with those dominated by *Moraxella catarrhalis, Streptococcus pneumoniae,* or *Haemophilus influenzae* emerging across multiple independent studies as associated with age[6] and RV-associated asthma exacerbation.[6,7] Further, longitudinal studies of pediatric populations without asthma have identified seasonal relationships among upper airway microbiota composition, viral upper respiratory infection, and sinusitis.[8,9] These data suggest that the upper airway microbiota is dynamically responsive to seasonal variation and could explain the heightened seasonal susceptibility to respiratory illness and exacerbation events in populations of children with asthma.

Airway immune responses clearly contribute to the relationship between upper respiratory infections and asthma exacerbations.[10] Using nasal epithelial transcriptional analysis, we previously identified specific gene expression modules (groups of coexpressed genes), which are differentially regulated during viral and nonviral exacerbations, including upregulation of those

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involved in cell differentiation and wound healing, eosinophil activation and mucus hypersecretion, extracellular matrix production, and epidermal growth factor receptor signaling.[10] Collectively, these findings support the concept that viruses, type II inflammatory immune responses, epithelial integrity, and upper airway microbiota represent important and potentially interactive contributors to asthma exacerbations.

We therefore hypothesized that the upper airway microbiota is dynamic and covaries with season. We further postulated that season- and age-aware algorithms are necessary to uncover relationships between upper airway microbiota and respiratory illness or exacerbation events in children with asthma. Using longitudinal nasal samples collected from participants in the MUPPITS-1 study of urban-residing children (6-17 years old) with exacerbation-prone asthma, we found that the upper airway microbiome indeed exhibits seasonal dynamics. Further, by leveraging seasonally adjusted analyses, we uncovered networks of upper airway microbes that interact with epithelial transcriptional modules during respiratory illness events to significantly increase risk of subsequent exacerbation.

METHODS

Samples obtained from children enrolled onto the Mechanisms Underlying Asthma Exacerbations Prevented and Persistent with Immune-based therapy (MUPPITS-1; NCT02502890) trial were used for this study, which was part of the Inner-City Asthma Consortium.[10] Children enrolled onto this study lived in 1 of 9 inner-city study sites in the United States within Boston, Chicago, Cincinnati, Dallas, Denver, Detroit, New York, St Louis, and Washington, DC; were between 6 and 17 years old; had doctor-diagnosed asthma; had experienced at least 2 exacerbations in the previous year; and had eosinophil counts greater than 150 cells/mm^3. In addition, eligible participants required treatment with at least 1 puff twice daily of either fluticasone 250 μg or fluticasone/salmeterol (Advair, Glaxo Smith Klein, Middlesex, United Kingdom) 250/50 μg for those less than or greater than 12 years old, respectively, and were nonsmokers. Children provided an initial baseline visit sample at enrollment and were then observed for upper respiratory illness symptoms and asthma exacerbations for either 6 months or 2 respiratory illness visits, whichever came first. Exacerbations were defined as a need for systemic corticosteroid therapy or hospitalization.

A clinic visit with nasal sampling occurred within 3 days of the start of reported upper respiratory illness symptoms for each illness (V1a and V2a, respectively). For this study, baseline and V1a samples were used. For each illness event, a subsequent asthma exacerbation was recorded if there was a requirement for systemic corticosteroids up to 10 days after the onset of respiratory illness symptoms. Nasal mucus samples collected at baseline and V1a were processed for microbiota (16S ribosomal RNA [rRNA] and internal transcribed spacer region 2 [ITS2] analyses); nasal lavage samples collected in parallel underwent RNA sequencing (RNA-Seq) analyses as previously described.[10] Predefined exacerbation-associated transcriptional modules identified in the parent study[10] were used in this investigation. In brief, gene expression data were clustered using weighted correlation network analysis into modules. These modules were then annotated using the Database for Annotation, Visualization, and Integrated Discovery. STRING identified known or predicted protein–protein interactions. Summary annotations for each module were derived from a manual inspection of a module's cell type assignment, functional enrichments, and interaction network.

This study was approved by the institutional review boards of all institutions. All participants or their legal guardians provided written informed consent before enrollment onto the study.

Nasal blow collection and processing

The sample collection protocol was developed by the Inner-City Asthma Consortium. Samples were collected under the supervision of a physician. Deep Sea saline nasal spray was administered into one nostril, and the child expelled the liquid from the nostril into a small plastic bag, then repeated in the opposite nostril. After the procedure, 1.5 mL of Addipak sterile saline (Hudson RCI, Temecula, Calif) was added to the bag, and the sample was mixed in and maintained in the refrigerator for additional processing within 24 hours. During additional processing, the sample was transferred completely from the plastic bag into M4RT viral transport media (Thermo Fisher Scientific, Waltham, Mass) and vortexed vigorously for 1 minute; 500 μL was formed into aliquots for virus analysis at the University of Madison. Subsequently, 1000 μL was formed into aliquots in 3 tubes stored on site, 1 aliquot of which was shipped to the University of California, San Francisco, for 16S rRNA and ITS2 biomarker sequencing.

