

Characteristics and Mechanisms of a Sphingolipid-associated Childhood Asthma Endotype

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Abstract

Rationale: A link among sphingolipids, 17q21 genetic variants, and childhood asthma has been suggested, but the underlying mechanisms and characteristics of such an asthma endotype remain to be elucidated.

Objectives: To study the sphingolipid-associated childhood asthma endotype using multiomic data.

Methods: We used untargeted liquid chromatography–mass spectrometry plasma metabolomic profiles at the ages of 6 months and 6 years from more than 500 children in the COPSAC₂₀₁₀ (Copenhagen Prospective Studies on Asthma in Childhood) birth cohort focusing on sphingolipids, and we integrated the 17q21 genotype and nasal gene expression of SPT (serine palmitoyl-CoA transferase) (i.e., the rate-limiting enzyme in *de novo* sphingolipid synthesis) in relation to asthma development and lung function traits from infancy until the age 6 years. Replication was sought in the independent VDAART (Vitamin D Antenatal Asthma Reduction Trial) cohort.

Measurements and Main Results: Lower concentrations of ceramides and sphingomyelins at the age of 6 months were associated with an increased risk of developing asthma before age 3, which was also observed in VDAART. At the age of 6 years, lower concentrations of key phosphosphingolipids (e.g., sphinganine-1-phosphate) were associated with increased airway resistance. This relationship was dependent on the 17q21 genotype and nasal SPT gene expression, with significant interactions occurring between the genotype and the phosphosphingolipid concentrations and between the genotype and SPT expression, in which lower phosphosphingolipid concentrations and reduced SPT expression were associated with increasing numbers of at-risk alleles. However, the findings did not pass the false discovery rate threshold of <0.05.

Conclusions: This exploratory study suggests the existence of a childhood asthma endotype with early onset and increased airway resistance that is characterized by reduced sphingolipid concentrations, which are associated with 17q21 genetic variants and expression of the SPT enzyme.

Keywords: sphingolipids; childhood asthma; lung function

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At a Glance Commentary

Scientific Knowledge on the

Subject: Experimental studies have suggested that sphingolipid synthesis is implicated in asthma pathogenesis through genetically regulated activity of SPT (serine palmitoyl-CoA transferase) (i.e., the rate-limiting enzyme in *de novo* sphingolipid synthesis). Recently, a study of school-aged children with asthma showed a lower amount of sphingolipid formation, in which 17q21 asthma risk variants were shown to decrease sphingolipid synthesis. These findings suggest that sphingolipid synthesis is affected genetically in childhood asthma, but the underlying mechanisms and clinical characteristics remain to be elucidated.

What This Study Adds to the Field:

This exploratory study consisting of a discovery cohort and an independent replication cohort used metabolomic data to investigate sphingolipid concentrations in early life and integrated 17q21 genotype data and nasal SPT gene expression data to study the development of childhood asthma and lung function traits. The findings suggest the existence of a sphingolipid-associated childhood asthma endotype with an early onset of symptoms and increased airway resistance by the age of 6 years, which is characterized by a reduction in sphingolipid concentrations that is already present in infancy and is associated with 17q21 genetic variants and expression of the SPT enzyme.

Asthma is a chronic inflammatory airway disease that has increased in prevalence among children in Westernized societies (1). The disease is believed to originate in early life from gene–environment interactions (2) that lead to the onset of chronic inflammatory processes, which manifest as intermittent or more

persistent troublesome respiratory symptoms later in childhood (3, 4). It may therefore be useful to understand the perturbed pathways and biochemical mechanisms involved in early life before the debut of symptoms to aid in both primary and secondary prevention.

Metabolomic data are suitable for studying early-onset disease mechanisms because they represent a snapshot of the ongoing biological pathways and metabolic status of an individual, which enables uncovering subtle phenotype differences and perturbed metabolic pathways that are essential for biomarker discovery and development of novel treatment modalities. Metabolomics has previously been applied in epidemiological studies of asthma in children and adults (5, 6), showing alterations in lipids, steroids, amino acids, bile acids, and metabolites related to immune responses and oxidative stress (5, 7–13). Longitudinal birth cohort studies with metabolomic data are particularly well suited to investigating the early-life origins of asthma, especially in the context of using discovery and replication cohorts.

