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Respiratory syncytial virus and rhinovirus bronchiolitis are associated with distinct metabolic pathways

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Short title: Respiratory virome, metabolome, and microbiome

Summary

While bronchiolitis is considered a relatively homogenous viral infection, these results provide novel evidence to support the emerging hypothesis that RSV and RV bronchiolitis may have different mechanisms that involve a complex interplay between virus, microbiome, and host.
ABSTRACT

Background: Bronchiolitis, the leading cause of US infant hospitalizations, is most commonly caused by respiratory syncytial virus (RSV) followed by rhinovirus (RV). Conventional perception is that bronchiolitis is a single entity, albeit with different viral etiologies and degrees of severity.

Methods: We conducted a cross-sectional study of nasopharyngeal aspirates from 106 infants hospitalized with either RSV-only (n=80) or RV-only (n=26) bronchiolitis. We performed metabolomics analysis and 16S rRNA gene sequencing on all samples, and metagenomic sequencing on 58 of the 106 samples.

Results: Infants with RSV-only and RV-only infections had significantly different nasopharyngeal metabolome profiles (P<0.001) and bacterial metagenome profiles (P<0.05). RSV-only was associated with metabolites from a range of pathways and a microbiome dominated by Streptococcus pneumoniae. By contrast, RV-only was associated with increased essential and non-essential N-acetyl amino acids and high relative abundance of Haemophilus influenzae. These co-occurring species were associated with driving the bacterially-derived metabolic pathways. Multi-omic analysis showed that both the virus and the microbiome were significantly associated with the metabolic function in infants hospitalized with bronchiolitis.

Conclusion: Although study replication is necessary, these results highlight that bronchiolitis is not a uniform disease between RSV and RV infections, a result with future implications for prevention and treatment.

Key words: Bronchiolitis, respiratory syncytial virus, rhinovirus, metabolomics, microbiome.
INTRODUCTION

Bronchiolitis, a lower respiratory infection in infants, is an important public health problem worldwide and the leading cause of US infant hospitalizations [1]. Respiratory syncytial virus (RSV) and rhinovirus (RV) account for ~85% of the viruses causing severe bronchiolitis [2]. Although different viruses cause bronchiolitis, the American Academy of Pediatrics (AAP) recommends that clinicians not test children with bronchiolitis for viruses, since treatment does not vary by viral etiology [3]. Furthermore, due to the lack of clinical trials demonstrating consistently beneficial responses to pharmacotherapy, the 2014 AAP clinical practice guideline discusses bronchiolitis as a single, relatively homogenous entity [4,5].

Recently, however, we identified distinct clinical phenotypes of bronchiolitis, signaling that bronchiolitis probably is a heterogeneous condition [6]. Furthermore, compared to children with RSV bronchiolitis, children with RV bronchiolitis have a shorter hospital length-of-stay [2], are significantly more likely to be treated with systemic corticosteroids [7], and have an increased risk of childhood asthma [7,8]. Although indicative of heterogeneity, these associations between viral etiology and outcomes do not provide insight into the underlying pathobiology of bronchiolitis, which involves complex interplay between the virus, microbiome, and host. Thus, we hypothesized that RSV and RV infections would be associated with underlying metabolic differences - as manifested by differences in the airway microbiome and metabolome. To test this hypothesis, we applied metabolomic and metagenomic approaches to the nasopharyngeal aspirates (NPA) from infants hospitalized with bronchiolitis. This integrated multi-omic approach examined molecular differences between RSV and RV pathobiology while accounting for both microbial and host factors.
METHODS

Study design, setting, and participants

The study design, setting, participants, and methods of data collection are from the 35th Multicenter Airway Research Collaboration (MARC-35) [9]. Briefly, using a standardized protocol, site investigators at 17 sites across 14 U.S. states enrolled infants hospitalized with an attending physician diagnosis of bronchiolitis. Bronchiolitis was defined by the AAP guidelines – acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions [3]. In order to make the timing of sample collection more synchronous, we excluded infants with previous enrollment, those who were transferred to a participating hospital >24 hours after the original hospitalization and those who were consented >24 hours after hospitalization. We also excluded those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age <32 weeks. All patients were treated at the discretion of the treating physician. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or legal guardian.

