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Respiratory viruses are associated with serum metabolome among infants hospitalized for bronchiolitis: A multicenter study

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Abstract

Background: Bronchiolitis is the leading cause of infant hospitalizations in the United States. Growing evidence supports the heterogeneity of bronchiolitis. However, little is known about the interrelationships between major respiratory viruses (and their species), host systemic metabolism, and disease pathobiology.

Methods: In an ongoing multicenter prospective cohort study, we profiled the serum metabolome in 113 infants (63 RSV-only, 21 RV-A, and 29 RV-C) hospitalized with bronchiolitis. We identified serum metabolites that are most discriminatory in the RSV-RV-A and RSV-RV-C comparisons using sparse partial least squares discriminant analysis. We then investigated the association between discriminatory metabolites with acute and chronic outcomes.

Results: In 113 infants with bronchiolitis, we measured 639 metabolites. Serum metabolomic profiles differed in both comparisons ($P_{\text{permutation}} < 0.05$). In the RSV-RV-A comparison, we identified 30 discriminatory metabolites, predominantly in lipid metabolism pathways (eg, sphingolipids and carnitines). In multivariable models, these metabolites were significantly associated with the risk of clinical outcomes (eg, tricosanoyl sphingomyelin, OR for recurrent wheezing at age of 3 years = 1.50; 95% CI: 1.05-2.15). In the RSV-RV-C comparison, the discriminatory metabolites were also primarily involved in lipid metabolism (eg, glycerophosphocholines [GPCs], 12,13-diHome). These metabolites were also significantly associated with the risk of outcomes (eg, 1-stearoyl-2-linoleoyl-GPC, OR for positive pressure ventilation use during hospitalization = 0.47; 95% CI: 0.28-0.78).

Conclusion: Respiratory viruses and their species had distinct serum metabolomic signatures that are associated with differential risks of acute and chronic morbidities of bronchiolitis. Our findings advance research into the complex interrelations between viruses, host systemic response, and bronchiolitis pathobiology.

Abbreviations: ARI, acute respiratory infection; FDR, false discovery rate; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; MARC, Multicenter Airway Research Collaboration; PPV, positive pressure ventilation; RSV, respiratory syncytial virus; RV, rhinovirus; sPLS-DA, sparse partial least squares discriminate analysis.

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KEYWORDS

bronchiolitis, infant, respiratory syncytial virus, rhinovirus, rhinovirus species, serum metabolome

1 | INTRODUCTION

Bronchiolitis is the most common lower respiratory infection in infants.^{1,2} In addition to its acute morbidity (eg, the leading cause of infant hospitalization in the United States),³ infants with severe bronchiolitis (bronchiolitis requiring hospitalization) are at high risk of developing chronic morbidities, such as recurrent wheeze and asthma in childhood.² Severe bronchiolitis is predominantly caused by two viruses; the most common virus is respiratory syncytial virus (RSV), followed by rhinovirus (RV).² RVs are RNA viruses with >160 recognized genotypes that are classified into three species (A, B, and C).⁴

Bronchiolitis has been traditionally considered a single, relatively homogenous disease entity; its management and prognostication do not currently differ by viral etiology.^{1,2} However, several recent studies demonstrate the heterogeneity of bronchiolitis by infecting virus. For example, research has shown that RSV and RV infection are associated with differential acute (eg, disease severity)⁵ and chronic (eg, incident asthma)^{6,7} morbidity burdens, host response (through transcriptomics,^{8,9} cytokines,¹⁰ and metabolomics^{11,12}), and airway microbiome profiles.^{9,13,14} Despite the clinical and research relevance, it remains unclear how different viruses (including RV species) *systemically* contribute to downstream molecules that influence clinical outcomes in infants with bronchiolitis. Metabolomics addresses this knowledge gap through comprehensively profiling functional metabolites that are a function of the child's genetic make-up and environmental factors,² such as respiratory viruses.

In this context, by comprehensively profiling the serum metabolome from a nested subset of multicenter bronchiolitis cohort, we examined the relationships between respiratory viruses (including RV species), metabolome, and acute and chronic morbidities of bronchiolitis.

2 | METHODS

2.1 | Study design, setting, and participants

We analyzed data from the 35th Multicenter Airway Research Collaboration (MARC-35)—a multicenter prospective cohort study of infants hospitalized with bronchiolitis.^{6-13,15,16} Site investigators enrolled infants (age of <1 year) hospitalized with bronchiolitis at 17 sites across 14 US states (Table S1) during three consecutive bronchiolitis seasons (from November through April) in 2011-2014. Bronchiolitis was defined by the American Academy of Pediatrics guidelines¹ as an acute respiratory illness (ARI) with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions and was diagnosed by an attending physician. We excluded

Key Message

Little had been known about the interrelationships between rhinovirus species, host systemic metabolism, and disease pathobiology in infants with bronchiolitis. The current study found that, compared with RSV-only infection, serum metabolomic signatures differed in infants with RV-A or RV-C infection, and that discriminatory metabolites were primarily involved in lipid metabolism (eg, sphingolipids and carnitines) in the RV-A comparison, and arginine and other lipid mediators (eg, glycerophospholipids) in the RV-C comparison. These metabolites were also associated with clinical outcomes. Our findings advance research into the complex interrelations between viruses and host systemic response.

infants who were transferred to a participating hospital >24 hours after the original hospitalization or those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age of <32 weeks. All patients were treated at the discretion of the treating physicians. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian. The current analysis investigated infants with RSV or RV infection who underwent serum metabolomic testing. By design, infants with RV infection were over-represented in this substudy (Table S2).

2.2 | Data collection, virus testing, and serum metabolomic testing

Clinical data (patients' demographic characteristics, and family, environmental, and medical history, and details of the acute illness) were collected via structured interview and chart reviews using a standardized protocol.^{6-13,15,16} In addition to the clinical data, nasopharyngeal and serum specimens were collected by trained site investigators within 24 hours of hospitalization.¹⁵ These specimens underwent the following virus and metabolomic testing.

Nasopharyngeal samples were first tested for 17 respiratory viruses (including RSV and RV) by using real-time polymerase chain reaction (PCR) assays at Baylor College of Medicine (Houston, TX, USA). For RV detection, complementary DNA was generated using gene-specific primers for RV and single-plex real-time PCR was used. The details of the RV primers and probes have been described elsewhere.¹⁷ Next, to identify the RV species (A, B, and C), RV-positive

samples were genotyped by molecular typing assay using partial sequencing¹⁸ at the University of Wisconsin (Madison, WI, USA).

The details of metabolomic profiling may be found in Appendix S1. In brief, metabolomic profiling used ultrahigh-performance liquid chromatography-tandem mass spectrometry. Instrument variability was 4%, as determined by calculating the median relative standard deviation for the internal standards that were added to each sample before the sample was injected into the mass spectrometer.

2.3 | Outcomes

The clinical outcomes were acute severity of bronchiolitis (as measured by positive pressure ventilation (PPV) use), as well as chronic morbidities (as measured by recurrent wheezing by age of 3 years and asthma by age of 5 years). PPV use was defined as the use of continuous positive airway pressure and/or intubation—at any time during the index hospitalization.¹⁶ Recurrent wheezing was defined—according to the 2007 NIH asthma guidelines¹⁹—as having at least two corticosteroid-requiring exacerbations in 6 months or at least four wheezing episodes in 1 year that last at least 1 day and affect sleep. Asthma was defined using an established epidemiologic definition of asthma²⁰—that is, physician diagnosis of asthma by age of 5 years, plus either asthma medication use (eg, albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year.

2.4 | Statistical analyses

In the current analysis, we categorized the patients into three mutually exclusive groups: RSV-only (reference) and two RV species (A and C) groups, excluding infants with RSV infection with non-RV pathogen(s), those with RV-B infection, and those with coinfection of multiple RV species. RV-B infections were excluded because of its clinical insignificance in ARI⁵ and the small number of RV-B ($n = 3$) infection in the cohort. To investigate the association of respiratory viruses with serum metabolome, we compared metabolites between RSV and RV-A as well as between RSV and RV-C groups. RSV group was chosen as the reference given the relatively large sample size. First, to derive metabolites that best discriminate the virus groups, we performed sparse partial least squares discriminate analysis (sPLS-DA) with Lasso penalization and 5-fold cross-validation (repeated 50 times) to minimize potential overfitting, using R *mixOmics* package.²¹ We also tested for the between-virus differences by performing permutation test (2000 random permutations). For the downstream analyses, we selected the top 30 metabolites that had a high variable importance in each of the between-virus comparisons (ie, RSV vs RV-A and RSV vs RV-C comparisons).

Next, we computed Spearman's correlation between viral genomic load (measured as the inverse cycle threshold) and the selected 30 discriminatory metabolites. We then tested for the association of respiratory viruses (RSV as the reference group) with

metabolites by constructing multivariable linear regression models adjusting for five potential confounders (age, sex, prematurity, feeding status, and batch of metabolomic testing) and patient clustering within hospitals using generalized estimating equations. To identify the underlying causal relations between respiratory viruses and metabolites—for example, infection to unique RV species perturbs the downstream metabolites (or alerted metabolomic profile changes the risk of infection to specific virus), we utilized the PC algorithm implemented in R *pcalg* package. This causal structure learning approach recovers the underlying causal pathways through the conditional independence relationships in the empirical data. To detect biologically meaningful pathways, we also performed metabolite-set enrichment analysis using MetaboAnalyst 4.0.