A particular class of lipids containing a backbone of sphingoid bases (i.e., the sphingolipids), which includes phosphosphingolipids, ceramides, and sphingomyelins, has gained attention as an important player in asthma pathogenesis (14). A recent study showed decreased sphingolipid concentrations in children 5–17 years of age with nonallergic asthma, in whom 17q21 asthma risk variants were shown to decrease *de novo* sphingolipids synthesis (15). Furthermore, we previously found that high-dose vitamin D supplementation during pregnancy in VDAART (Vitamin D Antenatal Asthma Reduction Trial) was associated with increased plasma concentrations of sphingosine-1-phosphate in children with the low-risk 17q21 genotype at the age of 3 years. Furthermore, higher sphingolipid concentrations were associated with a decreased risk of asthma, with evidence of

interactions among the sphingosine-1-phosphate concentration, the vitamin D intervention, and the 17q21 genotype (16). These findings suggest the existence of a sphingolipid-associated childhood asthma endotype, but the underlying mechanisms and characteristics of such an endotype remain to be elucidated.

Here, we performed global metabolomic profiling of plasma samples from children in the population-based COPSAC₂₀₁₀ (Copenhagen Prospective Studies on Asthma in Childhood) mother–child cohort at the ages of 6 months and 6 years and investigated the association with the development of asthma and lung function traits to obtain mechanistic insights into asthma pathogenesis, focusing on the sphingolipid pathway. We integrated 17q21 genotype data and gene expression data for the SPT (serine palmitoyl transferase) enzyme to explore the regulation of the biochemical findings. Replication was sought in the independent VDAART birth cohort (17).

Methods

Study Populations

COPSAC₂₀₁₀. The COPSAC₂₀₁₀ population-based, mother–child cohort of 738 pregnant women and their 700 children has previously been described in detail (18). The pregnant women participated in two nested, double-blind, randomized control trials (RCTs) with n-3 long-chain polyunsaturated fatty acids and high-dose vitamin D. The study was approved by The National Committee on Health Research Ethics (H-B-2008-093) and the Danish Data Protection Agency (2015-41-3696), with oral and written consent being provided by parents before enrollment. The details and results of the RCTs have been previously published (19, 20).

VDAART. The VDAART mother–child cohort of 881 women with a

Data sharing statement: The COPSAC (Copenhagen Prospective Studies on Asthma in Childhood) biobank is publicly available at the Danish National Biobank (www.biobankdenmark.dk), and data will become available in the Danish Data Archive (www.sa.dk) upon request to the corresponding author.

COPSAC (Copenhagen Prospective Studies on Asthma in Childhood) is funded by private and public research funds, which are all listed on www.copsac.com. The Lundbeck Foundation, Danish State Budget, Danish Council for Strategic Research, Danish Council for Independent Research, and The Capital Region Research Foundation have provided core support for COPSAC. The study is further supported by the following NIH grants: R01 HL129735 and R01 HL141826. C.E.W. was supported by the Swedish Heart–Lung Foundation (Hjärt–Lungfonden 20170734; 20180290). This project has received funding from the European Research Council under the European Union’s Horizon 2020 research and innovation program (grant agreement No. 946228).

history of asthma, eczema, or allergic rhinitis and their 810 children also has an embedded RCT of high-dose vitamin D supplementation during pregnancy (17). The children were prospectively followed for asthma/recurrent wheezing until age 3. Plasma samples from a subset of children at the age of 1 year were used for metabolomic profiling analysis by Metabolon, Inc., and were used for replication (16).

Data Collection

Clinical outcomes. The COPSAC₂₀₁₀ children were followed prospectively for asthma/recurrent wheezing, lower respiratory tract infections, wheezing episodes, and acute severe exacerbations until the age of 6 years, at which time spirometry (FEV₁, FEV₁/FVC ratio), whole-body plethysmography (specific airway resistance [sRaw]), and bronchial responsiveness to methacholine (PD₂₀) were measured. In addition, the parents filled a daily diary from birth, recording troublesome lung symptoms and use of antiasthmatic treatments.

Metabolomics. Blood samples for plasma metabolomic analysis were collected in ethylenediaminetetraacetic acid tubes at the scheduled clinic visits at 6 months of age and 6 years of age. The sample extracts were analyzed using four liquid chromatography–mass spectrometry methods: two separated reverse-phase ultraperformance liquid chromatography electrospray ionization tandem mass spectrometry methods optimized for hydrophilic and hydrophobic compounds, one reverse-phase ultraperformance liquid chromatography tandem mass spectrometry method using basic optimized conditions, and one hydrophilic interaction liquid chromatography/ultraperformance liquid chromatography tandem mass spectrometry method. The metabolomic analysis of the plasma samples was performed by Metabolon, Inc., using their HD4 platform, which was also applied to the VDAART samples. The identification level follows the criteria described by Sumner and colleagues (21).

