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ethnicity-stratified analyses revealed that the effect in Latinos was driven mainly by Mexicans and other Latinos (see Table E7 in this article's Online Repository at www.jacionline.org).

In conclusion, the SNP rs67622929 was associated with severe asthma exacerbations in African Americans and Latinos and showed regulatory effects over *DNASE1L3* expression.

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Nasopharyngeal *Haemophilus* and local immune response during infant respiratory syncytial virus infection



To the Editor:

The accumulating evidence suggests that viral-bacterial interactions can affect short- and long-term outcomes of acute respiratory infections (ARIs) due to respiratory syncytial virus (RSV) in infancy. In particular, prior studies have found that in young children with RSV ARIs, a higher relative abundance of *Haemophilus* in the nasopharynx is associated with an increased viral load,¹ delayed viral clearance,² a different gene expression profile,^{3,4} and more severe disease.^{3,4} However, the mechanisms underlying these associations are largely unknown. To address this gap in knowledge, we examined the association of the nasopharyngeal relative abundance of *Haemophilus* with viral load and 52 local immune mediators in 105 infants with an RSV-only ARI (ie, no coinfections) who were enrolled in the Infant Susceptibility to Pulmonary Infections and Asthma following RSV Infection in Infancy (INSPIRE) study.

The INSPIRE cohort is a population-based, birth cohort of healthy, term infants born in middle Tennessee with biweekly surveillance of acute respiratory symptoms during their first winter viral season to capture their initial RSV ARI. Infants who met prespecified criteria for an ARI underwent an in-person visit, which included collection of a nasal wash that was used for RSV detection and viral load assessment by real-time RT-PCR. This nasal wash has also been used for characterization of the nasopharyngeal microbiome and local immune response through 16S ribosomal RNA (rRNA) sequencing and measurement of multiple local immune mediators (ie, cytokines, chemokines, and growth factors) by using Luminex xMAP technology (see Table E1 in the Online Repository at www.jacionline.org), respectively, as previously described.^{5,6} In brief, following bacterial DNA extraction, we amplified the V4 region of the 16S rRNA gene by using universal primers. The libraries were then sequenced on an Illumina MiSeq platform with 2 × 300-bp reads. We subsequently processed the 16S rRNA paired sequences by using the dada2 pipeline, after which we grouped sequences into amplicon sequence variants (ASVs) and assigned taxonomy using the SILVA reference database. We used the relative abundance of *Haemophilus* and *Haemophilus* ASVs (expressed as simple proportions) for statistical analyses. In addition, we used the Basic Local Alignment Search Tool to search the ASV sequences against a reference database and available whole metagenomic sequencing (WMS) from a selected number of nasal washes (n = 6) to identify *Haemophilus* species. The Luminex xMAP

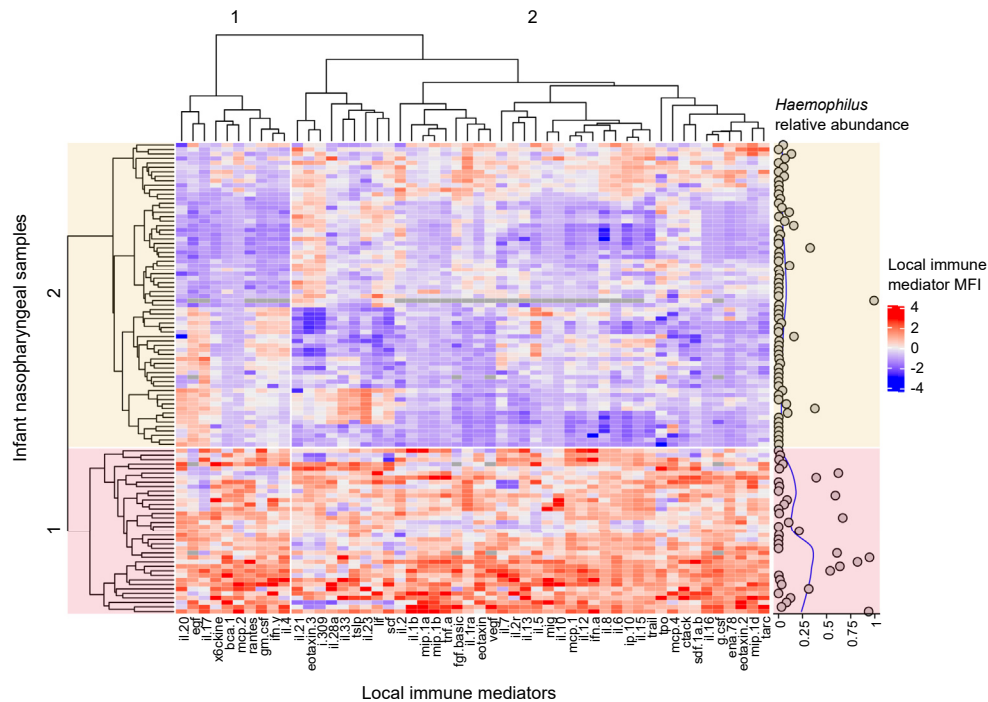


FIG 1. Heatmap of local immune mediators (columns) measured in nasopharyngeal samples (rows) collected during an ARI due to RSV in infancy. The nasopharyngeal relative abundance of *Haemophilus* in each sample with a corresponding fitted loess curve (blue line) is also shown (right panel). The columns and rows were hierarchically clustered by using the complete linkage method based on Spearman correlation coefficients or euclidean distances, respectively. The color of each cell represents the generalized log-transformed median fluorescence intensity (MFI) of the respective local immune mediator in an individual sample after subtracting the column mean and dividing it by its standard deviation as shown in the color scale (blue indicates lower, red indicates higher, and gray indicates missing). The heatmap shows 2 distinct clusters of samples (colored in red and yellow). Cluster 1 is characterized by higher MFIs of most local immune mediators, as well as by an increased nasopharyngeal relative abundance of *Haemophilus*.