Lastly, we also examined the association of discriminatory metabolites (Benjamini-Hochberg false discovery rate [FDR] of <0.05) with each of the three clinical outcomes (PPV, recurrent wheezing, and asthma). We constructed multivariable logistic regression models with generalized estimating equations adjusting for age, detected virus, potential batch effect, and patient clustering within sites. In the sensitivity analysis, we repeated the analysis using sole RV-A and RV-C infection data. To examine the relations between serum and nasopharyngeal metabolites, we examined the within-individual correlations of the discriminatory metabolites. We performed the analysis using R version 3.6.1.

3 | RESULTS

As part of a 17-center prospective cohort study, 140 infants with bronchiolitis underwent serum metabolomic testing. Of these, we excluded 23 infants with RSV infection with coinfection, 3 with RV-B infection, and 1 with coinfection of multiple RV species, leaving 113 infants (63 RSV-only, 21 RV-A, and 29 RV-C) eligible for the current analysis. The analytic (113 eligible infants) and non-analytic (903 infants who were not eligible) cohorts did not differ in patient characteristics, except for the overrepresentation of RV infection in the analytic cohort (Table S2). Overall, the median age was 3.0 (IQR, 1.3–5.9) months and 62% were male. Infants with RSV-only infection were younger than those with RV-A or RV-C ($P < .05$; Table 1). In contrast, there were no significant between-group differences in pre-hospitalization treatments (eg, systemic corticosteroids, antibiotics). While infants with RSV-only had the highest proportion of PPV use during the bronchiolitis hospitalization, those with RV-C group had the highest risk of developing recurrent wheezing by age of 3 years (both $P < .05$).

Serum metabolomic profiling identified a total of 639 metabolites from 87 subpathways within 7 superpathways. Based on the sPLS-DA, the overall metabolomic profiles distinctly clustered both in the RSV-RV-A ($P_{\text{permutation}} = 0.004$; Figure 1A) and in the RSV-RV-C comparisons ($P_{\text{permutation}} < 0.001$; Figure 1C). In the RSV-RV-A comparison, sPLS-DA selected 30 discriminatory metabolites—primarily involved in lipid metabolism—such

TABLE 1 Patient characteristics of infants hospitalized for bronchiolitis, according to respiratory virus group

	RSV (n = 63; 56%)	RV-A (n = 21; 19%)	RV-C (n = 29; 26%)	P-value
Baseline characteristics				
Age (month), median (IQR)	2.0 (1.0-4.5)	3.5 (2.3-6.6)	5.4 (3.3-8.0)	<.001
Male sex	39 (62)	13 (62)	19 (66)	.94
Race/ethnicity				
Non-Hispanic white	27 (43)	9 (43)	9 (31)	.89
Non-Hispanic black	15 (24)	4 (19)	8 (28)	
Hispanic	19 (30)	8 (38)	11 (38)	
Other or unknown	2 (3)	0 (0)	1 (3)	
Maternal smoking during pregnancy	6 (10)	5 (24)	3 (10)	.26
C-section delivery	24 (38)	9 (43)	9 (31)	.68
Prematurity (32-37 wks)	13 (21)	5 (24)	6 (21)	.95
History of eczema	7 (11)	4 (19)	6 (21)	.42
Mostly breastfed for the first 3 mo of age	30 (48)	10 (48)	15 (52)	.95
Cigarette smoke exposure at home	5 (8)	3 (14)	3 (10)	.63
Corticosteroid use before the index hospitalization	5 (8)	2 (10)	4 (14)	.69
Antibiotic use before the index hospitalization	17 (27)	1 (5)	8 (28)	.09
Clinical presentation				
Weight at presentation (kg), median (IQR)	5.2 (4.2-6.9)	6.1 (4.9-7.2)	7.5 (6.1-8.5)	.003
Respiratory rate at presentation (per minute), median (IQR)	48 (40-60)	46 (42-52)	50 (40-60)	.82
Oxygen saturation at presentation				
<90%	6 (10)	3 (14)	1 (3)	.58
90%-93%	12 (19)	4 (19)	4 (14)	
≥94%	43 (68)	13 (62)	24 (83)	
Missing	2 (3)	1 (5)	0 (0)	
Detected pathogen				
RSV	63 (100)	6 (29)	8 (28)	-
RV	0 (0)	21 (100)	29 (100)	-
Other pathogen ^a	0 (0)	7 (33)	10 (34)	<.001
No pathogen	0 (0)	0 (0)	0 (0)	-
Clinical outcomes				
Positive pressure ventilation during hospitalization ^b	26 (41)	5 (24)	8 (28)	.004
Hospital length of stay (day), median (IQR)	4 (2-8)	2 (1-3)	2 (1-3)	<.001
Recurrent wheezing by 3 y ^c	25 (40)	12 (57)	23 (79)	.002
Asthma by 5 y ^d	22 (35)	7 (33)	16 (55)	.16

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

Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; RV, rhinovirus.

^aAdenovirus, bocavirus, *Bordetella pertussis*, enterovirus, human coronavirus NL63, OC43, 229E, or HKU1, human metapneumovirus, influenza A or B virus, *Mycoplasma pneumoniae*, parainfluenza viruses 1-3.

^bDefined as use of invasive and/or non-invasive mechanical ventilation (eg, continuous positive airway pressure ventilation).

^cDefined by parental report of at least two corticosteroid-requiring breathing problems in 6 mo or at least four wheezing episodes in 1 y that last at least 1 d and affect sleep.

^dDefined by physician diagnosis of asthma by age of 5 y, plus either asthma medication use (eg, albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year.

as sphingolipids and carnitines (Table 2). In the RSV-RV-C comparison, while the most discriminatory metabolite was homo-arginine, the metabolites chiefly consisted of other families

of lipids—such as glycerophospholipids (eg, glycerophosphocholines [GPCs], glycerophosphoethanolamines [GPEs]) and 9,10/12,13-diHOMEs; Table 3). These differences between

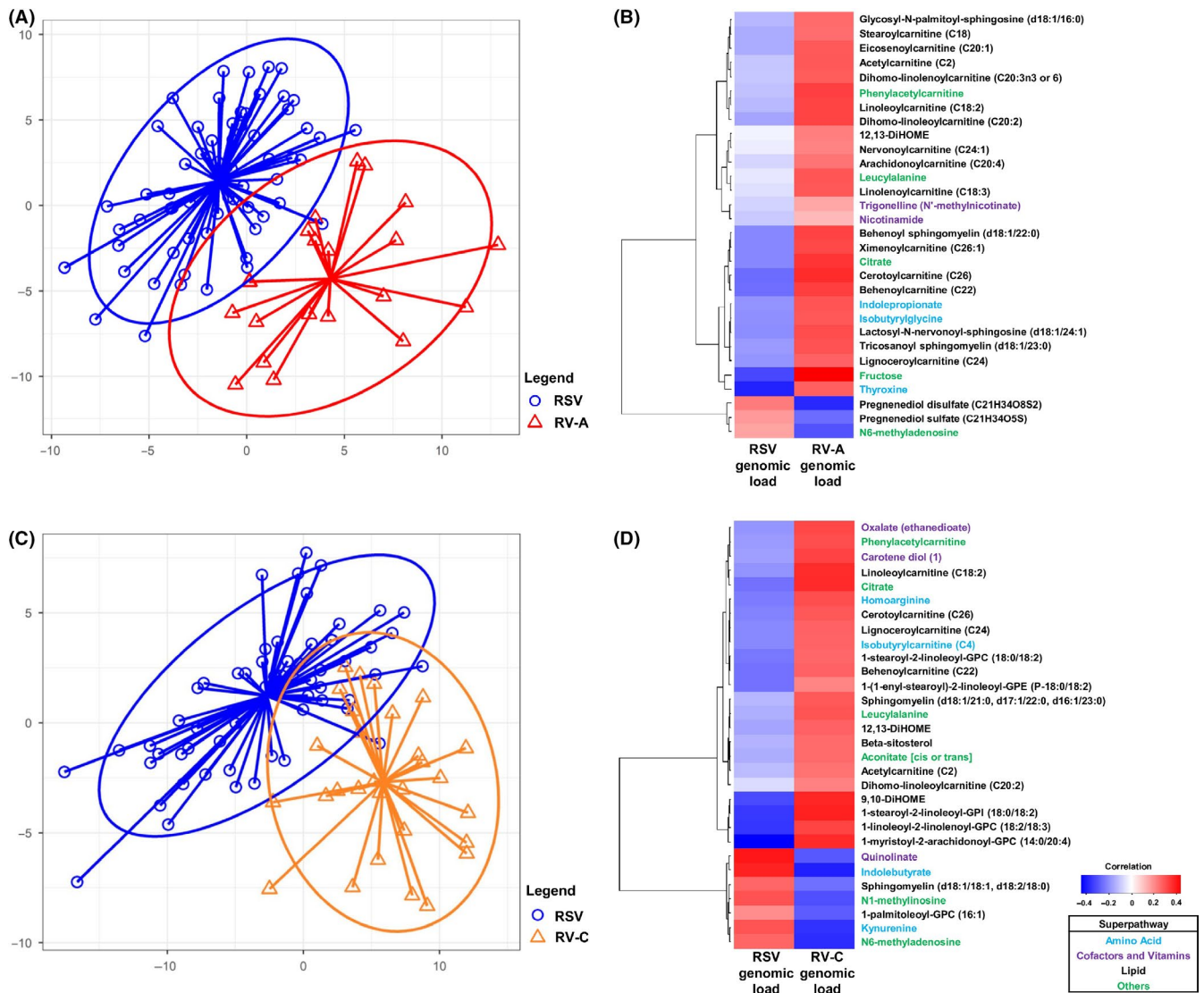


FIGURE 1 Associations of respiratory syncytial virus and rhinovirus species with serum metabolites in infants with severe bronchiolitis. A, Sparse partial least squares discriminant analysis (sPLS-DA) score plot of serum metabolome according to RSV-only (blue) vs RV-A (red) infection ($P_{\text{permutation}} = 0.004$ with 2000 random permutations). Each dot represents the serum metabolomic profile of a single infant, by plotting the component scores in the smaller subspace spanned by latent variables of sPLS-DA. The ellipses are 90% confidence intervals. The arrows start from the centroid of each virus group and end for each patient belonging to each group. B, Heat map of Spearman's correlations between the genomic load (measured as the inverse cycle threshold) of RSV and RV-A and the 30 most discriminatory serum metabolites in the corresponding sPLS-DA model (Table 2). Clustering is based on Euclidean distance and Ward's minimum variance linkage algorithm. The color bar indicates the correlation coefficients—red color indicates a positive correlation, while blue color indicates a negative correlation. Superpathway of each metabolite is color-coded for each function. C, sPLS-DA score plot in the comparison between RSV (blue) and RV-C (orange) infections ($P_{\text{permutation}} < 0.001$ with 2000 random permutations). D, Heat map of Spearman's correlations between the genomic load of RSV and RV-C and the 30 most discriminatory serum metabolites in the corresponding sPLS-DA model (Table 3). RSV, respiratory syncytial virus; RV, rhinovirus