Genotyping. Variation in the 17q21 region was assessed by genotyping the SNPs rs12936231, rs2305480, rs4065275, and rs7216389 using the Illumina Infinium HumanOmniExpressExome BeadChip Kit

at the AROS Applied Biotechnology A/S center.

Gene expression. Nasal-brushing transcriptomic data at 6 years of age (22) were generated from RNA extracted using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio Inc.), and cDNA libraries were created with the Illumina Nextera XT kit using the default instructions. The Agilent 2100 Bioanalyzer was used to determine concentrations. FastQC and MultiQC (Babraham Bioinformatics) were used to assess the read quality. The RNA-sequencing reads were mapped to the genome by using STAR (version 2.5.1) aligner software (23).

Data Analysis

The COPSAC₂₀₁₀ 6-month metabolomic data set included 577 children and 1,068 metabolites, whereas the 6-years data set comprised 513 children and 1,076 metabolites. Missing values were imputed metabolite-wise with half of the minimum value, and data were autoscaled before analysis. Data preprocessing is detailed in the online supplement.

Univariate regression analyses, consisting of linear regression, Cox proportional hazards regression, and logistic and quasi-Poisson modeling, were employed to relate the metabolite levels at 6 months of age and 6 years of age with the clinical outcomes by applying a multiple-testing false discovery rate (FDR) < 0.05 significance threshold. Metabolites in the sphingolipid pathway in COPSAC₂₀₁₀ were sought and replicated in VDAART in terms of the direction of association and significance at an FDR < 0.05 level.

Supervised multivariate analysis, specifically partial least squares discriminant analysis (PLS-DA), was employed to explore the association between the metabolite concentrations and sRaw. The sRaw measurements were split into quartiles and used for the analysis as a two-class problem comparing the lower and the upper quartile.

All models were adjusted for breastfeeding duration, z-scored child body mass index, and the pregnancy n-3 long-chain polyunsaturated fatty acid and high-dose vitamin D interventions.

Further details are outlined in the online supplement.

Results

Baseline characteristics and clinical outcome measures of the children with available plasma metabolomic profiling data by the ages of 6 months ($n = 577$) and 6 years ($n = 513$) are shown in Table E1 in the online supplement.

Metabolomic Profile at the Age of 6 Months and Clinical Outcomes

The plasma metabolomic profile at the age of 6 months was analyzed in relation to development of an early-onset asthma phenotype (i.e., symptom debut in the first 3 years of life) and in relation to asthma status by the age of 6 years (24). Using Cox regression analysis of asthma development until 3 years of age, we observed 150 out of 1,076 metabolites passing nominal significance ($P \leq 0.05$), with 27 of these 150 metabolites being sphingolipids. The nominally significant sphingolipids were ceramides and sphingomyelins containing long-chain saturated and monounsaturated fatty acids, and they were negatively associated with asthma development (i.e., lower concentrations increased the risk of developing early-onset asthma). However, neither of the sphingolipids passed a multiple-testing FDR < 0.05 threshold (Table 1). The sphingolipid that was most strongly associated with asthma was the ceramide glycosyl-*N*-stearoyl-sphingosine (d18:1/18:0): β -estimate, -0.52 (95% confidence interval [CI], -0.78 to -0.25), $P < 0.001$, FDR = 0.09 (see Kaplan-Meier curve in Figure 1).

In the VDAART plasma metabolomic data set for the age 1 year ($n = 469$), 23 of the 27 sphingolipids that were nominally significant in COPSAC₂₀₁₀ were detected. As shown in Table 1, 4 out of the 23 sphingolipids were significantly associated with development of asthma/wheezing before the age of 3 years ($P \leq 0.05$), and a further 4 showed a trend of association ($P \leq 0.10$). All of these sphingolipids showed the same direction of association as in COPSAC₂₀₁₀ (i.e., increasing risk with lower concentrations). The sphingolipids included the ceramide glycosyl-*N*-stearoyl-sphingosine (d18:1/18:0) and seven sphingomyelins, including stearoyl sphingomyelin (d18:1/18:0), sphingomyelin (d18:1/20:1, d18:2/20:0)*,

Table 1. Sphingolipids at the Age of 6 Months Associated with Early-Onset Asthma Development before 3 Years of Age: Discovery in COPSAC₂₀₁₀ and Replication in VDAART