Virus detection and specification

Respiratory viruses were assessed in nasal mucus samples obtained by the above-described nasal blow technique at respiratory illness events as previously described[11] using multiplex PCR, including the identification of all common respiratory viruses. Partial sequencing of RV allowed for further classification into species and types.[12]

Nasal 16S rRNA gene and ITS2 sequencing

Nasal blow samples obtained at baseline and respiratory illness clinic visits underwent 16S rRNA gene and ITS2 sequencing. DNA was extracted from all samples using a modified cetyltrimethylammonium bromide buffer extraction protocol as previously described.[13] Extracted DNA was amplified for the 16S rRNA gene V4 region using 515F and 806R primer pairs as previously described by Caporaso et al.[14] After sequencing on the NextSeq 500 platform (Illumina, San Diego, Calif), bacterial reads were demultiplexed by barcode and then assembled using FLASH v1.2.7,[15] and low-quality reads were discarded using QIIME 1.9.1.[16] Sequences with more than 2 consecutive bases having a Q-score of less than 30 were truncated. Sequences were dereplicated and binned into operational taxonomic units (OTUs) using a 97% sequence similarity threshold using USEARCH.[17] OTUs were removed if they were determined to be chimeric or of nonbacterial origin using QIIME. Negative controls provided information about background signal, and OTUs were removed if they were in more than half of negative controls and most of the samples. Any remaining negative control read counts were subtracted from samples using the maximum read count across negative controls. Upper airway bacterial sequence data were representatively rarefied at 20,722 reads per sample, a level selected to optimize sample count and community coverage.

Using extracted DNA, ITS2 of the rRNA gene was also amplified using fITS7 and ITS4, designed for the Illumina MiSeq platform and processed as previously described.[18] Fungal microbiota sequence data underwent similar procedures after sequencing with the following modifications. Adapter sequences were removed and sequences were quality trimmed using 'cutadapt'[19] before assembling paired-end reads with FLASH. ITSx extractor was used to identify sequences as specifically fungal and not of plant origin.[20] Any sequences not passing this filter were removed. Taxonomy was assigned to OTUs using UNITE.[21] Samples were retained if they had at least 50 fungal reads, and variance-stabilized transformations were used to normalize for differences in library depth and retain most samples.[22]

Statistical analyses

To relate clinical events with changes in season, generalized additive mixed models and generalized additive models were fitted to identify significant change throughout the year using the 'mgcv' package in R. For relationships with respiratory illness and exacerbation, a generalized additive mixed model was used with months as a spline and the option for a cyclic cubic regression spline; exacerbation or respiratory illness events were the dependent variable, and a logit regression was used. If 2 events occurred in the same month, only 1 event was noted. A random effect for subject was also included. This analysis used a participant-month data set (around 1300 observations over 208 individuals). Analyses of virus infection were cross-sectional, where a cyclic cubic regression spline was used for the dependent months variable, as well as logit

regression on a participant level data set for each visit type, with the assumption that the lack of visit did not necessarily indicate the lack of virus infection. Therefore, the "denominator" in the associated plot is the number of visits that occurred within that month.

Analyses with microbiome, fungal, and transcriptome relationships were analyzed similarly, except that the first principal coordinate (PC) was used as the dependent variable in a Gaussian regression. When data were available for fewer than 10 months, a smaller number of knots (evenly distributed cut points between which a continuous curve is fitted to the data) were used in the analysis. The estimated degrees of freedom statistic describes the nonlinearity in the curve over time, with larger numbers representing greater variability in the fitted line. Significant *P* values indicate the probability of a nonlinear trend. Predicted values were obtained from the model using the 'predict' function and represented using the 'ggplot2' package in R. To ensure model fit, we reduced data for mixed models to months where individuals contributed at least 5 days of observation. For associations with transcriptome modules, the second principal coordinate was used, as the first was primarily explained by differences in cell counts.[10] To identify the specific bacteria, fungi, and gene expression modules that related to the principal coordinate, Spearman correlations were used and the correlation coefficient plotted.

Nasal bacterial and fungal microbiota distance matrices were calculated using Bray Curtis and Canberra distance matrices, and bacterial microbiota distance matrices were also calculated by Unweighted and Weighted UniFrac. Bacterial and fungal distance matrices were calculated using 'phyloseq' in R.[23] Principal coordinates were calculated using the 'ape' package.

Permutational analyses of variance (PERMANOVA) were performed using the 'adonis2' function from the 'vegan' package,[24] and 999 permutations with marginal tests (by = "margin"), including exacerbation, virus detection, age, sex, and study site in the same model. $P < .05$ was considered significant, with trends being described at $P < .1$. Differentially abundant OTUs were identified by DESeq2 in R,[25] filtering to OTUs present in at least 10% of samples, and using size factors calculated from positive counts and a local fit type. Relationships with age included additive adjustments for sex, study site, and season, while season-specific outcome models also included adjustments for exacerbation and virus detection. *P* values were corrected for multiple comparisons using the Benjamini-Hochberg correction, and false discovery rate–corrected $P < .2$ was considered significant.

For analyses of OTU networks, nasal OTUs were filtered out if they were present in less than 10% of samples, and correlations between OTUs were identified using SparCC[26] from the SpiecEasi package in R. A correlation of 0.5 or more indicated a connection, and networks were identified using a cluster_fast_greedy approach from the 'igraph' package,[27] which uses the random walker method. Nineteen networks were identified (see Table E5 in this article's Online Repository at www.jacionline.org). To create a network-specific OTU table, OTUs were collapsed by summing counts of OTUs within each network for each sample. Any taxa that did not have any connections were agglomerated into an "unassigned" network and are not presented in Table E5.