qualitative virus statuses (presence vs absence of corresponding virus) were consistent with the correlations between quantitative virus status (viral genomic load [measured as the inverse cycle threshold value]) and metabolite intensities (Figure 1B and D). In the multivariable models, the between-virus differences remained significant for 23 metabolites when comparing with RV-A (eg, sphingolipids, carnitines) and 20 metabolites when comparing with RV-C (eg, homo-arginine, glycerophospholipids) ($\text{FDR} < 0.05$; Tables 2 and 3). These results were consistent in

the sensitivity analysis using *sole* RV data (Tables S3 and S4). In contrast, there was no significant within-individual correlation between the intensity of serum discriminatory metabolites and those in nasopharyngeal metabolites in either between-virus comparison (all $P > .05$; Figure S1).

The metabolite-set enrichment analysis also identified differentially enriched pathways—for example, mitochondrial β -oxidation of long-chain saturated fatty acid and oxidation of branched-chain fatty acids pathways in the RSV-RV-A comparison, as well as arachidonic

TABLE 2 Multivariable associations of respiratory syncytial virus and rhinovirus A with serum metabolites

Metabolites ^a	Superpathway	Subpathway	Coefficient ^b	Standard error ^b	P-value ^b	FDR ^b
Tricosanoyl sphingomyelin (d18:1/23:0)	Lipid	Sphingolipid metabolism	0.34	0.06	<.001	<0.001
Fructose	Carbohydrate	Fructose, mannose, and galactose metabolism	0.58	0.10	<.001	<0.001
Behenoyl sphingomyelin (d18:1/22:0)	Lipid	Sphingolipid metabolism	0.19	0.04	<.001	<0.001
Lignoceroyl-carnitine (C24)	Lipid	Fatty acid metabolism	0.42	0.08	<.001	<0.001
Leucylalanine	Peptide	Dipeptide	1.34	0.29	<.001	<0.001
Cerotoylcarnitine (C26)	Lipid	Fatty acid metabolism	0.54	0.12	<.001	<0.001
Isobutyrylglycine	Amino acid	Leucine, isoleucine, and valine metabolism	0.76	0.18	<.001	<0.001
Pregnenediol disulfate (C21H34O8S2)	Lipid	Pregnenolone steroids	−0.91	0.23	<.001	<0.001
Dihomo-linoleoylcarnitine (C20:2)	Lipid	Fatty acid metabolism	0.44	0.12	<.001	<0.001
Nervonoylcarnitine (C24:1)	Lipid	Fatty acid metabolism	0.33	0.09	<.001	<0.001
Behenoylcarnitine (C22)	Lipid	Fatty acid metabolism	0.47	0.13	<.001	<0.001
Ximenoylcarnitine (C26:1)	Lipid	Fatty acid metabolism	0.50	0.14	<.001	0.001
Glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	Lipid	Hexosylceramides	0.27	0.08	<.001	0.002
Lactosyl-N-nervonoyl-sphingosine (d18:1/24:1)	Lipid	Lactosylceramides	0.20	0.06	.001	0.003
Pregnenediol sulfate (C21H34O5S)	Lipid	Pregnenolone steroids	−0.74	0.24	.002	0.004
Arachidonoylcarnitine (C20:4)	Lipid	Fatty acid metabolism	0.55	0.18	.002	0.004
Dihomo-linolenoylcarnitine (C20:3n3 or 6)	Lipid	Fatty acid metabolism	0.50	0.17	.003	0.005
Citrate	Energy	TCA cycle	0.30	0.10	.003	0.005
Stearoylcarnitine (C18)	Lipid	Fatty acid metabolism	0.33	0.11	.003	0.005
N6-methyladenosine	Nucleotide	Purine metabolism, adenine containing	−0.38	0.13	.004	0.005
Thyroxine	Amino acid	Tyrosine metabolism	0.32	0.11	.005	0.005
Linoleoylcarnitine (C18:2)	Lipid	Fatty acid metabolism	0.40	0.17	.02	0.03
Phenylacetylcarnitine	Peptide	Acetylated peptides	1.04	0.49	.04	0.04
Eicosenoylcarnitine (C20:1)	Lipid	Fatty acid metabolism	0.31	0.18	.07	0.09
Indolepropionate	Amino acid	Tryptophan metabolism	1.16	0.66	.08	0.09
Acetylcarnitine (C2)	Lipid	Fatty acid metabolism	0.21	0.12	.08	0.09
Trigonelline (N'-methylnicotinate)	Cofactors and vitamins	Nicotinate and nicotinamide metabolism	0.56	0.33	.09	0.10
Linolenoylcarnitine (C18:3)	Lipid	Fatty acid metabolism	0.41	0.26	.12	0.13
12,13-DiHOME	Lipid	Fatty acid, dihydroxy	0.43	0.30	.15	0.15
Nicotinamide	Cofactors and vitamins	Nicotinate and nicotinamide metabolism	0.31	0.27	.25	0.25

Abbreviations: FDR, false discovery rate; TCA, tricarboxylic acid.

^aThe 30 metabolites that have a high variable importance in projection coefficients of component one in sparse partial least squares discriminate models.

^bThe multivariable linear models adjusted for age, sex, prematurity, feeding status, and potential batch effect, as well as clustering within hospitals (with the use of generalized estimating equations). The coefficient represents the between-virus difference in the metabolite intensity (the respiratory syncytial virus group as the reference).

acid, arginine, and glycerophospholipid metabolism pathways in the RSV-RV-C comparison (Figure S2). Additionally, the causal structure learning demonstrated that most metabolites were observed downstream of viruses—suggesting that viruses influenced the observed perturbations of most metabolites (Figure S3).

Several discriminatory metabolites were significantly associated with acute and chronic clinical outcomes (Tables 4 and 5). In the RSV-RV-A comparison, for example, lignoceroyl-carnitine intensity was associated with a significantly lower risk of PPV use (OR: 0.20; 95% CI: 0.08–0.48; $P < .001$). In contrast, tricosanoyl sphingomyelin was associated with a higher risk of developing recurrent wheeze (OR: 1.50; 95% CI: 1.05–2.15; $P = .03$) and linoleoyl-carnitine with a higher risk of asthma (OR: 1.70; 95% CI: 1.03–2.81; $P = .04$). In the RSV-RV-C comparison, for example, 1-stearoyl-2-linoleoyl-GPC and 1-(1-enyl-stearoyl)-2-linoleoyl-GPE were associated with a lower risk of PPV use (both $P < .05$). In contrast, homo-arginine was associated with a higher risk of recurrent wheezing (OR: 1.94; 95% CI: 1.06–3.53; $P = .03$).

4 | DISCUSSION

In this multicenter prospective cohort of infants with severe bronchiolitis, we tested for respiratory viruses (and their species) and comprehensively profiled the serum metabolome in a subset of 113 children. We found that overall serum metabolomic profiles differed by infecting virus. We also identified that discriminatory metabolites were primarily involved in lipid metabolism (eg, sphingolipids and carnitines) in the RSV-RV-A comparison, and arginine and other lipid mediators (eg, glycerophospholipids) in the RSV-RV-C comparison. The causal structure learning approach indicated that virus infections perturbed these metabolites (*not vice versa*). Additionally, these discriminatory metabolites were also significantly associated with the risk of acute and chronic morbidities of bronchiolitis. In contrast, there was no significant relation between the intensity of serum (systemic) discriminatory metabolites and those in nasopharyngeal airway (local) metabolites. To the best of our knowledge, this is the first investigation that has examined the interrelations between respiratory viruses, host systemic metabolism, and clinical outcomes in children with bronchiolitis.

For decades, bronchiolitis has been considered a single disease with similar mechanisms and clinical characteristics.² However, in agreement with the current study, growing evidence in infants with bronchiolitis supports between-virus differences in clinical presentation,²² acute and chronic morbidity,^{5–7} upper airway microbiome,^{9,13,14} microRNA,⁸ transcriptome,⁹ cytokine,¹⁰ and metabolomic^{11,12} profiles. In prior analyses *without* RV species-level resolution, nasopharyngeal¹¹ and circulating¹² metabolomic profiles differed between RSV and RV infection. Additionally, our group previously reported between-virus differences in the nasopharyngeal metabolome.²³ For example, in the RSV vs RV-A comparison, the discriminatory nasopharyngeal metabolites consisted mainly of nucleic and amino acids (such as guanine and cysteine-glutathione

disulfide). Interestingly, these discriminatory metabolites found in the upper airway were uniquely different from the serum discriminatory metabolites in the current study, suggesting that these infection-induced host local and systemic responses may involve different downstream molecules in infants with bronchiolitis. The present study with RV species-level characterization on the serum (circulating) metabolome corroborates these prior reports and extends them by comprehensively examining the relationship of respiratory virus (and species) with host *systemic* response in infants with severe bronchiolitis and demonstrating their relationships with both acute and chronic morbidities.

