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Nasopharyngeal lipidomic endotypes of infants with bronchiolitis and risk of childhood asthma: A multicenter prospective study

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Abstract

Background: Bronchiolitis is the leading cause of hospitalization of U.S. infants and an important risk factor for childhood asthma. Recent evidence suggests that bronchiolitis is clinically heterogeneous. We sought to derive bronchiolitis endotypes by integrating clinical, virus, and lipidomics data, and to examine their relationship with subsequent asthma risk.

Methods: This is a multicenter prospective cohort study of infants (age <12 months) hospitalized for bronchiolitis. We identified endotypes by applying clustering approaches to clinical, virus, and nasopharyngeal airway lipidomic data measured at hospitalization. We then determined their longitudinal association with the risk for developing asthma by age six years by fitting a mixed-effects logistic regression model. To account for multiple comparisons of the lipidomics data, we

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computed the false discovery rate (FDR). To understand the underlying biological mechanism of the endotypes, we also applied pathway analyses to the lipidomics data.

Results: Of 917 infants with bronchiolitis (median age, 3 months), we identified clinically- and biologically-meaningful lipidomic endotypes: A) cinical^{classic}lipid^{mixed} (n=263), B) clinicalseverelipidsphingolipids-high (n=281), C) clinicalmoderatelipidphospholipids-high (n=212), and D) clinical^{atopic}lipid$^{sphingolipids\text{-}low}$ (n=161). Endotype A infants were characterized by "classic" clinical presentation of bronchiolitis. Profile D infants were characterized by a higher proportion of parental asthma, IgE sensitization, and rhinovirus infection, and low sphingolipids (e.g., sphingomyelins, ceramides). Compared with endotype A, profile D infants had a significantly higher risk of asthma (22% vs. 50%; unadjusted OR, 3.60; 95%CI, 2.31–5.62; P<0.001). Additionally, endotype D had a significantly lower abundance of polyunsaturated fatty acids (e.g., docosahexaenoic acid [DHA]; FDR=0.01). The pathway analysis revealed that sphingolipid metabolism pathway was differentially-expressed in endotype D (FDR=0.048).

Conclusions: In this multicenter prospective cohort study of infants with bronchiolitis, integrated clustering of clinical, virus, and lipidomic data identified clinically- and biologically-distinct endotypes that have a significantly differential risk for developing asthma.

INTRODUCTION

Bronchiolitis is the leading cause of hospitalization in U.S. infants, accounting for ~110,000 hospitalizations annually [1]. In addition to the substantial acute disease burden, the literature has also highlighted its chronic morbidities [2]. Indeed, of these infants hospitalized for bronchiolitis, approximately 30% will develop childhood asthma [3–6].

Although bronchiolitis has traditionally been thought of as a single disease entity with a similar mechanism [7], growing evidence supports the heterogeneity of this condition [8]. For example, recent epidemiologic studies have reported and validated clinically-distinct subgroups of bronchiolitis [9] with a different risk of the development of recurrent wheeze [10] and asthma [11, 12]. Yet, these subgroups (phenotypes) were identified only through clinical characteristics. The pathobiological mechanisms underlying the clinical heterogeneity remains to be elucidated. This insufficient understanding of pathobiology of bronchiolitis during infancy—a critical period for airway development—has hindered efforts to develop targeted asthma prevention strategies in this high-risk population. Lipidomics is well-suited to address this limitation by comprehensively profiling lipids, which not only comprise the major structural basis of cell membranes but also have active roles in biological signaling [13, 14]. Recent research has suggested that their dysregulation is responsible for the pathobiology of various diseases, including bronchiolitis [15–17] and asthma [18–22]. However, no study has yet investigated biologically-distinct molecular subgroups (endotypes) of infant bronchiolitis based on their lipidomic signatures.

To address this knowledge gap, we analyzed data from a multicenter prospective cohort of infants hospitalized for bronchiolitis to 1) identify distinct nasopharyngeal lipidomic endotypes of bronchiolitis by applying an integrated clustering approach, and 2) determine their association with the subsequent development of recurrent wheeze and asthma.

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 [23, 24]. 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 Supplemental Methods.

Briefly, MARC-35 investigators at 17 sites across 14 U.S. states (Supplemental Table E1) enrolled infants (age <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. 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 retraction [7]. We excluded infants with a preexisting heart and lung disease, immunodeficiency, immunosuppression, or gestational age <32 weeks. All patients were treated at the discretion of the treating physicians.

Of 921 infants enrolled into the longitudinal cohort, the current analysis investigated 917 infants who underwent nasopharyngeal lipidomic testing. All study procedures were approved by the responsible bioethics committee at each participating hospital and were in accordance with the World Medical Association Declaration of Helsinki. A written informed consent was obtained from the parent or guardian.

Data collection

Clinical data (demographic characteristics, family, environmental, and medical history; and details of the acute illness) were collected via structured interview and chart reviews [15]. In addition to the clinical data, investigators also collected nasopharyngeal airway specimens at hospitalization using a standardized protocol [25, 26]. These specimens underwent 1) viral testing of 17 respiratory viruses (including respiratory syncytial virus [RSV], rhinovirus) using real-time polymerase chain reaction (RT-PCR) assays at Baylor College of Medicine (Houston, TX, USA) [25, 26], 2) complex lipidomic profiling at Metabolon (Durham, NC, USA), and 3) RNA sequencing (transcriptome profiling; n=243) at the University of Maryland (Baltimore, MD, USA) [19].

Nasopharyngeal complex lipidomic profiling

Lipids were extracted from the nasopharyngeal specimens by a modified Bligh-Dyer extraction using methanol/water/dichloromethane in the presence of deuterated internal standards [27]. The extracts were dried under nitrogen and reconstituted in ammonium acetate dichloromethane: methanol. The extracts were transferred to vials for infusion-mass spectrometry (MS) analysis, performed on a Shimadzu liquid chromatograph (LC) with nano PEEK tubing (Shimadzu Scientific Instruments, Kyoto, Kyoto, Japan) and the SCIEX SelexIon-5500 QTRAP (SCIEX, Toronto, ON, Canada). The specimens were analyzed by both positive and negative mode electrospray. Individual lipid species were then quantified

by taking the ratio of the signal intensity of each target compound to that of its internal standard, then multiplying by the concentration of internal standard added to the specimen.

Nasopharyngeal transcriptomic profiling

The details of RNA extraction, RNA-sequencing, quality control, and transcriptome profiling are described in the Supplemental Methods. Briefly, after total RNA extraction, DNase treatment, and ribosomal RNA reduction, we performed RNA-sequencing with NovaSeq6000 (Illumina, San Diego, CA, USA) using an S4 100PE Flowcell (Illumina, San Diego, CA, USA). All RNA-sequencing samples had high sequence coverage after quality control. The transcript abundances were estimated with Salmon using the human genome hg38 and the mapping-based mode [28].

Outcome measures

The primary outcome was the development of asthma by six years of age. Asthma was defined using a commonly used epidemiologic definition: physician diagnosis of asthma, with either asthma medication use (e.g., inhaled bronchodilators, inhaled corticosteroids, leukotriene receptor antagonists) or asthma-related symptoms (e.g., wheezing, nocturnal cough) in the preceding year [29]. The secondary outcome was the development of recurrent wheeze by three years of age. Recurrent wheeze was 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 sleep [30].

