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Metabolome subtyping of severe bronchiolitis in infancy and risk of childhood asthma

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Abstract

Background: Infants with bronchiolitis are at increased risk for developing asthma. Growing evidence suggests bronchiolitis is a heterogeneous condition.

Objective: To identify biologically-distinct subgroups based on the metabolome signatures (metabotypes) in infants with severe bronchiolitis and to examine their longitudinal relationships with asthma development.

Methods: In a multi-center prospective cohort study of infants (aged <12 months) hospitalized for bronchiolitis, the nasopharyngeal airway metabolome was profiled at hospitalization. Using

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a clustering approach, we identified mutually-exclusive metabolotypes. We also examined their longitudinal association with the risk of developing asthma by age five years.

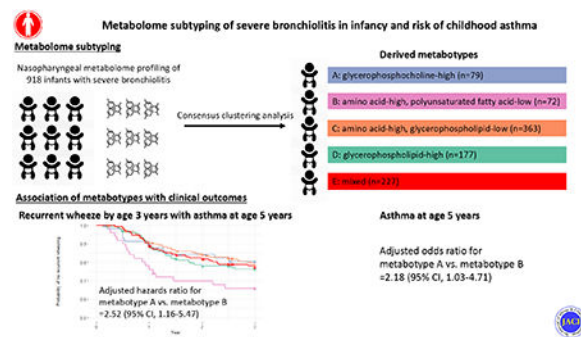
Results: Of 918 infants hospitalized for bronchiolitis (median age, 3 months), we identified 5 distinct metabolotypes—characterized by their nasopharyngeal metabolome profile: A) glycerophosphocholine-high; B) amino acid-high, polyunsaturated fatty acid (PUFA)-low; C) amino acid-high, glycerophospholipid-low; D) glycerophospholipid-high; and E) mixed. Compared with metabolotype A infants (who clinically resembled “classic” bronchiolitis), metabolotype B infants had a significantly higher risk for developing asthma (23% vs. 41%; OR_{adjusted}, 2.22; 95% CI, 1.07-4.69). The pathway analysis showed that metabolotype B had enriched amino acid (e.g., methionine, histidine, glutathione) and α -linolenic/linoleic acid metabolism pathways (all FDR $<5 \times 10^{-14}$). Finally, the transcriptome analysis revealed that metabolotype B infants had up-regulated interferon- α and interleukin-6/Janus kinase/signal transducer activator of transcription (IL-6/JAK/STAT3) pathways and down-regulated fatty acid metabolism pathways (both FDR <0.05).

Conclusion: In this multicenter prospective cohort study of infant severe bronchiolitis, the clustering analysis of metabolome data identified biologically-distinct metabolotypes, including one characterized by high inflammatory amino acids and low PUFAs that is at significantly increased risk for developing asthma.

Capsule Summary:

Among infant with severe bronchiolitis, clustering analysis of the nasopharyngeal airway metabolome identified biologically distinct metabolotypes, including one characterized by high inflammatory amino acids and low PUFAs that is at increased risk for developing asthma.

Graphical Abstract



Keywords

severe bronchiolitis; heterogeneity; subtype; asthma; metabolome; inflammatory amino acids; polyunsaturated fatty acids; transcriptome

INTRODUCTION

Bronchiolitis is the most common lower respiratory tract infection among infants, accounting for ~110,000 hospitalizations (“severe bronchiolitis”) in the U.S. annually ¹.

In addition to the acute morbidity, its chronic morbidity burden is also substantial—e.g., of these infants with severe bronchiolitis, approximately 30% develop recurrent wheeze and asthma in childhood^{2–7}.

Although bronchiolitis was traditionally considered a single disease entity with similar pathobiology⁷, there is growing evidence suggesting that bronchiolitis is a heterogeneous condition^{8–10}. For example, recent epidemiological studies have identified⁸ and validated⁹ the presence of multiple phenotypes that have differential risks for developing recurrent wheeze. However, these phenotypes were identified only based on major clinical characteristics. The pathobiological mechanisms underlying the heterogeneity remains unclear. This insufficient understanding of pathobiology of bronchiolitis during infancy—a critical period of airway development—has hindered efforts to develop targeted bronchiolitis treatment and asthma prevention strategies. Metabolomics is well-suited to address this knowledge gap by broadly profiling small molecules (metabolites), which are a function of an individual's genetic makeup and environmental factors¹¹. Regardless, no study has yet investigated the biologically-distinct subgroups of bronchiolitis based on metabolome signatures (“metabotypes”¹²).

In this context, we aimed to identify distinct nasopharyngeal airway metabotypes by applying an unsupervised clustering approach to the metabolome data from a multicenter prospective study of infants with severe bronchiolitis. We also sought to investigate the longitudinal relationships of the metabotypes with recurrent wheeze and childhood asthma. This study provides further evidence that “bronchiolitis” represents several diseases with distinct biological mechanisms and differential risks of chronic airway morbidities in childhood.

METHODS

Study Design, Setting, and Participants

We analyzed data from a multicenter prospective cohort study of infants hospitalized for bronchiolitis—the 35th Multicenter Airway Research Collaboration (MARC-35) study^{13, 14}. MARC-35 is coordinated by the Emergency Medicine Network (EMNet, www.emnet-usa.org), an international research collaboration with 247 participating hospitals. Details of the study design, setting, participants, data collection, testing, and statistical analysis may be found in the Supplementary Methods. Briefly, MARC-35 investigators at 17 sites, across 14 U.S. states, enrolled infants (aged <1 year) who were hospitalized with an attending physician diagnosis of bronchiolitis during three bronchiolitis seasons (November 1 to April 30) from 2011 to 2014 (Table E1). The diagnosis of bronchiolitis was made according to the American Academy of Pediatrics bronchiolitis guidelines, defined as acute respiratory illness with a combination of rhinitis, cough, tachypnoea, wheezing, crackles, or retraction¹⁵. We excluded infants with a preexisting heart and 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 participating hospital approved the study with a written informed consent obtained from parent or guardian.

Data Collection

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^{13, 14}. After the index hospitalization for bronchiolitis, trained interviewers began interviewing parents/legal guardians by telephone at 6-month intervals in addition to medical record review by physicians. All data were reviewed at the Emergency Medicine Network Coordinating Center at Massachusetts General Hospital (Boston, MA, USA)¹⁶. Nasopharyngeal specimens were collected within 24 hours of hospitalization using a standardized protocol¹⁴. The details of the data collection and measurement methods are described in the Supplementary Methods.