data were processed by using a method involving use of median fluorescence intensities of individual beads instead of the usual standard curve-based data processing method to increase the sensitivity and accuracy of high-throughput immunoassays.⁶ To examine the unadjusted and adjusted associations of the relative abundance of *Haemophilus* and *Haemophilus* ASVs with RSV viral load and the local immune mediator median fluorescence intensities, we used Spearman correlation and multivariable linear regression, respectively. Our primary models included the infant's age at the time of the RSV ARI, sex, and race and ethnicity as covariates. We also created secondary models replacing race and ethnicity with maternal asthma. The statistical analyses were conducted with R software. We used the Benjamini and Hochberg method to control for multiple comparisons when appropriate. One parent of each infant provided informed consent for the infant's participation. The institutional review board of Vanderbilt University Medical Center approved this study. Further details are also available in the Online Repository (available at www.jacionline.org).

Of the 309 infants with RSV-only ARIs who were enrolled in the INSPIRE study, 105 (~34%) had at least 1 nasal wash with 16S rRNA sequencing and immune mediator data available (obtained as part of separate projects^{5,6}) and were thus included in our study. The median age at the RSV ARI in these 105 infants was 4.90 months (interquartile range [IQR] = 3.06-6.15 months).

The majority of these infants were male, white non-Hispanic, and born via vaginal delivery (see Table E2 in the Online Repository at www.jacionline.org). Compared with the infants included in the study, those not included were more likely to be born via vaginal delivery and to have federal or state insurance (see Table E2).

The median viral load, as measured by the number of cycle threshold values, was 23.81 (IQR = 20.78-26.91) in all infants. There was no association between the nasopharyngeal relative abundance of *Haemophilus* and the viral load in unadjusted (Spearman $\rho = -0.04$; $P = .7$) or adjusted (β coefficient = -1.82 ; 95% CI = $-5.76-2.13$; $P = .4$) analyses.

Using unsupervised methods, we identified 2 clusters of infants based on their nasopharyngeal immune response patterns (Fig 1). Cluster 1 mostly comprised infants with higher local immune mediator levels when compared with cluster 2. Cluster 1 also mainly included infants with a higher relative abundance of *Haemophilus* in their nasopharynx (Fig 1), with the median relative abundance of cluster 1 (0.03 [IQR = 0.003-0.39]) being approximately 15 times higher than that of cluster 2 (0.002 [IQR = 0-0.03]) ($P = .001$ with use of the Mann-Whitney U test).

In unadjusted analyses, the nasopharyngeal relative abundance of *Haemophilus* was positively correlated with the levels of 39 of the 52 local immune mediators (~75%) after control for multiple comparisons (Fig 2, A and see Table E3 in the Online Repository at www.jacionline.org). These associations remained significant