Statistical analysis

The study objectives were 1) to identify biologically-distinct lipidomic endotypes among infants hospitalized for bronchiolitis (clustering), and 2) to relate them to subsequent clinical outcomes (association). The analytic workflow is summarized in Figure 1. The details of the statistical analysis can be found in the Supplemental Methods.

First, we computed a distance matrix for each of the three datasets (clinical, virus, and lipidomic data). Based on a *priori* knowledge [15, 19, 24, 31, 32], we choose clinical (age, sex, birth weight, history of breathing problems, lifetime antibiotics use, lifetime corticosteroid use, parental asthma, parental eczema, IgE sensitization, and intensive care use) and virus (RSV, rhinovirus species A and C, and virus genomic load) variables. We analyzed lipidomic data for 982 lipid species. We then derived mutually-exclusive clusters for each dataset by using a consensus clustering algorithm [33]. To choose an optimal number of clusters for each dataset, we used a combination of the consensus matrix, consensus cumulative distribution function, cluster consensus value, cluster size, and clinical and biological plausibility (Supplemental Figures E1–3) [15, 19, 24, 31, 32, 34]. Second, we combined these clusters (i.e., the clinical, virus, and lipidomic clusters) to derive a fused matrix, computed a Gower distance, and derived mutually-exclusive endotypes [33]. Third, to interpret the endotypes clinically and biologically, we developed chord diagrams on the relationship of the endotypes with major clinical and virus variables as well as a heatmap of the major lipid classes. Fourth, to determine the longitudinal association of the endotypes with asthma risk, we constructed a mixed-effects logistic regression model accounting for patient clustering within hospitals. As there was no significant between-endotype difference

in the proportion of the outcome missingness (n=59; P=0.85), we conducted a complete case analysis. To determine the association with the rate of recurrent wheeze, we modeled the time to the outcome by fitting a Cox proportional hazards model. Patients who did not have an outcome were censored at their last follow-up interview during the 36-month follow-up period. We verified the proportionality of hazards assumption through examining the Schoenfeld residuals. Fifth, to examine the function of each endotype, we conducted three analyses: i) between-endotype examinations of fatty acids, ii) metabolic pathway analysis by comparing the reference endotype with each of the other endotypes with the Kyoto Encyclopedia of Genes and Genomes (KEGG) as the reference [35], and iii) *integrated* transcriptomic-lipidomic pathway analysis in a subset of subject (n=243) using MetaboAnalyst 5.0 [36].

Lastly, in the sensitivity analysis, we first examined the endotype-asthma associations after excluding infants with a previous breathing problem. Second, we also examined the robustness of the endotype-outcome association by repeating the analysis using a different number of endotypes. We performed the statistical analysis using R version 3.6.1. (R Foundation, Vienna, Austria). We computed the Benjamini-Hochberg false discovery rate (FDR) that allows for the interpretation of statistical significance in the context of multiple hypothesis testing [37].

RESULTS

Of 921 infants enrolled into the MARC-35 longitudinal cohort, the current study focused on 917 infants hospitalized for bronchiolitis who underwent nasopharyngeal lipidomic testing. Among the current cohort, the median age was 3 (interquartile range [IQR], 2–6) months, 60% were male, and 44% were non-Hispanic white. Overall, 89% had RSV, 20% had rhinovirus, and 15% underwent intensive care use during the hospitalization (Table 1).

Multi-modal clustering identified distinct lipidomic endotypes among infants with bronchiolitis

After integrating the cluster data derived from each of the clinical, virus, and lipidomic datasets (Figure 1 and Supplemental Figures E1–3), we applied the consensus clustering approach. The combination of the consensus matrix, consensus cumulative distribution function, cluster consensus value, endotype size (n=161–281), and clinical and biological plausibility found that a 4-class model was an optimal fit (Supplemental Figure E4), with the four lipidomic endotypes called A, B, C, and D. The four distinct endotypes were chiefly characterized by their clinical and lipidomic characteristics: A) $\text{clinical}^{\text{classic}}\text{lipid}^{\text{mixed}}$ (n=263; 29%), B) $\text{clinical}^{\text{severe}}\text{lipid}^{\text{sphingolipids-high}}$ (n=281; 31%), C) $\text{clinical}^{\text{moderate}}\text{lipid}^{\text{phospholipids-high}}$ (n=212; 23%), and D) $\text{clinical}^{\text{atopic}}\text{lipid}^{\text{sphingolipids-low}}$ (n=161;18%).

Between the endotypes, several clinical characteristics were significantly different (P<0.05; Table 1 and Figure 2). Figure 3A summarizes between-endotype differences in lipid classes (all FDR<0.05). Endotype A ($\text{clinical}^{\text{classic}}\text{lipid}^{\text{mixed}}$) infants were characterized by a young age, a high proportion of males, a low proportion of breathing problem history, parental history of asthma and eczema, and IgE sensitization, and a high proportion of RSV

infection, with a mixed lipidomic profile. In many clinical aspects, endotype A resembled "classic" bronchiolitis. Endotype B (clinical^{severe}lipid$^{sphingolipids\text{-}high}$) was the most severe endotype with a high proportion of girls, intensive care use, prolonged hospital length-of-stay, and RSV infection, with the highest abundance of sphingolipids (including ceramides) (Figures 2A and 3A). Endotype C (clinical^{moderate}lipid$^{phospholipids\text{-}high}$) infants were characterized by an intermediate severity among the four endotypes (moderate severity), with the highest abundance of phospholipids (Figures 2B and 3A). Lastly, endotype D (clinical^{atopic}lipid$^{sphingolipids\text{-}low}$) infants were characterized by a high proportion of breathing problem history, eczema, parental history of asthma and eczema, and IgE sensitization, and a high proportion of rhinovirus infection, with the lowest abundance of sphingolipids and the highest abundance of lysophosphatidylcholine (LPC) (Figures 2C and 3A).

Lipidomic endotypes had differential risks of chronic airway morbidities

To investigate the longitudinal association of the lipidomic endotypes with the clinical outcomes, we compared the outcome risks between endotype A (who resemble classic bronchiolitis) and each of the other endotypes. Compared to endotype A, endotype D had a significantly higher risk of developing asthma (22% vs. 50%; odds ratio [OR], 3.60; 95%CI, 2.31–5.62; P<0.001; Figure 4). For the recurrent wheeze outcome, the Kaplan-Meier curves significantly differed between the endotypes ($P_{log\text{-}rank}$=0.01; Figure 5). Compared to endotype A, endotype D had a significantly higher rate (30% vs. 43%; hazard ratio [HR], 1.65; 95% CI, 1.19–2.27; P=0.003; Figure 4). In contrast, the risk of asthma and the rate of recurrent wheeze were not significantly different in endotype B or C infants.

Lipidomic endotypes had distinctly different functional characteristics

The lipidomic endotypes of infant bronchiolitis had distinctly different lipid classes (all FDR<0.05; Figure 3A) and fatty acid profiles (all FDR<0.05 except for hexacosanoic acid; Figure 3B). For example, compared to endotype A, endotype D had a lower abundance of ceramides and sphingomyelins (FDR≤0.10; Figure 6A) and polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA) (FDR<0.05; Figure 6B). Likewise, the metabolic pathway analysis revealed that the sphingolipid metabolism pathway was differentially enriched between endotypes A and D (FDR<0.05; Figure 6C). Similarly, the integrated transcriptomic-lipidomic analysis also revealed a between-endotype difference in the sphingolipid and α-linolenic acid (an ω−3 fatty acid) metabolism pathways (both FDR<0.05; Figure 6D).