Metabolite	Subpathway	β -Estimate for COPSAC ₂₀₁₀	CI	P Value	FDR P Value	β -Estimate for VDAART	P Value	FDR P Value
Glycosyl- <i>N</i> -stearoyl-sphingosine (d18:1/18:0)	Ceramides	-0.516	-0.777 to -0.254	0.0001	0.09	-0.598	0.03	0.93
Glycosyl ceramide (d18:1/20:0, d16:1/22:0)*		-0.448	-0.687 to -0.208	0.0003	0.24	-0.409	0.15	0.93
Glycosyl- <i>N</i> -behenoyl-sphingadienine (d18:2/22:0)*		-0.319	-0.558 to -0.081	0.009	1	—	—	—
Glycosyl- <i>N</i> -(2-hydroxynervonoyl)-sphingosine (d18:1/24:1)*		-0.454	-0.726 to -0.182	0.001	1	—	—	—
<i>N</i> -stearoyl-sphingosine (d18:1/18:0)*		-0.282	-0.515 to -0.048	0.02	1	-0.115	0.50	0.95
Ceramide (d16:1/24:1, d18:1/22:1)*		-0.225	-0.437 to -0.012	0.04	1	—	—	—
Sphingomyelin (d18:1/19:0, d19:1/18:0)*	Sphingomyelins	-0.428	-0.647 to -0.21	0.0001	0.12	-0.286	0.13	0.93
Sphingomyelin (d18:2/21:0, d16:2/23:0)*		-0.421	-0.643 to -0.199	0.0002	0.19	-0.290	0.17	0.93
Stearoyl sphingomyelin (d18:1/18:0)		-0.391	-0.61 to -0.172	0.0005	0.41	-0.951	0.02	0.93
Sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)		-0.368	-0.603 to -0.133	0.002	1	-0.526	0.06	0.93
Sphingomyelin (d18:1/20:0, d16:1/22:0)*		-0.330	-0.568 to -0.092	0.007	1	-0.333	0.38	0.93
Sphingomyelin (d18:1/20:1, d18:2/20:0)*		-0.293	-0.505 to -0.081	0.007	1	-1.487	0.005	0.71
Sphingomyelin (d18:1/18:1, d18:2/18:0)		-0.311	-0.541 to -0.08	0.008	1	-0.994	0.02	0.93
Sphingomyelin (d18:2/18:1)*		-0.303	-0.53 to -0.075	0.009	1	-0.622	0.08	0.93
Sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)*		-0.273	-0.482 to -0.064	0.01	1	-0.449	0.17	0.93
Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)*		-0.269	-0.477 to -0.062	0.01	1	-0.248	0.35	0.93
Sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)*		-0.307	-0.556 to -0.057	0.02	1	-0.168	0.35	0.93
Sphingomyelin (d17:2/16:0, d18:2/15:0)*		-0.276	-0.505 to -0.047	0.02	1	-0.053	0.76	0.98
Sphingomyelin (d18:2/23:1)*		-0.266	-0.491 to -0.042	0.02	1	-0.247	0.33	0.93
<i>N</i> -palmitoyl-sphinganine (d18:0/16:0)		0.219	0.032 to 0.407	0.02	1	-0.146	0.32	0.93
Sphingomyelin (d18:1/14:0, d16:1/16:0)*		-0.253	-0.471 to -0.036	0.02	1	-0.111	0.70	0.97
Sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*		-0.245	-0.456 to -0.034	0.02	1	-0.443	0.40	0.93
Sphingomyelin (d18:0/20:0, d16:0/22:0)*		-0.266	-0.501 to -0.031	0.03	1	-0.269	0.19	0.93
Sphingomyelin (d18:1/20:2, d18:2/20:1, d16:1/22:2)*		-0.232	-0.449 to -0.016	0.04	1	-0.417	0.14	0.93
Sphingomyelin (d18:0/18:0, d19:0/17:0)*		-0.266	-0.519 to -0.013	0.04	1	-0.275	0.07	0.93
Palmitoyl sphingomyelin (d18:1/16:0)		-0.210	-0.413 to -0.007	0.04	1	-1.301	0.06	0.93
Sphingomyelin (d18:1/25:0, d19:0/24:1, d20:1/23:0, d19:1/24:0)*	-0.245	-0.488 to -0.001	0.05	1	-0.208	0.14	0.93	

Definition of abbreviations: CI = confidence interval; COPSAC₂₀₁₀ = Copenhagen Prospective Studies on Asthma in Childhood; FDR = false discovery rate; VDAART = Vitamin D Antenatal Asthma Reduction Trial.

sphingomyelin (d18:1/18:1, d18:2/18:0), sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0), sphingomyelin (d18:0/18:0, d19:0/17:0)*, sphingomyelin (d18:2/18:1)*, and

palmitoyl sphingomyelin (d18:1/16:0). However, none of these sphingolipids were replicated in VDAART at a multiple-testing FDR < 0.05 threshold.