Nasopharyngeal Metabolome Profiling—The details of metabolome profiling are described in Supplementary Methods. Briefly, the metabolome profiling was conducted by Metabolon (Durham, NC, USA) using liquid chromatography–tandem mass spectrometry. All specimens were blinded to the laboratory and processed in a random order. Instrument variability was 4%, as determined by calculating the median relative standard deviation for the internal standards. We used the metabolome data of 278 nasopharyngeal metabolites from 76 sub-pathways within seven super-pathways.

Nasopharyngeal Transcriptome Profiling—The details of RNA extraction, RNAseq, quality control, and transcriptome profiling are described in Supplementary Methods. Briefly, after total RNA extraction, DNase treatment, and rRNA reduction, we performed RNAseq with Illumina NovaSeq6000 (Illumina, San Diego, CA, USA). All RNAseq samples had high sequence coverage after quality control. The transcript abundances were estimated with *Salmon*¹⁷ using the human genome (hg38) and the mapping-based mode.

Outcome Measures

The primary outcome was asthma at age five years. The secondary outcome was the development of recurrent wheeze by age three years stratified by asthma status, to account for the heterogeneity of recurrent wheeze according to prior research¹⁶. The definition of asthma was based on a commonly-used epidemiologic definition of asthma^{16, 18}—physician-diagnosis of asthma by age five years, plus either asthma medication use (e.g., albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year. The definition of recurrent wheeze was based on the U.S. asthma guidelines—defined as having at least two corticosteroid-requiring exacerbations in six months or at least four wheezing episodes in one year that last at least one day and affect sleep¹⁹.

Statistical Analysis

In the current study, our objectives were to identify biologically-distinct severe bronchiolitis subgroups based on the metabolome signatures (clustering/description) and to relate them to the outcomes (association). The analytic workflow is summarized in Figure 1.

Data Normalization and Clustering to Identify Metabotypes—We first adjusted the metabolome data for potential batch effect by using empirical Bayes models (ComBat method)²⁰. Then, we normalized the data using total sum scaling method to account for

potentially differential dilutions. To identify individuals with a similar metabolome signature (metabotype), we applied consensus clustering with partitioning around medoids algorithm. We determined the optimal number of clusters by a combination of the average silhouette score, consensus matrix heatmap (Figure E1), and biological plausibility^{11, 21, 22}.

Association of Metabotypes with Clinical Outcomes—To investigate the longitudinal association of metabotypes with asthma status at age five years (binary outcome), we fitted unadjusted and adjusted logistic regression models. To investigate the relation of metabotypes with the development of recurrent wheeze (time-to-event) outcomes, we constructed Cox proportional hazards models. Patients were censored at time of the recurrent wheeze development, loss to follow-up, or age 36-months, whichever occurred first. For both models, we adjusted for potential confounders (age, sex, parent history of asthma, number of previous breathing problems, respiratory syncytial virus infection and rhinovirus infection) based on *a priori* knowledge^{13, 14, 16, 23, 24}.

To examine the robustness of inferences, we conducted a series of sensitivity analyses. First, we repeated the outcome regression model by restricting the study sample to those without previous breathing problems. Second, we also examined the robustness of the metabotype-outcome associations by repeating the analysis with a different number of metabotypes. We also computed E-values to examine the robustness of the causal inference by using the R *Evalue* package²⁵. The E-value indicates the minimum strength of association that an unmeasured confounder(s) would need to have with both the exposure and outcome to fully explain away a specific association, conditional on the measured covariates²⁵. For example, an E-value of 2.5 means that unmeasured confounders that were associated with both the metabotypes and outcome by an odds ratio of 2.5 could explain away the estimate, but weaker confounding could not.

Pathway Analyses—To identify biologically-meaningful pathways, we conducted a metabolite-set enrichment analysis by comparing the reference metabotype with each of the other metabotypes using MetaboAnalyst 4.0²⁶ and the Small Molecule Pathway Database²⁷ library as the reference. To investigate whether gene expression for specific biological pathways are enriched, we conducted a functional class scoring analysis using R *fgsea* package²⁸. To detect differentially-expressed pathways by integrating the metabolome and transcriptome data, we performed a Wilcoxon pathway enrichment analysis using the Integrated Molecular Pathway Level Analysis (IMPALA) method²⁹.

All statistical analyses were performed using R version 4.0.2. All P-values are two-tailed, with $P < 0.05$ considered statistically significant. We further calculated the false discovery rate (FDR) that allows for the interpretation of statistical significance in the context of multiple hypothesis testing. The FDR value was controlled using Benjamini-Hochberg method³⁰.

RESULTS

Of 921 infants with severe bronchiolitis enrolled into this longitudinal cohort, the current study included 918 infants (>99%) who had information for the nasopharyngeal airway

metabolome. Among the analytic cohort, the median age was 3 (IQR, 2-6) months, 40% were female, and 44% were non-Hispanic white. Subsequently, 32% developed recurrent wheeze by age 3 years and 27% developed asthma by age 5 years.

Identification of Metatypes and Their Characteristics

By applying the consensus clustering approach on the nasopharyngeal airway metabolome data, we identified five distinct metatypes (Figure E1): A) glycerophosphocholine (GPC)-high (8.6%); B) amino acid-high, polyunsaturated fatty acid (PUFA)-low (7.8%); C) amino acid-high, glycerophospholipid (GP)-low (39.5%); D) GP-high (19.3%); and E) mixed (24.7%) (Figure 2). Across these metatypes, several clinical characteristics (e.g., age, number of previous breathing problems, and infecting virus) were significantly different ($P<0.05$; Table 1).

Descriptively, infants with a metatype A were characterized by “classic” clinical presentation of bronchiolitis (e.g., young age and a low proportion of previous breathing problems, lifetime corticosteroid use, and parental asthma and eczema) and high abundance of GPC (Table 1, Figure 2). Metatype B was characterized by a high proportion of lifetime corticosteroid use and parental asthma, a high abundance of amino acids and hydroxyoctadecadienoic acid (HODE) metabolites, and low abundance of PUFAs (Figures 2–3, Figure E2). Metatype C was characterized by a high proportion of rhinovirus-C infection, high abundance of amino acids, and low abundance of GP (Figure 2, Figure E3). Metatype D was characterized by a high proportion of parent history of eczema and IgE sensitization, and high abundance of GP (Figure 2, Figure E4). Lastly, the metatype E was characterized by a high proportion of intensive treatment use, and a mixed metabolite profile (Figure 2, Figure E5).