For the comparisons of endotype A with each of the other endotypes (B and C), the detailed functional differences are summarized in Supplemental Figures E5 and 6. For example, the pathway analyses demonstrated that endotype B (clinical^{severe}lipid$^{sphingolipids\text{-}high}$) had a differentially-enriched sphingolipid metabolism pathway and that endotype C (clinical^{moderate}lipid$^{phospholipids\text{-}high}$) had a differentially-enriched glycerophospholipid metabolism pathway (both FDR<0.05).

Sensitivity analysis demonstrated the robustness of the findings

In the analysis limiting to infants without a previous breathing problem, the endotype-outcome associations remained consistent (Supplemental Table E2). For example, compared with endotype A, profile D infants had a significantly higher risk of asthma (OR, 3.64; 95%CI, 2.18–6.08; P<0.001). Next, different numbers of endotypes were also examined. The alluvial plot (Supplemental Figure E7) demonstrates consistency with the original endotypes across the different numbers chosen. With the use of 5-class models (endotypes 1–5), for example, endotype 1 had 100% concordance with the original endotype A and the endotype 5 had 100% concordance with endotype D (Supplemental Table E3). Additionally, compared to endotype 1 (concordant with endotype A), endotype 5 (concordant with endotype D) had a significantly higher risk of asthma (19% vs. 53%; OR, 4.72; 95%CI, 2.86–7.81; P<0.001; Supplemental Figure E8). Likewise, in the rate of developing recurrent wheeze, the Kaplan-Meier curves significantly differed between the endotypes ($P_{log\text{-}rank}$=0.01; Supplemental Figure E9). Lastly, compared to endotype 1, endotype 5 had a significantly higher rate (28% vs. 41%; HR, 1.70; 95% CI, 1.18–2.46; P=0.005; Supplemental Figure E8).

DISCUSSION

By applying a clustering approach to the integrated clinical, virus, and nasopharyngeal lipidomic data from a multicenter prospective cohort of 917 infants hospitalized for bronchiolitis, we identified four endotypes. These endotypes not only had distinct lipidomic profiles but also invoked differential risks of chronic airway morbidities—the development of recurrent wheeze and asthma. In particular, compared with infants with an endotype A ($cinical^{classic}lipid^{mixed}$), those with an endotype D ($clinical^{atopic}$ $lipid^{sphingolipids\text{-}low}$) had a significantly higher risk. Furthermore, endotype D had a unique fatty acid profile with low PUFAs (e.g., DHA). The sensitivity analysis indicated the robustness of our inference. To the best of our knowledge, this is the first investigation that has identified clinically-meaningful and biologically-distinct lipidomic endotypes among infants with bronchiolitis and demonstrated their longitudinal relationships with the risk of chronic respiratory outcomes.

Epidemiological studies have indicated heterogeneity of bronchiolitis by demonstrating reproducible clinical subtypes (phenotypes) based on clinical data [10–12]. For example, a recent analysis of three cohort studies of bronchiolitis in the U.S. and Finland has revealed that the phenotype characterized by a history of breathing problems, allergic predisposition, and rhinovirus infection had a significantly increased risk for asthma [12] —which is in line with the present analysis. Additionally, previous reports using global metabolomics (i.e., not lipidomics) have suggested pathobiological roles of complex lipids in the pathobiology of both acute respiratory infections [17, 38–42] and asthma [19, 43, 44]. For example, concordant with the endotype B ($clinical^{severe}lipid^{sphingolipids\text{-}high}$) observed in the current study, a metabolomic analysis of 144 infants with bronchiolitis has reported a positive association of sphingolipids in the upper airway with higher bronchiolitis severity [15]. Furthermore, consistent with the endotype D ($clinical^{atopic}lipid^{sphingolipids\text{-}low}$) observed in the current study, an analysis of plasma metabolome has reported that lower

levels of ceramides and sphingomyelins at age six months were associated with a higher risk of asthma-like symptoms before age three years in two birth cohort studies from Denmark and the U.S [18]. Moreover, another global metabolomic analysis of infants with bronchiolitis has shown that the "metabotype" characterized by a low abundance of PUFAs (e.g., α-linolenic acid) had the highest risk for developing asthma by five years [19]. These studies have collectively suggested important roles of functional lipids in the pathogenesis of childhood asthma. The current multicenter prospective study builds on these previous reports, and extends them by comprehensively investigating airway lipids and demonstrating biologically-distinct bronchiolitis endotypes that have differential risks for developing asthma.

The exact mechanisms underlying the observed endotypes—particularly endotype D characterized by rhinovirus infection, unique lipidomic profiles (e.g., lower sphingolipid and PUFA levels and higher LPC levels), and the highest asthma risk—warrant further clarification. First, studies have suggested that sphingolipids play important roles in the airway immune response, contributing to asthma pathogenesis [19, 45] For example, the inhibition of serine palmitoyl-CoA transferase—the rate-limiting enzyme of *de novo* sphingolipid biosynthesis—in the airways alters the pulmonary sphingolipid composition (e.g., decreased ceramides) and results in bronchial hyperreactivity [19]. Second, sphingolipid biosynthesis is regulated by the *ORMDL3* gene in the 17q21 locus—one of the most widely replicated loci in the asthma GWAS literature [46]. Its overexpression results in decreased sphingolipid levels [17] and increased asthma risks [47]. Consistently, an epidemiologic study has also shown an interaction between 17q21 variants and rhinovirus infection in early childhood on the risk of asthma development [48]. Third, research has also suggested immune modulating roles of PUFAs (e.g., ω−3 PUFAs, including DHA and eicosapentaenoic acid [EPA]) in asthma pathobiology [49]. For example, DHA and EPA are precursors for protectins and resolvins, which bring about a programmed resolution of the inflammatory process [50, 51]. Observational studies and randomized controlled trials have also shown a protective effect of PUFAs on asthma development [52–54]—e.g., an inverse association of plasma PUFA levels and dietary PUFA intake with the risk of allergic sensitization and asthma-like symptoms at age 3 years [52]. Supplementation with ω−3 long-chain PUFAs during the third trimester of pregnancy significantly reduced the risk of persistent wheeze or asthma in offspring [53]. Lastly, we also observed that LPC level is highest in endotype D. Consistently, observational studies have reported that LPC—a pro-inflammatory lipid mediator—is elevated in the bronchoalveolar lavage fluids of patients with asthma [55, 56]. Additionally, mouse models also demonstrated that intranasal LPC exposure increases Th2 cytokine levels, airway inflammation, and hyperresponsiveness [57]. Notwithstanding the complexity of these potential mechanisms, our findings—in conjunction with the existent literature—should facilitate further research into the mechanisms underlying the bronchiolitis-asthma link and the development of early and targeted interventions (e.g., supplementation of DHA) for asthma prevention.