Apart from sphingolipids, homocitrulline showed a strong positive association with development of asthma before the age of 3 years in COPSAC₂₀₁₀

(β -estimate, 0.28 [95% CI, 0.16–0.4], $P < 0.001$, FDR = 0.01), but homocitrulline was not associated with early-onset asthma/wheezing in VDAART ($P = 0.99$, FDR = 1).

There was no association between the metabolome at the age of 6 months and asthma by the age 6 of years and the number of wheezing episodes, lower respiratory tract infections, acute exacerbations of wheezing, or any of the lung function outcomes (see Figure E1).

Metabolomic Profile at 6 Years and Clinical Outcomes

The plasma metabolomic profiles at the age of 6 years were investigated in relation to asthma status and lung function measurements at the same time point.

The univariate cross-sectional analyses of the association between the metabolome and asthma at 6 years showed no significant associations (Figure E2). Furthermore, in a Spearman correlation analysis, the plasma concentrations of the 8 sphingolipids at the age of 6 months, which showed similar results in relation to early-onset asthma in COPSAC₂₀₁₀ and VDAART, were not correlated with the corresponding plasma concentrations at the age of 6 years (Table E2).

Among the lung function measurements, the univariate analyses showed significant associations for sRaw but not for FEV₁, the FEV₁/FVC ratio, or the PD₂₀ of methacholine (Figure E3). For sRaw ($n = 495$), a total of 73 metabolites were nominally significant ($P \leq 0.05$), with 2 of these being sphingolipids. The significant sphingolipids were among the top six metabolites most strongly associated with sRaw in the univariate linear models.

They were both phosphosphingolipids and showed an inverse association, with lower concentrations resulting in increasing airway resistance (sphinganine-1-phosphate: β -estimate, -0.04 [95% CI, -0.06 to -0.02], $P < 0.001$, FDR = 0.16; sphingosine-1-phosphate: β -estimate, -0.03 [95% CI, -0.05 to -0.01], $P = 0.002$, FDR = 1).

To validate the univariate findings, a multivariate PLS-DA model was applied to investigate the association between the overall patterns of metabolites and sRaw, which was analyzed by comparing children with an sRaw measurement in the first and fourth quartiles. The final PLS-DA model consisted of two components and 37 metabolites, had a cross-validated area under the curve = 0.85, a cross-validated classification error = 0.23, and a P value = 0.005 (based on 100 permutations) for separating children with low versus high sRaw (Figures 2 and E4). The PLS-DA model showed results comparable to those of the univariate analyses, with lower amounts of the phosphosphingolipids sphinganine-1-phosphate and sphingosine-1-phosphate in children with higher sRaw.

sRaw, Phosphosphingolipids, 17q21 Genotype, and Nasal Gene Expression of SPT

It has been shown that 17q21 SNPs associated with childhood asthma outcomes are linked to an increased transcription of *ORMDL3* (25, 26), which is believed to affect the enzyme activity of SPT in *de novo* sphingolipid synthesis, particularly involving phosphosphingolipids. Therefore, we investigated this relationship using four

SNPs in the 17q21 region previously shown to increase expression of *ORMDL3* (27): rs12936231 (28), rs7216389 (29), rs2305480 (29), and rs4065275 (28). Subsequently, we used transcriptomic data from nasal brushings to evaluate possible interactions among the expression of the *SPTLC1*, *SPTLC2*, *SPTLC3*, *SPTSSA*, and *SPTSSB* genes coding for the SPT enzyme, the expression of the *ORMDL3* gene encoding the *ORMDL3* protein, phosphosphingolipid levels, and 17q21 genotypes.

First, when analyzing for an interaction between 17q21 genotypes and the amounts of sphinganine-1-phosphate and sphingosine-1-phosphate in relation to sRaw at the age of 6 years, we observed that rs12936231 showed a significant interaction with sphinganine-1-phosphate ($P = 0.043$) (Table E3). Our rs12936231 genotype-stratified analysis showed that this interaction was due to lower sphinganine-1-phosphate concentrations in children carrying increasing numbers of the high-risk allele (Figure 3).