Association of Metatypes with Chronic Respiratory Outcomes

To investigate longitudinal association of the metatypes with clinical outcomes, we compared the outcome risks between infants with a metatype A (clinically “classic” bronchiolitis) and those with each of the other metatypes (Table 2). Compared with metatype A infants, metatype B infants had a significantly higher risk of developing asthma (23.4% vs. 40.6% [binary outcome]; OR, 2.24; 95% CI, 1.10-4.63; $P=0.03$). This association remained significant after adjusting for the confounders (OR_{adj}, 2.18; 95% CI, 1.03–4.71; $P=0.04$; E-value=2.32). By contrast, those with a metatype C, D, or E did not have significantly differential risks.

Likewise, compared with metatype A infants, metatype B infants had a significantly higher rate of recurrent wheeze that resulted in asthma (19.0% vs. 34.0%; HR_{adj}, 2.52; 95% CI, 1.16–5.47; $P=0.02$; E-value=3.18; Table E2). By contrast, for the rate of recurrent wheeze that did not result in asthma, there were no significant differences between the metatypes A and B (20.3% vs. 19.5%; HR_{adj}, 0.88; 95% CI, 0.36–2.17; $P=0.78$).

Distinct Biological Characteristics of Metatypes

Based on principal component analysis, the global metabolome profiles were distinct between the reference (metatype A) and highest asthma risk (metatype B) groups

(Figure 3A). To better understand their functional difference, we conducted pathway analyses using the nasopharyngeal metabolome and transcriptome data. In the metabolite-set enrichment analysis, the differentially-enriched (FDR<0.05) pathways for metabotype B infants included amino acid (e.g., methionine, betaine, histidine, glutathione) and fatty acid (α -linolenic acid and linoleic acid) metabolism pathways (all FDR< 5×10^{-14} ; Figure 3B). Additionally, in the gene-set enrichment analysis of the transcriptome data, infants with a metabotype B had differentially-enriched pathways, such as up-regulated interferon- α (IFN- α) and interleukin-6/Janus kinase/signal transducers and activators of transcription-3 (IL-6/JAK/STAT3) pathways as well as down-regulated fatty acid metabolism pathway. The integrated analysis of both metabolome and transcriptome data also identified differentially-enriched pathways in metabotype B, such as metabolism of lipids (FDR= 5×10^{-27} ; Figure E2). For the comparisons of metabotype A vs. the other metabolotypes (C, D, and E), the detailed functional differences are summarized in Figures E3–E5.

Sensitivity Analysis

In the subgroup analysis restricting the study sample to the infants without previous breathing problems, compared with metabotype A, metabotype B had a non-significantly higher risk of developing asthma (OR_{adj}, 1.76; 95%CI, 0.75-4.16; $P=0.19$; Table E3). Second, we also examined different numbers of metabolotypes. The alluvial plot (Figure E6) showed a consistency of the original 5 metabolotypes (A-E) across the different numbers of metabolotypes. With the use of 6 metabolotypes, for example, the metabotype 1 had 98.6% concordance with the original metabotype A and the metabotype 2 had 96.5% concordance with the original metabotype B (Table E4). Similar to the primary analysis, the metabolite-set enrichment analysis demonstrated that, compared to metabotype 1 (which is concordant with metabotype A), metabotype 2 (which is concordant with metabotype B) infants also had differentially-enriched metabolism of amino acids and PUFAs (Figure E7). In addition, compared to metabotype 1, metabotype 2 infants had a non-significantly but consistently higher risk of asthma (23.9% vs. 41.8%; OR_{adj}, 2.19; 95%CI, 0.97-5.01; $P=0.06$; Table E5) and significantly higher rate of recurrent wheeze that resulted in asthma (20.4% vs. 37.8%; HR_{adj}, 2.55; 95%CI, 1.13-5.76; $P=0.02$; E-value=3.21; Table E6).

DISCUSSION

By applying a clustering approach to the nasopharyngeal airway metabolome data from a multicenter prospective cohort study of 918 infants with severe bronchiolitis, we identified five biologically-distinct metabolotypes. In particular, compared to infants with metabotype A (who resemble “classic” bronchiolitis), those with a metabotype B (characterized by a high proportion of parental asthma, high abundance of amino acids, and low abundance of PUFAs) had a significantly higher risk for developing recurrent wheeze and asthma. To the best of our knowledge, this is the first study that has identified biologically-distinct airway metabolotypes in infants with severe bronchiolitis and demonstrated their longitudinal relations with the risk of chronic respiratory outcomes.

Bronchiolitis has been traditionally viewed as a single disease condition with similar mechanisms. Indeed, the current U.S. guidelines for diagnosis and management of

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bronchiolitis rely on this assumption^{15,31}. Yet, concordant with the results of the present study, a growing body of evidence supports the complexity of bronchiolitis, as reflected by heterogeneous clinical characteristics^{8,9}, transcriptome^{32–34}, microRNA³³, cytokine^{35,36}, and upper airway microbiome^{37–39}. Downstream of these transcriptional and translational processes, the metabolome—the global collection of small molecules (e.g., sugars, amino acids, lipids)—provides “proximal reports” of a system’s functional state¹¹. Recent studies have suggested the relationship of the metabolome with disease severity among infants with severe bronchiolitis^{13,14,24,40–42}. For example, among 140 infants with severe bronchiolitis in the U.S., *serum* metabolome profiles (e.g., glutathione) was associated with a higher severity of illness²⁴. Furthermore, in a single-center study of 52 infants with bronchiolitis in Italy, urine amino acids (e.g., methionine) were associated with a higher risk of recurrent wheeze during the 2-year follow-up period⁴³. These earlier reports have supported the heterogeneity of bronchiolitis and discovered potential mechanisms that underlie the bronchiolitis-chronic respiratory outcomes link. The current study—with a sample size many times larger than any other prior study—corroborates earlier findings and extends them by identifying biologically-distinct severe bronchiolitis metabolotypes that have differential risks of developing recurrent wheeze and asthma.