LIMITATIONS

This study has several potential limitations. First, bronchiolitis involves lower airway inflammation in addition to that of upper airways. Although the current study relied on

nasopharyngeal airway data, research has demonstrated that upper airway specimens offer a reliable representation of inflammatory profiles in the lower airways [58]. Additionally, the use of upper airway specimens is favored because bronchoscopy or other lower airway sampling methods are invasive in these infants. Second, the nasopharyngeal specimens were obtained at the time of bronchiolitis hospitalization. While sequential lipidomic testing would also be instrumental, the goal of the current study was to identify lipidomic endotypes of bronchiolitis. Even with the single–time point data, the study successfully identified biologically-meaningful endotypes that have differential risks of asthma development. Third, our data did not include detailed nutritional information (e.g., supplemental DHA use by the mother and infants), which may have contributed to the endotypes. Fourth, the current study did not have non-bronchiolitis controls. Nonetheless, the objective of the study was not to assess lipidomic endotypes related to incident bronchiolitis but to examine the relationship of endotypes with subsequent asthma risk among infants hospitalized for bronchiolitis [4]. Fifth, although this hypothesis-generating study derives novel and well-calibrated hypotheses that guide future experiments, our inferences warrant further validation. Lastly, while the study sample consists of racially/ethnically- and geographically-diverse infants, the generalization of our findings to other populations (e.g., infants with mild-to-moderate bronchiolitis) requires caution. Regardless, our observations remain directly relevant to the 110,000 infants hospitalized yearly in the U.S.—a patient population with large morbidity [1].

CONCLUSIONS

By integrating the clinical, virus, and nasopharyngeal airway lipidomic data from a multicenter prospective cohort study of 917 infants hospitalized for bronchiolitis, we identified four clinically-meaningful and biologically-distinct lipidomic endotypes. Specifically, compared with the endotype that clinically resembles “classic” bronchiolitis, lipidomic endotype D —which was characterized by a higher proportion of previous breathing problems, history of eczema, parental asthma, IgE sensitization, and rhinovirus infection, and specific lipidomic profile (e.g., low sphingolipids and PUFAs)—had a higher risk for developing recurrent wheeze and asthma. Our data lend significant support to the emerging concept that bronchiolitis is a heterogeneous syndrome involving different pathobiological mechanisms. For clinicians, our observations provide an evidence base for early identification of infants at high risk for childhood asthma. For researchers, our findings should advance research into the development of endotype-specific strategies for asthma prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CI	Confidence interval
DHA	Docosahexaenoic acid
EMNet	Emergency Medicine Network
EPA	Eicosapentaenoic acid
FDR	False discovery rate
GPI	Glycosylphosphatidylinositol
IQR	Interquartile range
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatograph
LPC	Lysophosphatidylcholine
MARC	Multicenter Airway Research Collaboration
MS	Mass spectrometry
OR	Odds ratio
PUFA	Polyunsaturated fatty acid
RSV	Respiratory syncytial virus
RT-PCR	Real-time polymerase chain reaction

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Key messages

What is already known on this topic:

Emerging evidence suggests that bronchiolitis is a heterogeneous condition. Although subgroups (phenotypes) were identified only through clinical characteristics, little is known about its biologically-distinct subgroups (endotypes) based on the molecular signatures and their longitudinal relationships with asthma development.

What this study adds:

Integrated clustering of clinical, virus, and nasopharyngeal lipidomic data in infants with bronchiolitis revealed biologically-distinct endotypes (e.g., an endotype with low sphingolipids and PUFAs) that have differential risks for asthma development.

How this study might affect research, practice or policy:

Our observations not only lend significant support to the emerging concept that bronchiolitis is a heterogeneous syndrome involving different pathobiological mechanisms but also provide an evidence base for early identification of infants at high risk for asthma.

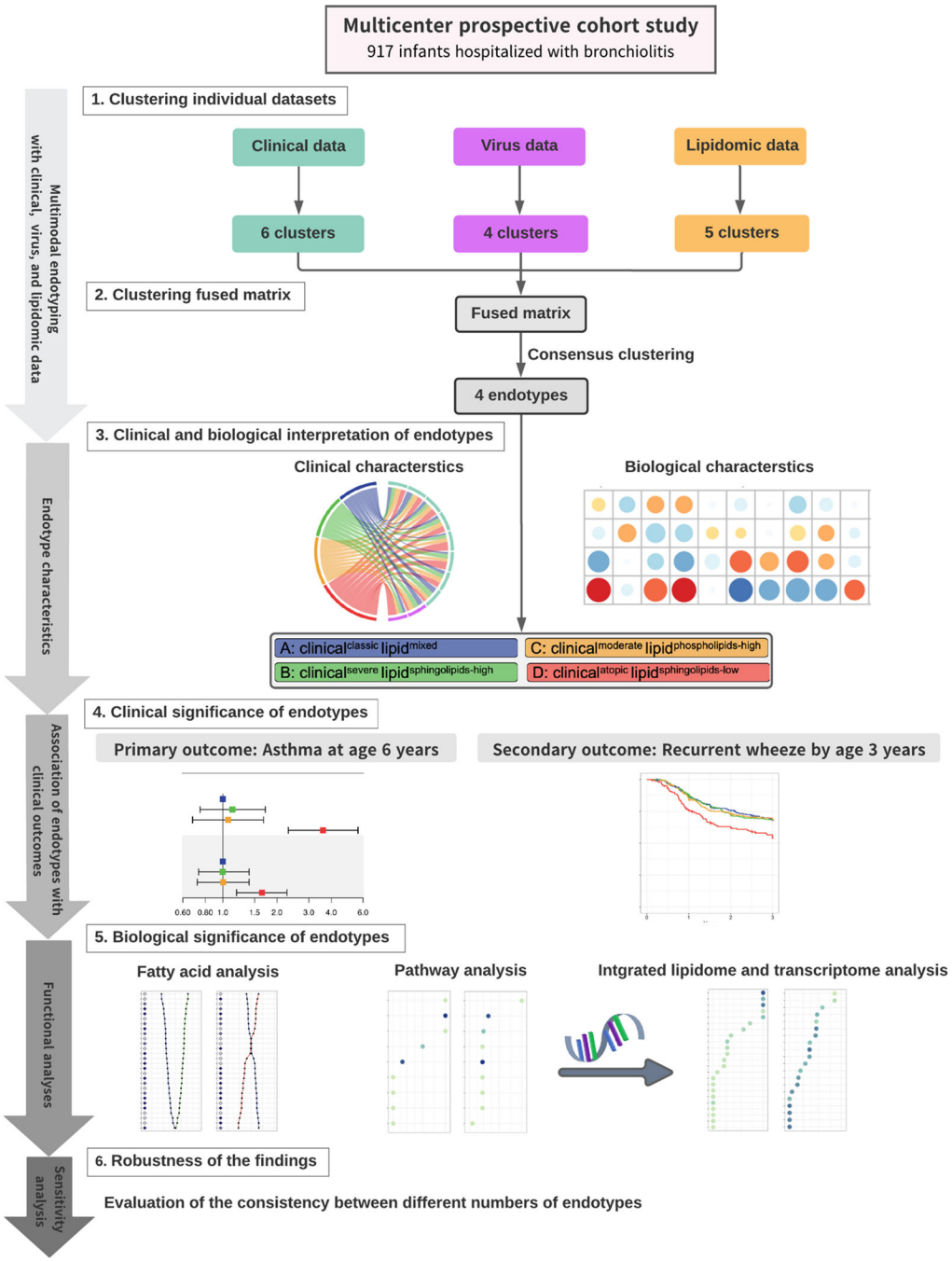

Figure 1. Analytic workflow of lipidomic endotyping

The analytic cohort consists of 917 infants hospitalized for bronchiolitis in a multicenter prospective cohort study—MARC-35. At enrolment, the nasopharyngeal airway specimens were collected for lipidomic and transcriptomic profiling.

