Associations of Nasopharyngeal Metabolome and Microbiome with Severity among Infants with Bronchiolitis

A Multiomic Analysis

Christopher J. Stewart¹, Jonathan M. Mansbach², Matthew C. Wong¹, Nadim J. Ajami¹, Joseph F. Petrosino¹, Carlos A. Camargo, Jr.³, and Kohei Hasegawa³

¹Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas; and ²Department of Medicine, Boston Children's Hospital, and ³Department of Emergency Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

ORCID ID: 0000-0002-6033-338X (C.J.S.).

Abstract

Rationale: Bronchiolitis is the most common lower respiratory infection in infants; however, it remains unclear which infants with bronchiolitis will develop severe illness. In addition, although emerging evidence indicates associations of the upper-airway microbiome with bronchiolitis severity, little is known about the mechanisms linking airway microbes and host response to disease severity.

Objectives: To determine the relations among the nasopharyngeal airway metabolome profiles, microbiome profiles, and severity in infants with bronchiolitis.

Methods: We conducted a multicenter prospective cohort study of infants (age <1 yr) hospitalized with bronchiolitis. By applying metabolomic and metagenomic (16S ribosomal RNA gene and whole-genome shotgun sequencing) approaches to 144 nasopharyngeal airway samples collected within 24 hours of hospitalization, we determined metabolome and microbiome profiles

and their association with higher severity, defined by the use of positive pressure ventilation (i.e., continuous positive airway pressure and/or intubation).

Measurements and Main Results: Nasopharyngeal airway metabolome profiles significantly differed by bronchiolitis severity ($P < 0.001$). Among 254 metabolites identified, a panel of 25 metabolites showed high sensitivity (84%) and specificity (86%) in predicting the use of positive pressure ventilation. The intensity of these metabolites was correlated with relative abundance of *Streptococcus pneumoniae*. In the pathway analysis, sphingolipid metabolism was the most significantly enriched subpathway in infants with positive pressure ventilation use compared with those without ($P < 0.001$). Enrichment of sphingolipid metabolites was positively correlated with the relative abundance of *S. pneumoniae*.

Conclusions: Although further validation is needed, our multiomic analyses demonstrate the potential of metabolomics to predict bronchiolitis severity and better understand microbe–host interaction.

Keywords: bronchiolitis; metabolomics; microbiome; sphingolipids; infants

Bronchiolitis, an acute lower respiratory viral infection, is an important public health problem in the United States and worldwide. Indeed, bronchiolitis is the leading cause of infant hospitalizations

in the United States, accounting for 18% of infant hospitalizations (~130,000 hospitalizations annually) (1, 2). Although approximately 40% of children develop clinical bronchiolitis in the first 2 years

of life (3), its severity ranges from a minor nuisance to fatal (4). Studies have reported several clinical risk factors for higher severity (e.g., prematurity, comorbidity); however, it remains unclear which children

(Received in original form January 10, 2017; accepted in final form May 30, 2017)

Supported by National Institutes of Health grants U01 AI-087881, R01 AI-114552, R01 AI-108588, R21 HL-129909, R01 AI-127507, and UG3 OD-023253. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions: Conception and design, C.J.S., J.M.M., C.A.C., and K.H. Acquisition of data, C.J.S., J.M.M., N.J.A., J.F.P., C.A.C., and K.H. Analysis and interpretation of data, C.J.S., J.M.M., M.C.W., and K.H. Drafting or revising the article, C.J.S., J.M.M., C.A.C., and K.H. Acquisition of data, C.J.S., J.M.M., N.J.A., J.F.P., C.A.C., and K.H. Final approval of the manuscript, C.J.S., J.M.M., M.C.W., N.J.A., J.F.P., C.A.C., and K.H.

Correspondence and requests for reprints should be addressed to Christopher J. Stewart, Ph.D., Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, MS BCM385, Houston, TX 77030. E-mail: christopher.stewart@bcm.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 196, Iss 7, pp 882–891, Oct 1, 2017

Copyright © 2017 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201701-0071OC on May 21, 2017

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Bronchiolitis disease severity varies significantly among infants, which cannot be explained by currently known risk factors. Emerging evidence indicates that bronchiolitis pathobiology involves a complex interplay among viruses, airway microbiome, and host immunity. However, a detailed assessment of the airway metabolome and bacterial metagenome (microbiome) has not been performed.

What This Study Adds to the

Field: In this multicenter cohort study, infants with more severe bronchiolitis (defined by the use of positive pressure ventilation) had significantly altered nasopharyngeal airway metabolome profiles compared with those with less severe bronchiolitis. Increased sphingolipid metabolism was correlated with higher relative abundance of *Streptococcus pneumoniae* and was associated with higher bronchiolitis severity. Our data highlight microbe–host interactions in the airway of infants, which may provide a novel therapeutic strategy to treat infants with bronchiolitis.

with bronchiolitis will develop severe illness (5), such as bronchiolitis requiring positive pressure ventilation (PPV) support.

Emerging evidence indicates that bronchiolitis pathobiology involves a complex interplay among viruses, airway microbiome, and host immunity (6, 7). For instance, dominance of *Streptococcus* or *Haemophilus* in the upper airway has been associated with higher disease severity (7–9). Yet, little is known about molecular mediators of this interplay. Metabolomics, the systematic analysis of functional small molecules, present pathobiologic profiles that encompass microbial and host interactions (10–12). For several complex infectious and inflammatory diseases, such as sepsis (13), asthma (14, 15), and cystic fibrosis (16, 17), metabolomic approaches have identified new biomarkers and novel pathobiologic pathways. However, to date, no study has applied a metabolomics approach to infants with bronchiolitis.