We selected MARC-35 samples for metabolomic analysis to examine severity of illness [10] as well as RSV-only vs RV-only infections. Given the cost of this testing and the volume of sample required, we not only limited the overall sample size for testing, but also selected nasopharyngeal samples with sufficient volume for the metabolome and metagenomic WGS testing. For the present analysis we analyzed the 106 samples with RSV-only or RV-only infections. Of the 106 samples, all had 16S rRNA gene sequencing data and 58 had metagenomic whole genome shotgun (WGS) sequencing data. Detailed descriptions of the sample preparation, data acquisition and processing, and statistical analysis may be found in the Online Supplement (Supplementary Methods).
Sample collection and testing

Sample collection: NPA samples were collected by trained site investigators and stored at −80 °C using a standardized protocol [2]. All samples were handled and processed identically.

Virology testing: Viral genomic load was determined as previously described [11]. A Ct value <40 was considered positive. C_T-values provide a semi-quantitative measure of viral load, with an inverse linear relationship between viral load and C_T-values. Thus, the inverse of the C_T-values (i.e., viral load) was applied for analysis.

Metabolomic profiling: Metabolomics was performed by Metabolon (Durham, NC) using ultra performance liquid chromatography tandem-mass spectrometry (UPLC-MS/MS) in both positive and negative ionization mode. All samples were blinded to Metabolon and processed in a random order. Sample preparation was carried out as described previously [12].

Bacterial 16S rRNA gene profiling: Bacterial genomic DNA was extracted from the nasopharyngeal samples using MOBIO PowerSoil DNA Isolation Kit (Mo Bio Laboratories; Carlsbad, CA) as described previously [13]. The sequencing methods were adapted from those developed for the NIH-Human Microbiome Project [14] using the V4 region (515F and 806R [15]) and sequenced on the MiSeq platform (Illumina; San Diego, CA) using the 2 x 250 bp paired-end protocol. Resulting reads were quality filtered using strict merging criteria and biom tables were rarified to 1,500 reads per sample.

Metagenomic whole genome shotgun sequencing: Metagenomic WGS sequencing was available for 58 nasopharyngeal samples[10]. Individual libraries constructed from each sample were sequenced using the 2x100 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.
Statistical analyses

The primary exposure was RSV-only or RV-only infection and the primary outcome was the nasopharyngeal airway metabolome. Orthogonal partial least squares-discriminatory analysis (OPLS-DA) was performed using MetaboAnalyst 3.0 and validated using 2000 random permutations [16]. Significant metabolites were determined using two-tailed Welch’s t-test and adjusted for multiple comparisons with the false discovery rate (FDR) algorithm [17]. Linear regression models adjusted for 7 potential confounding variables (age, sex, preterm, antibiotics, systemic corticosteroids pre-hospitalization, positive pressure ventilation (PPV), and hospital site) and were FDR corrected. MixOmics [18] was implemented in R version 3.3 [19] based on sparse partial least squares regression (sPLS) and was performed in canonical mode with LASSO penalization. FishTaco (functional shifts taxonomic contributors) was used to determine which species was associated with “reducing” or “driving” the significantly altered functions [20] and model-based integration of metabolite observations and species abundances (MIMOSA) was applied to determine biologically feasible correlations between the WGS bacterial KEGG orthologs (KOs) and the resulting metabolites of likely bacterial origin [21].

RESULTS

Study population

Nasopharyngeal metabolomic and microbiome data were generated from 106 infants enrolled into the MARC-35 study, a 17-center cohort study of 1,016 infants hospitalized with bronchiolitis [9]. Compared with the non-analytic MARC-35 cohort (n=910), patients in the current analytic (n=106) cohort were younger and more likely to require PPV, defined as continuous positive pressure ventilation and/or endotracheal intubation (Supplementary Table 1). The 106 infants in the analytic cohort had a median age at hospitalization of 3 months (interquartile range; IQR, 1–5 months) and 61% were male. Eighty (75%) were infected with
RSV-only and 26 (25%) were infected with RV-only (i.e., no other co-infecting virus was detected among 16 viruses tested). Patient characteristics did not differ significantly between the two virus groups, with the exception of higher proportion of non-Hispanic white and greater use of PPV in the RSV-only group (Table 1).