The mechanisms underlying these interrelations remain to be elucidated. In the RSV-RV-A comparison, sphingolipids and carnitines were discriminatory metabolites that are associated with differential risks of acute and chronic morbidities. Sphingolipids are not only major structural component of cellular membranes but also lipid mediators that play important roles in immune response and inflammation.²⁴ Consistent with our observations, research reported that sphingolipid metabolism is associated with bronchiolitis severity¹⁶ and childhood asthma (eg, *ORMDL3* within the 17q21 locus regulating *de novo* sphingolipid synthesis).²⁵ A recent study using human epithelial cell models showed that a *ORMDL3* knockdown inhibits RV-A16 replication, endoplasmic reticulum stress, and IFN- β expression.²⁶ Furthermore, previous research using newborn screening data also reported associations of acylcarnitines—metabolites that play critical role in fatty acid metabolism—with risks of wheezing by age of 3 years.²⁷ Another experimental model also reported that RV-A infection perturbs both sphingolipid and carnitine levels, thereby leading to infection-induced metabolic reprogramming.²⁸

In the RSV-RV-C comparison, our data demonstrated that glycerophospholipids from the plasmalogen pathway metabolites (eg, GPE) were not only identified as discriminatory metabolites but also associated with lower acute disease severity. Consistently, plasmalogens are known to function as anti-inflammatory mediators and have protective effects against oxidation of surfactant lipids.²⁹ Additionally, studies reported decreased levels of plasmalogens in premature infants with bronchopulmonary dysplasia.³⁰ We also found the most discriminatory metabolite—homo-arginine—was associated with a higher risk of chronic outcome. Arginine metabolism via the arginase and nitric oxide synthase pathways is important in the maintenance of airway tone and responsiveness in asthma pathobiology.³¹ Altered arginine metabolism leads to nitric oxide deficiency and airway dysfunction in patients with asthma.³¹

The current study has potential limitations. First, the study did not have “healthy controls.” However, the study objective was *not* to compare metabolomic profiles between healthy infants and those with bronchiolitis, *but* rather to examine the relations of respiratory viruses with serum metabolome within infants with bronchiolitis. Second, the metabolome was profiled at a single time point. Yet, the findings using the data in the early course of bronchiolitis are biologically and clinically significant. Third, pre-hospitalization treatments (eg, systemic corticosteroids, antibiotics)

TABLE 3 Multivariable associations of respiratory syncytial virus and rhinovirus C with serum metabolites

Metabolites ^a	Superpathway	Subpathway	Coefficient ^b	Standard error ^b	P-value ^b	FDR ^b
Homo-arginine	Amino acid	Urea cycle; arginine and proline metabolism	0.71	0.15	<.001	<0.001
Linoleoylcarnitine (C18:2)	Lipid	Fatty acid metabolism	0.36	0.08	<.001	<0.001
9,10-DiHOME	Lipid	Fatty acid, dihydroxy	0.64	0.14	<.001	<0.001
1-stearoyl-2-linoleoyl-GPC (18:0/18:2)	Lipid	Phosphatidylcholine	0.24	0.06	<.001	<0.001
Oxalate (ethanedioate)	Cofactors and vitamins	Ascorbate and aldarate metabolism	0.41	0.11	<.001	<0.001
1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)	Lipid	Phosphatidylcholine	1.14	0.31	<.001	0.001
1-myristoyl-2-arachidonoyl-GPC (14:0/20:4)	Lipid	Phosphatidylcholine	0.59	0.16	<.001	0.002
Phenylacetylcarnitine	Peptide	Acetylated peptides	1.02	0.29	<.001	0.002
1-stearoyl-2-linoleoyl-GPI (18:0/18:2)	Lipid	Phosphatidylinositol	0.52	0.15	<.001	0.003
1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)	Lipid	Plasmalogen	0.36	0.11	.001	0.003
Isobutyrylcarnitine (C4)	Amino acid	Leucine, isoleucine, and valine metabolism	0.57	0.18	.002	0.006
Sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)	Lipid	Sphingolipid metabolism	0.40	0.15	.007	0.02
Beta-sitosterol	Lipid	Sterol	0.67	0.26	.01	0.02
Leucylalanine	Peptide	Dipeptide	1.09	0.43	.01	0.02
12,13-DiHOME	Lipid	Fatty acid, dihydroxy	0.47	0.19	.01	0.03
Aconitate	Energy	TCA cycle	0.32	0.13	.01	0.03
Dihomo-linoleoylcarnitine (C20:2)	Lipid	Fatty acid metabolism	0.29	0.13	.02	0.04
Indolebutyrate	Amino acid	Tryptophan metabolism	-0.63	0.28	.03	0.04
Quinolate	Cofactors and vitamins	Nicotinate and nicotinamide metabolism	-0.20	0.09	.03	0.04
1-palmitoleoyl-GPC (16:1)	Lipid	Lysophospholipid	-0.28	0.13	.03	0.047
Citrate	Energy	TCA cycle	0.30	0.14	.04	0.06
Acetylcarnitine (C2)	Lipid	Fatty acid metabolism	0.33	0.17	.04	0.06
N6-methyladenosine	Nucleotide	Purine metabolism, adenine containing	-0.22	0.11	.04	0.06
Carotene diol (1)	Cofactors and vitamins	Vitamin A metabolism	0.81	0.41	.05	0.06
Behenoylcarnitine (C22)	Lipid	Fatty acid metabolism	0.36	0.19	.05	0.07
Sphingomyelin (d18:1/18:1, d18:2/18:0)	Lipid	Sphingolipid metabolism	-0.21	0.11	.06	0.07
Kynurenine	Amino acid	Tryptophan metabolism	-0.12	0.07	.07	0.07
Lignoceroylcarnitine (C24)	Lipid	Fatty acid metabolism	0.23	0.13	.07	0.08
Cerotoylcarnitine (C26)	Lipid	Fatty acid metabolism	0.21	0.17	.21	0.22
N1-methylinosine	Nucleotide	Purine metabolism, xanthine/inosine containing	0.01	0.06	.89	0.89

Abbreviations: FDR, false discovery rate; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; GPI, glycosylphosphatidylinositol; TCA, tricarboxylic acid.

^aThe 30 metabolites that have a high variable importance in projection coefficients of component one in sparse partial least squares discriminate models.

^bThe multivariable linear models adjusted for age, sex, prematurity, feeding status, and potential batch effect, as well as clustering within hospitals (with the use of generalized estimating equations). The coefficient represents the between-virus difference in the metabolite intensity (the respiratory syncytial virus group as the reference).

TABLE 4 Multivariable associations of serum discriminatory metabolites (respiratory syncytial virus vs rhinovirus A) with clinical outcomes

Metabolites ^a	Superpathway	Positive pressure ventilation ^b			Recurrent wheezing ^c			Asthma ^d		
		OR ^e	95% CI	P-value	OR ^e	95% CI	P-value	OR ^e	95% CI	P-value
Tricosanoyl sphingomyelin (d18:1/23:0)	Lipid	0.99	0.56-1.75	.97	1.50	1.05-2.15	.03	1.15	0.77-1.73	.50
Fructose	Carbohydrate	1.14	0.78-1.66	.49	0.58	0.41-0.84	.004	0.96	0.68-1.37	.83
Behenoyl sphingomyelin (d18:1/22:0)	Lipid	0.77	0.46-1.29	.32	1.54	1.06-2.23	.02	1.07	0.70-1.64	.76
Lignoceroylcarnitine (C24)	Lipid	0.20	0.08-0.48	<.001	0.99	0.68-1.44	.95	0.73	0.45-1.17	.19
Leucylalanine	Peptide	2.08	0.90-4.85	.09	0.77	0.55-1.08	.14	1.16	0.65-2.09	.61
Cerotoylcarnitine (C26)	Lipid	0.41	0.19-0.93	.03	1.01	0.53-1.94	.97	1.15	0.55-2.40	.71
Isobutyrylglycine	Amino acid	1.01	0.45-2.28	.98	1.77	1.15-2.73	.009	1.50	0.97-2.32	.07
Pregnenediol disulfate (C21H34O8S2)	Lipid	0.95	0.56-1.59	.84	0.67	0.47-0.96	.03	1.00	0.65-1.54	.99
Dihomo-linoleoylcarnitine (C20:2)	Lipid	0.42	0.15-1.20	.10	1.11	0.66-1.86	.69	1.22	0.63-2.37	.55
Nervonoylcarnitine (C24:1)	Lipid	0.52	0.25-1.09	.08	1.26	0.70-2.25	.44	0.88	0.47-1.65	.70
Behenoylcarnitine (C22)	Lipid	0.14	0.02-0.81	.03	1.32	0.52-3.37	.56	0.68	0.39-1.18	.17
Ximenoylcarnitine (C26:1)	Lipid	0.51	0.25-1.05	.07	1.17	0.73-1.89	.51	1.04	0.45-2.40	.93
Glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	Lipid	1.25	0.45-3.48	.67	1.15	0.71-1.86	.58	0.71	0.39-1.32	.28
Lactosyl-N-nervonoyl-sphingosine (d18:1/24:1)	Lipid	1.51	0.84-2.72	.17	1.41	0.98-2.01	.06	1.15	0.74-1.77	.53
Pregnenediol sulfate (C21H34O5S)	Lipid	0.72	0.46-1.11	.14	0.66	0.50-0.88	.004	0.88	0.53-1.46	.62
Arachidonoylcarnitine (C20:4)	Lipid	0.42	0.16-1.09	.08	1.00	0.64-1.54	.99	1.09	0.56-2.10	.81
Dihomo-linolenoylcarnitine (C20:3n3 or 6)	Lipid	0.38	0.20-0.70	.002	1.11	0.74-1.68	.61	1.14	0.61-2.15	.68
Citrate	Energy	0.46	0.17-1.25	.13	1.05	0.58-1.9	.88	1.08	0.60-1.95	.80
Stearoylcarnitine (C18)	Lipid	1.02	0.61-1.71	.93	1.47	0.90-2.42	.12	1.00	0.64-1.56	.99
N6-methyladenosine	Nucleotide	0.85	0.44-1.65	.64	0.81	0.54-1.21	.30	0.75	0.56-1.01	.06
Thyroxine	Amino acid	0.45	0.26-0.80	.01	0.87	0.47-1.62	.66	0.84	0.59-1.20	.34
Linoleoylcarnitine (C18:2)	Lipid	0.39	0.12-1.24	.11	1.72	1.28-2.32	<.001	1.70	1.03-2.81	.04
Phenylacetylcarnitine	Peptide	0.32	0.07-1.55	.16	0.97	0.59-1.6	.91	1.38	0.89-2.13	.15