1. Clustering individual datasets: We first computed a distance matrix and identified mutually exclusive clusters for each of the clinical, virus, and lipidomic datasets by applying consensus clustering algorithms.

2. Clustering fused matrix: By integrating these derived clusters from three datasets, we generated a fused matrix and computed a Gower distance. By applying a consensus clustering algorithm to the fused matrix, we identified four mutually exclusive lipidomic endotypes. To choose an optimal number of profiles, we used a combination of consensus matrix, consensus cumulative distribution function, cluster consensus value, endotype size, and clinical and biological plausibility.

3. Examining clinical and biological characteristics of endotypes: To interpret the lipidomic endotypes clinically and biologically, we developed chord diagrams on major clinical and virus variables and a heatmap of 14 major lipid classes.

4. Determining clinical significance of endotypes: To examine clinical significance of the endotypes, we determined the longitudinal relationship of the lipidomic profiles with the risk for developing asthma (the primary outcome) and recurrent wheeze (the secondary outcome). We constructed logistic regression models for asthma development and Cox proportional hazards models for recurrent wheeze.

5. Investigating the biological significance of each endotype: We conducted three analyses: i) between-endotype examinations of fatty acids, ii) metabolic pathway analysis, and iii) *integrated* transcriptomic-lipidomic pathway analysis.

6. Examining the robustness of the findings: In the sensitivity analysis, we also examined the concordance between different numbers of lipidomic endotypes.

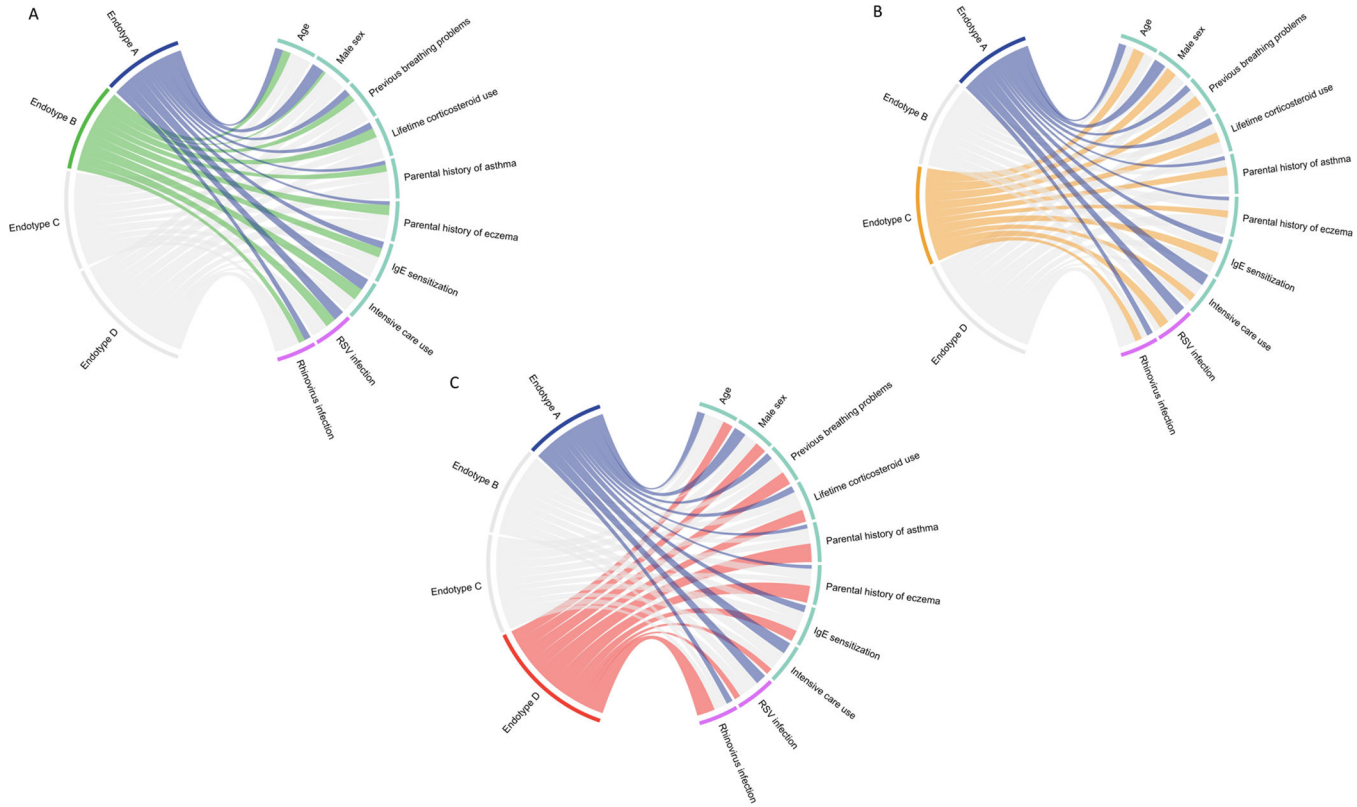

Figure 2. Major clinical and virus characteristics according to the lipidomic endotypes among infants hospitalized for bronchiolitis

The ribbons connect from the individual lipidomic profiles to the major clinical and virus characteristics. The width of the ribbon represents the proportion of infants within the profile who have the corresponding clinical or virus characteristic, which was scaled to a total of 100%. For example, in panel C, the endotype A infants (blue) had a high proportion of males, a low proportion of previous breathing problems, parental history of asthma and eczema, and IgE sensitization, and a high proportion of RSV infection. In contrast, endotype D (red) infants had a high proportion of previous breathing problems, parental history of asthma and eczema, and rhinovirus infection.