To address these knowledge gaps, we used nasopharyngeal airway samples from a multicenter prospective study of infants hospitalized for bronchiolitis to profile the airway metabolome and microbiome, and to determine their relationship to disease severity with focusing on the use of PPV. The current study also used 16S ribosomal RNA (rRNA) gene sequence data published previously (6, 8, 18).

Methods

Study Design, Setting, and Participants

As part of a 17-center, prospective cohort study of 1,016 infants (age <1 yr) hospitalized for bronchiolitis, based on *a priori* defined study aim, the current investigation analyzed the data of 144 infants with nasopharyngeal metabolomic and microbiome testing and determined their relation to disease severity. This cohort, called the MARC-35 (35th Multicenter Airway Research Collaboration) (6), was coordinated by the Emergency Medicine Network, a research collaboration comprised of 245 hospitals. The study design, setting, participants, and methods of data collection have been reported previously (6, 8). In brief, MARC-35 site investigators at 17 sites across 14 U.S. states enrolled infants hospitalized with an attending physician diagnosis of bronchiolitis during three consecutive bronchiolitis seasons from November 1 to April 30 (2011–2014). Bronchiolitis was defined by the American Academy of Pediatrics guidelines: acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions (19). We excluded infants who were transferred to a participating hospital more than 24 hours after the original hospitalization; those who were consented more than 24 hours after hospitalization; or those with known heart-lung disease, immunodeficiency, or gestational age less than 32 weeks. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Nasopharyngeal Airway Sample Collection

Nasopharyngeal samples were collected by trained site investigators using the same

standardized protocol used in a previous cohort study of children with bronchiolitis (20, 21). Briefly, 1 ml of normal saline was instilled into one naris, and mucus was removed by means of an 8F suction catheter. The procedure was performed once on each nostril and, after sample collection from both nares, 2 ml of normal saline was suctioned through the catheter to clear the tubing and ensure that a standard volume of aspirate was obtained. All sites used the same collection equipment (Medline Industries, Mundelein, IL) and collected the samples within 24 hours of hospitalization. The nasopharyngeal sample was immediately placed on ice and then stored at -80°C until the samples were tested for nasopharyngeal airway metabolome and microbiome.

Metabolome Testing

Metabolome testing used 125 μl of nasopharyngeal sample and was performed by Metabolon (Durham, NC). All samples were blinded to Metabolon and processed in a random order. Metabolome profiling used ultra-high-performance liquid chromatography–tandem mass spectrometry. Details of the sample preparation, metabolome profiling, identification of compounds, and quality control may be found in the METHODS section of the online supplement (22).

Microbiome Testing

Microbial DNA was extracted from the nasopharyngeal samples using MO BIO PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) as described previously (8). The 16S rRNA gene sequencing data were generated in earlier work (8). The sequencing methods were adapted from those developed for the National Institutes of Health Human Microbiome Project (23, 24). In brief, the 16S rDNA V4 region was amplified by polymerase chain reaction using barcoded Illumina adapter-containing primers 515F and 806R (25) and sequenced on the MiSeq platform (Illumina, San Diego, CA) using the 2×250 bp paired-end protocol yielding pair-end reads that overlap almost completely. Demultiplexing of reads was performed using USEARCH v7.0.1090 (26) and quality filtering was performed as previously described (8). Resulting

operational taxonomic unit tables were rarified to 1,500 reads per sample.

To examine the encoded metabolic potential of nasopharyngeal airway microbiome, the current study also applied a metagenomic whole-genome shotgun sequencing to 70 nasopharyngeal samples. Individual libraries constructed from each sample were sequenced using the 2×100 bp paired-end read protocol on the HiSeq platform (Illumina). The process of quality filtering, trimming, and demultiplexing was performed using a pipeline developed at the Baylor College of Medicine that used several publicly available tools, such as Casava v1.8.3 (Illumina) for the generation of fastqs, Trim Galore and cutadapt for adapter and quality trimming, and PRINSEQ for sample demultiplexing. In addition, Bowtie2 v2.2.1 was used to map reads to custom databases for bacteria, viruses, human, and vectors, and remove nonbacterial reads from the dataset. For bacterial reads, the highest identity match was chosen. If there were multiple top hits, the lowest common ancestor was determined, but these reads do not contribute to the analysis. Reads whose genomic coordinates overlapped with known KEGG orthologs (KO; K numbers) were tabulated. Coding sequences from reference genomes that have not been specifically annotated by KEGG were aligned to all known KOs. Any coding sequence that had more than 70% identity and more than 70% query coverage to a known KO was assigned to that KO. This in effect created links between new genomes and the KEGG database. KEGG modules (M numbers) were calculated step-wise and determined to be complete if 65% of the reaction steps were present per detected species and for the metagenome as a whole. Pathways were constructed for each taxa and metagenome by calculating the minimum set through MinPath (27) resulting from the gene orthologs present.

Details of the microbial DNA extraction, 16S rRNA gene sequencing, and metagenomic whole-genome shotgun sequencing may be found in the online supplement. Microbiome data have been deposited in the NCBI BioProject (ID PRJNA356075).

Statistical Analyses

The primary outcome measure was the use of PPV, defined as the use of continuous positive airway pressure and/or intubation,

at any time during the index hospitalization (20, 28). The analysis followed a workflow to determine the relationships among the nasopharyngeal airway metabolome profiles, microbiome profiles, and use of PPV (see Figure E1 in the online supplement). Full analytical methodology is described in the online supplement. First, to examine the differences in nasopharyngeal metabolomic profile between infants with PPV use and those without, orthogonal partial least squares–discriminatory analysis (OPLS-DA) was performed (29) and validated by permutation testing (2,000 permutations). Individual metabolites that significantly differ by PPV use were determined using two-tailed Welch's *t* test in MetaboAnalyst 3.0 (30) and multivariable linear regression in R version 3.3 (31). The models adjusted for potential confounders (age, sex, history of prematurity, history of antibiotic use before the enrollment, use of systemic corticosteroids during the prehospitalization visit, virus, and hospital site). *P* values were adjusted for multiple comparisons with the false discovery rate algorithm (32).