Second, we investigated whether this relationship could be observed in the gene expression levels for genes related to SPT and *ORMDL3*, phosphosphingolipid levels, and sRaw. The interaction models between the 17q21 genotypes and SPT gene expression in relation to sRaw showed significant interactions between *SPTLC1* expression and all four of the 17q21 SNPs. In addition, *SPTSSA* expression was lower in children carrying rs12936231 risk alleles (Figure 4A). Models stratified by 17q21 genotype showed that this interaction was explained by increasing sRaw with decreasing SPT expression. These associations were significant between *SPTLC1* expression and 17q21 genotypes with two risk alleles, with similar patterns of association occurring between *SPTSSA* and *SPTLC3* expression and 17q21 genotypes (Figure 4B).

Finally, we investigated whether concentrations of sphinganine-1-phosphate and sphingosine-1-phosphate were affected by SPT expression and 17q21 genotype, which could explain the negative association between sRaw and phosphosphingolipid levels. The analyses overall showed significant interactions between *SPTSSB* expression and the 17q21 SNPs (Figure 5A). In 17q21 genotype-stratified models, we observed that the interactions were explained by an association between phosphosphingolipid concentrations and *SPTSSB* expression only among children

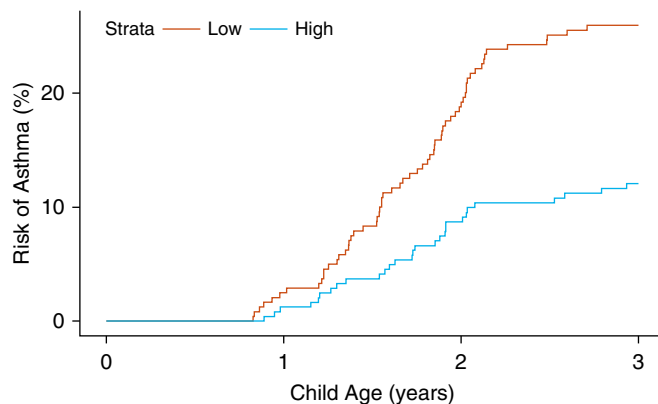
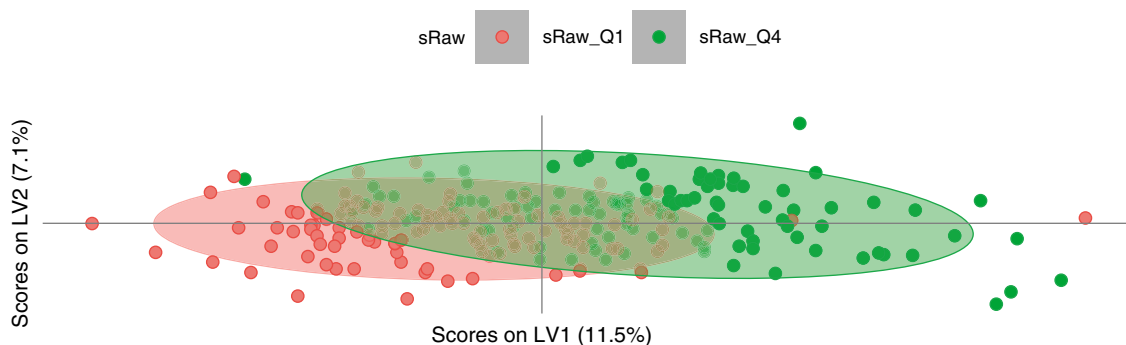


Figure 1. Kaplan-Meier curve of the association between the ceramide glycosyl-*N*-stearoyl sphingosine (d18:1/18:0) dichotomized by the median (low and high) and the development of asthma in the first 3 years of life.