Infants with RSV-only or RV-only have significantly different metabolomic profiles

Metabolomic analysis detected a total of 254 metabolites. When comparing infants with RSV-only bronchiolitis to those with RV-only bronchiolitis, the metabolomic profiles clustered distinctly based on the viral etiology (Figure 1A). This difference was validated using 2000 permutations (P<0.001). Since the use of PPV was significantly greater in infants with RSV-only and may influence the metabolites (Table 1), we removed the infants who underwent PPV from the analysis and still found significant differences in the metabolomic profiles between RSV-only and RV-only infants (P < 0.001). Metabolites associated with RSV-only were from a range of super-pathways, including carbohydrate metabolism (N-acetylglucosamine-N-acetylgalactosamine), lipid metabolism (mevalonolactone), amino acid metabolism (5-oxoproline), and energy metabolism (2-methylcitrate-homocitrate) (Figure 1B). By contrast, metabolites associated with RV-only infection consisted primarily of N-acetyl metabolites from the amino acid super-pathway, specifically, N-acetylthreonine, N-acetylleucine, N-acetylisoleucine, N-acetylphenyalanine, N-acetylvaline, N-acetylglutamate, N-acetytyrosine, and N-acetylysine (Figure 1B).

Of the 254 detected metabolites, there were 31 metabolites that significantly differed between RSV-only and RV-only infections by relative intensity, with 25 metabolites significant even after adjustment for seven covariates, including race/ethnicity and PPV (FDR P<0.05; Table 2). These associations between the qualitative viral differences (i.e., viral presence or absence) and all 254 detected metabolites were also supported by correlations between quantitative viral differences (i.e., genomic load of RSV and RV based on inverse C_T values) and all detected metabolites.
(Figure 1C). In other words, not only were the metabolites different based on the infecting virus, but also the amount of virus correlated with increased metabolite intensity. Of the samples with detectable virus, the median viral load Ct values for RSV was 23.0 (IQR 21.5 – 26.4) and for RV was 26.4 (IQR 24.6– 28.1).

Infants with RSV-only or RV-only infection have significantly different bacterial functional potential primarily associated with *S. pneumoniae* and *H. influenzae*, respectively

Since RSV and RV infect an airway colonized with bacteria, we conducted metagenomic WGS sequencing to determine the functional potential of the microbiome (i.e., the presence, but not necessarily expression, of genes in microbial genomes). From the 106 nasopharyngeal samples, 58 were included in the metagenomic analyses (39 infants from RSV-only and 19 infants from RV-only). There were no demographic differences between the RSV-only and RV-only groups in the metagenomic subset, although infants with RSV-only infection were more likely to undergo PPV (Supplementary Table 2). Infants with either RSV-only or RV-only infection had significantly different metagenomic (metabolomic potential) profiles (permutation *P*=0.043) (Figure 2).

We performed further analysis of the metagenomic data using FishTaco which determines taxa that are associated with “reducing” or “driving” functional shifts [20]. This analysis showed that nine bacterially-derived metabolic pathways were significantly different between infants with RSV-only and RV-only infection (Figure 2B). Among infants with RSV-only, *S. pneumoniae* relative abundance was associated with driving the functional shift in all nine pathways. By contrast, *Moraxella catarrhalis* was associated with driving four of the nine pathways and reducing the remaining five pathways. Among infants with RV-only infection, *H. influenzae* was associated with driving all nine pathways and *Prevotella melaninogenica* was associated with reducing all nine pathways. The more granular module-level analysis was comparable to the broader pathway-level analysis, except energy metabolism modules were
reduced by *S. pneumoniae* and *M. catarrhalis* in RSV-only bronchiolitis, and by *H. influenzae* in RV-only bronchiolitis (Supplementary Figure 1). Furthermore, in infants with RSV-only, *S. pneumoniae* was associated with driving carbohydrate and lipid metabolite modules while *Streptococcus mitis* was associated with reducing these modules, demonstrating that species within the same genus may influence modules differently.

Multi-omic analysis showed the virus and dominant bacteria were both associated with the metabolomic profiles

The three dominant bacterial OTUs (operational taxonomic unit) from the 16S rRNA gene profiling (representing 80% of the total bacterial community) were characterized by metagenomic data as *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* species. Examining viral-bacterial associations in the current analytic cohort, we found that RSV-only infection was associated with a high relative abundance of *S. pneumoniae* and RV-only infection was associated with high relative abundance of *H. influenzae* (Supplementary Figure 2). However, given the dominance of all three bacteria (i.e., *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*) and previously published viral-microbial associations from the entire MARC-35 cohort [9], we included all three dominant species in the analysis.