To determine the predictivity of individual metabolites as a marker for PPV use, receiver operating characteristic curves were generated by linear support vector machine classification with Monte Carlo cross validation. In each Monte Carlo cross validation, two-thirds of the samples were used to examine the feature importance based on weighted coefficients, and the important features (metabolites) were used to build classification models, which were validated using the one-third of samples left out. In addition, to examine pathways that are differentially enriched in infants who underwent PPV, pathway enrichment analysis was performed. Next, MixOmics (33) was implemented in R to determine the correlation between bacterial taxa and the intensity of metabolites of interest with the use of sparse partial least squares regression in canonical mode (34). Furthermore, to determine the severity-related differences in metabolic potential of the nasopharyngeal microbiome overall and bacterial genera, two-tailed Welch's *t* test was used to compare the metagenomic KOs. Lastly, to determine biologically feasible correlations between the metagenomic KOs and the measured metabolites of likely bacterial origin, the Model-based Integration of Metabolite

Observations and Species Abundances method was used (35).

Results

Study Population and Analysis Workflow

As part of a 17-center prospective cohort study of 1,016 infants hospitalized for bronchiolitis, the current investigation analyzed 144 infants with sufficient amount of nasopharyngeal airway sample for metabolome and microbiome testing. The analytic and nonanalytic cohorts had no significant differences in most patient characteristics ($P > 0.05$) (see Table E1), except the analytic cohort had a relatively higher proportion of rhinovirus infection (28% vs. 20%; $P = 0.04$). Of 144 infants in the analytic cohort, the median age was 3 months (interquartile range, 1–6 mo) and 25 (17%) underwent PPV during their hospitalization. Infants who underwent PPV were younger and more likely to have respiratory syncytial virus infection compared with those without (both $P < 0.05$) (Table 1).

Metabolomics Analysis of Individual Metabolites Showed That Nasopharyngeal Metabolomic Profiles Significantly Differed by PPV Use among Infants with Bronchiolitis

Metabolomic analysis detected a total of 254 metabolites, from 62 subpathways, contained within eight superpathways, in the nasopharyngeal airway of infants with bronchiolitis. The metabolomic profiles (i.e., normalized intensity of individual metabolites, irrespective of subpathways and superpathway) clustered distinctly between infants with PPV use and those without PPV use in OPLS-DA (see Figure E2). The significant difference was validated using permutation testing ($P < 0.001$). The individual metabolites that discriminated the infants with PPV use from those without included all 20 detected metabolites from the sphingolipid metabolism subpathway (Figure 1). By contrast, 5-oxoproline, trans-urocanate, 13-HODE-9-HODE, and 15-HETE were negatively associated with the risk of PPV use.

The intensity of these individual metabolites in the infants' nasopharyngeal airway differed by PPV use, with no single

Table 1. Characteristics of 144 Infants Hospitalized for Bronchiolitis by Use of Positive Pressure Ventilation

Characteristics	Use of Positive Pressure Ventilation (n = 25)	No Positive Pressure Ventilation Use (n = 119)	P Value
Age, mo, median (IQR)	1.4 (1.0–3.0)	3.0 (1.4–5.7)	0.02
Male sex	14 (56)	74 (62)	0.56
Race/ethnicity			0.21
Non-Hispanic white	15 (60)	55 (46)	
Non-Hispanic black	2 (8)	22 (18)	
Hispanic	5 (20)	36 (30)	
Other	3 (12)	6 (5)	
Maternal smoking during pregnancy	2 (8)	10 (8)	0.98
Prematurity (32–37 wk)	4 (16)	27 (23)	0.46
C-section delivery	9 (36)	44 (37)	0.78
Postnatal smoke exposure at home	0 (0)	16 (13)	0.052
Number of siblings, median (IQR)	1 (0–5)	1 (0–5)	0.45
History of antibiotic use before enrollment	6 (24)	41 (34)	0.3
History of corticosteroid use before enrollment	3 (12)	19 (16)	0.61
Virology			
RSV infection	23 (92)	85 (71)	0.03
Rhinovirus infection	3 (12)	37 (31)	0.053
Neither RSV nor rhinovirus*	2 (8)	3 (3)	0.21
Positive pressure ventilation use			
Noninvasive positive pressure ventilation	14 (56)	0 (0)	–
Invasive positive pressure ventilation	18 (72)	0 (0)	–
Both	7 (28)	0 (0)	–

Definition of abbreviations: IQR = interquartile range; RSV = respiratory syncytial virus.

Data are n (%) of infants unless otherwise indicated.

*Includes parainfluenza virus types 1, 2, and 3; influenza virus types A and B; 2009 novel H1N1; human metapneumovirus; coronaviruses NL-65, HKU1, OC43, and 229E; adenovirus; human bocavirus type 1; and enterovirus.

metabolite ubiquitously increased among all infants with PPV use and decreased among all infants without PPV use, or *vice versa*. Thus, to determine the predictivity of individual metabolites as a marker for higher severity (i.e., use of PPV) at the point of hospitalization, we used multivariate receiver operating characteristic curve analysis (Figure 2A). The receiver operating characteristic curve for the top 5, 10, 15, 25, 50, and 100 metabolites showed that the area under the curve improved with a larger number of metabolites. Model 4 (25 metabolites) and Model 6 (100 metabolites) achieved the highest accuracy with both having a sensitivity of 84% and specificity of 86% (see Table E2). Given the equivalent accuracy, Model 4 was selected as most appropriate. Of these 25 metabolites, 12 predicted a higher risk of PPV use and 13 predicted a lower risk (Figure 2B). The model performance was validated using

permutation testing ($P < 0.001$ with 1,000 random permutations).