Note: Bold results are statistically significant.

Abbreviations: CI, confidence interval; OR, odds ratio.

^aThe discriminatory metabolites that have a false discovery rate of < 0.05 in the virus-metabolite model (Table 2) are selected.

^bDefined as use of invasive and/or non-invasive mechanical ventilation (eg, continuous positive airway pressure ventilation).

^cDefined by parental report of at least two corticosteroid-requiring breathing problems in 6 mo or at least four wheezing episodes in 1 y that last at least 1 d and affect sleep.

^dDefined by physician diagnosis of asthma by age of 5 y, plus either asthma medication use (eg, albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year.

^eThe multivariable logistic regression models adjusted for age and potential batch effect, as well as clustering within hospitals (with the use of generalized estimating equations). Odds ratio is calculated per each standard deviation of corresponding metabolite intensity.

might have altered some of the measured metabolites. However, the proportion of these treatments was not different between the virus groups. Therefore, it is unlikely that they served as confounders. Fourth, as with any observational study, causal inference may be confounded by unmeasured factors, such as nutrition

and between-hospital differences in intensive care resources. However, we statistically accounted for patient clustering at the hospital level. Fifth, although we applied approaches to minimize overfitting of data (eg, via Lasso penalization and cross-validations), an external validation is warranted. Lastly, while the cohort

TABLE 5 Multivariable associations of serum discriminatory metabolites (respiratory syncytial virus vs rhinovirus C) with clinical outcomes

Metabolites ^a	Superpathway	Positive pressure ventilation ^b			Recurrent wheezing ^c			Asthma ^d		
		OR ^e	95% CI	P-value	OR ^e	95% CI	P-value	OR ^e	95% CI	P-value
Homo-arginine	Amino acid	0.40	0.18-0.89	.02	1.94	1.06-3.53	.03	0.81	0.60-1.09	.17
Linoleoylcarnitine (C18:2)	Lipid	0.62	0.31-1.22	.16	1.51	1.12-2.03	.007	1.64	1.17-2.29	.004
9,10-DiHOME	Lipid	0.91	0.48-1.73	.77	1.50	0.94-2.38	.09	1.03	0.63-1.67	.91
1-stearoyl-2-linoleoyl-GPC (18:0/18:2)	Lipid	0.47	0.28-0.78	.004	1.00	0.67-1.48	.99	0.85	0.52-1.39	.52
Oxalate (ethanedioate)	Cofactors and vitamins	0.58	0.36-0.94	.03	0.94	0.65-1.35	.74	0.89	0.55-1.44	.64
1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)	Lipid	0.65	0.37-1.14	.13	0.89	0.58-1.37	.59	1.06	0.66-1.70	.82
1-myristoyl-2-arachidonoyl-GPC (14:0/20:4)	Lipid	0.35	0.18-0.68	.002	1.01	0.71-1.42	.97	1.16	0.59-2.31	.66
Phenylacetylcarnitine	Peptide	0.60	0.23-1.57	.30	0.86	0.46-1.61	.64	0.99	0.48-2.03	.98
1-stearoyl-2-linoleoyl-GPI (18:0/18:2)	Lipid	0.39	0.23-0.67	.001	0.88	0.55-1.41	.60	1.01	0.55-1.85	.98
1-(1-enyl-stearyl)-2-linoleoyl-GPE (P-18:0/18:2)	Lipid	0.34	0.17-0.67	.002	1.04	0.56-1.95	.89	0.71	0.46-1.09	.12
Isobutyrylcarnitine (C4)	Amino acid	0.32	0.09-1.16	.08	1.04	0.55-1.96	.90	0.98	0.58-1.64	.93
Sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)	Lipid	0.71	0.45-1.12	.14	1.23	0.87-1.72	.24	1.09	0.71-1.67	.70
Beta-sitosterol	Lipid	0.74	0.45-1.22	.24	1.56	1.08-2.25	.02	0.98	0.71-1.34	.88
Leucylalanine	Peptide	1.50	0.69-3.25	.31	1.32	0.86-2.04	.21	0.48	0.32-0.73	<.001
12,13-DiHOME	Lipid	0.47	0.16-1.36	.16	1.15	0.67-1.96	.61	0.94	0.67-1.34	.75
Aconitate	Energy	0.56	0.24-1.29	.17	1.28	0.91-1.79	.16	0.89	0.49-1.64	.72
Dihomo-linoleoylcarnitine (C20:2)	Lipid	0.72	0.44-1.20	.21	0.98	0.71-1.35	.90	1.36	0.96-1.94	.08
Indolebutyrate	Amino acid	3.81	1.58-9.20	.003	0.90	0.47-1.73	.76	0.92	0.56-1.49	.72
Quinolinate	Cofactors and vitamins	1.05	0.75-1.46	.79	1.08	0.61-1.93	.79	1.26	0.83-1.90	.28
1-palmitoleoyl-GPC (16:1)	Lipid	0.95	0.60-1.53	.84	0.89	0.51-1.54	.68	1.12	0.87-1.44	.38

Note: Bold results are statistically significant.

Abbreviations: CI, confidence interval; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; GPI, glycosylphosphatidylinositol; OR, odds ratio.

^aThe discriminatory metabolites that have a false discovery rate of < 0.05 in the virus-metabolite model (Table 3) are selected.

^bDefined as use of invasive and/or non-invasive mechanical ventilation (eg, continuous positive airway pressure ventilation).

^cDefined by parental report of at least two corticosteroid-requiring breathing problems in 6 mo or at least four wheezing episodes in 1 y that last at least 1 d and affect sleep.

^dDefined by physician diagnosis of asthma by age of 5 y, plus either asthma medication use (eg, albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year.

^eThe multivariable logistic regression models adjusted for age and potential batch effect, as well as clustering within hospitals (with the use of generalized estimating equations). Odds ratio is calculated per each standard deviation of corresponding metabolite intensity.

consisted of a racially/ethnically diverse multicenter sample, the inferences may not be generalizable beyond infants hospitalized for bronchiolitis. Regardless, our data are directly relevant to >100 000 infants with severe bronchiolitis in the United States each year.³

5 | CONCLUSIONS

Based on the multicenter prospective data of infants with severe bronchiolitis, we found that, compared with RSV-only infection, serum metabolomic signatures differed in infants with RV-A or RV-C infection. The discriminatory metabolites—primarily involved in lipid metabolism—were associated with acute and chronic morbidities of bronchiolitis. Our data facilitate further investigations into the complex interrelations between respiratory viruses (including their species) and host systemic metabolism as well as their integrated contributions to clinical outcomes. For clinicians, our inferences lend additional support to the growing evidence that bronchiolitis is not a homogeneous disease condition. Furthermore, our observations should also offer new avenues for bronchiolitis management (eg, modification of metabolic pathways) as well as primary prevention strategies of childhood asthma (eg, through early identification of infants at high risk of asthma) in this large population.

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CONFLICT OF INTEREST

Dr Bochkov has a patent methods of propagating rhinovirus C in previously unsusceptible cell lines with royalties paid and a patent adapted rhinovirus C with royalties paid. Dr Gern reports personal fees from Regeneron, personal fees and stock options from Meissa Vaccines Inc, and personal fees from MedImmune/AstraZeneca and Ena Therapeutics, outside the submitted work. In addition, Dr Gern has a patent methods of propagating rhinovirus C in previously unsusceptible cell lines pending and a patent adapted rhinovirus C pending. The other authors have indicated that they have no financial relationships relevant to this article to disclose.