A



B

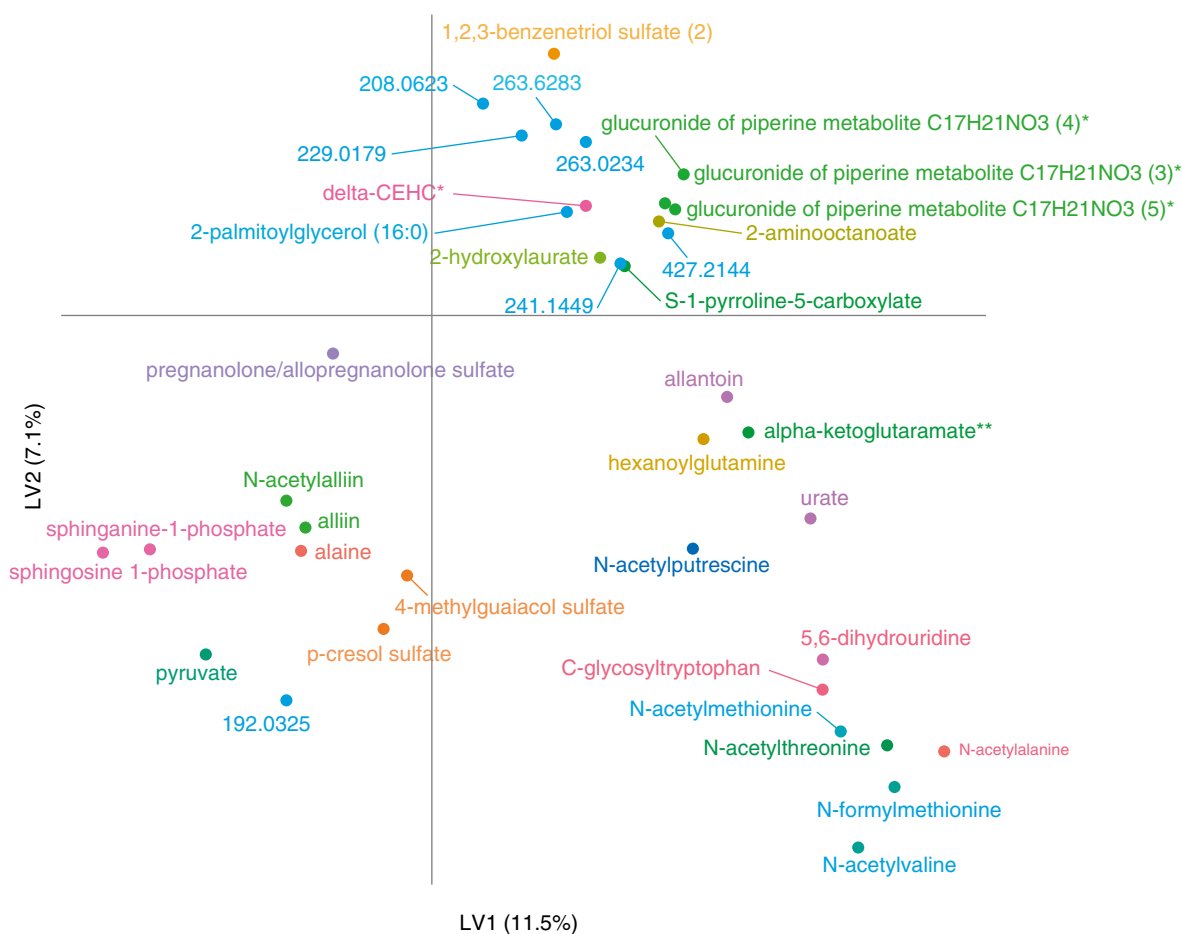


Figure 2. Partial least squares discriminant analysis of the metabolome at the age 6 years versus the sRaw at the age of 6 years. (A) Scores plot. (B) Loadings plot. (C) Spearman Corr map of the metabolites at the age of 6 years selected in the partial least squares discriminant analysis model. Corr = correlation; delta-CEHC = $C_{14}H_{18}O_4$; LV = latent variable; Q = quartile; sRaw = specific airway resistance.

without risk alleles. A similar trend was observed for the *SP TLC2* expression (Figure 5B).

Discussion

This exploratory study suggests that changes in the sphingolipid pathway across two time

points in early life (i.e., 6 months and 6 years) are associated with the risk of developing early-onset asthma and increased airway resistance, which involves both the *de novo* and the salvage sphingolipid pathways. The findings were specific for airway resistance

measured by plethysmography and were not present for FEV₁ or bronchial hyperreactivity, which may suggest that a perturbed sphingolipid metabolism is associated with inflammation, particularly in the smaller airways in young children.