Metabolomics Analysis of Metabolic Pathways Showed That Sphingolipid Metabolism Is Significantly Enriched among Infants Who Underwent PPV

Previous analysis of individual metabolites, irrespective of the subpathway, demonstrated that the metabolomic profiles differ by PPV use and that 25 metabolites accurately predicted PPV use. Although these 25 individual metabolites were the most predictive, a total of 103 nasopharyngeal airway metabolites (from six superpathways) significantly differed between infants with PPV use and those without (see Figure E3). Moving beyond the analysis of individual metabolites, we next investigated metabolic pathway enrichment to determine which metabolic subpathways were significantly associated with risks of PPV use.

In accordance with the OPLS-DA (Figure 1), pathway enrichment analysis demonstrated that the sphingolipid metabolism subpathway (superpathway: lipid) was the most significantly associated with risks of PPV use ($P < 0.001$) (see Figure E4). Indeed, all 20 detected metabolites from this pathway were significantly more abundant in infants with PPV use compared with those without. Even after adjusting for potential confounders, 19 out of the 20 metabolites remained significant (all $P < 0.05$) (Figure 3; see Table E3). To address the possibility of reverse causation (i.e., the use of PPV enhanced sphingolipid metabolism), we repeated the analysis with the use of another severity outcome: hospital length-of-stay greater than or equal to 3 days (8). In this sensitivity analysis, the association between up-regulated sphingolipid metabolism and higher disease severity persisted, with 17 out of 20 sphingolipid metabolites remaining significant with adjustment for potential confounders (all $P < 0.05$) (see Table E3).

Although many metabolites do not currently have corresponding KEGG compound identifiers, 5 of the 20 significant sphingolipid metabolites had corresponding KEGG compound numbers and mapped throughout the KEGG sphingolipid metabolism pathway (see Figure E5). Other subpathways with significant enrichment included the phosphatidylethanolamine ($P < 0.001$), plasmalogen ($P < 0.001$), and phosphatidylcholine ($P < 0.001$) from the lipid superpathway, and the glycine, serine, and threonine metabolism ($P = 0.02$) subpathway from the amino acid superpathway (see Figures E3 and E4).

Microbiome and Metabolomic Analysis Showed That *Streptococcus* Relative Abundance Positively Correlates with Intensities of Metabolites That Predict Risks of PPV Use and with Enrichment of Sphingolipid Metabolism Pathway

Taxonomic data generated by 16S rRNA gene sequencing and metagenomic whole-genome shotgun were comparable, showing that *Haemophilus*, *Moraxella*, and *Streptococcus* genera dominated nasopharyngeal airway of infants with bronchiolitis (see Figures E6A and E6B).

Figure 1. Orthogonal partial least squares–discriminatory analysis loadings plot of nasopharyngeal airway metabolomics among infants with bronchiolitis ($n = 144$). This loadings plot combines the covariance (x -axis) and correlation (y -axis) loading profiles resulting from orthogonal partial least squares–discriminatory analysis model. This corresponds to combining the contribution (covariance) with the effect and reliability (correlation) for the model variables (metabolites) with regard to the risk of positive pressure ventilation use. In this figure, we selected and labeled metabolites with higher covariance and correlation that are significantly associated with a higher risk of positive pressure ventilation use (red) or lower risk (blue) in the subsequent analysis. Green circles represent individual metabolites. A score scatter plot may be found in the online supplement (see Figure E2). HETE = hydroxyeicosatetraenoic acid; HODE = hydroxyoctadecadienoic acid.

The relative abundance of these genera was also comparable between sequencing approaches (see Figure E7). The metagenomic sequencing ($n = 70$) (see Table E4) permitted species-level identification and further demonstrated that *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* specifically dominated the nasopharyngeal airway (see Figure E6C).

To determine the potential microbe–host interaction in the airway of infants with bronchiolitis, we examined the correlations between the relative abundance of the three dominant bacterial genera (*Streptococcus*, *Moraxella*, and *Haemophilus*) with the relative intensity of selected metabolites that were identified in the previous analyses (metabolites in Model 4 and sphingolipid metabolism). The relative abundance of *Streptococcus* was positively correlated with most (11 of 12) metabolites predicting high risks of PPV

use from Model 4 (Figure 4A). By contrast, the relative abundance of *Moraxella* and *Haemophilus* was generally negatively correlated with relative intensity of the same metabolites. These correlations were comparable in the sphingolipid metabolism pathway analysis, in which the relative abundance of *Streptococcus* was also positively correlated with the relative intensity of all metabolites from the sphingolipid metabolism pathway (Figure 4B).

Bacterial Gene Orthologs Are Distinct between Infants with PPV Use and Those Without

Lastly, the metagenome was analyzed to investigate potential differences in the metabolic potential of the nasopharyngeal microbiome according to use of PPV. Metagenomic sequencing identified a total of 285 KOs that were differentially enriched between infants with PPV and those without

(173 KOs vs. 112 KOs, respectively; all $P < 0.05$) (see Table E5). KOs significantly enriched in infants with PPV use were primarily from *Streptococcus* sp. (33 KOs), *Rothia* sp. (33 KOs), and *Klebsiella* sp. (27 KOs). In addition, six KOs from *Haemophilus* sp. were also significantly enriched among infants with PPV use. Conversely, all 112 KOs significantly enriched in infants without PPV use were from *M. catarrhalis*.