To delineate the specifics of these relationships in the context of season and clinical outcomes, we examined associations of these networks with 4 transcriptome modules previously identified in this cohort, including those associated with exacerbation (ie, epithelial mothers against decapentaplegic homolog 3 [*SMAD3*]-related cell differentiation, eosinophil activation, and mucus hypersecretion, extracellular matrix production, and *EGFR* signaling modules).

To identify instances where the combination of microbiota networks and gene expression modules associated with distinct risk of subsequent exacerbation, we utilized interaction terms in logistic regression models with exacerbation as the dependent variable, and pairwise gene expression modules and microbiome networks as interaction terms (ie, exacerbation ~ gene expression module × microbiome network). Interaction models are adjusted for age, sex, and season. For these models, study site increased the degrees of freedom, reduced the models' ability to converge, and did not change any effect estimates by more than 10%, and thus it was not used for adjustments. Models with significant interaction terms were stratified by the median count of the bacterial network to understand the nature of significant interactions. In other words, in the context of a large abundance of a specific microbiota network, a gene expression module is associated with the risk of exacerbation. Interactions were considered significant if the *P* value for the overall interaction effect was less than .05 or if the *P* value for a specific median stratum was less than .05 with an overall interaction *P* value was less than .1. Plots for visualization of interactions were developed using the 'emmip' function from the 'emmeans' package in R.[28]

Amplicon sequencing data sets generated and analyzed under the current study are available in the European Nucleotide Archive under accession number PRJEB42394. Transcriptomics data sets are available from the original publication (PMID:30962590). Participant-level metadata are available upon request.

RESULTS

Clinical and upper airway microbiota features covary with season

Relationships among season and clinical events, upper airway microbiome, and nasal epithelial transcriptome were initially assessed independently. Among 208 children with asthma enrolled onto the study, a total of 164 respiratory illnesses and 143 exacerbations were reported. Consistent with previous reports,[7-9,29,30] incidence of respiratory illnesses and exacerbations exhibited seasonal trends (generalized additive mixed model for respiratory illnesses; $P < .001$, Fig 1, *A;* $P = .039$, Fig 1, *B*), with the highest frequency in fall and winter and the lowest frequency in the summer months. An increase in both respiratory illnesses and exacerbations was evident from late summer through late fall. As expected, detection of any respiratory virus (picornavirus, coronavirus, influenza, or others) also varied by season; this was true during baseline visits in the absence of illness (visit 0; generalized additive model $P = .012$) and also during respiratory illness events (visit 1a; $P = .003$; Fig 1, *C*). More specifically, in respiratory illness samples, RV-A and RV-B, but not RV-C, exhibited significant seasonality (RV-A $P = .015$, RV-B $P = .008$, Fig 1, *D*), peaking in late summer and fall.

Likewise, 16S rRNA nasal microbiota composition varied with season in both baseline and respiratory illness samples (baseline n = 181, $P = .001$; respiratory illness n = 97, $P = .016$, respectively, Fig 1, *E*). Distinct upper airway microbiota were evident in late spring and early fall compared to other seasons, primarily driven by increased relative abundance of multiple *Moraxella* taxa in spring and several *Staphylococcus* in the fall (see Fig E1 in the Online Repository at www.jacionline.org). Upper airway fungal microbiota did not relate to season in the absence of a respiratory illness (visit 0 n = 108, $P = .742$, Fig 1, *F*). However, a significant relationship between season and variation in fungal microbiota composition was observed in respiratory illness samples (visit 1a n = 57, $P = .006$, Fig 1, *F*). Illnesses experienced in the spring associated with *Malassezia* enrichment, while those in the fall were associated with enrichment of allergenic fungi, *Candida,* and *Cladosporium* (see Fig E2 in the Online Repository).

Nasal transcriptomes were also assessed for relationships with season within baseline and respiratory illness samples. For baseline samples, a suggestion of variation throughout the year did not quite reach statistical significance (visit 0 Principal Coordinate [PC] 2, $P = .071$, n = 94, Fig 1, *G*), though in the late spring months, in parallel with elevated *Moraxella* abundance, increases in chromatin modification, integrin, and keratinization modules were evident (see Fig E3 in the Online Repository at www.jacionline.org). Respiratory illnesses were associated with epithelial transcriptional modules related to antigen presentation (Fig E3), although no association between season and epithelial transcription was identified in these samples (visit 1a PC2, $P = .149$,