There are several potential mechanisms linking severe bronchiolitis—in particular metabolotype B (amino acid-high, PUFA-low)—with subsequent respiratory outcomes. First, studies have shown inflammatory amino acids (such as betaine, methionine, and histidine) involved in DNA methylation in the asthma pathobiology. Specifically, betaine provides the methyl groups for synthesizing methionine, which further generates S-adenosylmethionine (SAM). SAM is a key methyl donor, which contributes to DNA methylation⁴⁴. Indeed, DNA methylation is vital in the activation of the immune response in patients with asthma. For example, a previous study showed an increased DNA methylation level at the IL-4 promoter in cells from patients with asthma after house dust mite stimulation⁴⁵. Second, the role of PUFAs (e.g., ω -3 PUFAs) as immune modulators has been demonstrated in relation to asthma⁴⁶. For example, α -linolenic acid (an ω -3 PUFA) is converted to eicosapentaenoic acid, which inhibits arachidonic acid thereby suppressing production of eicosanoid inflammatory mediators. Eicosapentaenoic acid also functions as a precursor for pro-resolving mediators (such as resolvins, protectins, and maresins)⁴⁷. These mediators have important anti-inflammatory properties with protective effects on asthma⁴⁶. Observational studies and randomized controlled trials have also shown a protective effect of PUFAs on development of asthma. For example, the supplement of ω -3 PUFAs during pregnancy are associated with a reduced risk of wheezing illness⁴⁸ and asthma^{48,49} in the offspring. Similarly, lower ω -3 PUFA intake is associated with a higher incidence of asthma in children⁵⁰. Third, in the current study, we also found a high level of 9- and 13-HODE in the metabolotype B. Emerging evidence suggests that HODE plays a role in the pathobiology of asthma⁵¹. For example, Panda *et al.* reported, in allergic asthma mouse and human bronchial epithelial cell models, that 13-HODE increases pNF- κ B levels and reduces GR- α transcripts, thereby inducing corticosteroid resistance⁵¹. Lastly, our transcriptome analysis also found that metabolotype B had up-regulated IFN- α and IL-6/JAK/STAT3 pathways, which are involved in asthma pathobiology⁵². Notwithstanding the complexity of these mechanisms, we believe that the identification of severe bronchiolitis subtypes

based on metabolome data (or metabotypes) and their longitudinal relations with chronic respiratory outcomes are important. Our findings, in conjunction with the existent literature, should advance research into the development of subtype-specific strategies for bronchiolitis treatment and asthma prevention.

Our study has several potential limitations. First, bronchiolitis involves inflammation of the lower airways, in addition to the upper airways. While our data relied on nasopharyngeal specimens, research has shown that upper airway sampling provides reliable representation of inflammatory profiles in the lower airways⁵³. Furthermore, the use of upper airway specimens is preferable as bronchoscopy or other methods of lower airway sampling would be too invasive in young infants with bronchiolitis. Second, the nasopharyngeal specimens were obtained at a single timepoint. While longitudinal molecular data are also instrumental, the study objective was to identify metabotypes of severe bronchiolitis. Even with single-time point data, we successfully identified biologically-distinct metabotypes that are longitudinally associated with subsequent respiratory outcomes. Third, the current analysis was designed not to examine asthma phenotypes but to use the epidemiological definition of asthma at age five years^{16, 18}. The cohort is being followed up not only to minimize misclassification due to asthma development at a later age, but also to perform comprehensive phenotyping and endotyping of children who develop asthma. Fourth, our causal inference might have been confounded by unmeasured factors (e.g., host genetics, nutrition, inborn errors of metabolism). However, the relatively large E-values implied that confounders would have been strong to explain away our inference. Fifth, the current study used untargeted metabolomics procedure and it is important to conduct independent replication using targeted metabolomics technique to improve the profiling resolution and replicate our findings. Lastly, despite the study cohort consisting of racially/ethnically- and geographically-diverse infants, our inferences may not be generalizable to those with mild-to-moderate bronchiolitis, and hence warrant external validation. Nonetheless, our data remain directly relevant for the 110,000 infants hospitalized yearly in the U.S.¹, a population with substantial morbidity burden.

In conclusion, by applying an unsupervised clustering approach to the nasopharyngeal airway metabolome data from a multicenter prospective cohort study of 918 infants with severe bronchiolitis, we identified five biologically-distinct and clinically-meaningful metabotypes. Specifically, the metabotype characterized by a high abundance of inflammatory amino acids and low abundance of PUFAs had the highest risk for developing asthma. Our data lend significant support to the concept that “bronchiolitis” is a heterogenous syndrome with different biological mechanisms. These observations should facilitate further investigations into the development of metabotype-targeted strategies for bronchiolitis treatment and asthma prevention. Furthermore, future examinations of the relationship between bronchiolitis metabotypes and asthma endotypes (e.g., based on the genome, transcriptome, and metabolome) will also provide a new avenue for the development of endotypes-specific prevention strategies for asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

DNA	Deoxyribonucleic acid
FDR	False discovery rate
GP	Glycerophospholipid
GPC	Glycerophosphocholine
GR-α	Glucocorticoid receptor-alpha
HODE	Hydroxyoctadecadienoic acid
IFN-α	Interferon-alpha
IL-4	Interleukin-4
IL-6	Interleukin-6
IMPALA	Integrated Molecular Pathway Level Analysis
IQR	Interquartile range
JAK	Janus kinase
MARC-35	The 35th Multicenter Airway Research Collaboration
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OR	Odds ratio
PUFA	Polyunsaturated fatty acid
RSV	Respiratory syncytial virus
SAM	S-adenosylmethionine
STAT3	Signal transducers and activators of transcription-3

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Key Messages:

- Growing evidence suggests that bronchiolitis is a heterogeneous syndrome.
- Using data of the nasopharyngeal airway metabolome from a multicenter cohort of infants with severe bronchiolitis, unsupervised clustering analysis identified five biologically distinct “metabotypes”.
- These metabotypes were also clinically meaningful. For example, the metabotype characterized by high abundance of inflammatory amino acids and low abundance of PUFAs had the highest risk for developing asthma.