Among the 254 metabolites, the relative abundance of *S. pneumoniae* was positively correlated with metabolites from sphingolipid metabolism (Supplementary Figure 3). However, sphingolipid metabolites were not associated with RSV-only infections. Consistent with these results, the network analysis showed that the relative abundance of *S. pneumoniae* had only weak positive correlations with metabolites significantly increased in RSV-only infection (Figure 3). By contrast, *H. influenzae* was positively correlated with the N-acetyl metabolites from amino acid metabolism that are increased in RV-only infections, and was negatively correlated with all other metabolites (Figure 3 and Supplementary Figure 3). The abundance of *M. catarrhalis* was positively correlated with metabolites from a range of
pathways, but none of the metabolites associated with either RSV-only or RV-only infections (Figure 3 and Supplementary Figure 3).

Permutational multivariate analysis of variance using distance matrices (adonis) was utilized to test if either the virus or bacterial species had a greater association with the metabolomic profiles [22]. As the adonis function is based on sequential sum of squared deviations from a centroid, the order of the factors (i.e., if the virus was entered first or second in the model) was tested using both permutations. The virus (RSV-only or RV-only) was statistically significant when entered as the first factor ($P = 0.04$), but not when entered as the second factor ($P = 0.14$). Similarly, the bacterial species (dominance by *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, or a mixed community) was statistically significant when entered as the first factor ($P = 0.049$), but not when entered as the second factor ($P = 0.10$). These results suggest that both the viral etiology and the bacterial species are associated with the metabolomic profiles and that both are relevant in infants hospitalized with bronchiolitis.

**Significant metabolites were host-derived based on computational analysis**

We employed MIMOSA to ascertain which metabolites were either bacterially-derived or host-derived, based on metagenome orthology. Although there were significant metagenomic (metabolomic potential) differences between infants with RSV-only and those with RV-only infection, MIMOSA analysis of the 254 metabolites estimated that 19 metabolites (7%) were derived from bacteria (Supplementary Table 3). None of the 31 metabolites that significantly differed between infants with RSV-only and RV-only infection were bacterially-derived (Table 2). Taken together, these results suggest that most metabolites that distinguish RSV-only from RV-only infections are host-derived.
DISCUSSION

In this novel multi-omic analysis of nasopharyngeal airway samples collected from infants hospitalized with bronchiolitis, RSV-only and RV-only infections were associated with significantly different metabolic function. RSV-only bronchiolitis had significantly increased metabolites from a range of pathways, whereas infants with RV-only bronchiolitis had increased N-acetyl amino acids. Moreover, higher viral genomic load positively correlated with higher intensity of the metabolites associated with RSV and RV infection. RSV and RV co-occurred with bacterial species (e.g., RSV with *S. pneumoniae* and RV with *H. influenzae*), and the associated metagenomes significantly differed between RSV-only and RV-only infections. Thus, these data demonstrate, for the first time, that RSV and RV are associated with different metabolic pathways and that the associated bacterial functional capacity is derived primarily from *S. pneumoniae* in RSV bronchiolitis and *H. influenzae* in RV bronchiolitis.

Although the clinical phenotype of bronchiolitis due to different viruses is considered indistinguishable by some experts [23], comparing epidemiologic data of children with RV to those with RSV bronchiolitis demonstrates that children with RV have different demographics [2,24], medical histories [25], hospital treatment [25], short-term outcomes [6], and long-term outcomes [7,25]. The present results provide the first multi-omic comparison demonstrating pathobiological differences between RSV and RV bronchiolitis. Higher genomic load of RSV and RV was positively correlated with a higher intensity of the metabolites that distinguished RSV from RV. Although this finding is intriguing given the association between higher RSV genomic load and increased severity of illness [26], the same genomic load-severity association has not been found in RV [27]. While the relevance of genomic viral load requires additional validation, given the epidemiological and now pathobiological differences between RSV and RV, future clinical trials for infants with bronchiolitis should consider stratifying infants based on viral etiology.
RV-only infected infants hospitalized with bronchiolitis had altered amino acid metabolism, particularly related to N-acetyl metabolism. *H. influenzae*, which co-occurred with RV, was also associated with these metabolites. Nα-acetylation is the process of irreversibly transferring an acetyl group from acetyl co-enzyme A to the α-amino group of an amino acid [28]. It is among the most abundant co- and post-translational protein modifications in eukaryotes, but is less common in bacteria [29]. The functional implication of acetylation in RV bronchiolitis remains unclear. Notably, in asthma, there is an increase in histone acetyltransferase activity that is treatable with corticosteroids [30]. Although speculative, the association between early childhood RV wheezing illnesses and increased risk of school age asthma [31–33] may be explained in part by the altered host amino acid metabolism in RV bronchiolitis seen in the present results.