At the individual metabolite level, Model-based Integration of Metabolite Observations and Species Abundances analysis demonstrated the metagenomic KOs were significantly correlated with 15 measured metabolites, primarily amino acids (see Table E6). Notably, the abundance of seryl-tRNA synthetase (KO1875) from *S. pneumoniae* was correlated with the intensity of serine (substrate of sphingosine). However, there was no significant correlation between the abundance of metagenomic KOs and the intensity of the metabolites that were significantly altered by PPV use (e.g., metabolites from sphingolipid metabolism), suggesting that the PPV-related measured metabolites (and pathways) are likely host derived.

Discussion

In a multicenter prospective cohort of infants hospitalized for bronchiolitis, we used three complementary methodologies to characterize the nasopharyngeal airway metabolome, microbiome composition, and metagenome in samples collected at hospitalization. This is the first multiomic analysis integrating these distinct methodologies to determine the association of airway metabolome and microbiome with severity of illness in infants with bronchiolitis. We found that the nasopharyngeal airway metabolome profiles significantly differed by PPV use. A panel of 25 selected metabolites provided high sensitivity (84%) and specificity (86%) in predicting the use of PPV. Strikingly, all detected metabolites from the sphingolipid metabolism subpathway were discriminatory for PPV use by OPLS-DA, and sphingolipid metabolism was the most significantly enriched subpathway among infants with PPV use, with 19 out of 20 metabolites within this pathway significant even

Figure 2. Support vector machine biomarker analysis predicting the risk of positive pressure ventilation (PPV) use among infants with bronchiolitis ($n = 144$). (A) Linear support vector machine receiver operating characteristic curves showing increased area under the curve with increased numbers of metabolites. After Model 4 (light blue, 25 metabolites), the prediction was not improved materially, as per Table E2 in the online supplement. Model 4 had a sensitivity of 84% and specificity of 86%. (B) Average importance of metabolites from Model 4 (25 metabolites) in predicting the use (or nonuse) of PPV based on support vector machine feature ranking. AUC = area under the curve; CI = confidence interval; HETE = hydroxyeicosatetraenoic acid; HODE = hydroxyoctadecadienoic acid; Var. = number of metabolites included.

after adjusting for potential confounders. These findings were further validated using length of hospitalization as another marker for severity. The data also demonstrated the association between higher abundances of *Streptococcus* and enriched sphingolipid metabolites, suggesting that the alterations of these metabolites are the result of a combination of microbial and metabolic activity. Sphingolipids are not only integral components of the eukaryotic cell

Figure 3. Bar plots of the 20 metabolites from the sphingolipid metabolism subpathway by use of positive pressure ventilation (PPV). Bars represent the average scaled intensity and error bars represent the standard error of the mean. After adjusting for potential confounders, the intensity of 19 out of 20 metabolites within the sphingolipid metabolism pathway were significantly different between infants who underwent PPV and those who did not. $*P < 0.05$.

Figure 4. Canonical correlation between nasopharyngeal airway microbiota and metabolites associated with positive pressure ventilation (PPV) use ($n = 144$). Relative abundance for the three dominant genera is based on 16S ribosomal RNA gene sequencing. *Red text* indicates metabolites that are associated with a higher risk of PPV use; *blue text* indicates metabolites that are associated

membrane, but also have molecular signaling functions with important roles in inflammation, immune response to infections, stress response, cell proliferation, and apoptosis (36–38). Although the current study is the first to apply metabolomics to infant nasopharyngeal samples, metabolites associated with sphingolipid metabolism have been reported to increase in a range of existing studies investigating pulmonary disorders in childhood and adulthood (36, 37). For example, sphingolipids were primarily significantly increased in sputum from adults with cystic fibrosis compared with healthy control subjects (17). In addition, sphingolipids directly promoted airway inflammation and were increased in the bronchoalveolar lavage fluid in patients with asthma compared with healthy control subjects (39, 40). Furthermore, multiomic analysis of plasma in children with asthma also demonstrated altered metabolic functioning, primarily caused by increased sphingolipid metabolism (15).

In a different line of inquiry, novel therapeutic agents have also targeted sphingolipids. It has been shown in an asthma murine model that inhalation of a sphingosine kinase inhibitor reduced inflammation and airway hyperresponsiveness, and improved immune responses (41). In a more recent murine study, inhibition of sphingosine kinase suppressed proinflammatory nuclear factor- κ B and reduced airway inflammation (42). Although these previously mentioned studies do not directly investigate bronchiolitis, they link sphingolipid metabolism to inflammatory-mediated pathogenesis of airway diseases, and thereby support a potential role of sphingolipids in the pathobiology of bronchiolitis.

Amplicon sequencing of upper airway samples from children with respiratory infection has demonstrated an association between microbial dominance in the airway and illness severity. *Moraxella* has been associated with decreased severity of illness in infants hospitalized with bronchiolitis (8), whereas *Streptococcus* or *Haemophilus* have been associated with increased severity (7, 9, 43) and reduced microbiome stability (44). Consistent with these studies, our multiomic analysis

demonstrated that the abundance of *Moraxella* was generally negatively correlated with the intensity of metabolites associated with PPV use, and *M. catarrhalis* contributed to all metagenomes significantly enriched among infants without PPV use. By contrast, the relative abundance of *Streptococcus* was positively correlated with the intensity of PPV-associated metabolites. These bacteria-metabolite correlations persisted for both the individual metabolites (Model 4) and the sphingolipid metabolism subpathway.