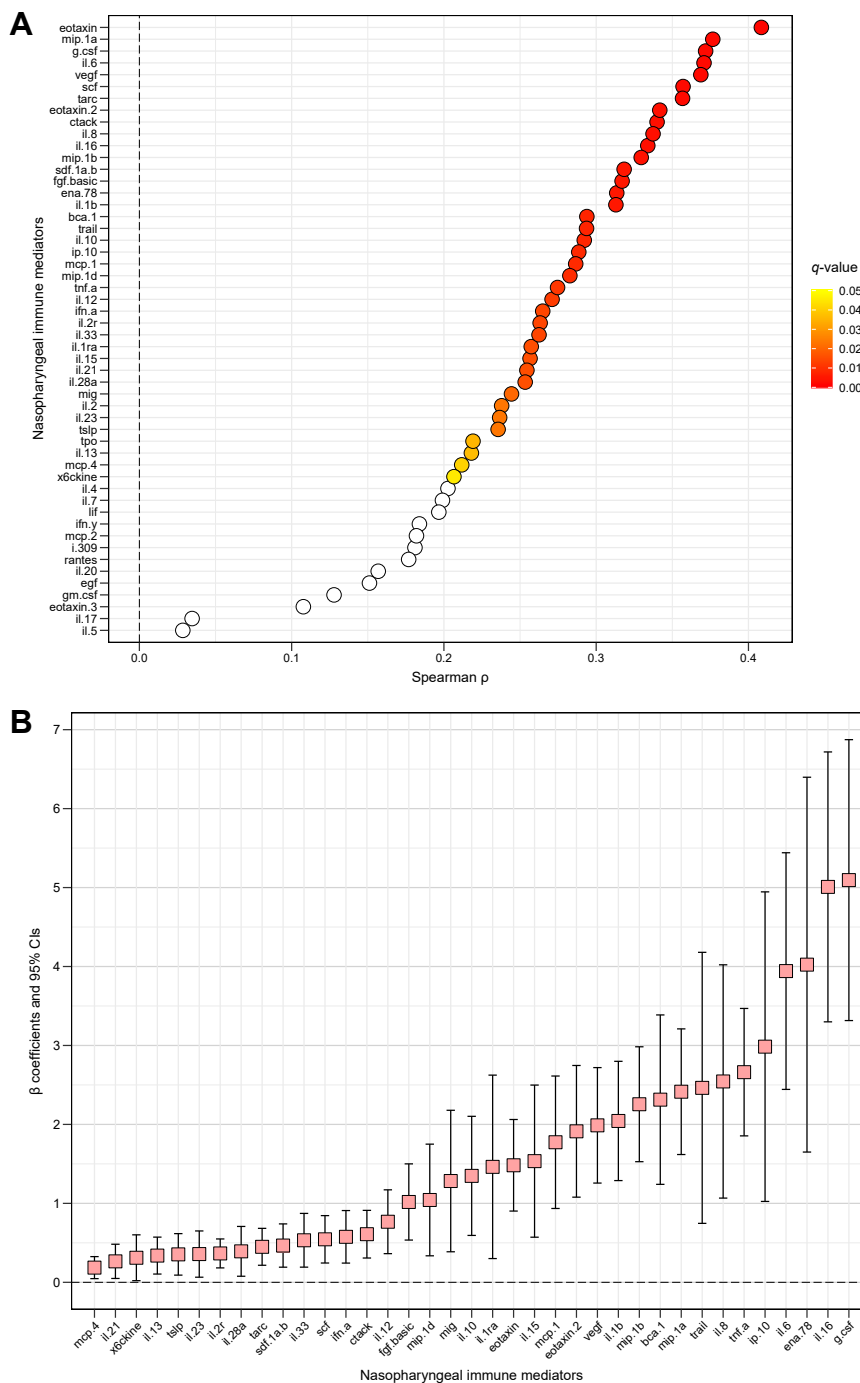


FIG 2. The unadjusted and adjusted association of the nasopharyngeal relative abundance of *Haemophilus* with each measured local immune mediator's median fluorescence intensity during an RSV ARI in infancy. **A**, Unadjusted analyses performed by using Spearman correlation. The Spearman correlation coefficients (ρ) are indicated by circles and colored by their respective *P* values after controlling for multiple comparisons using the Benjamini and Hochberg method (ie, *q* values). The nonsignificant *q* values of 0.05 or more are shown in white, whereas those less than 0.05 are shown according to the color scale (yellow indicates higher, red indicates lower). **B**, Adjusted analyses performed by using multivariate linear regression. The β coefficients are indicated by squares, and their corresponding 95% CIs are indicated by the adjacent black lines. Each multivariable model included the infant's age at the time of RSV ARI, race and ethnicity, and sex as covariates. The β coefficient can be interpreted as the increase in that local immune mediator's generalized-log-transformed median fluorescence intensity per 1% increase in the nasopharyngeal relative abundance of *Haemophilus* during an RSV ARI after control for these covariates. Only those immune mediators with *q* values that were less than 0.05 in unadjusted analyses and remained significant in adjusted analyses are shown.

for 37 of the 52 local immune mediators (~71%) after adjustment for potential confounders in our primary and secondary models (Fig 2, B and see Table E3). In general, the nasopharyngeal relative abundance of *Haemophilus* was associated with higher levels of those local immune mediators with antiviral and/or proinflammatory functions and those that are important in the chemotaxis, differentiation, proliferation, and survival of numerous immune cells (eg, TNF- α , IFN- α , IL-1 β , IL-6, IL-8, IL-12, IL-21, and IL-28A). In contrast, nasopharyngeal relative abundance of *Haemophilus* was not associated with the levels of the main type 1 (eg, IFN- γ) or type 3 (eg, IL-17A) local immune mediators. In regard to type 2 immune responses, there was a positive association with IL-13, but not with IL-4 or IL-5, which may be related to the temporal expression of these cytokines. The nasopharyngeal relative abundance of *Haemophilus* was also associated with IL-33 (Fig 2). Interestingly, IL-33 can induce a type 2 immune response either through activating ILC2 or CD4⁺ T_H2 cells and animal studies have shown that IL-33 is important for RSV pathophysiology.⁷

To explore whether this effect was attributable to a single *Haemophilus* species, we repeated the aforementioned statistical analyses for the most abundant *Haemophilus* ASVs. In all infants, a total of 81 *Haemophilus* ASVs were identified, with 4 of them accounting for approximately 95% of all *Haemophilus* sequences. The nasopharyngeal relative abundance of 2 of these 4 *Haemophilus* ASVs had a direction of associations similar to those noted in the genus-level analyses (with 1 of these being correlated with the levels of 28 of the 52 local immune mediators [~54%] after control for multiple comparisons [see Fig E1 in the Online Repository at www.jacionline.org]). When we used the Basic Local Alignment Search Tool and the available WMS data, both of these *Haemophilus* ASVs most likely corresponded to *Haemophilus influenzae* strains (see the Results section in the Online Repository at www.jacionline.org).

Several members of the genus *Haemophilus* are commonly found in the nasopharynx of healthy infants, although usually at low relative abundances. The aggregated evidence suggests that early-life RSV ARIs can lead to a rapid increase in the nasopharyngeal relative abundance of *Haemophilus* throughout infancy (a critical period of lung and immune development) and that this could in turn affect the pathophysiology of RSV disease.¹⁻⁴ In our study, we have shown that the relative abundance of *Haemophilus* (most likely *H influenzae*) in the nasopharynx during an RSV ARI in infancy is positively associated with multiple local immune mediators, particularly those with antiviral and proinflammatory functions, which could be a mechanism through which these viral-bacterial interactions influence short- and long-term RSV-related outcomes. One prior study found that a nasopharyngeal microbial profile dominated by *Haemophilus* was associated with delayed viral clearance during RSV ARI in infancy²; although exactly how the combination of antiviral and proinflammatory immune responses affect viral clearance during RSV ARI in infancy is unknown. To our knowledge, only 1 other study has examined the effect of nasopharyngeal *Haemophilus* on the local immune response during an RSV ARI in infancy. That study was smaller, used ELISA-based immunoassays, and included only 4 of the 52 local immune mediators examined by us, finding similar results for IL-8.¹ Likewise, 2 *in vitro* studies found similar results for IL-6 and IL-8, which also supports our findings.^{8,9}