The bacterial derivation (vs host) of 7% of detected metabolites was comparable to a previous report in adult sputum showing 4% of detected metabolites were microbial [34]. The KEGG orthologs from *H. influenzae* were associated with the generation of essential amino acids, but neither *S. pneumoniae* nor other microbes correlated with the generation of amino acids. Indeed, *S. pneumoniae* cannot synthesize amino acids [35], producing peptidases, proteases, and utilizing cell wall transporters to uptake amino acids [36]. Therefore, in addition to dietary intake, *H. influenzae* provides access to essential amino acids, which may have contributed to the observed increase in RV-associated N-acetyl metabolites. *S. pneumoniae* and *H. influenzae* were responsible for the same bacterial metabolic pathway functions in RSV and RV, respectively. Additionally, we found cases of *S. pneumoniae* driving and *S. mitis* reducing the same modules, suggesting important effects of different species within the same genera [20]. Taken together, these results suggest that both the virus and bacteria have important influences on the metabolomic profiles, as supported by the adonis results. Thus, future bronchiolitis studies may need to account for both viral and bacterial communities and clinicians may need to begin thinking of “viral” bronchiolitis as being due to a complex interplay among the infecting virus, microbiome, and host response.
The current results offer novel findings and additional evidence of microbial (virus and bacteria) and host interaction in the pathobiology of bronchiolitis as investigated within the MARC-35 cohort. We have previously found that infants with RV bronchiolitis have shorter hospital length-of-stay [37,38] and associations with *Haemophilus* and *Moraxella* microbial dominance when compared with RSV, which is associated with microbial dominance by *Streptococcus* [9]. And although *Haemophilus* and *Streptococcus* are associated with increased severity of illness [13,39] and *Moraxella* with less severe bronchiolitis [13], the results from the MARC studies underscore the complex bacterial, viral, and host interaction in bronchiolitis pathobiology [40,41].

The current study has several potential limitations. First, bronchiolitis is mostly a disease of the lower airways and while nasopharyngeal samples may not provide a reliable representation of the lung microbiome [42] they are more easily obtained than lower airway samples [43]. Additionally, nasopharyngeal samples have further technical and ethical justification [43]. Second, the cross-sectional study design prevented temporal monitoring of viral and bacterial abundance and function, thus subsequent studies would benefit from longitudinal sampling, including, ideally, the collection of samples prior to hospitalization and samples from non-infectious control infants. Third, antibiotic data was limited to use prior to enrollment, but neither the exact antibiotic used nor the duration of administration was analyzed. However, there were no significant differences in antibiotic use prior to enrollment between infants with RSV-only and those with RV-only infections as shown in Table 1 (P = 0.75). Fourth, significant financial expense of the techniques employed limited the number of samples that could be analyzed. Thus, the present results require validation not only in the full MARC-35 cohort, but also in an independent cohort. Indeed, such temporal and validation analyses would be necessary for biomarker identification. Finally, from an analytic standpoint, not all significant metabolites are present in the KEGG metabolic network model, meaning MIMOSA would be unable to analyze them. Thus, it is possible that some metabolites considered host-derived are products of novel microbial metabolism. Notwithstanding these limitations, the
current study provides novel evidence to support the emerging hypothesis that RSV and RV induce bronchiolitis through distinct metabolic pathways.