Abbreviations: IgE, immunoglobulin E; RSV, respiratory syncytial virus

A. Comparison of endotype A (blue) with endotype B (green)

B. Comparison of endotype A (blue) with endotype C (orange)

C. Comparison of endotype A (blue) with endotype D (red)

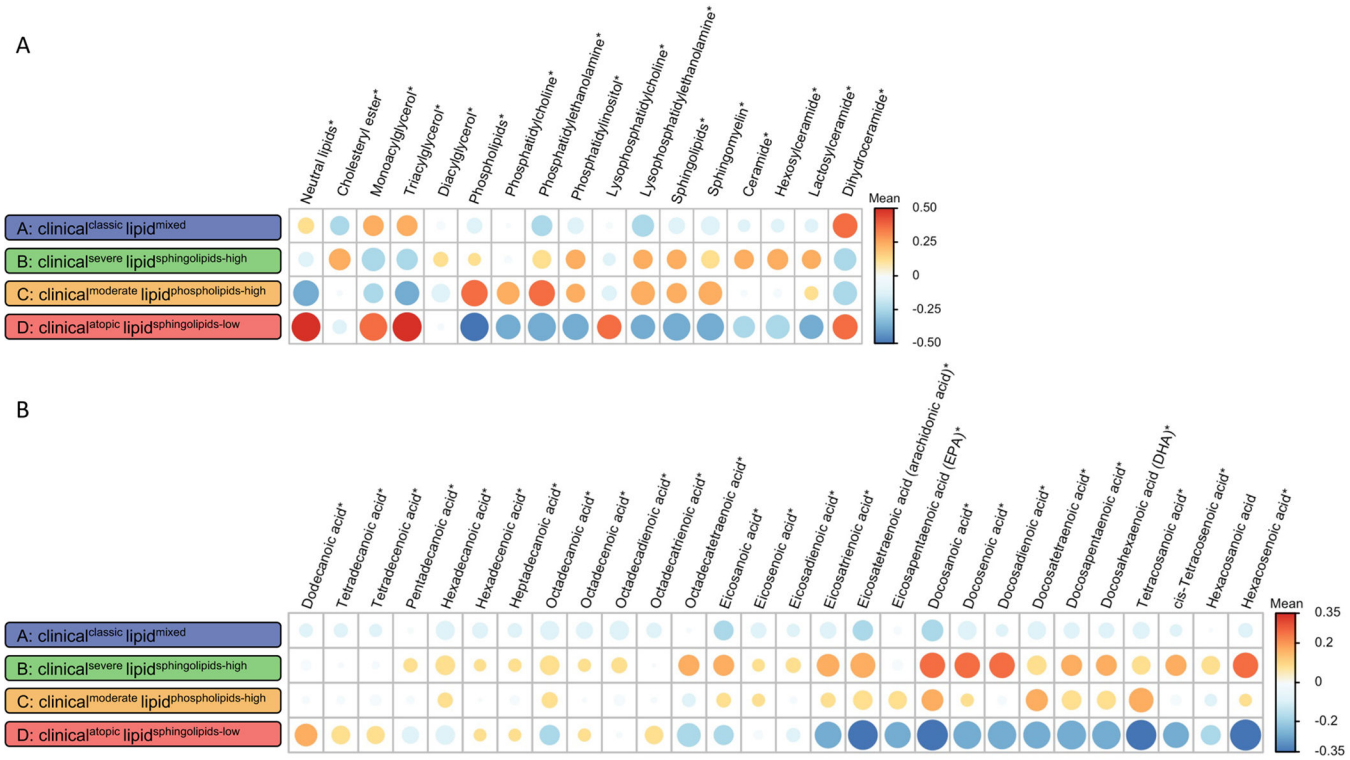

Figure 3. Lipid classes and fatty acids according to the lipidomic endotypes among infants hospitalized for bronchiolitis

Heatmaps show the mean values for the corresponding **A)** lipid classes and **B)** fatty acids in each of the four lipidomic endotypes. The areas of circles and colors represent the mean value of the corresponding value. Each variable is standardized by using auto-scaling. The between-endotype differences were examined by the Kruskal-Wallis test.

* False discovery rate <0.05

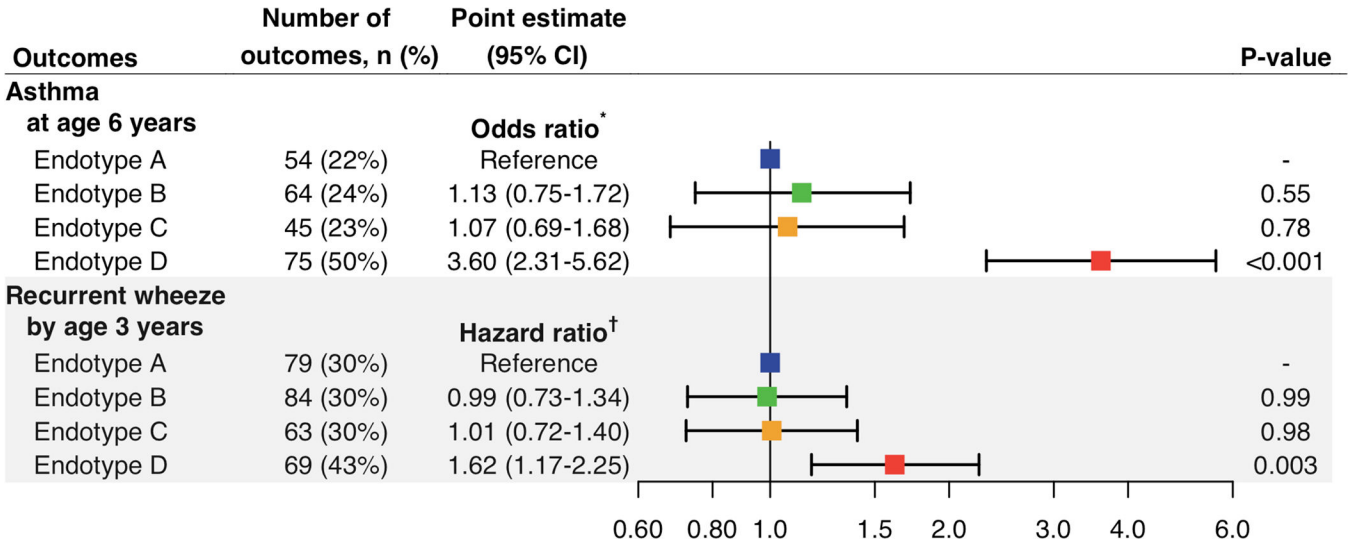

Figure 4. Association of the lipidomic endotypes among infants hospitalized for bronchiolitis with the risk for developing asthma and recurrent wheeze

* To examine the association of the lipidomic endotypes (endotype A as the reference) with the risk for developing asthma, a mixed-effects logistic regression model accounting for potential patient clustering within the hospitals was fit.

† To examine the association between the endotypes (endotype A as the reference) and the rate of recurrent wheeze, a mixed-effects Cox proportional hazards model was constructed.