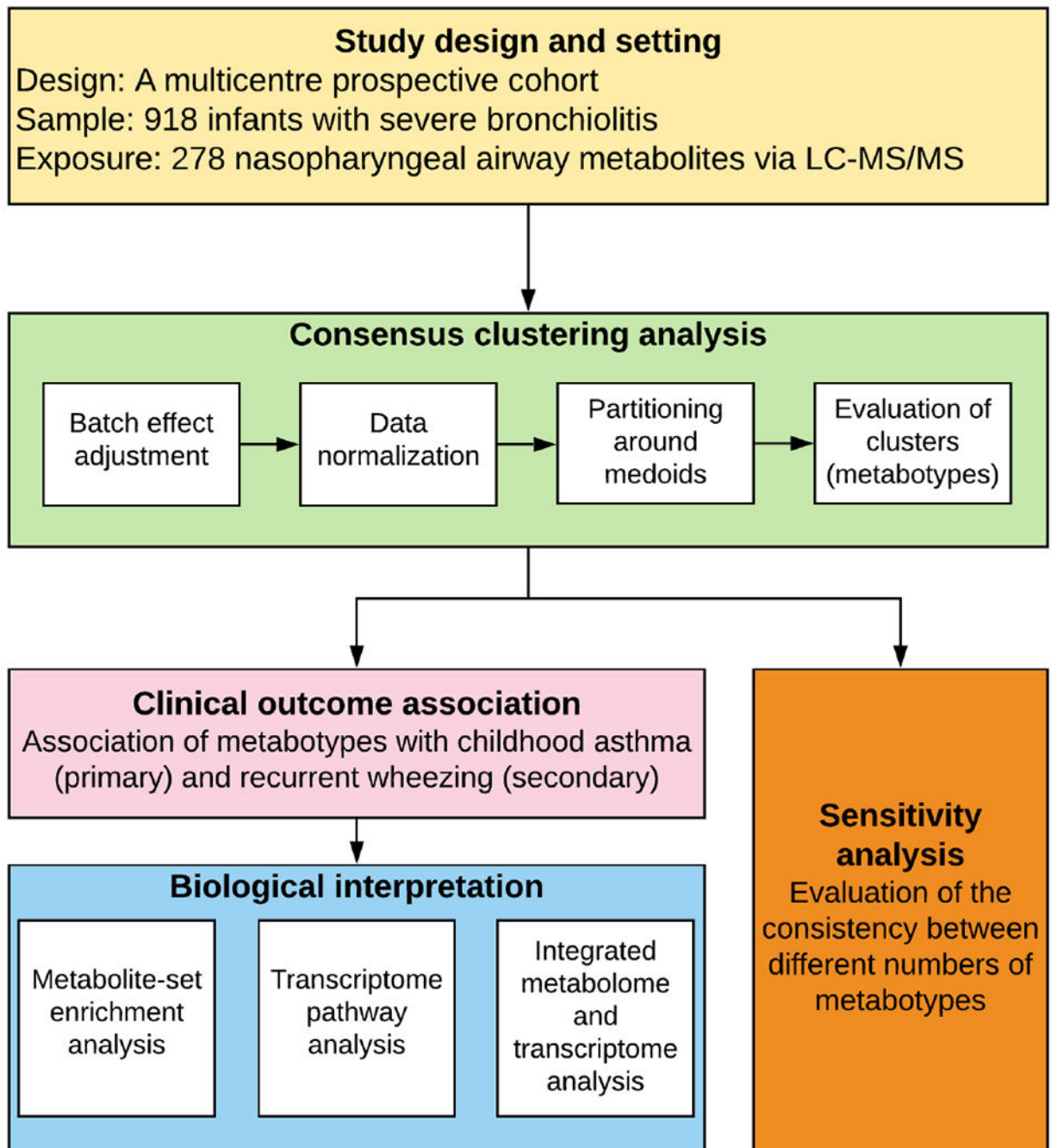


Figure 1. Analytic workflow

The analytical cohort consists of 918 infants hospitalized for bronchiolitis (severe bronchiolitis) in a multicenter prospective cohort study—the 35th Multicenter Airway Research Collaboration (MARC-35). The nasopharyngeal metabolome data were adjusted for the potential batch effect and normalized by total sum scaling before clustering analysis. To identify metabotypes, the partitioning around medoids algorithm was used in consensus clustering. To choose the optimal number of metabotypes, a combination of the average silhouette score, consensus matrix heatmap, and biological plausibility was used. The

between-metabotype differences in the metabolome profile were visualized using a heatmap. The association between the metabotypes and the risk of developing childhood asthma (binary outcome) was estimated by fitting logistic regression models; the rate of developing recurrent wheeze (time-to-event outcome) was estimated by fitting Cox proportional hazards models. To examine the distinct function of each metabotype, 1) metabolite set enrichment analysis examining the metabolome data, 2) gene set enrichment analysis examining the nasopharyngeal transcriptome data, and 3) Wilcoxon pathway enrichment analysis integrating the metabolome and transcriptome data were conducted. In the sensitivity analysis, the concordance between the different numbers of metabotypes was also examined. Abbreviation: LC-MS/MS, liquid chromatography-tandem mass spectrometry