Our study has multiple strengths, including the population-based design of the parent study, the close surveillance during the infants' first winter viral season to capture their initial RSV ARI, the use of next-generation sequencing and high-throughput immune assays, and the use of statistical analysis adjusting for potential confounders. We also acknowledge several limitations. First, we decided to focus on *Haemophilus* because of the multiple prior studies assessing the whole nasopharyngeal microbiome that have identified this taxon as a key determinant of RSV-related outcomes¹⁻⁴ even though it is certainly possible that *Haemophilus* could be just a marker for other bacterial pathogens or that other taxa could also have important or stronger immunomodulatory effects. Second, our sample size was small, and it may not have been powered to detect certain associations. The number of nasal washes with WMS data was also limited; therefore, the WMS analyses should be regarded as exploratory, as it is possible that we did not capture all relevant *Haemophilus* species. Third, it is possible that some of the associations found were driven by a small number of observations, as the nasopharyngeal relative abundance of *Haemophilus* was overall low in most infants. However, several of the statistical tests that we used are robust to the effect of outliers. Furthermore, the effect of a particular species on a microbial ecosystem is not directly related to its abundance. Thus, *Haemophilus* could be a crucial taxon affecting the RSV-related local immune response in infancy, even at low nasopharyngeal relative abundances. Lastly, our study included only a subset of RSV-infected infants from the INSPIRE cohort, and it had a cross-sectional design. Thus, there is a possibility of selection bias and/or reverse causation (ie, a possibility that the levels of certain local immune mediators are what affect the relative abundance of *Haemophilus* in the nasopharynx). In spite of these limitations, our study adds to the small but increasing literature on the effect of RSV and *Haemophilus* interactions on common childhood respiratory diseases.

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Coagulation factors induce human skin mast cell and basophil degranulation via activation of complement 5 and the C5a receptor



To the Editor:

Chronic spontaneous urticaria (CSU), also called chronic idiopathic urticaria, is a common skin disorder characterized by daily or almost daily recurring wheal and flare, with itch occurring anywhere on the body for more than 6 weeks in association with degranulation of skin mast cells and basophils.^{1,2} The involvement of both exogenous factors (such as infections and psychological stress) and endogenous factors has been suggested. The latter includes the presence of autoantibodies against IgE or the high-affinity IgE receptor and autoantigens for IgE, such as thyroid peroxidase, double-stranded DNA, and IL-24 in sera of the patients.^{3,4} Moreover, the increase in blood-coagulating and fibrinolytic activities has been shown in patients with moderate-to-severe CSU.^{5,6} However, the precise mechanisms of mast cell and basophil activation in CSU has remained unclear. We previously demonstrated that human umbilical vein endothelial cells (HUVECs) synergistically expressed tissue factor (TF) on their surface subsequent to treatment with histamine and LPS *in vitro*.⁷ We also showed that TF expression of peripheral monocytes in patients with CSU is significantly enhanced compared with that in healthy donors.⁸ Both TF expressed on HUVECs and human peripheral

monocytes activated the extrinsic coagulation pathway and produced active forms of coagulation factors with serine protease activity, such as factor Xa and thrombin (IIa), resulting in the gap formation between HUVECs via the protease-activated receptor (PAR) PAR1.⁷ Leakage of plasma containing the activated coagulation factors with serine protease activity is predicted to activate skin mast cells and basophils expressing PARs, and consequentially develop clinical edema formation. However, actual degranulation of human mast cells and basophils by activated coagulation factors has never been reported. Here, we have demonstrated that the activated extrinsic coagulation factors do not activate human skin mast cells (hsMCs) and basophils by themselves but induce degranulation of hsMCs and basophils via the production of complement C5a acting on the C5a receptor (C5aR).

We first studied the effect of PAR1 and PAR2 agonists and various activated factors produced in the extrinsic coagulation pathway and consequent fibrinolytic pathways. As expected, hsMCs expressed mRNA of PAR1 and marginally expressed PAR2 (see Fig E1 in this article's Online Repository at www.jacionline.org). However, neither TRAP-6 (a PAR1 agonist) nor AC55541 (a PAR2 agonist) nor the activated extrinsic coagulation factors VIIa, Xa, and IIa nor the fibrinolytic factor plasmin induced degranulation of hsMCs by themselves (Fig 1, A). We then studied degranulation of hsMCs in response to the complement activation products C3a and C5a. As shown in Fig 1, B, C5a, but not C3a, induced degranulation of hsMCs. Because serine proteases, such as coagulation and/or fibrinolysis factors, may convert C5 into C5a and C5b, we investigated the effect of C5 in the presence of factor VIIa, factor Xa, factor IIa, or plasmin on the degranulation of hsMCs. Although neither C5 nor factor VIIa nor factor Xa nor factor IIa induced degranulation of hsMCs by themselves, C5 did induce degranulation of hsMCs in the presence of factor Xa, factor IIa, or plasmin, suggesting that C5a converted by factor Xa, factor IIa, or plasmin from C5 induces degranulation of hsMCs (Fig 1, C, D). C5a cleaved from C5 by factor Xa and factor IIa induced histamine release from hsMCs as well (see Fig E2 in this article's Online Repository at www.jacionline.org). However, C3 induced a slight degranulation of hsMCs even in the presence of factor Xa and factor IIa (Fig 1, C). Moreover, nafamostat mesylate, a serine protease inhibitor, reduced C5 plus IIa- and C5 plus plasmin-induced degranulation of hsMCs in a concentration-dependent manner. This suggests that serine protease activity of activated coagulation and/or fibrinolysis factors is essential and sufficient for the conversion of C5 to C5a and consequent activation of hsMCs (Fig 1, E). Furthermore, C5a-, C5 plus IIa-, and C5 plus plasmin-induced degranulation of hsMCs were clearly inhibited by pretreatment for 15 minutes with a C5a receptor (C5aR) antagonist, W-54011, in a concentration-dependent manner (Fig 1, F). These results suggest that activated coagulation factors cleave C5 into C5a, which then induces degranulation of hsMCs via C5aR on the surface of mast cells.