AUTHOR CONTRIBUTION

Michimasa Fujiogi: Formal analysis (lead); Writing-original draft (lead); Writing-review & editing (lead). **Carlos A Camargo Jr:** Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Writing-review & editing (equal). **Yoshihiko Raita:** Formal analysis (supporting); Writing-review & editing (supporting). **Yury**

A Bochkov: Investigation (equal); Writing-review & editing (supporting). **James Gern:** Investigation (equal); Writing-review & editing (supporting). **Jonathan Mansbach:** Investigation (equal); Writing-review & editing (supporting). **Pedro A Piedra:** Investigation (equal); Writing-review & editing (supporting). **Kohei Hasegawa:** Conceptualization (equal); Formal analysis (supporting); Funding acquisition (equal); Project administration (lead); Supervision (equal); Writing-review & editing (lead).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Appendix S1**Respiratory viruses are associated with serum metabolome
among infants hospitalized for bronchiolitis: A multicenter study**

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Supplemental Methods**Supplemental References**

Table S1. Principal investigators at the 17 participating sites in MARC-35

Table S2. Comparisons between analytic and non-analytic cohorts in MARC-35

Table S3. Multivariable associations of respiratory syncytial virus and *sole* rhinovirus-A with serum metabolites

Table S4. Multivariable associations of respiratory syncytial virus and *sole* rhinovirus-C with serum metabolites

Figure S1. Correlation between serum and nasopharyngeal airway metabolites in infants with severe bronchiolitis

Figure S2. Metabolite set enrichment analysis in between-virus comparisons

Figure S3. Inferred causal relations between respiratory viruses and selected serum metabolites

21 **SUPPLEMENTAL METHODS**22 **Serum metabolomic profiling**23 ***Sample preparation for metabolomics testing***

24 Metabolomic profiling used 100 µl of serum sample and was performed by Metabolon
25 (Durham, NC). All samples were blinded to Metabolon and processed in a random order. Sample
26 preparation was carried out as described previously.¹ Recovery standards were added prior to the
27 first step in the extraction process for quality control purposes. Proteins were precipitated with
28 methanol under vigorous shaking for two minutes (Glen Mills Genogrinder 2000; Clifton, NJ)
29 followed by centrifugation. The resulting extract was divided into five fractions: two (i.e., early
30 and late eluting compounds) for analysis by ultra-high performance liquid
31 chromatography-tandem mass spectrometry (UPLC-MS/MS) positive ionization, one for
32 analysis by UPLC-MS/MS negative ionization, one for the UPLC-MS/MS polar platform
33 (negative ionization), and one sample was reserved for backup.

34 Three types of controls were analyzed in concert with the experimental samples:
35 samples generated from a pool from a small portion of each experimental sample of interest
36 served as technical replicate throughout the data set; extracted water samples served as process
37 blanks; and a cocktail of standards spiked into every analyzed sample allowed instrument
38 performance monitoring. The median relative standard deviation for the standards that were
39 added to each sample—a measure of instrument variability—was <5%.

40

41 ***Ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS)***

42 All methods utilized a Waters ACQUITY UPLC (Milford, MA) and a Thermo Scientific
43 Q-Exactive (Waltham, MA) high resolution/accurate MS interfaced with a heated electrospray

ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. The first aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μ m) using water and methanol, containing 0.05% perfluoropentanoic acid and 0.1% formic acid. The second aliquot was also analyzed using acidic positive ion conditions, but it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% perfluoropentanoic acid and 0.01% formic acid and was operated at an overall higher organic content. The third aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, including 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μ m) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data files were archived and extracted as described below.

Compound identification, quantification, and data duration

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using QUICS software.² Identification of known chemical entities was based on comparison to metabolomic library entries of commercially available purified standards. Peaks were quantified using area-under-the-curve. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences by the median value for each run-day, therefore, setting the medians to 1.0 for each run. Missing values were imputed with the observed minimum for that particular compound.

77 **SUPPLEMENTAL REFERENCES**

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81 small-molecule complement of biological systems. *Anal Chem.* 2009;81:6656–67.
- 82 2. Dehaven CD, Evans AM, Dai H, Lawton KA. Organization of GC/MS and LC/MS
83 metabolomics data into chemical libraries. *J Cheminform.* 2010;2:9.

84

85 **Table S1. Principal investigators at the 17 participating sites in MARC-35**

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Michelle D. Stevenson, MD, MS	Norton Children's Hospital, Louisville, KY
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87 **Table S2. Comparisons between analytic and non-analytic cohorts in MARC-35**

	Analytic cohort (n=113)	Non-analytic cohort (n=903)	P-value
Baseline characteristics			
Age (month), median (IQR)	3.0 (1.4-5.9)	3.2 (1.6-6.0)	0.44
Male sex	71 (63)	523 (58)	0.59
Race/ethnicity			0.82
Non-Hispanic white	45 (40)	385 (43)	
Non-Hispanic black	27 (24)	212 (23)	
Hispanic	38 (34)	270 (30)	
Other or unknown	3 (3)	36 (4)	
Maternal smoking during pregnancy	14 (12)	133 (15)	0.55
C-section delivery	42 (37)	306 (34)	0.66
Prematurity (32-37 weeks)	24 (21)	162 (18)	0.47
History of eczema	17 (15)	132 (15)	0.99
Mostly breastfed for the first 3 month of age	55 (49)	368 (41)	0.32
Cigarette smoke exposure at home	11 (10)	145 (16)	0.11
Corticosteroid use before the index hospitalization	23 (20)	124 (14)	0.08
Clinical presentation			
Weight at presentation (kg), median (IQR)	6.0 (4.4-7.8)	6.1 (4.8-7.7)	0.42
Respiratory rate at presentation (per minute), median (IQR)	48 (40-60)	48 (40-60)	0.98
Oxygen saturation at presentation			0.72
<90%	10 (9)	81 (9)	
90-93%	20 (18)	135 (15)	
≥94%	80 (71)	669 (74)	
Detected pathogen			
RSV	77 (68)	744 (82)	<0.001
RSV-only	63 (56)	523 (58)	0.74
RV	50 (44)	164 (18)	<0.001
Other pathogen*	17 (15)	218 (24)	0.04

88 Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; RV, rhinovirus

89 * Adenovirus, bocavirus, *Bordetella pertussis*, enterovirus, human coronavirus NL63, OC43,
90 229E, or HKU1, human metapneumovirus, influenza A or B virus, *Mycoplasma pneumoniae*,
91 parainfluenza virus 1-3.

92 **Table S3. Multivariable associations of respiratory syncytial virus and *sole* rhinovirus-A with serum metabolites**

Metabolites*	Coefficient[†]	Standard error[†]	P-value[†]	FDR[†]
Fructose	0.68	0.12	<0.001	<0.001
Cerotoylcarnitine (C26)	0.58	0.10	<0.001	<0.001
Behenoylcarnitine (C22)	0.47	0.13	<0.001	<0.001
Dihomo-linoleoylcarnitine (C20:2)	0.46	0.13	<0.001	<0.001
Tricosanoyl sphingomyelin (d18:1/23:0)	0.35	0.06	<0.001	<0.001
Isobutyrylglycine	0.79	0.18	<0.001	<0.001
Lactosyl-N-nervonoyl-sphingosine (d18:1/24:1)	0.21	0.04	<0.001	<0.001
Behenoyl sphingomyelin (d18:1/22:0)	0.20	0.04	<0.001	<0.001
Lignoceroylcarnitine (C24)	0.42	0.08	<0.001	<0.001
5-bromotryptophan	0.74	0.18	<0.001	<0.001
Ximenoylcarnitine (C26:1)	0.56	0.15	<0.001	<0.001
Leucylalanine	1.77	0.53	0.001	0.002
Thyroxine	0.38	0.12	0.001	0.002
N6-methyladenosine	-0.44	0.15	0.003	0.006
3-hydroxy-2-ethylpropionate	-0.50	0.17	0.003	0.006
Arachidonoylcarnitine (C20:4)	0.62	0.21	0.004	0.008
N-acetylglucosaminylasparagine	0.86	0.31	0.006	0.01
Phenylacetate	0.97	0.36	0.006	0.01
Glycerol 3-phosphate	0.50	0.20	0.01	0.02
Phenylalanylglycine	0.93	0.38	0.01	0.02
2'-deoxyuridine	1.25	0.51	0.01	0.02
Dihomo-linolenoylcarnitine (C20:3n3 or 6)	0.52	0.21	0.02	0.02
Phenylacetylcarnitine	1.31	0.54	0.02	0.02
Eicosenoylcarnitine (C20:1)	0.28	0.12	0.02	0.02
Linoleoylcarnitine (C18:2)	0.44	0.20	0.03	0.03
Cysteine-glutathione disulfide	0.86	0.45	0.05	0.06
Glutarate (C5-DC)	0.56	0.33	0.08	0.09
5-methylthioadenosine (MTA)	0.47	0.29	0.10	0.11

Nicotinamide	0.47	0.34	0.17	0.17
Linolenoylcarnitine (C18:3)	0.36	0.26	0.18	0.18

93 Abbreviation: FDR, false discovery rate

94 * The 30 metabolites that have a high variable importance in projection coefficients of component one in sparse partial least squares
95 discriminate models.