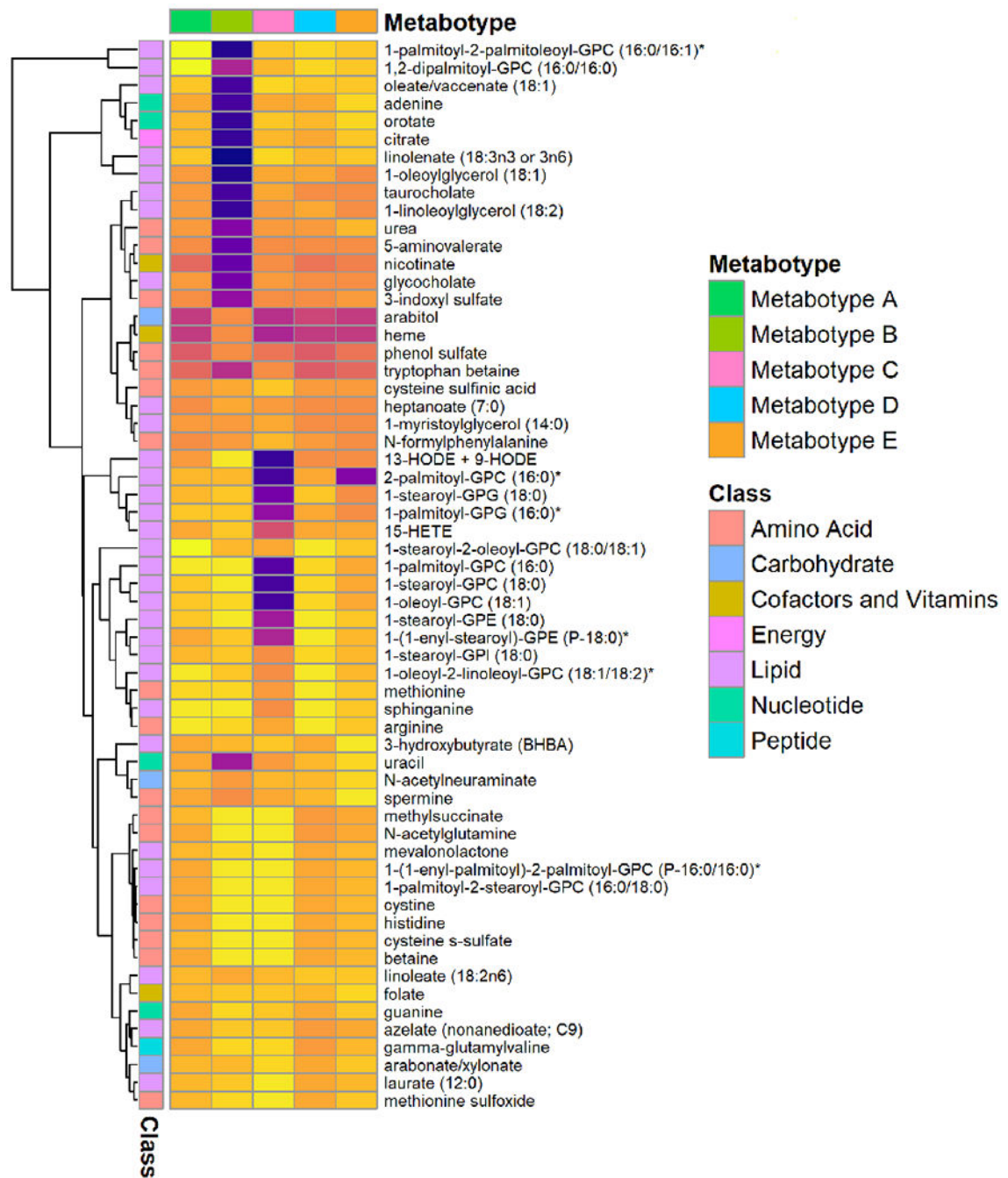


Figure 2. Abundance of selected nasopharyngeal metabolites between five metabotypes of severe bronchiolitis

The normalized metabolomics data (50 metabolites with highest variance and top 10 metabolites in the metabolite-set enrichment analysis) are used to visualize the between-metabotype differences. The top row represents five different metabotypes (A-E). The first column on the left represents a major metabolite class (e.g., amino acids, lipids) for the corresponding metabolite. The tree on the left represents hierarchical clustering of metabolites. Each cell represents the median value of normalized metabolite data within each metabotype—the yellow color represents a high value, and dark purple color represents

a low value. Asterisk on some metabolites indicates a compound that has not been confirmed based on a standard, but we are confident in its identity (by Metabolon, Inc).

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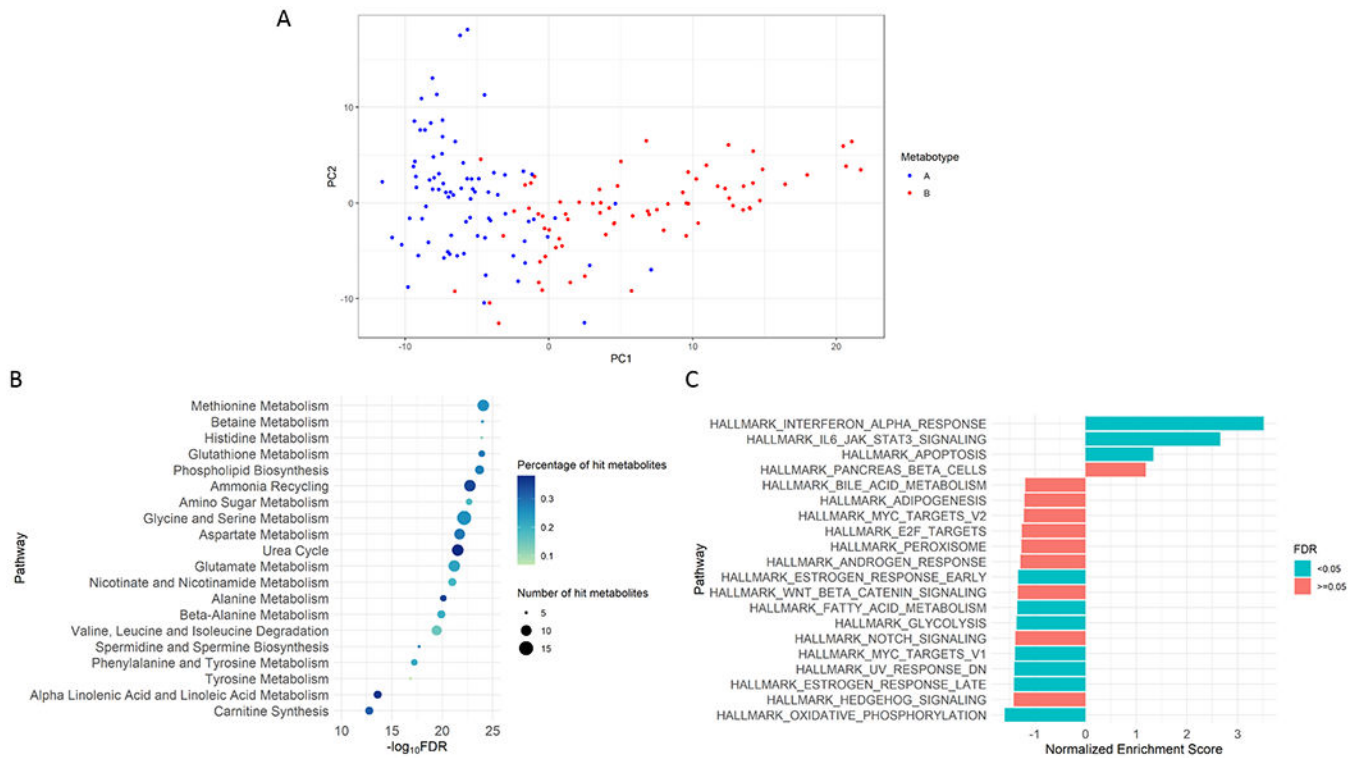