Human basophils also express C3aR, C5aR, and a small level of PAR1 and PAR2 at mRNA levels (see Fig E3 in this article's Online Repository at www.jacionline.org). We then investigated the effect of C3a and C5a on histamine release from human peripheral basophils in PBMCs. As shown in Fig 2, A, C5a, but not C3a, induced histamine release from human peripheral basophils. Moreover, neither C5 nor the coagulation factors Xa

METHODS

Overview of infant susceptibility to pulmonary infections and asthma following RSV exposure study

The INSPIRE cohort is a large (N = 1949) population-based birth cohort of previously healthy, term infants born between June and December that was designed so that the first ARI due to RSV in infancy could be captured.^{E1} Eligible infants were enrolled mainly during a well-child visit at a participating general pediatric practice throughout the middle Tennessee region. The catchment zone encompassed urban, suburban, and rural areas.

To capture all RSV ARIs of infants enrolled in the INSPIRE study (including the first RSV ARI, which is usually the most severe^{E2}), we conducted intensive passive and active surveillance during each infant's first RSV season (November to March in our region^{E3,E4}) by (1) performing biweekly telephone, e-mail, and/or in-person follow-up; (2) frequently educating parents and reminding them to call us at the onset of any acute respiratory symptom; and (3) approaching all infants who were seen at 1 of the participating pediatric practices for an unscheduled visit. An ARI was defined as parental report of (1) at least 1 of the major symptoms or diagnoses (wheezing, difficulty in breathing, and presence of a positive RSV test) or (2) any 2 of the minor symptoms or diagnoses (fever, runny nose/congestion/snotty nose, cough, ear infection, and hoarse cry). If an infant met these prespecified criteria, we then conducted an in-person respiratory illness visit that included the collection of a nasal wash. The nasal wash was collected by gently flushing 5 mL of sterile saline solution into 1 of the infant's nares. After sampling, the nasal washes were aliquoted and snap-frozen at -80°C until further processing. Data were collected and managed by using the Research Electronic Data Capture tool hosted at Vanderbilt University.^{E5} One parent of each infant provided informed consent for participation. The institutional review board of Vanderbilt University approved this study. The detailed methods for the INSPIRE study have been reported previously.^{E1}

Overview of the study population

For our study, on the basis of our hypothesis of interest, we included only infants enrolled in the INSPIRE study who had at least 1 RSV ARI during their first RSV season. Because we have previously shown that specific respiratory viruses can be associated with distinct nasopharyngeal bacterial signatures,^{E6} we further limited eligibility to infants with RSV-only ARIs (ie, no coinfections). Of the 309 infants with RSV-only ARIs who were enrolled in the INSPIRE study, 105 (~34%) had at least 1 nasal wash with available 16S rRNA sequencing and immune mediator data (obtained as part of separate studies^{E6-E9}) and were thus included in our study. For infants with data from at least 1 RSV ARI, only data available from the earliest RSV ARI were included in the statistical analyses.

Detection of RSV and viral load assessment

The detection of RSV in nasal washes was performed by real-time RT-PCR. For this, TaqMan low-density array cards using virus-specific primers and probes were run on the Applied Biosystems StepOnePlus Real-Time PCR platform using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems) as previously described.^{E10,E11} The cycle threshold values were used for the assessment of viral load (where a lower cycle threshold value indicates a higher viral load).

Characterization of the nasopharyngeal abundance of *Haemophilus*

We extracted bacterial DNA from 100 to 200 μL of nasal wash solution by using the Qiagen PowerSoil Kit. Construction of libraries was performed as previously described.^{E6-8,E12} Amplicons targeting the V4 region of the bacterial 16S rRNA were generated by combining 7 μL of template, 12.5 μL of MyTaq HS Mix (Bioline), 0.75 μL of dimethyl sulfoxide (Sigma),

1 μL of PCR-Certified Water (Teknova), and 2 μL of each 10 μM primer before each round of PCR. During the first round of PCR, the target region was amplified with the primers 515F (5'-GTGCCAGCHGCGYCGGT-3') and 806R (5'-GGACTACNNGGTTCTAAT-3'), with an initial denaturing step at 95°C for 3 minutes. This was followed by 10 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 second and a final extension at 72°C for 5 minutes. During the second round of PCR, 20 cycles with the same cycling conditions as before were performed to add Illumina adaptors, standard Illumina sequence primer region, a 12-bp barcode, and random nucleotides to increase sequence diversity.

Each amplified sample was run on a 1.2% agarose gel to confirm reaction success. Amplicons were cleaned and normalized with the SeqPrep Normalization Kit (ThermoFisher). Normalized amplicons were pooled and cleaned with 1X AMPure XP beads (Beckman Coulter). The pool was run on a 1.5% agarose gel, and the target size band was extracted and cleaned with the NucleoSpin Gel and PCR Cleanup Kit (Macherey-Nagel). The pool was then sequenced on an Illumina MiSeq platform with $2 \times 300\text{-bp}$ reads.

A PCR and an extraction negative control and 2 samples with known taxonomic composition (provided by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Biological and Emerging Infections [BEI] program^{E13}) were amplified and sequenced concurrently with the samples. The 2 BEI control reagents obtained through BEI Resources included (1) Genomic DNA from Microbial Mock Community B (Staggered, Low Concentration), v5.2L, for 16S rRNA Gene Sequencing, HM-783D, and (2) Genomic DNA from Microbial Mock Community B (Even, Low Concentration), v5.1L, for 16S rRNA Gene Sequencing, HM-782D. After sequencing, only a small fraction of 16S rRNA sequences were found in the negative controls compared with in the infant samples, and the bacterial sequences recovered had little overlap with the infant samples. Both BEI controls returned a taxonomic profile similar to their expected taxon distributions.

We subsequently processed the 16S rRNA sequences by using the dada2 pipeline by following its standard operating procedure (available at: <https://benjjneb.github.io/dada2/tutorial.html>, last accessed November 21, 2019).^{E14} To this end, sequences were grouped into ASVs and taxonomy was assigned by using the SILVA reference database.^{E15} Sequences were subsequently processed through the R software package decontam to remove any suspected contaminants that were found in the negative control samples.^{E16} *Haemophilus* genus and ASV abundances were calculated as relative abundances by using simple proportions.