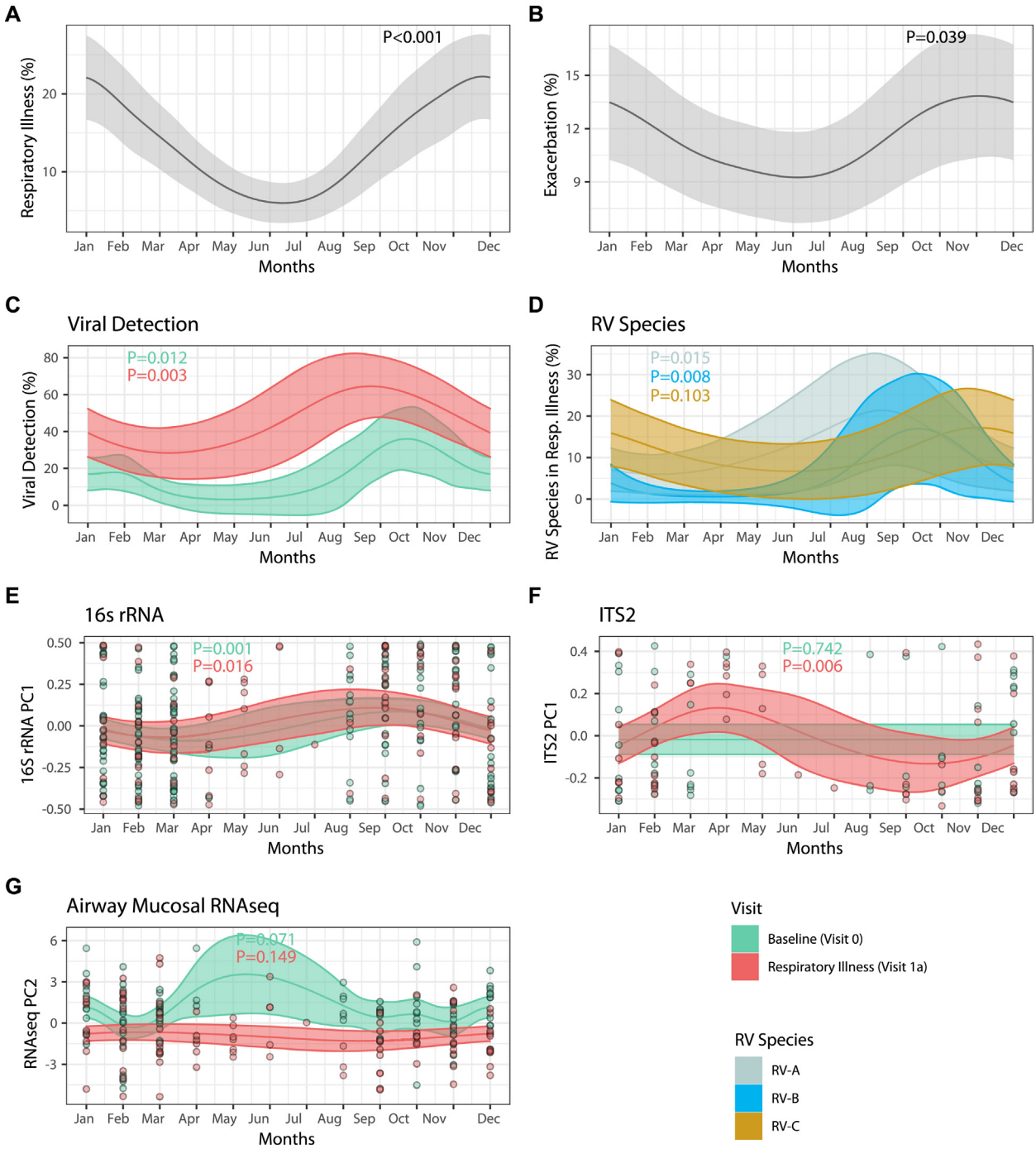

FIG 1. Variation in (**A**) respiratory illness (respiratory illness events as a proportion of total individuals observed each month), (**B**) exacerbation (exacerbation events as a proportion of total individuals observed each month), (**C**) virus detection (proportion of samples with detectable virus), (**D**) RV species during respiratory illness events, (**E**) nasal bacterial composition (16S rRNA, PC1), (**F**) mycobiome (ITS2, PC1), and (**G**) nasal transcriptome (airway mucosal RNA-Seq, PC2) across seasons in baseline (visit 0) and respiratory illness (visit 1a) samples. *PC*, Principal coordinate; *ITS*, Internal Transcribed Spacer; *RV*, Rhinovirus.

n = 97, Fig 1, *G*), suggesting that host transcriptional response to respiratory infection is relatively conserved across seasons. Relationships with PC1 were examined but did not reach statistical significance and were primarily characterized by differences in cell types (see Fig E4 in the Online Repository).

Baseline nasal microbiota relates to time to respiratory illness

Consistent with previous reports,[6] older children in this study exhibited nasal enrichment of several *Staphylococcus* and *Corynebacterium* members, while younger children exhibited

increased *Moraxella, Haemophilus,* and *Alloiococcus* taxa in their baseline samples (n = 181; Fig 2, *A;* see Table E1 in the Online Repository at www.jacionline.org). Among children with a baseline sample, those who reported a first respiratory illness visit did so between 3 and 202 days after the baseline visit (n = 93). While nasal bacterial microbiota composition at baseline was not associated with the time to respiratory illness (Unweighted UniFrac, PERMANOVA $R^2 = 0.014$, $P = .13$, adjusted for age and season of sampling), it was significantly associated with time to exacerbation (Weighted UniFrac, $R^2 = 0.034$, $P = .04$, adjusted for age and season). These data indicate that age- and season-adjusted analyses are necessary to reveal relationships between upper airway microbiota and length of time to exacerbation in children with asthma.

Fungus composition at baseline (n = 53) was primarily associated with the age of the child (Bray Curtis, PERMANOVA, $R^2 = 0.044$, $P = .001$) after adjustment for exacerbation, virus detection, sex, and study site ($P > .4$ for all). Age-associated fungi included a single *Malassezia* taxon (associated with older children) and distinct members of *Ascochyta, Malassezia, Cladosporium,* and *Verticillium* associated with younger-age participants (Fig 2, *B;* see Table E2 in the Online Repository at www.jacionline.org). This subset of children returned for a respiratory illness visit between 5 and 192 days after the baseline visit, but fungal communities at baseline were not associated with time to first illness (Bray Curtis, PERMANOVA $R^2 = 0.021$, $P = .242$; after adjustment for age, study site, virus positivity, and season). A single *Cladosporium* taxon (OTU8, the same *Cladosporium* OTU associated with younger children in the study), was found to be enriched in children who experienced a longer duration to respiratory illness (OTU8 $\log_2$ fold change = 0.106, false discovery rate–corrected $P = .006$), after adjustment for age, study site, and season of sample collection.