Our multiomic analysis brings together microbiome and metabolome data and yielded findings that are concordant with recent studies exploring respiratory infections. In adults with cystic fibrosis, *Streptococcus* abundance in sputum was correlated with the relative intensity of metabolites involved in sphingolipid metabolism, including ceramide (18:2/16:0) and sphingomyelin (16:1/16:0) (16). A separate study investigating adults with *S. pneumoniae* and *H. influenzae* pneumonia and control subjects showed that plasma sphingolipids were primarily responsible for discrimination between patients with pneumonia and control subjects (45). Interestingly, our data also demonstrated that the metagenome-derived function of nasopharyngeal microbiome is not directly correlated with the intensity of metabolites that are associated with PPV use. This suggests that the significantly discriminant metabolites and pathways (e.g., sphingolipid pathway) are unlikely to be microbiome-derived, but rather produced by the host. Although further work is necessary to confirm this, most bacteria do not produce sphingolipids, which is especially true for aerobic bacteria, such as those found in the airways (46). Nevertheless, it is notable that the metagenomic data demonstrated *S. pneumoniae* to generate serine, a substrate of sphingosine, the fundamental building block of all sphingolipids. Thus, although the sphingolipid metabolites are host

derived, exogenous serine generated by *S. pneumoniae* may contribute to the significantly increased sphingolipid metabolism in the airway of infants with bronchiolitis.

The nature of the observed airway microbiome composition-metabolome severity relations warrants clarification. It is possible that the airway microbiome contributed to higher bronchiolitis severity by modulating host cellular function and metabolism (e.g., sphingolipid metabolism). Alternatively, after a change in host metabolism certain species (e.g., *S. pneumoniae*) were able to proliferate (47–49). In addition, reverse causation is possible (i.e., more severe bronchiolitis not only led to an overgrowth of specific bacteria in the airway niche, but also altered host cellular metabolism). We also recognize that these mechanisms are not mutually exclusive. Our data should facilitate further research to elucidate the underlying mechanisms linking the microbes, host immune response, and altered metabolism in the airway to bronchiolitis pathogenesis.

Potential Limitations

The current study has several potential limitations. First, nasopharyngeal samples were used because of technical and ethical restrictions in sampling lower airways from young infants. However, studies have shown that upper airway sampling provides reliable representation of the lung microbiome (50, 51) and gene expression profiles (as a proxy for cellular function) in children (52). Second, this study was not able to directly prove causality, but depletion of sphingolipid metabolism prevents disease onset in murine models of inflammatory-mediated respiratory diseases, supporting our data for the role of this subpathway in bronchiolitis severity (41, 42). Third, although the current study demonstrated the findings to be robust by performing analytical validation, external validation is necessary

to confirm the observations. Fourth, the inferences using 16S rRNA gene sequencing data were potentially limited because of their compositionality and may have led to false positives in correlation and statistical analyses (53, 54).

Fifth, although the intensity of sphingolipids was significantly associated with risks of PPV use independently from age, history of antibiotic use, and other adjusted covariates, it is possible that some observations might have been confounded by age. In addition, our binary adjustment of antibiotic use assumes any exposure to antibiotics to be comparable, regardless of type and duration. Potential effects of antibiotic type and time course were not accounted for in our models. Sixth, we did not have information from a “control” group, such as infants with noninfectious disease. Yet, the objective of the current study was not to examine the role of metabolome and microbiome on the development of bronchiolitis but to determine their relationships with disease severity. Finally, longitudinal studies would advance understanding of temporal changes in bacterial taxa and metabolites before bronchiolitis onset. Nonetheless, investigations into disease severity among infants hospitalized with bronchiolitis remain an important research focus given the high incidence (the leading cause of infant hospitalization).

Conclusions

This multiomics analysis of nasopharyngeal samples collected prospectively from a multicenter cohort of infants hospitalized for bronchiolitis demonstrated that the metabolomic profiles differ by use of PPV (clinically important marker of disease severity). We also found that, among 254 metabolites identified, a panel of 25 metabolites showed high sensitivity and specificity in discerning the use of PPV. Although external validation of the present findings is required, the ability to predict disease severity, specifically the need for respiratory support, has important

Figure 4. (Continued). with a lower risk of PPV use. (A) Canonical correlation between the relative abundance of dominant bacterial genera and the 25 metabolites from Model 4. *Streptococcus* abundance was positively correlated with the intensity of most metabolites from Model 4 that are associated with a higher risk of PPV use, such as mannitol-sorbitol, methyl-4-hydroxybenzoate, and 3-(4-hydroxyphenyl)lactate. (B) Canonical correlation between the relative abundance of three dominant bacterial genera and the 20 metabolites within the sphingolipid metabolism pathway. *Streptococcus* abundance was positively correlated with the intensity of all 20 metabolites within the pathway. HETE = hydroxyeicosatetraenoic acid; HODE = hydroxyoctadecadienoic acid; sPLS = sparse partial least squares regression.

implications for the clinical management of infants with bronchiolitis. Furthermore, our data demonstrated that the sphingolipid metabolism pathway was the most significantly enriched subpathway among infants who underwent PPV and that its enrichment was positively correlated with the abundance of *Streptococcus*. Emerging data suggest several important roles that sphingolipid metabolism may play in

airway disease pathogenesis. Our findings support further experimental investigations to define the function of sphingolipids in acute respiratory infections, which may provide a novel therapeutic strategy to treat infants with severe bronchiolitis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Ashley Sullivan, Courtney Tierney, and Janice Espinola, at the Emergency Medicine Network Coordinating Center (Boston, MA), and all of the site investigators and study staff for their valuable contributions to the 35th Multicenter Airway Research Collaboration study. They also thank Dr. Alkis Togias at the National Institutes of Health (Bethesda, MD) for helpful comments about the study results. Finally, they thank the participating families for making all of this possible.