Identification of nasopharyngeal *Haemophilus* species

To identify the nasopharyngeal *Haemophilus* species, we used both (1) the Basic Local Alignment Search Tool (BLAST) and (2) available WMS data from a selected number of nasal washes (n = 6).^{E17}

To compare *Haemophilus* ASVs against a reference database with BLAST, we used the nucleotide sequence database (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome [last accessed November 21, 2019]) and specified the organism as *Haemophilus* (taxid:724).

For the WMS, we first depleted eukaryotic DNA from the genomic DNA extracted as already described for 16S rRNA gene sequencing using the NEBNext Microbiome Enrichment Kit (New England Biolabs). Next, we constructed dual-indexed sequencing libraries with the Nextera XT DNA Library Prep Kit (Illumina). Equimolar amounts of each library were then pooled, and sequencing was performed on an Illumina NextSeq platform with $2 \times 150\text{-bp}$ reads. For the initial processing of the WMS data set, we used FastQC to assess the sequence quality.^{E18} The adapter trimming and removal of low-quality data were performed with Trimmomatic.^{E19} Following this, we removed human DNA by aligning sequences to the Genome Research Consortium Human Build 38 reference assembly with the Burrows-Wheeler Aligner.^{E20,E21} Lastly, we assigned taxonomy by using GOTTCHA with the software's default parameters.^{E22} For the detection of *Haemophilus* species, no minimum cutoff for the relative abundance was instituted.

Measurement of nasopharyngeal immune mediator levels

The nasopharyngeal immune mediators were measured by using 2 multiplex magnetic bead-based assays (Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel II Premixed 23 Plex Kit [MilliporeSigma] and Cytokine 30-Plex Human Panel [Thermo Fisher Scientific]) as previously described.^{E9} These panels measure a total of 53 immune mediators (ie, cytokines, chemokines, and growth factors) that have been associated with clinical outcomes in allergy, immunology, and pulmonary research (see Table E1). The assays were all conducted in a Luminex MAGPIX platform using the xMAP technology as per the manufacturer's instructions. Two replicates of each sample assay were performed on 96-well plates. One blank well was used as a negative control and to estimate the background. One immune mediator (ie, hepatocyte growth factor) had a large number of values below the lower detectable limit (~50%) and was thus excluded from further statistical analyses. The Luminex xMAP data were processed by using the method described by Won et al, which uses median fluorescence intensities of individual beads instead of the usual standard curve-based data-processing method to increase the sensitivity and accuracy of high-throughput immunoassays.^{E9,E23} The immune mediator median fluorescence intensities were generalized log₂-transformed before statistical analyses to stabilize the data variance.^{E9}

RESULTS

In the 105 RSV-infected infants included in the study, a total of 81 *Haemophilus* ASVs were identified, and 4 of these accounted for approximately 95% of all *Haemophilus* sequences. The 2 *Haemophilus* ASVs with the highest nasopharyngeal relative abundance, ASV 0003 and ASV 0007, accounted for approximately 87% of all *Haemophilus* sequences. These 2 *Haemophilus* ASVs also had a direction of associations similar to those noted in the genus-level analyses. After control for multiple comparisons, the nasopharyngeal relative abundance of 1 of these ASVs (*Haemophilus* ASV 0003) was correlated with the levels of 28 of the 52 local immune mediators (~54%), whereas the nasopharyngeal relative abundance of the other ASV (*Haemophilus* ASV 0007) was correlated only with the level of 1 of the 52 local immune mediators (~2%) (Fig E1).

To identify *Haemophilus* species, we first used BLAST to search against the nucleotide sequence database.^{E17} The ASV 0003 sequence was a 100% match with 2 classified *Haemophilus* species (*H influenzae* [79 of 112 hits (~71%)] and *Haemophilus haemolyticus* [31 of 112 hits (~28%)]); *e* value = 4e-132 for both species). Likewise, the ASV 0007 sequence was a 100% match with 2 classified *Haemophilus* species (*H influenzae* [114 of 120 hits (~95%)] and *Haemophilus aegyptius* [2 of 120 hits (~2%)]); *e* value = 4e-132 for both species). To further differentiate between these *Haemophilus* species, we then used the WMS data that were available for 6 of the infants included in the study. Two of these infants had *Haemophilus* detected by 16S rRNA sequencing, and in both of them, the only *Haemophilus* species detected by WMS was *H influenzae*, suggesting that the *Haemophilus* ASVs 0003 and 0007 corresponded to *H influenzae* strains.

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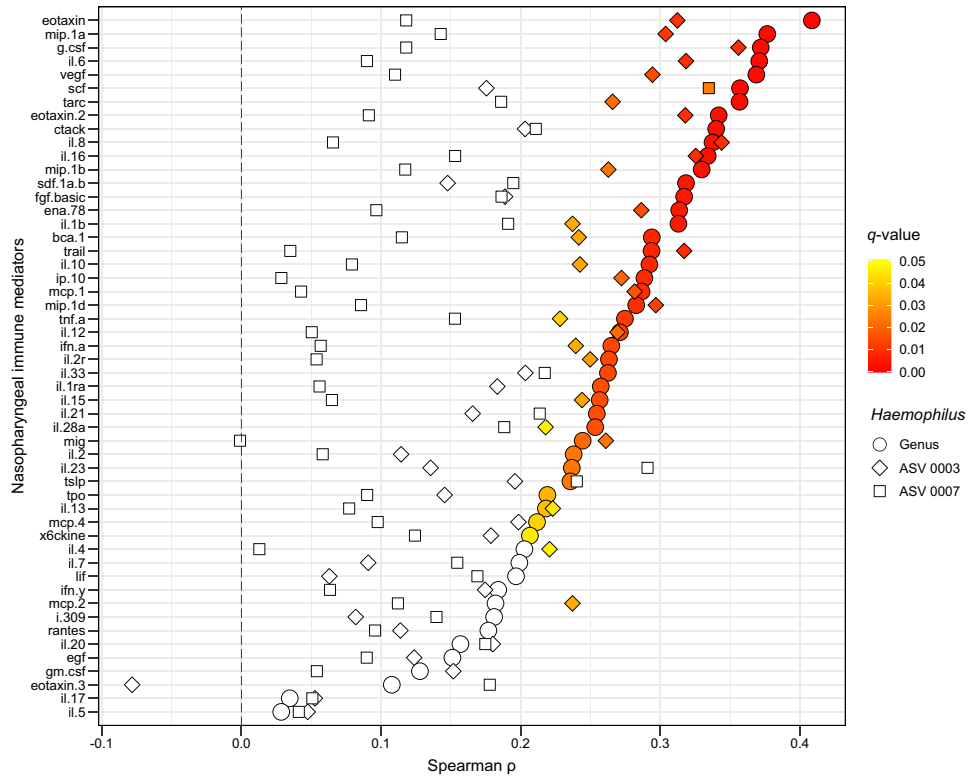