Currently, most clinicians and researchers regard bronchiolitis as a relatively homogenous clinical entity. Although replication is needed, these data demonstrate the underlying heterogeneity of bronchiolitis by showing that RSV and RV are associated with different metabolic pathways, and that both the virus and the co-occurring bacterial species play a role in the host metabolism. Bronchiolitis is also currently considered a viral condition, but these data provide further evidence that bronchiolitis involves a complex interplay among virus, microbiome, and host. Indeed, these results not only challenge long-standing ideas about bronchiolitis, but also, given the lack of effective pharmacotherapies for this very common condition [3–5], have important implications for the future development of novel treatments.
FOOTNOTE

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CONFLICT OF INTERESTS

Dr. Mansbach has provided bronchiolitis-related consultation for Regeneron. Drs. Ajami and Petrosino own shares at Diversigen Inc., a microbiome research company. Dr. Piedra provided bronchiolitis-related consultation for Gilead, Novavax, and Regeneron. The other authors have no financial relationships relevant to this article to disclose.
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### Table 1 – Characteristics of infants hospitalized with either respiratory syncytial virus-only (RSV-only) or rhinovirus-only (RV-only) bronchiolitis, n=106

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RSV-only (n=80)</th>
<th>RV-only (n=26)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in months, median (IQR)</td>
<td>2.7 (1-5)</td>
<td>2.7 (1.4-7.9)</td>
<td>0.64</td>
</tr>
<tr>
<td>Male sex</td>
<td>47 (59%)</td>
<td>18 (69%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>43 (54%)</td>
<td>11 (42%)</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic black</td>
<td>11 (14%)</td>
<td>6 (23%)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>21 (26%)</td>
<td>8 (31%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>5 (6%)</td>
<td>1 (4%)</td>
<td></td>
</tr>
<tr>
<td>Maternal smoking during pregnancy</td>
<td>5 (6%)</td>
<td>4 (15%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Prematurity (32-37 weeks)</td>
<td>16 (20%)</td>
<td>7 (27%)</td>
<td>0.46</td>
</tr>
<tr>
<td>C-section delivery</td>
<td>29 (36%)</td>
<td>10 (38%)</td>
<td>0.87</td>
</tr>
<tr>
<td>Postnatal smoke exposure at home</td>
<td>9 (12%)</td>
<td>0 (0%)</td>
<td>0.97</td>
</tr>
<tr>
<td>Number of siblings, median (IQR)</td>
<td>1 (1-2)</td>
<td>1 (1-2)</td>
<td>0.54</td>
</tr>
<tr>
<td>History of antibiotic use prior to enrolment</td>
<td>22 (28%)</td>
<td>8 (31%)</td>
<td>0.75</td>
</tr>
<tr>
<td>History of corticosteroid use prior to enrolment</td>
<td>11 (14%)</td>
<td>3 (12%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Positive pressure ventilation</td>
<td>16 (23%)</td>
<td>0 (0%)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Abbreviations: RSV, respiratory syncytial virus; RV, rhinovirus; IQR, interquartile range.
Table 2 – Metabolites associated with respiratory syncytial virus-only (RSV-only) and rhinovirus-only (RV-only) infections among infants hospitalized for bronchiolitis

<table>
<thead>
<tr>
<th>Increased in</th>
<th>Metabolite</th>
<th>Sub-Pathway</th>
<th>Super-Pathway</th>
<th>KEGG Compound</th>
<th>P Value</th>
<th>Adjusted P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>N-acetylglcosamine-N-acetylgalactosamine</td>
<td>Aminosugar Metabolism</td>
<td>Carbohydrate</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mevalonolactone</td>
<td>Mevalonate Metabolism</td>
<td>Lipid</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cystine</td>
<td>Methionine, Cysteine, SAM and Taurine Metabolism</td>
<td>Amino Acid</td>
<td>C00491</td>
<td>0.033</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Palmitic-amide</td>
<td>Fatty Acid, Amide</td>
<td>Lipid</td>
<td>0.001</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-methylcitrate-homocitrate</td>
<td>TCA Cycle</td>
<td>Energy</td>
<td>0.001</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caprate (10:0)</td>
<td>Medium Chain Fatty Acid</td>
<td>Lipid</td>
<td>C01571</td>
<td>0.018</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Diglycerol</td>
<td>Chemical</td>
<td>Xenobiotics</td>
<td></td>
<td>0.025</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Beta-hydroxyisovalerate</td>
<td>Leucine, Isoleucine and Valine Metabolism</td>
<td>Amino Acid</td>
<td></td>
<td>0.015</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>Pregnen-diol-disulfate</td>
<td>Steroid</td>
<td>Lipid</td>
<td>0.018</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Laurate (12:0)</td>
<td>Medium Chain Fatty Acid</td>
<td>Lipid</td>
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<td>0.002</td>
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<td>Amino Acid</td>
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<td>&lt;0.001</td>
<td>0.002</td>
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<td>0.002</td>
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<td>Fatty Acid, Dicarboxylate</td>
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<td>Amino Acid</td>
<td>C02710</td>
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<td>Leucine, Isoleucine and Valine Metabolism</td>
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<td>Lipid</td>
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<td>N-acetyltaurine</td>
<td>Methionine, Cysteine, SAM and Taurine Metabolism</td>
<td>Amino Acid</td>
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**FIGURE LEGENDS**