Nasal microbiota during respiratory illness exhibits season-specific relationships with clinical outcomes

Season-specific relationships with nasal microbiota, virus detection, and exacerbations were observed in respiratory illness samples. Specifically, nasal bacterial microbiota composition of respiratory illness samples in the fall (n = 34) associated with virus detection (Weighted UniFrac, PERMANOVA, $R^2 = 0.089$, $P = .017$) and with subsequent exacerbations ($R^2 = 0.078$, $P = .027$) in a multivariable model that also included study site, age, and sex ($P > .2$ for site, age, and sex; Table I). In the fall, bacteria associated with respiratory illnesses and subsequent exacerbations included several *Moraxella* and *Haemophilus* members, which were enriched both in virus-positive respiratory illnesses and those that progressed to exacerbations (DESeq2, Fig 3, *A* and *B;* see Tables E3 and E4 in the Online Repository at www.jacionline.org). Nasal bacterial microbiota detected in respiratory illnesses during winter (n = 33) primarily covaried with virus detection (Weighted UniFrac, PERMANOVA, $R^2 = 0.097$, $P = .008$), study site ($R^2 = 0.325$, $P = .050$), and age ($R^2 = 0.11$, $P = .009$), but not with exacerbations or child's sex ($P > .15$ for each; Table I) in a multivariable model. Relationships between these variables and spring (n = 22) or summer (n = 8) upper airway microbiota did not reach statistical significance, likely as a result of the smaller sample sizes available during these seasons. Relationships using the same multivariable model and including all samples were primarily related to age, with no significant relationships with exacerbation or virus infection observed (Table I). Taken together, these data suggest that season-specific bacterial interactions influencing respiratory illness and exacerbations in children with asthma are strongest in the fall.

During respiratory illnesses (n = 58), fungal composition primarily associated with season (Bray Curtis; PERMANOVA; $R^2 = 0.076$, $P = .008$), and to a lesser extent age ($R^2 = 0.025$, $P = .055$). Neither baseline nor illness fungal composition associated with exacerbation risk.

Interactions between microbial networks and immune transcriptional modules influence asthma exacerbation risk

We next hypothesized that interactions between exacerbation-associated epithelial transcriptional modules and specific networks of cocolonizing microbes in the upper airways during respiratory illness influence the risk of subsequent exacerbation in a season-specific manner. Before this study, host transcriptional modules from RNA-Seq of paired nasal lavage samples were developed using weighted correlation network analysis; these modules were found to discriminate viral and nonviral exacerbations.[10] Because of our sample size, we focused specifically on the 4 host transcriptional modules previously found to be associated with exacerbation events in this cohort:[10] epithelial *SMAD3*-related cell differentiation, eosinophil activation and mucus hypersecretion, extracellular matrix production, and epidermal growth factor receptor signaling. Bacterial (16S rRNA) data also underwent network analysis to reduce data dimensionality. This process identified 19 bacterial networks (N1-19; Table E5); interactions between these bacterial networks and the 4 exacerbation-associated transcriptome modules were assessed using logistic regression.

Seasonally adjusted analysis of respiratory illness samples (n = 97) revealed that *SMAD3* interacted with 2 bacterial networks (N1 and N3) to significantly modify the risk of subsequent exacerbation. N1 primarily comprised multiple *Veillonella, Streptococcus, Neisseria,* and *Haemophilus* OTUs (interaction $P = .050$), while N3 comprised entirely of *Staphylococcus* OTUs (interaction $P = .059$). Specifically, increased expression of *SMAD3* among children with higher abundance of the N1 bacterial network was associated with increased exacerbation risk (odds ratio [OR] = 14.71, 95% confidence interval [CI] = 1.50-144.14, $P = .018$; Fig 4, *A,* adjusted for age and season). Children who possessed a reduced abundance of the N3 network in the context of increased *SMAD3* expression also exhibited increased risk of exacerbation (OR = 39.17, 95% CI = 2.44-626.48, $P = .008$, Fig 4, *B,* adjusted for age and season), suggesting that low abundance or loss of this nasal bacterial network may promote exacerbation. Importantly, these 2 bacterial networks were not inversely related or mutually exclusive (χ^2 $P = .747$). When bacterial networks were not considered in the relationship between *SMAD3* and exacerbation, odds ratios were substantially lower (OR = 3.22, 95% CI = 1.28-8.07, $P = .01$), and in interaction models unadjusted for season, interaction P values were nonsignificant ($P = .11$ and .18). Thus, these findings provide evidence that interactions between specific upper airway microbial networks and host transcriptional modules during respiratory illness increase the risk of exacerbation in a season-specific manner.