FIG E1. The unadjusted associations of the nasopharyngeal relative abundance of the top 2 most frequent *Haemophilus* ASVs with each measured local immune mediator’s median fluorescence intensity (MFI) during an ARI due to RSV in infancy. The unadjusted analyses were conducted by using Spearman correlation. The Spearman correlation coefficients (ρ) for each *Haemophilus* ASV are indicated by shapes (diamond indicates ASV 0003, squares indicate ASV 0007) and colored by their respective *P* values after control for multiple comparisons by using the Benjamini and Hochberg method (ie, *q* values). The nonsignificant *q* values or 0.05 or higher are shown in white, whereas those that are less than 0.05 are shown according to the color scale (yellow indicates higher, red indicates lower). The estimates for the unadjusted associations at the genus level are also shown as a reference (circles). The local immune mediator MFIs were generalized log-transformed before all statistical analyses.

TABLE E1. The nasopharyngeal immune mediators measured during an ARI due to RSV in infants that were included in the study

● BCA.1	● IL-7	● MIP-1 α
● CTACK	● IL-8	● MIP-1 β
● EGF	● IL-10	● MIP-1d
● ENA-78	● IL-12	● RANTES
● Eotaxin	● IL-13	● SCG
● Eotaxin.2	● IL-15	● SDF-1a.b
● Eotaxin.3	● IL-16	● TARC
● FGF.basic	● IL-17A	● TNF. α
● G-CSF	● IL-20	● TPO
● GM-CSF	● IL-21	● TRAIL
● HGF*	● IL-23	● TSLP
● INF a	● IL-28A	● VEGF
● IFN- γ	● IL-33	● 6Ckine
● IL-1 β	● IP-10	
● IL-1RA	● I-309	
● IL-2	● LIF	
● IL-2R	● MCP-1	
● IL-4	● MCP-2	
● IL-5	● MCP-4	
● IL-6	● MIG	

The nasopharyngeal immune mediators are presented in alphabetical order.

*The levels of HGF were undetectable in approximately 50% of the nasopharyngeal samples, and because of this, this immune mediator was excluded from statistical analyses.

TABLE E2. Baseline characteristics of the infants enrolled in the INSPIRE study with an RSV-only ARI included (n = 105) and not included (n = 204) in our study

	Included (n = 105)	Not included (n = 204)
Age at the time of the RSV ARI (mo)	4.90 (3.06-6.15)	4.62 (2.45-5.98)
Female sex	45 (42.86%)	96 (47.06%)
Race and ethnicity		
Black non-Hispanic	12 (11.43%)	37 (18.14%)
White non-Hispanic	74 (70.48%)	134 (65.69%)
Hispanic	11 (10.48%)	16 (7.84%)
Other	8 (7.62%)	17 (8.33%)
Gestational age (wk)	39 (39-40)	39 (38-40)
Birth weight (g)	3507 (3149-3774)	3405 (3105-3714)
Birth by cesarean section	42 (40.00%)	59 (28.92%)
Ever any breast-feeding in infancy	85 (82.52%)	149 (76.80%)
Exposure to secondhand smoking <i>in utero</i> or before enrollment	21 (20.00%)	56 (27.45%)
Maternal asthma	17 (16.19%)	44 (21.57%)
Federal or state insurance at enrollment	39 (37.14%)	122 (59.80%)

Data are presented as medians (25th-75th percentiles) for continuous variables or number (%) for categorical variables and are calculated for children with complete data. $P < .05$ for the comparison between groups that was performed by using a Mann-Whitney U test or Pearson chi-square test, as appropriate.

TABLE E3. The unadjusted and adjusted associations of the nasopharyngeal relative abundance of *Haemophilus* with each measured local immune mediator's MFI during an RSV ARI in infancy