Figure 3. Principal component analysis and functional pathway analyses in the metabolotypes A vs. B comparison

a. Principal component analysis

Principal component analysis of metabolotypes A and B based on the first two principal components. The global metabolome profiles were distinct between the metabolotypes A and B.

b. Metabolite-set enrichment analysis

Shown metabolite pathways (based on the Small Molecule Pathway Database [SMPDB]) are 20 pathways with a smallest false discovery rate (FDR) that have the number of hits per pathway of 5 nasopharyngeal metabolites. SMPDB is a 99-metabolite set based on normal human metabolism pathways. The colour of each dot represents the proportion of hit metabolites; the size of each dot represents the number of hit metabolites.

c. Functional class scoring analysis

Shown biological pathways (based on the Molecular Signatures Database [MSigDB] hallmark gene sets) are 20 pathways with a highest absolute value of normalized enriched score. MSigDB hallmark collection contains 50 gene sets, which summarize and represent specific well-defined biological states or processes and display coherent expression. The analysis is based on nasopharyngeal transcriptome (through RNAseq) data. The colour of bars represents $FDR < 0.05$ (green) or $FDR \geq 0.05$ (orange).

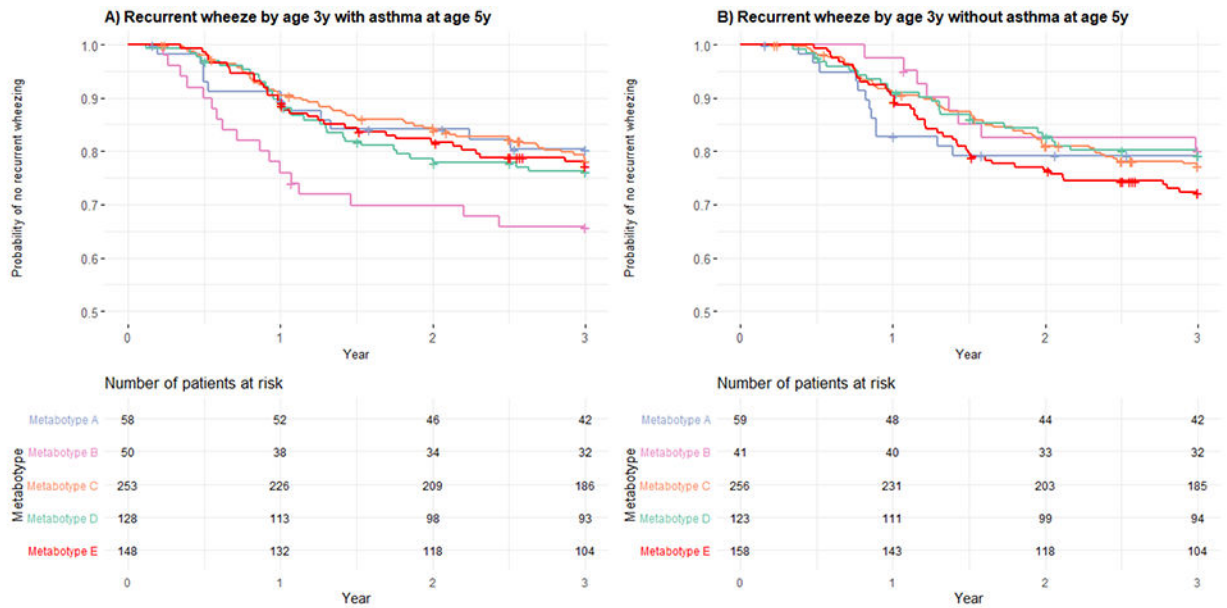


Figure 4. Kaplan-Meier curves for development of recurrent wheeze by age 3 years, according to severe bronchiolitis metabolotypes

a. Recurrent wheeze by age 3 years *with* asthma at age 5 years

Compared with metabotype A infants, the rate of developing recurrent wheeze was not significantly different in metabotype B, C, D, or E infants. However, the rate was significantly higher in metabotype B (amino acid-high/PUFA-low) infants (adjusted HR 2.52; 95% CI 1.16-5.47; P=0.02).

b. Recurrent wheeze by age 3 years *without* asthma at age 5 years

Compared with metabotype A infants, the rate of developing recurrent wheeze was not significantly different in metabotype B, C, D, or E. Corresponding hazards ratio estimates are presented in Table E2.

Table 1. Baseline characteristics and clinical course of infants, according to severe bronchiolitis metabolotypes