References

1. Nair H, Simões EAF, Rudan I, Gessner BD, Azziz-Baumgartner E, Zhang JSF, Feikin DR, Mackenzie GA, Moisi JC, Roca A, *et al.*; Severe Acute Lower Respiratory Infections Working Group. Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. *Lancet* 2013;381:1380–1390.
2. Hasegawa K, Tsugawa Y, Brown DFM, Mansbach JM, Camargo CA Jr. Trends in bronchiolitis hospitalizations in the United States, 2000–2009. *Pediatrics* 2013;132:28–36.
3. Meissner HC. Selected populations at increased risk from respiratory syncytial virus infection. *Pediatr Infect Dis J* 2003;22(Suppl 2): S40–S44, discussion S44–S45.
4. Meissner HC. Viral bronchiolitis in children. *N Engl J Med* 2016;374: 62–72.
5. Hasegawa K, Dumas O, Hartert TV, Camargo CA Jr. Advancing our understanding of infant bronchiolitis through phenotyping and endotyping: clinical and molecular approaches. *Expert Rev Respir Med* 2016;10:891–899.
6. Mansbach JM, Hasegawa K, Henke DM, Ajami NJ, Petrosino JF, Shaw CA, Piedra PA, Sullivan AF, Espinola JA, Camargo CA Jr. Respiratory syncytial virus and rhinovirus severe bronchiolitis are associated with distinct nasopharyngeal microbiota. *J Allergy Clin Immunol* 2016;137: 1909–1913.e4.
7. de Steenhuijsen Piters WAA, Heinonen S, Hasrat R, Bunsow E, Smith B, Suarez-Arrabal M-C, Chaussabel D, Cohen DM, Sanders EAM, Ramilo O, *et al.* Nasopharyngeal microbiota, host transcriptome, and disease severity in children with respiratory syncytial virus infection. *Am J Respir Crit Care Med* 2016;194:1104–1115.
8. Hasegawa K, Mansbach JM, Ajami NJ, Espinola JA, Henke DM, Petrosino JF, Piedra PA, Shaw CA, Sullivan AF, Camargo CA Jr; on behalf of the MARC-35 Investigators. Association of nasopharyngeal microbiota profiles with bronchiolitis severity in infants hospitalised for bronchiolitis. *Eur Respir J* 2016;48:1329–1339.
9. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, Holt BJ, Hales BJ, Walker ML, Hollams E, *et al.* The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 2015;17: 704–715.
10. Stewart CJ, Skeath T, Nelson A, Fernstad SJ, Marrs EC, Perry JD, Cummings SP, Berrington JE, Embleton ND. Preterm gut microbiota and metabolome following discharge from intensive care. *Sci Rep* 2015;5:17141.
11. Nobakht M, Gh BF, Aliannejad R, Rezaei-Tavirani M, Taheri S, Oskouie AA. The metabolomics of airway diseases, including COPD, asthma and cystic fibrosis. *Biomarkers* 2014;20:1–12.
12. Shenoy MK, Iwai S, Lin DL, Worodria W, Ayakaka I, Byanyima P, Kaswabuli S, Fong S, Stone S, Chang E, *et al.* Immune response and mortality risk relate to distinct lung microbiomes in patients with HIV and pneumonia. *Am J Respir Crit Care Med* 2017;195: 104–114.
13. Langley RJ, Tsalik EL, van Velkinburgh JC, Glickman SW, Rice BJ, Wang C, Chen B, Carin L, Suarez A, Mohny RP, *et al.* An integrated clinico-metabolomic model improves prediction of death in sepsis. *Sci Transl Med* 2013;5:195ra95.
14. Kelly RS, Dahlin A, McGeachie MJ, Qiu W, Sordillo J, Wan ES, Wu AC, Lasky-Su J. Asthma metabolomics and the potential for integrative omics in research and the clinic. *Chest* 2017;151: 262–277.
15. McGeachie MJ, Dahlin A, Qiu W, Croteau-Chonka DC, Savage J, Wu AC, Wan ES, Sordillo JE, Al-Garawi A, Martinez FD, *et al.* The metabolomics of asthma control: a promising link between genetics and disease. *Immun Inflamm Dis* 2015;3:224–238.
16. Quinn RA, Lim YW, Mak TD, Whiteson K, Furlan M, Conrad D, Rohwer F, Dorrestein P. Metabolomics of pulmonary exacerbations reveals the personalized nature of cystic fibrosis disease. *PeerJ* 2016;4:e2174.
17. Quinn RA, Phelan VV, Whiteson KL, Garg N, Bailey BA, Lim YW, Conrad DJ, Dorrestein PC, Rohwer FL. Microbial, host and xenobiotic diversity in the cystic fibrosis sputum metabolome. *ISME J* 2016;10:1483–1498.
18. Hasegawa K, Mansbach JM, Ajami NJ, Petrosino JF, Freishtat RJ, Teach SJ, Piedra PA, Camargo CA Jr. Serum cathelicidin, nasopharyngeal microbiota, and disease severity among infants hospitalized with bronchiolitis. *J Allergy Clin Immunol* 2017;139: 1383–1386.e6.
19. Ralston SL, Lieberthal AS, Meissner HC, Alverson BK, Baley JE, Gadomski AM, Johnson DW, Light MJ, Maraga NF, Mendonca EA, *et al.*; American Academy of Pediatrics. Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis. *Pediatrics* 2014;134:e1474–e1502.
20. Hasegawa K, Jartti T, Mansbach JM, Laham FR, Jewell AM, Espinola JA, Piedra PA, Camargo CA Jr. Respiratory syncytial virus genomic load and disease severity among children hospitalized with bronchiolitis: multicenter cohort studies in the United States and Finland. *J Infect Dis* 2015;211:1550–1559.
21. Mansbach JM, Piedra PA, Teach SJ, Sullivan AF, Forgey T, Clark S, Espinola JA, Camargo CA Jr; MARC-30 Investigators. Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. *Arch Pediatr Adolesc Med* 2012; 166:700–706.
22. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. Integrated, nontargeted ultrahigh performance liquid chromatography/ electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem* 2009;81: 6656–6667.
23. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS, *et al.* A framework for human microbiome research. *Nature* 2012;486: 215–221.
24. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* 2012;486: 207–214.
25. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 2012;6:1621–1624.
26. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–2461.
27. Ye Y, Doak TG. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Comput Biol* 2009;5:e1000465.