Abbreviation: CI, confidence interval

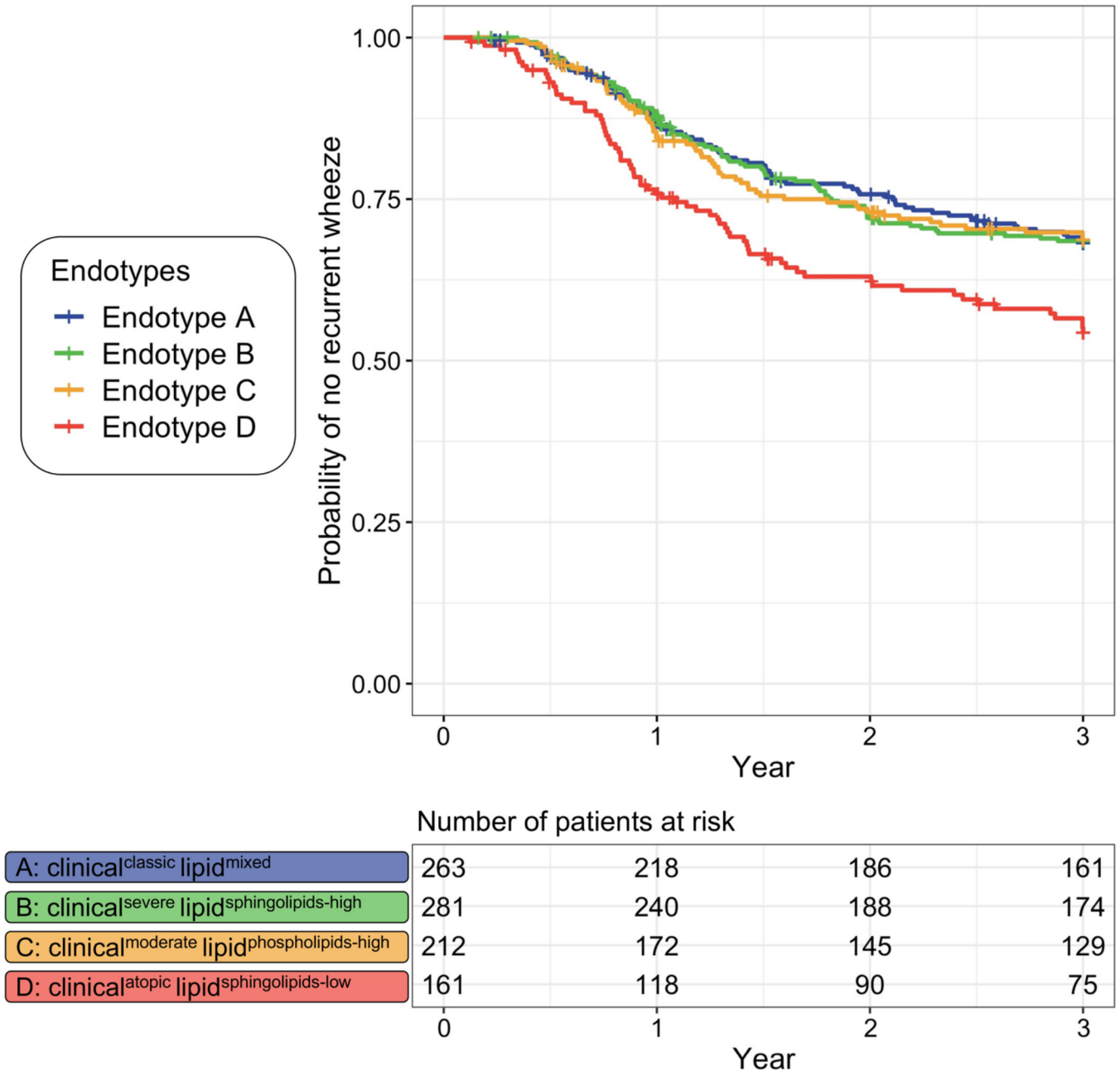

Figure 5. Kaplan-Meier curves for development of recurrent wheeze by age three years, according to the lipidomic endotypes, among infants hospitalized for bronchiolitis

Overall, the survival curves significantly differed between the endotypes ($P_{log\text{-}rank}$=0.01). Compared with endotype A infants (clinical^{classic}lipid^{mixed}), the rate of developing recurrent wheeze by age three years was not significantly different in endotype B or endotype C infants. In contrast, the rate was significantly higher in endotype D (clinical^{atopic}lipid$^{sphingolipids\text{-}low}$) infants (HR 1.65; 95% CI 1.19–2.27; P=0.003). The corresponding hazard ratio estimates are presented in Figure 4.

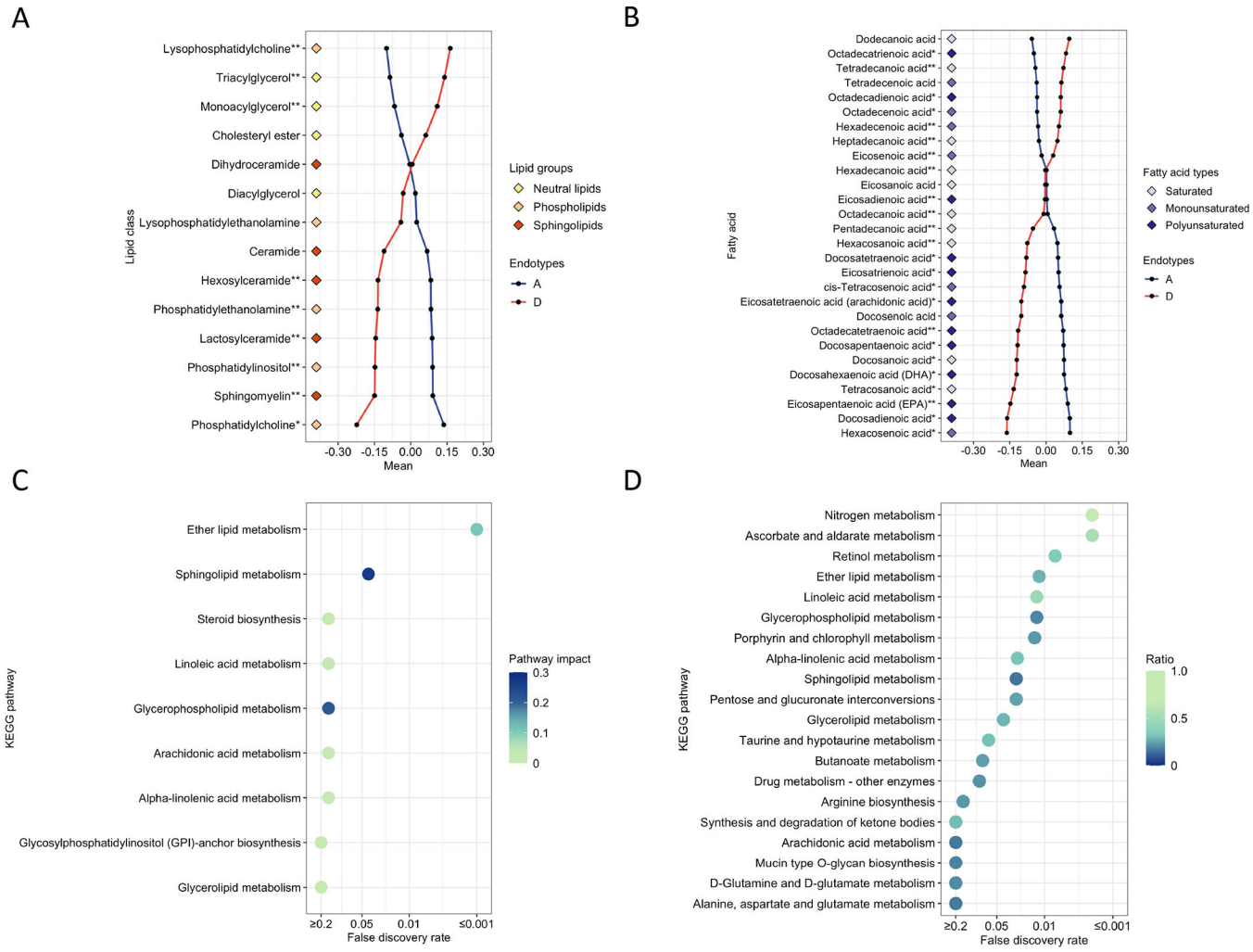

Figure 6. Between-endotype differences (A vs. D) in lipidomic profiles and pathways among infants hospitalized for bronchiolitis

A) Lipid classes: The mean values for the corresponding lipid classes in each of lipidomic endotypes (A vs. D) are plotted. Each lipid class is standardized by using auto-scaling. The between-endotype differences were examined by Wilcoxon rank-sum test.

B) Fatty acids: The mean values for the corresponding fatty acids in each of lipidomic endotypes (A vs. D) are plotted. Each fatty acid is standardized by using auto-scaling. The between-endotype differences were examined by Wilcoxon rank-sum test.

C) Metabolic pathway analysis: In the metabolite pathway analysis, all detected pathways (based on the Kyoto Encyclopedia of Genes and Genomes [KEGG]) are shown. The color of each dot represents the pathway impact. The pathway impact is calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway [59].

D) Integrated transcriptomic-lipidomic pathway analysis: In the integrated transcriptomic-lipidomic pathway analysis, 20 pathways (based on KEGG) with the lowest FDRs are selected. The color of each dot represents the ratio of hit lipids and genes for the corresponding pathways.

* False discovery rate (FDR) of <0.05 and ** FDR of ≤0.10 in panels A and B.
Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GPI, glycosylphosphatidylinositol; KEGG, Kyoto Encyclopedia of Genes and Genome

Table 1.