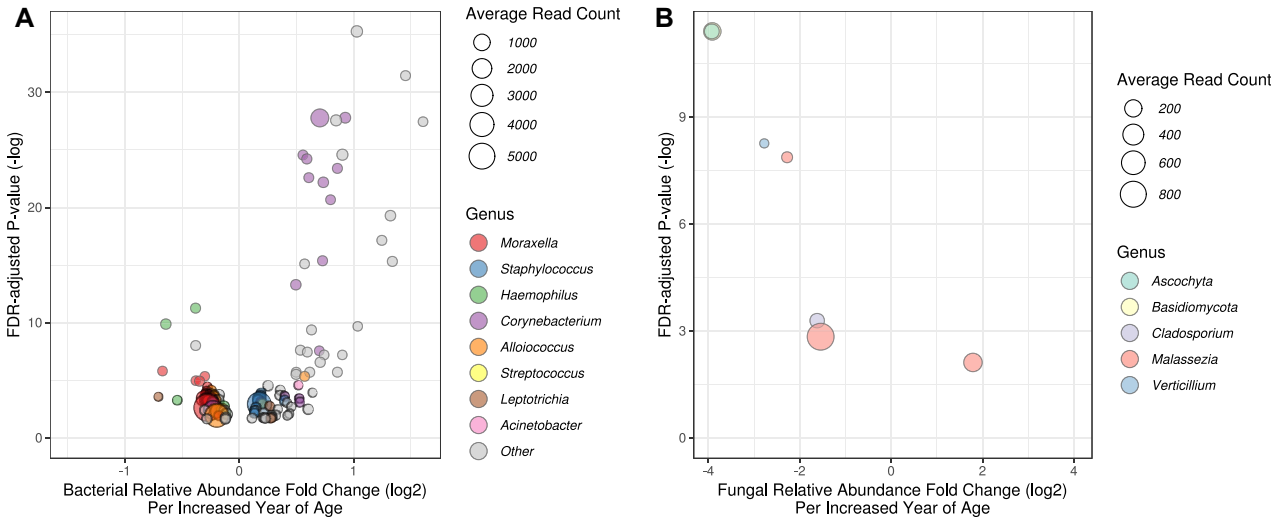

FIG 2. A, Specific bacterial taxa in baseline samples that associate with age (n = 181). *Moraxella* and *Haemophilus* decrease in relative abundance with increasing age, while *Corynebacterium* and *Staphylococcus* increase in abundance with increasing age. **B,** Fungus taxa in baseline samples related to age (n = 53). Positive fold changes reflect taxa that increase with child's age; negative fold changes, those that decrease with increasing age. Data are color coded by genus-level taxon identity; circle size indicates average relative taxon abundance. All findings are adjusted for sex, season of sample collection, and study site.

TABLE I. Variation in season-specific nasal microbiota during respiratory illness relates to clinical outcomes in children with asthma using Weighted UniFrac distance

	Overall (n = 97)		Fall (n = 34)		Winter (n = 33)	
Variable	R^2	***P* value**	R^2	***P* value**	R^2	***P* value**
Exacerbation	0.005	.747	0.078	.027	0.020	.455
Virus positive	0.013	.239	0.089	.017	0.097	.008
Age (years)	0.034	.017	0.025	.453	0.110	.009
Study site	0.111	.093	0.151	.785	0.325	.050
Sex	0.019	.093	0.036	.251	0.034	.194

R^2 values and *P* values were obtained from multivariable PERMANOVA; multivariable PERMANOVA included all variables listed.

DISCUSSION

Seasonal variations in respiratory illnesses and asthma exacerbations are well described.[4,31] Although asthma exacerbations occur throughout the year, they are most common in the fall, an observation validated in this cohort of inner-city children with asthma. While this pattern has traditionally been attributed to increased virus exposure after returning to school,[32] there are a number of other potential contributing factors to this seasonal variation. Human behavior covaries with season, including the proportion of time spent indoors and in close proximity to others, increasing the potential for horizontal transmission of cellular microbes as well as viruses. In parallel, atmospheric,[33] human, and animal microbiomes also exhibit seasonal variation,[34,35] as do several respiratory viruses.[36-38] The latter is consistent with observations made in this cohort, which indicate increased RV detection in late summer which peaks in fall and comprises successional waves of RV-A, -B, and -C.

Conditions in the upper airway also covary with seasonal changes in humidity, temperature, air pollution, allergen exposure, and virus infection.[39] In this analysis, while upper airway transcriptional response to respiratory illness was relatively conserved, transcriptional variation in baseline samples was associated with season, particularly summer, when modules involved in keratinization and integrins were expressed. Given that this season exhibits low rates of respiratory illness and exacerbation, it is possible these season-specific host responses protect against virus infection. An alternative possibility is that these transcriptional changes in the summer in children with asthma influence their upper airway microbiome and increase susceptibility to subsequent virus encounters in the late summer and fall season. Yet another possibility is that upper airway microbiota responds to the external environment, including pollution[40] and temperature.[41] One recent publication identified thermo-sensing abilities in *S pneumoniae* and *H influenzae* leading to altered expression of virulence factors under distinct temperature conditions.[41] Seasonal variation in nasal microbiota composition, especially in infants and children, has previously been described,[7,9,29,30] and the types of bacteria present in the upper airways during the fall season relate to risk of respiratory illness and exacerbation in children with asthma.[6]

These observations raised the possibility that the upper airway microbiome and microbial exposures may dynamically interact with each other and the host in a season-specific manner to influence respiratory illness susceptibility and exacerbation outcomes in children with asthma. This prompted an examination of microbiome features in season-specific and -adjusted analyses