Nasopharyngeal immune mediator	Sample size available for statistical analyses	Unadjusted analyses*			Adjusted analyses†					
		Spearman ρ	P value	q value	Model 1			Model 2		
					β coefficient	95% lower CI	95% upper CI	β coefficient	95% lower CI	95% upper CI
BCA.1	105/105	0.29	.00235	0.00682	2.31	1.24	3.39	2.22	1.1	3.33
CTACK	105/105	0.34	.00039	0.00224	0.61	0.31	0.91	0.59	0.29	0.89
EGF	101/105	0.15	.13148	0.14244	—	—	—	—	—	—
ENA.78	105/105	0.31	.00113	0.00402	4.02	1.65	6.40	3.69	1.26	6.13
Eotaxin	104/105	0.41	.00002	0.00087	1.48	0.90	2.06	1.41	0.81	2.01
Eotaxin.2	105/105	0.34	.00036	0.00224	1.91	1.08	2.75	1.79	0.93	2.64
Eotaxin.3	105/105	0.11	.27419	0.28516	—	—	—	—	—	—
FGFbasic	101/105	0.32	.00124	0.00402	1.02	0.54	1.50	0.92	0.43	1.41
G-CSF	101/105	0.37	.00013	0.00139	5.09	3.32	6.87	4.71	2.87	6.54
GM-CSF	104/105	0.13	.19593	0.20793	—	—	—	—	—	—
I-309	105/105	0.18	.06467	0.07473	—	—	—	—	—	—
INF- α	104/105	0.26	.00658	0.01338	0.58	0.24	0.91	0.51	0.16	0.86
INF- γ	104/105	0.18	.06165	0.07455	—	—	—	—	—	—
IL-10	104/105	0.29	.00262	0.00716	1.35	0.59	2.10	1.30	0.53	2.06
IL-12	104/105	0.27	.00539	0.01167	0.77	0.36	1.17	0.67	0.25	1.09
IL-13	104/105	0.22	.02618	0.03680	0.34	0.10	0.57	0.32	0.09	0.55
IL-15	104/105	0.26	.00856	0.01525	1.53	0.57	2.50	1.40	0.41	2.39
IL-16	105/105	0.33	.00050	0.00236	5.01	3.30	6.72	4.76	3.04	6.48
IL-17	104/105	0.03	.72713	0.74139	—	—	—	—	—	—
IL-1 β	104/105	0.31	.00122	0.00402	2.04	1.29	2.80	1.82	1.02	2.61
IL-1RA	104/105	0.26	.00836	0.01525	1.46	0.30	2.62	1.47	0.32	2.61
IL-2	104/105	0.24	.01502	0.02309	—	—	—	—	—	—
IL-20	105/105	0.16	.11007	0.12177	—	—	—	—	—	—
IL-21	105/105	0.25	.00880	0.01525	0.27	0.05	0.48	0.25	0.04	0.47
IL-23	105/105	0.24	.01510	0.02309	0.36	0.06	0.65	0.35	0.06	0.64
IL-28A	105/105	0.25	.00911	0.01528	0.39	0.08	0.71	0.41	0.10	0.72
IL-2R	104/105	0.26	.00695	0.01338	0.37	0.18	0.55	0.33	0.14	0.52
IL-33	105/105	0.26	.00683	0.01338	0.53	0.19	0.87	0.50	0.16	0.85
IL-4	104/105	0.20	.03910	0.05083	—	—	—	—	—	—
IL-.5	104/105	0.03	.77379	0.77379	—	—	—	—	—	—
IL-.6	104/105	0.37	.00011	0.00139	3.94	2.44	5.44	3.64	2.09	5.19
IL-7	104/105	0.20	.04281	0.05429	—	—	—	—	—	—
IL-8	104/105	0.34	.00046	0.00236	2.54	1.07	4.02	2.36	0.85	3.87
IL-10	104/105	0.29	.00297	0.00772	2.98	1.02	4.95	2.78	0.79	4.77
LIF	105/105	0.20	.04437	0.05493	—	—	—	—	—	—
MCP-1	104/105	0.29	.00319	0.00789	1.77	0.94	2.61	1.64	0.78	2.49
MCP-2	105/105	0.18	.06318	0.07467	—	—	—	—	—	—
MCP-4	105/105	0.21	.03017	0.04129	0.19	0.05	0.33	0.17	0.03	0.31
MIG	104/105	0.24	.01242	0.02018	1.28	0.39	2.18	1.11	0.18	2.05
MIP-1 α	104/105	0.38	.00008	0.00139	2.41	1.62	3.21	2.19	1.35	3.02
MIP-1 β	104/105	0.33	.00063	0.00275	2.26	1.53	2.98	2.12	1.37	2.88
MIP.1d	105/105	0.28	.00348	0.00822	1.04	0.34	1.75	0.99	0.29	1.69
RANTES	104/105	0.18	.07252	0.08197	—	—	—	—	—	—
SCF	105/105	0.36	.00018	0.00139	0.54	0.24	0.84	0.55	0.25	0.84
SDF-1a,b	105/105	0.32	.00093	0.00374	0.47	0.19	0.74	0.45	0.18	0.72
TARC	105/105	0.36	.00019	0.00139	0.45	0.22	0.68	0.43	0.19	0.66
TNF- α	104/105	0.27	.00478	0.01081	2.66	1.85	3.47	2.49	1.66	3.31
TPO	105/105	0.22	.02473	0.03572	—	—	—	—	—	—
TRAIL	105/105	0.29	.00236	0.00682	2.46	0.75	4.18	2.29	0.58	4.01
TSLP	105/105	0.24	.01555	0.02310	0.35	0.09	0.62	0.34	0.07	0.6
VEGF	101/105	0.37	.00015	0.00139	1.99	1.26	2.72	1.88	1.12	2.63
6Ckine	105/105	0.21	.03448	0.04598	0.31	0.02	0.60	0.29	0.002	0.59

MFI, Median fluorescence intensity.

The local immune mediator MFIs were generalized log–transformed before all statistical analyses.

*The unadjusted analyses were conducted by using Spearman correlation. The Spearman correlation coefficients (ρ) and their corresponding P values are shown. The P values after control for multiple comparisons with use of the Benjamini and Hochberg method (ie, q values) are also shown. The boldface cells indicate nonsignificant P and q values (ie, ≥ 0.05).

†The adjusted analyses were conducted by using multivariable linear regression. The β coefficients and their corresponding 95% lower and upper CIs are shown. Model 1 included the infant's age at the time of the RSV ARI, sex, and race and ethnicity as covariates. Model 2 included the infant's age at the time of the RSV ARI, sex, and maternal asthma as covariates. The β coefficient can be interpreted as the increase in that local immune mediator's generalized log–transformed MFI per 1% increase in the nasopharyngeal relative abundance of *Haemophilus* during an RSV ARI after control for the model covariates. Only the estimates for local immune mediators with q values that were less than 0.05 in the unadjusted analyses and remained significant in the adjusted analyses are shown.