96 † The multivariable linear models adjusted for age, sex, prematurity, feeding status, and potential batch effect, as well as clustering
97 within hospitals (with the use of generalized estimating equations). The coefficient represents the between-virus difference in the
98 metabolite intensity (the respiratory syncytial virus group as the reference).
99

100 **Table S4. Multivariable associations of respiratory syncytial virus and *sole* rhinovirus-C with serum metabolites**

Metabolites*	Coefficient [†]	Standard error [†]	P-value [†]	FDR [†]
1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)	1.56	0.34	<0.001	<0.001
Homoarginine	0.93	0.11	<0.001	<0.001
Valine	0.37	0.08	<0.001	<0.001
Oxalate (ethanedioate)	0.53	0.11	<0.001	<0.001
9,10-DiHOME	0.84	0.13	<0.001	<0.001
Threonate	0.50	0.08	<0.001	<0.001
1,2-dilinoleoyl-GPE (18:2/18:2)	1.21	0.29	<0.001	<0.001
Phenylalanylglycine	0.98	0.20	<0.001	<0.001
Isobutyrylcarnitine (C4)	0.82	0.16	<0.001	<0.001
4-guanidinobutanoate	0.90	0.17	<0.001	<0.001
Linoleoylcarnitine (C18:2)	0.40	0.09	<0.001	<0.001
Propionylcarnitine (C3)	0.90	0.15	<0.001	<0.001
1-myristoyl-2-arachidonoyl-GPC (14:0/20:4)	0.75	0.19	<0.001	<0.001
Leucylalanine	1.82	0.48	<0.001	<0.001
Isoleucine	0.41	0.10	<0.001	<0.001
1,2-dilinoleoyl-GPC (18:2/18:2)	1.06	0.26	<0.001	<0.001
2-oxoarginine	0.83	0.22	<0.001	<0.001
Indolebutyrate	-1.03	0.28	<0.001	<0.001
1-stearoyl-2-linoleoyl-GPI (18:0/18:2)	0.65	0.18	<0.001	0.001
Caprylate (8:0)	1.41	0.42	0.001	0.001
Isovalerate (i5:0)	0.76	0.24	0.002	0.002
2'-deoxyuridine	1.17	0.38	0.002	0.003
Beta-sitosterol	0.82	0.27	0.003	0.003
Sebacate (C10-DC)	0.95	0.33	0.004	0.01
Quinolinate	-0.32	0.11	0.005	0.01
Tyrosine	0.54	0.19	0.01	0.01
12,13-DiHOME	0.58	0.21	0.01	0.01
Behenoylcarnitine (C22)	0.54	0.22	0.01	0.02
Citrate	0.32	0.17	0.05	0.06

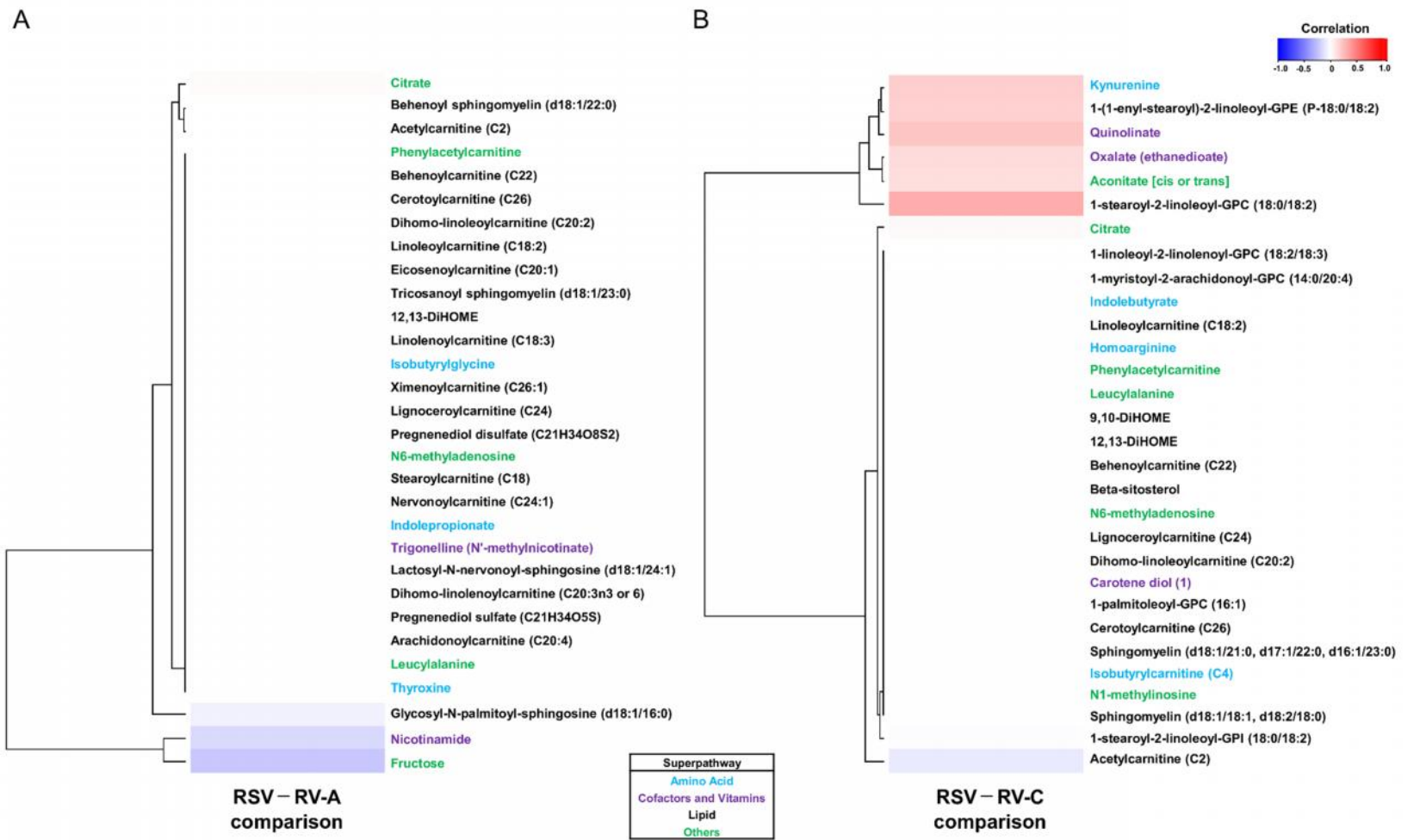
Lignoceroylcarnitine (C24)	0.29	0.18	0.11	0.11
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Abbreviations: FDR, false discovery rate; GPC, glycerophosphorylcholine; GPI, glycosylphosphatidylinositol; GPE, glycerophosphorylethanolamine

* The 30 metabolites that have a high variable importance in projection coefficients of component one in sparse partial least squares discriminate models.

† The multivariable linear models adjusted for age, sex, prematurity, feeding status, and potential batch effect, as well as clustering within hospitals (with the use of generalized estimating equations). The coefficient represents the between-virus difference in the metabolite intensity (the respiratory syncytial virus group as the reference).

Figure S1. Correlation between serum and nasopharyngeal airway metabolites in infants with severe bronchiolitis



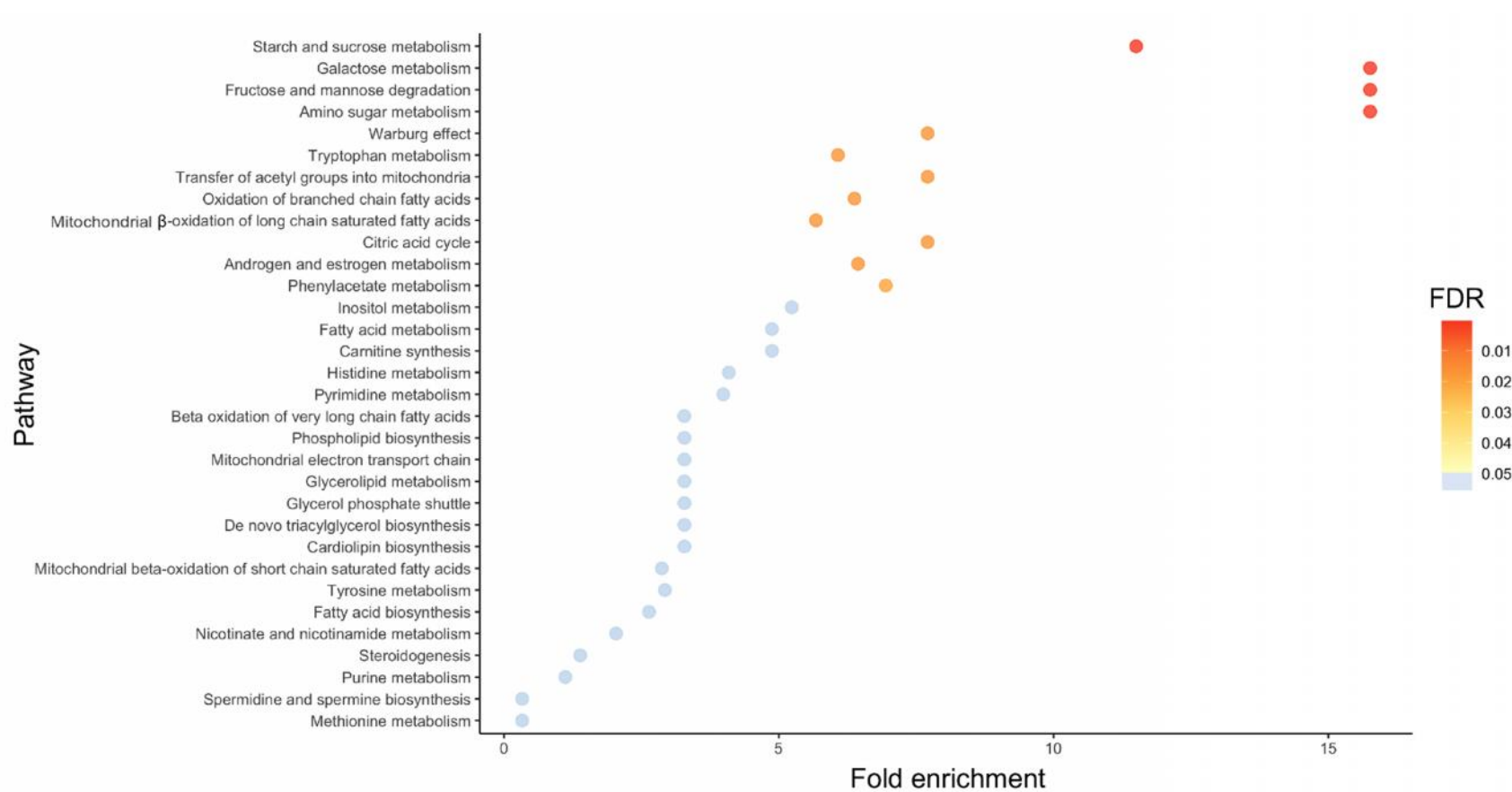
Heatmaps visualize the Spearman's correlations between the intensity of 30 most discriminatory serum metabolites and that of nasopharyngeal samples in the same infants with severe bronchiolitis. Metabolites are clustered by using hierarchical clustering with Euclidean distance and Ward's minimum variance linkage algorithm. The top 30 serum metabolites with a high variable