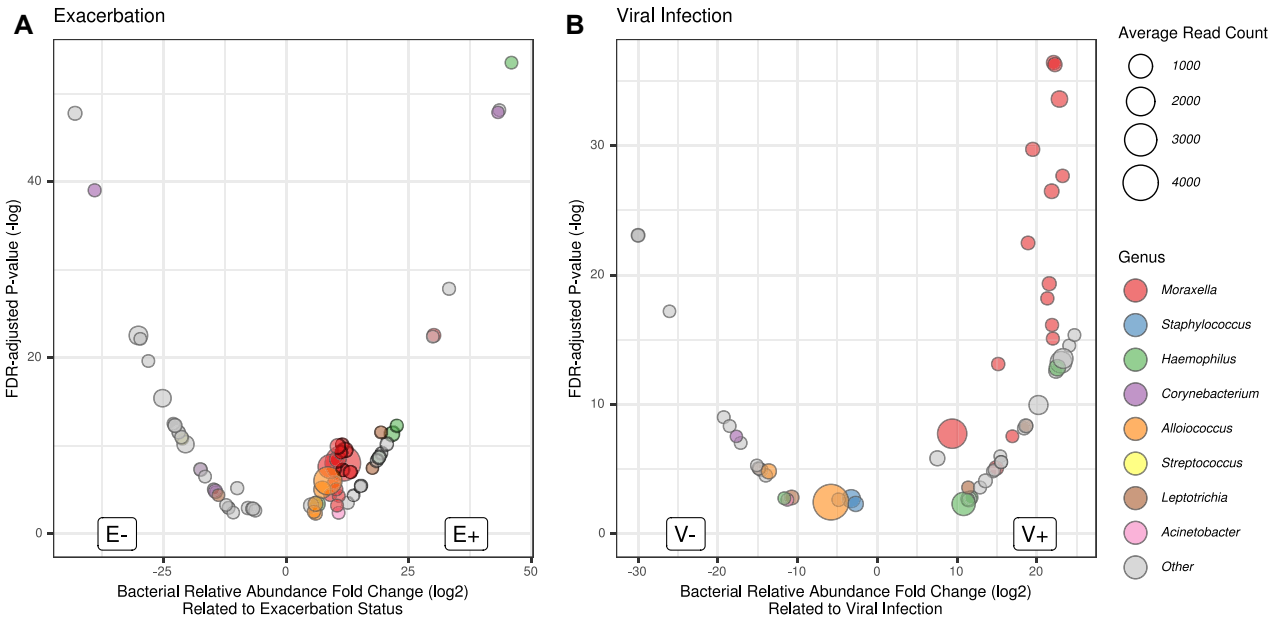

FIG 3. Specific bacterial taxa in fall respiratory illness samples (n = 34) relate to (**A**) subsequent exacerbation and (**B**) virus infection. Taxa are color coded by genus-level taxon identity; circle size indicates average relative taxon abundance. All findings are adjusted for exacerbation, virus infection, sex, season of sample collection, and study site.

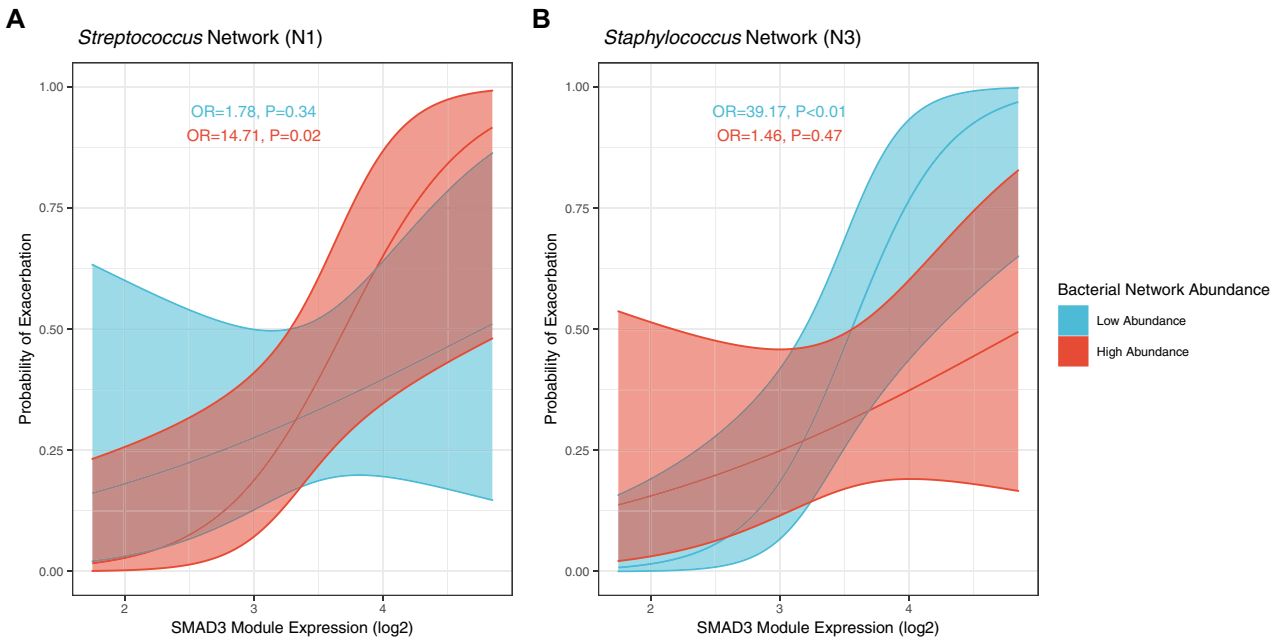

FIG 4. Interactions between *SMAD3* expression and 2 upper airway bacterial networks (N1 and N3) during respiratory illness events significantly increases the probability of subsequent exacerbation (age, sex and season of respiratory illness adjusted analysis). **A**, Interaction between *SMAD3* expression and elevated abundance of the N1 bacterial network increases risk of exacerbation. **B**, Interaction between *SMAD3* expression and reduced abundance of the N3 bacterial network increases risk of exacerbation. Slopes indicate the association between exacerbation and *SMAD3* expression among subsets of children differentiated by the median count of reads in each network, with 4558 reads in (*A*) and 7949 reads in (*B*).