28. Jartti T, Hasegawa K, Mansbach JM, Piedra PA, Camargo CA Jr. Rhinovirus-induced bronchiolitis: lack of association between virus genomic load and short-term outcomes. *J Allergy Clin Immunol* 2015;136:509–12.e11.
29. Trivedi DK, Iles RK. The application of SIMCA P+ in shotgun metabolomics analysis of ZIC[®]HILIC-MS spectra of human urine: experience with the Shimadzu IT-TOF and profiling solutions data extraction software. *J Chromatogr Sep Tech* 2012;3:145.
30. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0: making metabolomics more meaningful. *Nucleic Acids Res* 2015;43:W251–W257.
31. R Core Team. A language and environment for statistical computing. 2014 [accessed 2017 Jan]. Available from: <http://www.r-project.org/>
32. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 1995;57:289–300.
33. Lê Cao K-A, González I, Déjean S. integrOmics: an R package to unravel relationships between two omics datasets. *Bioinformatics* 2009;25:2855–2856.
34. Lê Cao K-A, Rossouw D, Robert-Granié C, Besse P. A sparse PLS for variable selection when integrating omics data. *Stat Appl Genet Mol Biol* 2008;7:35.
35. Noecker C, Eng A, Srinivasan S, Theriot CM, Young VB, Jansson JK, Fredricks DN, Borenstein E. Metabolic model-based integration of microbiome taxonomic and metabolomic profiles elucidates mechanistic links between ecological and metabolic variation. *mSystems* 2016;1:e00013-15.
36. Yang Y, Uhlig S. The role of sphingolipids in respiratory disease. *Thorax* 2011;5:325–344.
37. Ghidoni R, Caretti A, Signorelli P, Ghidoni R, Caretti A, Signorelli P. Role of sphingolipids in the pathobiology of lung inflammation. *Mediators Inflamm* 2015;2015:1–19.
38. Nixon GF. Sphingolipids in inflammation: pathological implications and potential therapeutic targets. *Br J Pharmacol* 2009;158:982–993.
39. Maceyka M, Spiegel S. Sphingolipid metabolites in inflammatory disease. *Nature* 2014;510:58–67.
40. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S, et al. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* 2001;15:1212–1214.
41. Nishiuma T, Nishimura Y, Okada T, Kuramoto E, Kotani Y, Jahangeer S, Nakamura S. Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1085–L1093.
42. Price MM, Oskeritzian CA, Falanga YT, Harikumar KB, Allegood JC, Alvarez SE, Conrad D, Ryan JJ, Milstien S, Spiegel S. A specific sphingosine kinase 1 inhibitor attenuates airway hyperresponsiveness and inflammation in a mast cell-dependent murine model of allergic asthma. *J Allergy Clin Immunol* 2013;131:501–11.e1.
43. Kloepper KM, Lee WM, Pappas TE, Kang TJ, Vrtis RF, Evans MD, Gangnon RE, Bochkov YA, Jackson DJ, Lemanske RF, et al. Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. *J Allergy Clin Immunol* 2014;133:1301–1307.
44. Biesbroek G, Tsivtsivadze E, Sanders EAM, Montijn R, Veenhoven RH, Keijser BJJ, Bogaert D. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med* 2014;190:1283–1292.
45. To KKW, Lee K-C, Wong SSY, Sze K-H, Ke Y-H, Lui Y-M, Tang BSF, Li IWS, Lau SKP, Hung IFN, et al. Lipid metabolites as potential diagnostic and prognostic biomarkers for acute community acquired pneumonia. *Diagn Microbiol Infect Dis* 2016;85:249–254.
46. Olsen I, Jantzen E. Sphingolipids in bacteria and fungi. *Anaerobe* 2001;7:103–112.
47. van Ooij C, Kalman L, van Ijzendoorn, Nishijima M, Hanada K, Mostov K, Engel JN. Host cell-derived sphingolipids are required for the intracellular growth of *Chlamydia trachomatis*. *Cell Microbiol* 2000;2:627–637.
48. Heung LJ, Luberto C, Del Poeta M. Role of sphingolipids in microbial pathogenesis. *Infect Immun* 2006;74:28–39.
49. Zaas DW, Duncan M, Rae Wright J, Abraham SN. The role of lipid rafts in the pathogenesis of bacterial infections. *Biochim Biophys Acta Mol Cell Res* 2005;1746:305–313.
50. Marsh RL, Kaestli M, Chang AB, Binks MJ, Pope CE, Hoffman LR, Smith-Vaughan HC. The microbiota in bronchoalveolar lavage from young children with chronic lung disease includes taxa present in both the oropharynx and nasopharynx. *Microbiome* 2016;4:37.
51. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman RG. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med* 2011;184:957–963.
52. Poole A, Urbanek C, Eng C, Schageman J, Jacobson S, O'Connor BP, Galanter JM, Gignoux CR, Roth LA, Kumar R, et al. Dissecting childhood asthma with nasal transcriptomics distinguishes subphenotypes of disease. *J Allergy Clin Immunol* 2014;133:670–678.
53. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis* 2015;26:27663.
54. Morton JT, Sanders J, Quinn RA, McDonald D, Gonzalez A, Vázquez-Baeza Y, Navas-Molina JA, Song SJ, Metcalf JL, Hyde ER, et al. Balance trees reveal microbial niche differentiation. *mSystems* 2017;2:e00162-116.