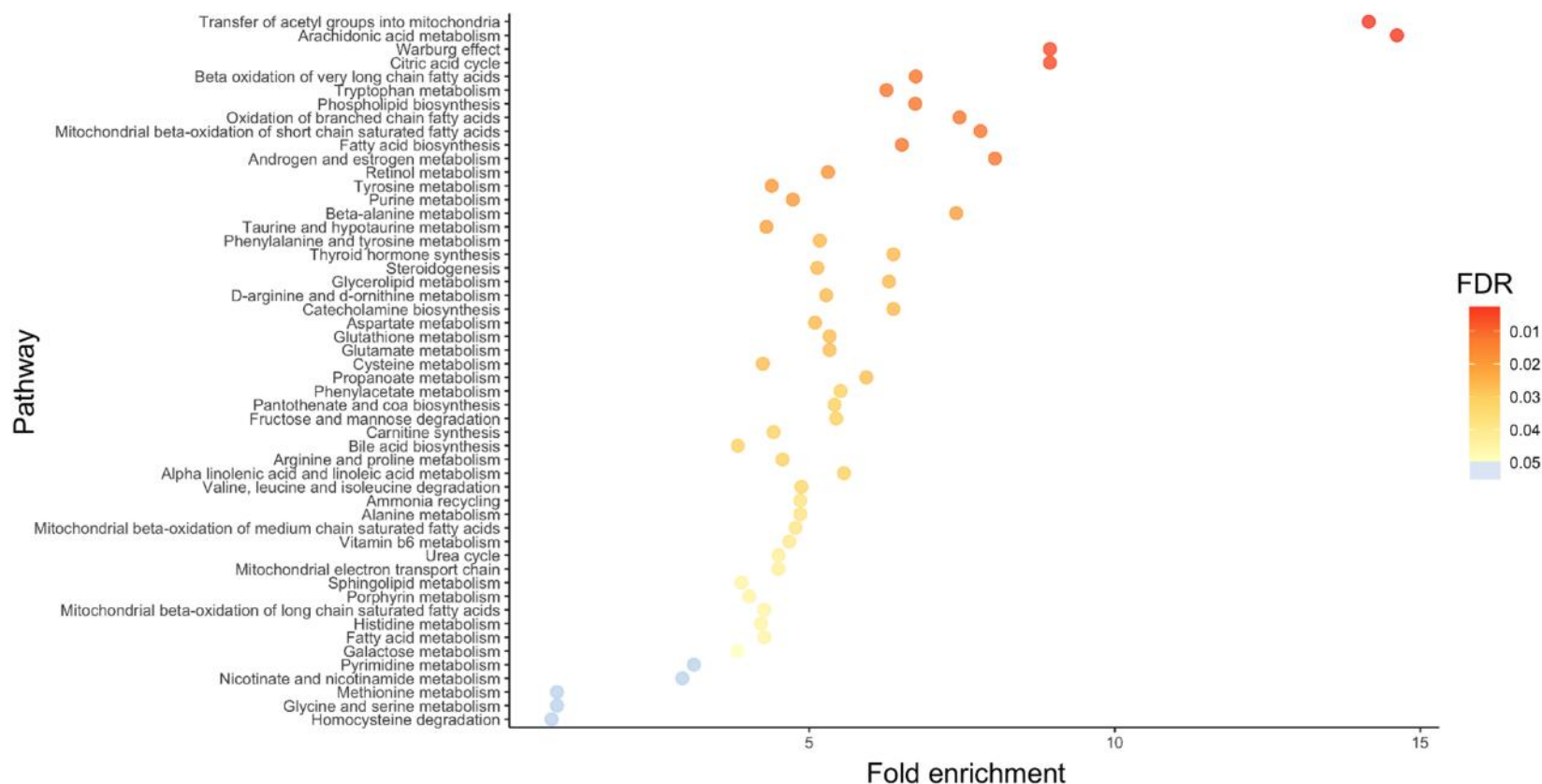
116 importance in projection by the corresponding sparse partial least squares discriminate analysis (sPLS-DA) (**Tables 2 and 3**) are
117 shown. The color bar indicates the correlation coefficients—red color indicates a positive correlation while blue color indicates a
118 negative correlation. White color indicates correlation coefficient of 0 or non-detection of the metabolite in the nasopharyngeal
119 sample. Superpathway of each metabolite is color-coded for each function.

120 **A)** Correlation in RSV—RV-A comparisons

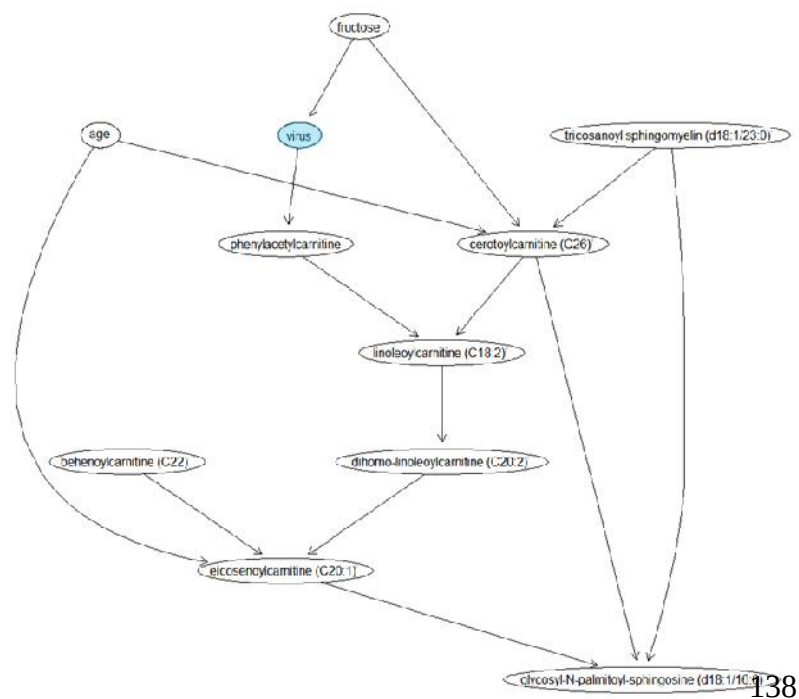
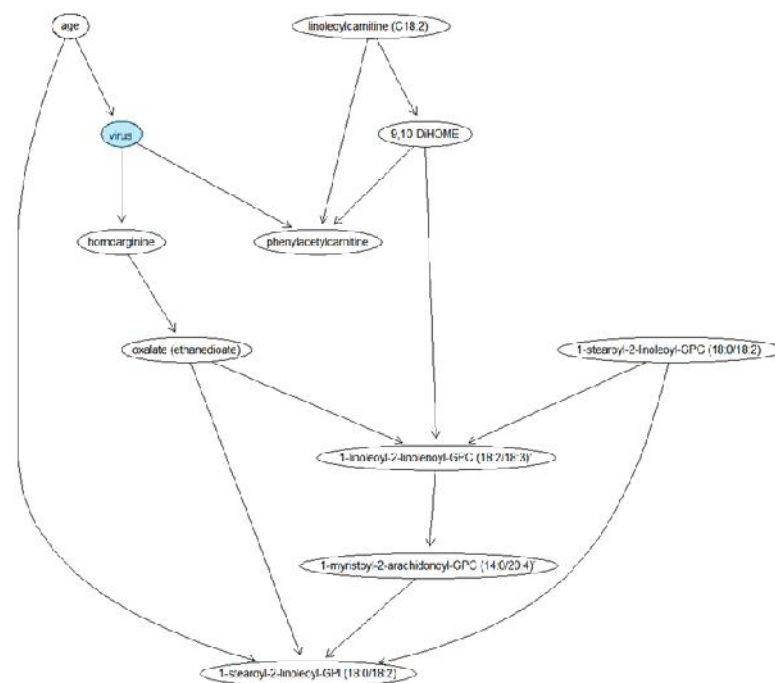
121 **B)** Correlation in RSV—RV-C comparisons

122

123 **Figure S2. Metabolite set enrichment analysis in between-virus comparisons**124 **A) Comparisons between RSV and RV-A infection**

127 **B) Comparisons between RSV and RV-C infection**

128
 129 Metabolite set enrichment analysis in between-virus comparisons used the Small Molecule Pathway Database library as the reference.
 130 Metabolites with a >1 variable importance in projection (VIP) coefficient by the corresponding sparse partial least squares
 131 discriminate analysis (sPLS-DA) are used in the analysis. X-axis represents fold enrichment that is calculated by dividing the observed
 132 Q statistic by the expected Q statistic. The color bar indicates the false discovery rate (FDR).
 133
 134 Abbreviations: FDR, false discovery rate; RSV, respiratory syncytial virus; RV, rhinovirus

Figure S3. Inferred causal relations between respiratory viruses and selected serum metabolites**A)****B)**

Inferred causal relations between respiratory viruses, patient's age, and most discriminatory serum metabolites are demonstrated in directed acyclic graphs (DAGs). The metabolites with nine lowest p-values by the multivariable linear models (**Tables 2 and 3**) are shown. These results suggested that viruses caused the observed perturbations of most metabolites.

A) Comparisons between RSV and RV-A infection

B) Comparisons between RSV and RV-C infection