Baseline characteristics and clinical course of infants hospitalized for bronchiolitis, according to l 1 ipidomic endotypes

Characteristics	Overall (n=917; 100%)	Endotype A (n=263; 29%)	Endotype B (n=281; 31%)	Endotype C (n=212; 23%)	Endotype D (n=161;18%)	P-value
Demographics						
Age (month), median (IQR)	3.2 (1.6–6.0)	2.7 (1.5–5.1)	2.9 (1.5–5.3)	4.4 (2.4–6.7)	3.5 (1.8–6.3)	<0.001
Male sex	550 (60)	216 (82)	55 (20)	155 (73)	124 (77)	<0.001
Race/ethnicity						0.08
Non-Hispanic white	399 (44)	107 (41)	125 (45)	101 (48)	66 (41)	
Non-Hispanic black	209 (23)	58 (22)	70 (25)	36 (17)	45 (28)	
Hispanic	275 (30)	82 (31)	77 (27)	71 (34)	45 (28)	
Other or unknown	34 (4)	16 (6)	9 (3)	4 (2)	5 (3)	
Prematurity (32–37 weeks)	171 (19)	54 (21)	50 (18)	37 (18)	30 (19)	0.81
Birth weight (kg), median (IQR)	3.26 (2.90–3.60)	3.30 (2.89–3.60)	3.20 (2.89–3.52)	3.39 (2.98–3.63)	3.24 (2.83–3.59)	0.04
Previous breathing problems * (count)						0.01
0	731(80)	222 (84)	233 (83)	164 (77)	112 (70)	
1	145 (16)	33 (13)	36 (13)	37 (18)	39 (24)	
≥2	41 (5)	8 (3)	12 (4)	11 (5)	10 (6)	
History of eczema	137 (15)	38 (14)	32 (11)	31 (15)	36 (22)	0.02
Lifetime antibiotic use	293 (32)	20 (8)	67 (24)	168(79)	38 (24)	<0.001
Lifetime corticosteroid use	138 (15)	28 (11)	44 (16)	34 (16)	32 (20)	0.07
Ever attended daycare	210 (23)	48 (18)	61 (22)	58 (27)	43 (27)	0.07
Cigarette smoke exposure at home	137 (15)	38 (14)	40 (14)	32 (15)	27 (17)	0.90
Maternal smoking during pregnancy	126 (14)	32 (12)	36 (13)	28 (13)	30 (19)	0.24
Maternal use of fish oil (at least once / week)	90 (10)	29 (11)	25 (9)	19 (9)	17 (11)	0.80
Parental history of asthma	303 (33)	41 (16)	77 (27)	69 (33)	116(72)	<0.001
Parental history of eczema	174 (19)	21 (8)	62 (22)	32 (15)	59 (37)	<0.001
Clinical presentation at index hospitalization						
Body weight (kg), median (IQR)	6.1 (4.8–7.7)	5.7 (4.7–7.2)	5.5 (4.5–7.3)	7.0 (5.3–8.3)	6.4 (5.2–8.0)	<0.001
Respiratory rate (per minute), median (IQR)	48 (40–60)	52 (40–60)	50 (40–60)	48 (40–58)	48 (40–60)	0.11
Oxygen saturation at presentation						0.18

Characteristics	Overall (n=917; 100%)	Endotype A (n=263; 29%)	Endotype B (n=281; 31%)	Endotype C (n=212; 23%)	Endotype D (n=161;18%)	P-value
<90%	85 (9)	22 (8)	29 (10)	24 (11)	10 (6)	
90–93%	135 (15)	39 (15)	44 (16)	36 (17)	16 (10)	
≥94%	677 (74)	196 (75)	203 (72)	146 (69)	132 (82)	
Laboratory data						
Blood eosinophilia (≥300 mcL)	129 (14)	29 (18)	45 (21)	25 (17)	30 (25)	0.32
IgE sensitization †	181 (20)	38 (14)	57 (20)	48 (23)	38 (24)	0.06
Clinical course						
Intensive care use ‡	140 (15)	47 (18)	52 (19)	25 (12)	16 (10)	0.03
Positive pressure ventilation use	49 (5)	16 (6)	20 (7)	8 (4)	5 (3)	0.20
Length of day ≥3 days	367 (40)	111 (42)	125 (45)	82 (40)	49 (30)	0.03
Phenotypes §						<0.001
Profile A	93 (10)	14 (5)	17 (6)	13 (6)	49 (30)	
Profile B	322 (35)	96 (37)	88 (31)	82 (39)	56 (35)	
Profile C	331 (36)	108 (41)	115 (41)	73 (3)4	35 (22)	
Profile D	171 (19)	45 (17)	61 (22)	44 (21)	21(13)	
Respiratory virus						
Any RSV	749 (82)	236 (90)	252 (90)	180 (85)	81 (50)	<0.001
Any rhinovirus	184(20)	39 (15)	42 (15)	35 (17)	68 (42)	<0.001
Other pathogen //	209 (23)	43 (16)	48 (17)	55 (26)	63 (39)	<0.001
Adenovirus	44 (5)	8 (3)	10 (4)	18 (9)	8 (5)	0.03
Bocavirus	46 (5)	10 (4)	14 (5)	11 (5)	11 (7)	0.59
Bordetella pertussis	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	0.18
Enterovirus	4 (4)	1 (<1)	1 (<1)	2 (1)	0 (0)	0.72
Human coronavirus (NL63, OC43, 229E, HKU1)	57 (6)	13 (5)	13 (5)	13 (6)	18 (11)	0.049
Human metapneumovirus	50 (6)	8 (3)	11 (4)	11 (5)	20 (12)	<0.001
Influenza A or B virus	13 (1)	4 (2)	4 (1)	3 (1)	2 (1)	0.99
Mycoplasma pneumoniae	11 (1)	3 (1)	3 (1)	3 (1)	2 (1)	0.99
Parainfluenza virus 1–3	26 (3)	4 (2)	5 (2)	8 (4)	9 (6)	0.06

Abbreviations: IQR, interquartile range; IgE, immunoglobulin E; RSV, respiratory syncytial virus.

Data are n (%) of infants unless otherwise indicated. Percentages may not equal 100, because of missing data.

To test for the between-endotype difference, χ^2, Fisher's exact, or Kruskal-Wallis test was used, as appropriate.

*Breathing problem is defined by cough (that wakes the infant up at night), wheezing, and/or dyspnea.

†Defined by having one or more positive values for allergen-specific IgE.

‡Defined as use of invasive and/or non-invasive positive pressure ventilation (e.g., continuous positive airway pressure ventilation) and/or intensive care unit admission.

§Bronchiolitis phenotypes were identified in a previous study [11]. Profile A was characterized by a history of breathing problems, allergic predisposition, and rhinovirus (non-RSV) infection, and had a significantly higher risk for asthma. Profile B resembled "classic" RSV-induced bronchiolitis. Profile C was comprised of the most severely ill group. Profile D was comprised of the least-ill group.

//Adenovirus, bocavirus, *Bordetella pertussis,* enterovirus, human coronavirus NL63, OC43, 229E, or HKU1, human metapneumovirus, influenza A or B virus, *Mycoplasma pneumoniae,* and parainfluenza virus 1–3. As 33 infants had co-infection with ≥2 infecting agents, the total number is not equal to 209.