**Figure 1. Nasopharyngeal airway metabolomic profiles in infants with respiratory syncytial virus-only (RSV-only) or rhinovirus-only (RV-only) bronchiolitis.** Analysis includes all infants \( n = 106 \) and 254 detected metabolites. 

A) Orthogonal partial least squares discriminant analysis (OPLS-DA) score scatter plot of samples classified according to RSV-only (red) or RV-only (green) infection. Each dot represents the metabolomic profile of one infant. Ellipses show 95% confidence interval. Metabolomic profiles were significantly different between RSV and RV \( (p_{\text{permuation}} < 0.001) \). 

<table>
<thead>
<tr>
<th>Metabolite</th>
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<th>P-value</th>
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<td>4-hydroxyphenylpyruvate</td>
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<td>Glycerol 3-phosphate</td>
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<td>Folate</td>
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<td>0.034</td>
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* Linear regression models adjusting for age, race/ethnicity, history of prematurity, history of antibiotic use prior to the enrollment, use of systemic corticosteroids during the prehospitalization visit, use of PPV, and hospital site.

Abbreviations: RSV, respiratory syncytial virus; RV, rhinovirus; KEGG, Kyoto encyclopedia of genes and genomes
correlations of the viral genomic load (inverse C_T value) of RSV and RV infections with the metabolomic profiles. Only the top 25 (of all 254 metabolites) most strongly correlated metabolites are shown. Canonical correlations are based on sparse partial least squares (sPLS). Colored squares on the left column denote metabolites significantly increased in RSV-only infection (red), significantly increased in RV-only infection (green), and not significant (gray), as per Table 2.

**Figure 2. Metabolic potential of the bacterial community among infants with respiratory syncytial virus-only (RSV-only) or rhinovirus-only (RV-only) infection.** Analysis includes metagenomic infant subset (n = 58) and all KEGG orthologs (KOs; n = 3,883). **A** Orthogonal partial least squares discriminant analysis (OPLS-DA) grouped by RSV-only (red) or RV-only (green) infection. Each dot represents the metagenomic profile of one infant. Metagenomic profiles were significantly different between RSV and RV (Permutation P = 0.043). **B** FishTaco analysis determined the contribution of bacterial species to metabolic pathways (determined from KOs) that were significantly different between RSV-only and RV-only infections. Plots are divided by the nine significant pathways. In each plot, the bars with negative enrichment (left of the line) show the association between bacterial species reducing function and bars with positive enrichment (right of the line) show the association between bacterial species driving function. For each pathway, RV-only samples are on the top row and RSV-only samples are the bottom row.

**Figure 3. Association of infants hospitalized with respiratory syncytial virus-only (RSV-only) and rhinovirus-only (RV-only) bronchiolitis with the nasopharyngeal microbiota.** Analysis includes all infants (n = 106). Network plot showing all correlations greater than 0.5 between the relative abundance of the three dominant bacterial genera (yellow) and the 31 significant metabolites in RSV (red) or RV infection (green). Nodes are colored red or green
according to the significantly increased metabolite in RSV or RV infection. The metabolite names are colored according to the super-pathway. Edges are colored by canonical sparse partial least squares (sPLS) correlations. Relative abundance for the three dominant genera is based on 16S rRNA gene sequencing. OTUs were identified to species level based on metagenomic data.
Chemical carcinogenesis
Lipopolysaccharide biosynthesis
Ubiquinone and other terpenoid-quinone biosynthesis
Peroxisome Linoleic acid metabolism
Bisphenol degradation
Sulfur metabolism
Biosynthesis of siderophore group nonribosomal peptides
Bacterial chemotaxis
Haemophilus influenzae
Moraxella catarrhalis
Prevotella melaninogenica
Other taxa (201)

RSV driving
RSV reducing
Haemophilus influenzae
Streptococcus pneumoniae
Moraxella catarrhalis
Prevotella melaninogenica
Other taxa (201)