Characteristics	Metabotype A (n=79, 8.6%)	Metabotype B (n=72, 7.8%)	Metabotype C (n=363, 39.5%)	Metabotype D (n=177, 19.3%)	Metabotype E (n=227, 24.7%)	P-value
Demographics						
Age (month), median (IQR)	2 (1-3)	3 (2-5)	3 (2-6)	3 (2-6)	4 (2-7)	<0.001
Female sex	36 (45.6)	27 (37.5)	147 (40.5)	74 (41.8)	83 (36.6)	0.63
Race/ethnicity						0.13
Non-Hispanic white	28 (35.4)	35 (48.6)	167 (46.0)	68 (38.4)	101 (44.5)	
Non-Hispanic black	15 (19.0)	17 (23.6)	89 (24.5)	45 (25.4)	44 (19.4)	
Hispanic	34 (43.0)	19 (26.4)	90 (24.8)	59 (33.3)	73 (32.2)	
Other or unknown	2 (2.5)	1 (1.4)	17 (4.7)	5 (2.8)	9 (4.0)	
Prematurity (32-37 weeks)	14 (17.7)	12 (16.7)	67 (18.5)	39 (22.0)	39 (17.2)	0.76
Birth weight (kg), median (IQR)	3.35 (2.95-3.60)	3.33 (2.90-3.60)	3.26 (2.90-3.58)	3.20 (2.86-3.54)	3.32 (2.98-3.63)	0.51
Mode of birth (caesarean delivery)	24 (31.2)	25 (35.2)	126 (35.1)	65 (36.9)	71 (31.8)	0.81
Previous breathing problems (count)						0.001
0	69 (87.3)	58 (80.6)	293 (80.7)	152 (85.9)	160 (70.5)	
1	6 (7.6)	9 (12.5)	61 (16.8)	18 (10.2)	51 (22.5)	
2	4 (5.1)	5 (6.9)	9 (2.5)	7 (4.0)	16 (7.0)	
Previous ICU admission	1 (1.3)	2 (2.8)	4 (1.1)	2 (1.1)	6 (2.6)	0.56
Child history of eczema	5 (6.3)	8 (11.1)	57 (15.7)	24 (13.6)	43 (18.9)	0.07
Lifetime antibiotic use*	24 (30.4)	22 (30.6)	111 (30.6)	49 (27.7)	87 (38.3)	0.19
Lifetime corticosteroid use*	8 (10.1)	13 (18.1)	52 (14.3)	27 (15.3)	38 (16.7)	0.61
Ever attended daycare	10 (12.7)	18 (25.0)	86 (23.7)	31 (17.5)	65 (28.6)	0.02
Cigarette smoke exposure at home	7 (8.9)	12 (16.7)	55 (15.2)	28 (15.8)	35 (15.4)	0.62
Maternal smoking during pregnancy	10 (13.0)	11 (15.7)	50 (13.9)	26 (14.8)	29 (12.9)	0.97
Parental history of asthma	24 (30.4)	25 (34.7)	119 (32.8)	54 (30.5)	82 (36.1)	0.77
Parental history of eczema	11 (13.9)	14 (19.4)	70 (19.3)	39 (22.0)	41 (18.1)	0.64
Clinical presentation						
Weight (kg), median (IQR)	4.99 (4.20-6.17)	5.80 (4.70-7.15)	6.10 (4.70-7.60)	5.90 (4.80-7.60)	6.90 (5.30-8.32)	<0.001
Respiratory rate (per minute), median (IQR)	48 (42-60)	48 (39-60)	48 (40-60)	52 (42-62)	50 (40-60)	0.26

Characteristics	Metabotype A (n=79, 8.6%)	Metabotype B (n=72, 7.8%)	Metabotype C (n=363, 39.5%)	Metabotype D (n=177, 19.3%)	Metabotype E (n=227, 24.7%)	P-value
Oxygen saturation						0.50
<88%	6 (7.7)	7 (10.1)	19 (5.3)	10 (5.7)	10 (4.5)	0.50
88%-89.9%	1 (1.3)	5 (7.2)	14 (3.9)	7 (4.0)	6 (2.7)	
90%-93.9%	9 (11.5)	7 (10.1)	51 (14.3)	30 (17.2)	38 (17.2)	
94%	62 (79.5)	50 (72.5)	272 (76.4)	127 (73.0)	167 (75.6)	
Blood eosinophilia (4%)	9 (12.7)	10 (17.9)	38 (12.0)	10 (6.2)	15 (7.8)	0.06
Any IgE sensitization	16 (20.3)	15 (20.8)	64 (17.6)	47 (26.6)	39 (17.2)	0.13
Aeroallergen sensitization	0 (0.0)	1 (1.4)	6 (1.7)	3 (1.7)	4 (1.8)	0.85
Food allergen sensitization	16 (20.3)	14 (19.4)	60 (16.5)	44 (24.9)	36 (15.9)	0.14
Clinical course						
Positive pressure ventilation use [‡]	4 (5.1)	2 (2.8)	14 (3.9)	14 (7.9)	15 (6.6)	0.23
Intensive treatment use [‡]	11 (13.9)	8 (11.1)	49 (13.5)	29 (16.4)	44 (19.4)	0.28
Length-of-stay (day), median (IQR)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-4)	2 (1-3)	0.24
Respiratory virus						
Any RSV infection	66 (83.5)	61 (84.7)	294 (81.0)	155 (87.6)	173 (76.2)	0.05
Any rhinovirus infection	13 (16.5)	9 (12.5)	79 (21.8)	27 (15.3)	57 (25.1)	0.04
Rhinovirus-A	6 (7.6)	5 (6.9)	31 (8.5)	15 (8.5)	31 (13.7)	0.20
Rhinovirus-B	3 (3.8)	0 (0.0)	5 (1.4)	2 (1.1)	5 (2.2)	0.36
Rhinovirus-C	6 (7.6)	3 (4.2)	41 (11.3)	8 (4.5)	23 (10.1)	0.05

Abbreviations: ICU, intensive care unit; IgE, immunoglobulin E; IQR, interquartile range; RSV, respiratory syncytial virus

Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100, because of rounding and missingness.

* Any systemic antibiotic or corticosteroid use from birth up to the index hospitalization for bronchiolitis.

[‡] Infants with severe bronchiolitis who underwent continuous positive airway ventilation and/or mechanical ventilation.

[‡] Infants with severe bronchiolitis who were admitted to ICU and/or who underwent positive pressure ventilation.

Unadjusted and adjusted associations of severe bronchiolitis metabolites in infants with development of childhood asthma

Table 2.

Metabotypes	Childhood asthma*					
	Unadjusted model			Adjusted model [†]		
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value	P-value	
Metabotype A (GPC-high)	1 [Reference]	–	1 [Reference]	–	–	
Metabotype B (amino acid-high, PUFA-low)	2.24 (1.10-4.63)	0.03	2.18 (1.03-4.71)	0.04	0.04	
Metabotype C (amino acid-high, GP-low)	1.14 (0.65-2.08)	0.66	1.07 (0.58-2.02)	0.84	0.84	
Metabotype D (GP-high)	1.23 (0.66-2.34)	0.52	1.25 (0.65-2.49)	0.51	0.51	
Metabotype E (mixed)	1.12 (0.62-2.11)	0.72	0.87 (0.46-1.72)	0.69	0.69	

Abbreviations: CI, confidential interval; GP, glycerophospholipid; GPC, glycerophosphocholine; PUFA, polyunsaturated fatty acid.

* Asthma was defined as physician-diagnosis of asthma by age 5 years, plus either asthma medication use (e.g., albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year. To examine the association between severe bronchiolitis metabolites (metabotype A as the reference) and the risk of developing childhood asthma, logistic regression model was fit.

[†]The multivariable logistic regression models adjusted for potential confounders, including patient's age, sex, parent history of asthma, number of previous breathing problems, respiratory syncytial virus infection, and rhinovirus infection.