that associated with respiratory illness and subsequent exacerbation. Fall respiratory illness microbiota were enriched for *Haemophilus* and *Moraxella* in the context of both virus infection and subsequent exacerbation, which is consistent with our previous study that longitudinally profiled the upper airway bacterial microbiota of over 400 children with asthma specifically during the fall season.[6] Thus, the presence of specific bacterial genera in the upper airway microbiota during fall respiratory illnesses

increased the likelihood of subsequent exacerbation and virus detection, suggesting that their interaction with host response to virus infection relate to these outcomes.

Host transcriptome analyses provide insights into the host response during respiratory illnesses and subsequent exacerbations. Our data indicate that combining this information with networks of coassociated bacteria in season-adjusted interaction models permits identification of children at significantly higher risk of asthma exacerbation. Specifically, those possessing a network of *Streptococcus, Haemophilus, Neisseria, Prevotella,* and other genera (N1) or those lacking a *Staphylococcus* network (N3) were found to have increased risk of exacerbation associated with increased epithelial *SMAD3*-related cell differentiation module expression. Although causative directionality is currently unknown, the *SMAD3* module is composed of several genes including *RRAD*[42] and *TRPV4*[43] that regulate the uptake of calcium. Previous studies have demonstrated that *Staphylococcus aureus* forms less-structured biofilms with increasing calcium availability[44,45] and that calcium improves macrophage response to *H influenzae.*[46] Additionally, the *SMAD3* module also includes the *CLU* gene, which encodes clusterin, a protein associated with cellular debris clearance.[47] Intriguingly, independent studies have shown that clusterin binds efficiently to *S aureus,*[48] improving species-specific bacterial adhesion and reducing the bacteria load required to produce a pathogenic response. These findings support the hypothesis that specific upper airway bacterial networks may leverage host responses to alter their abundance and pathogenicity in the upper airways of children with asthma, thus enhancing disease activity and risk of subsequent exacerbation.

This study provides initial insights into specific host–microbe interactions in the upper airways that promote exacerbation. Legacy effects of these interactions imprinted via epigenetic modifications in key immune cell populations poses a plausible mechanism for the chronicity of asthma. Indeed, previous studies have identified methylation patterns associated with T-cell maturation, T_H2 immunity, and oxidative stress (a key antimicrobial response) in children with asthma,[49] providing data that epigenetic marks in these children relate to asthma development. The role of DNA methylation in the differentiation and function of Treg cells is also well described,[50,51] and in parallel, a recent study found that gut bacteria with enhanced methylation signatures exhibit greater Treg cell induction.[52] How asthma-associated epigenetic modifications in the upper airways are influenced by interactions with colonizing microbes remains unknown but is an active area of new and potentially fruitful research.

While this analysis provides important insights into seasonal variation the upper airway microbiome and its relationship with respiratory illnesses and exacerbations, there are a few limitations to consider. First, children enrolled onto the MUPPITS-1 study reside in urban environments and had active asthma; as such, these findings may not be generalizable to a broader population. Also, the number of samples with bacterial and fungal data available for respiratory illness events, especially those in specific seasons, was limited; a larger sample size may further elucidate these interactions. The study also leveraged DNA amplicon–based microbiota profiles providing information on which microbes exist in the upper airways, limiting our ability to assess microbial activities that relate to increased exacerbation risk. However, the use of RNA-based microbiome analyses to capture transcriptionally active microbes on the upper airway mucosal surface should offer a more accurate view of microbial activities that relate to host immune responses and clinical outcomes. In addition, dual RNA-Seq, capturing both host and microbial transcriptional activity in parallel, could greatly enhance our understanding of the relationships we described. Finally, our findings surrounding microbe–host interactions are correlative and do not prove causality; further studies deconstructing these interactions to delineate mechanisms that govern susceptibility to respiratory infection and exacerbation are clearly required.

These findings indicate that age and seasonal dynamics in upper airway microbiomes represent important factors to consider in analyses of respiratory microbiomes. The study also identifies specific networks of upper airway microbes that interact with host transcriptional responses to significantly increase risk of subsequent exacerbation and that this relationship is also strongly dependent on season. Therefore, host responses in concert with cooperative networks of airway-associated bacteria may promote exacerbation in a season-dependent manner.

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Key messages

- **Asthma exacerbation, respiratory illness, virus infection, RV species, and upper airway microbiota composition all exhibit seasonal dynamics.**
- **During fall respiratory illnesses, nasal microbiota composition relates to exacerbation and virus infection.**
- **Specific networks of upper airway bacteria present during respiratory illness interact with a host gene expression module to increase risk of exacerbation, suggesting that exacerbation risk associated with gene expression may be microbiome dependent.**

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