C

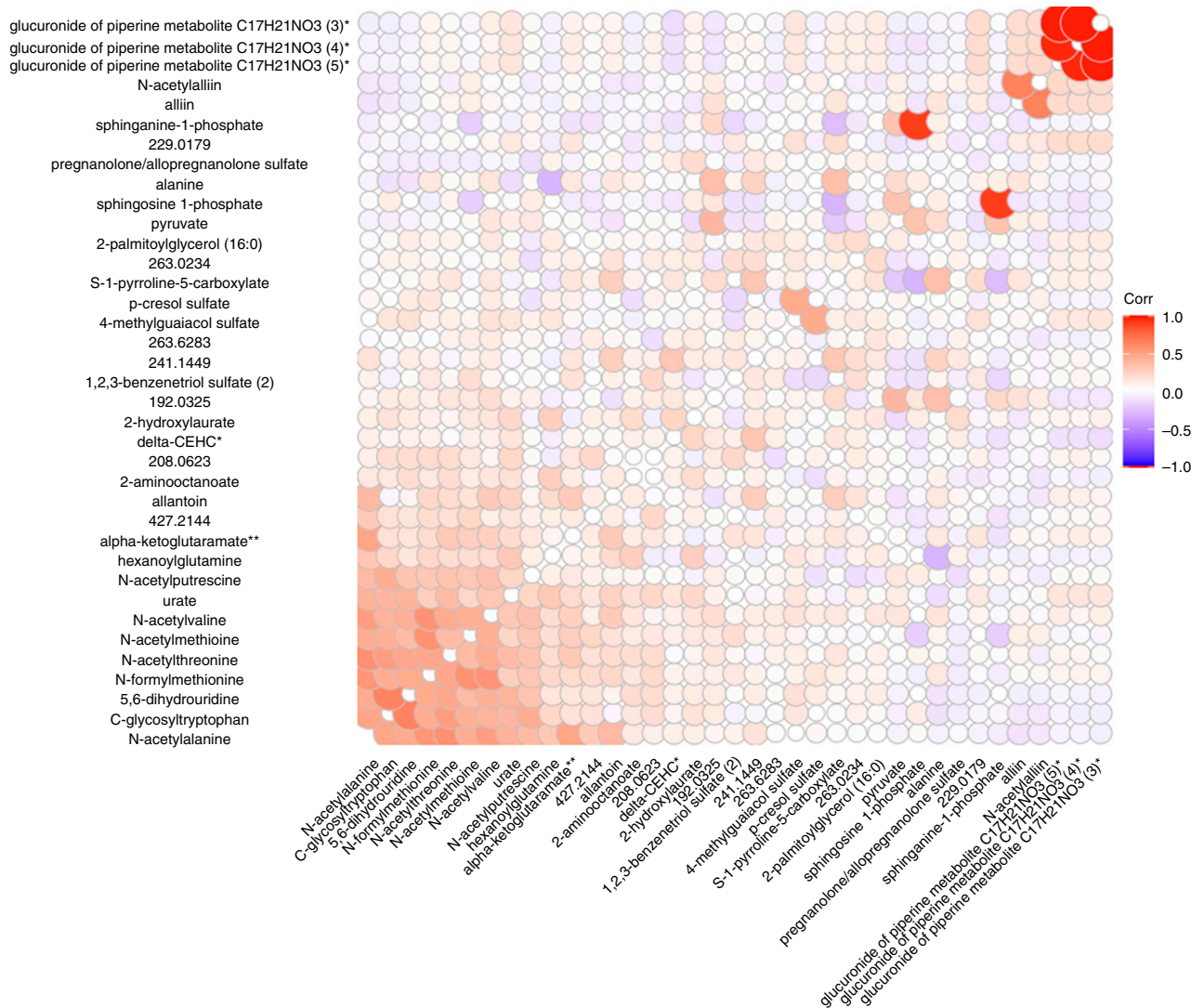


Figure 2. (Continued).

For the age of 6 months, we found that decreased amounts of ceramides and sphingomyelins were associated with the development of asthma/wheezing before the age of 3 years. This is possibly explained by the formation/degradation of complex sphingolipids from the action of specific enzymes, such as kinases, phosphatases, and lyases, present in the sphingolipid pathway. However, we cannot discriminate whether the observed phenomenon is due to a lower formation of ceramides from the *de novo* pathway or whether it is due to a general effect on the salvage pathway because none of the significant sphingolipids that we observed to be associated with early-onset

asthma/wheezing are produced solely in the *de novo* pathway. On the other hand, for children at the age of 6 years, we observed that children with higher airway resistance had lower plasma concentrations of the phosphosphingolipids (i.e., sphinganine-1-phosphate and sphingosine-1-phosphate), which are present in the *de novo* and salvage pathways, respectively. This latter observation may indicate suboptimal sphingolipid production in susceptible individuals, which is similar to what we observed during infancy at the age of 6 months before disease onset. However, the phosphosphingolipids associated with

airway resistance by the age of 6 years are different from the sphingolipids at the age of 6 months that were associated with an early onset of symptoms. Furthermore, despite our observation of similar associations in the discovery and replication cohorts, the sphingolipids did not pass an FDR < 0.05 multiple-testing threshold, and these results should therefore be interpreted with caution.

Sphingolipid metabolites are involved in several cellular functions, including immune responses, growth, and differentiation (30, 31). Studies of sphingolipids in different cellular and animal airway models, particularly

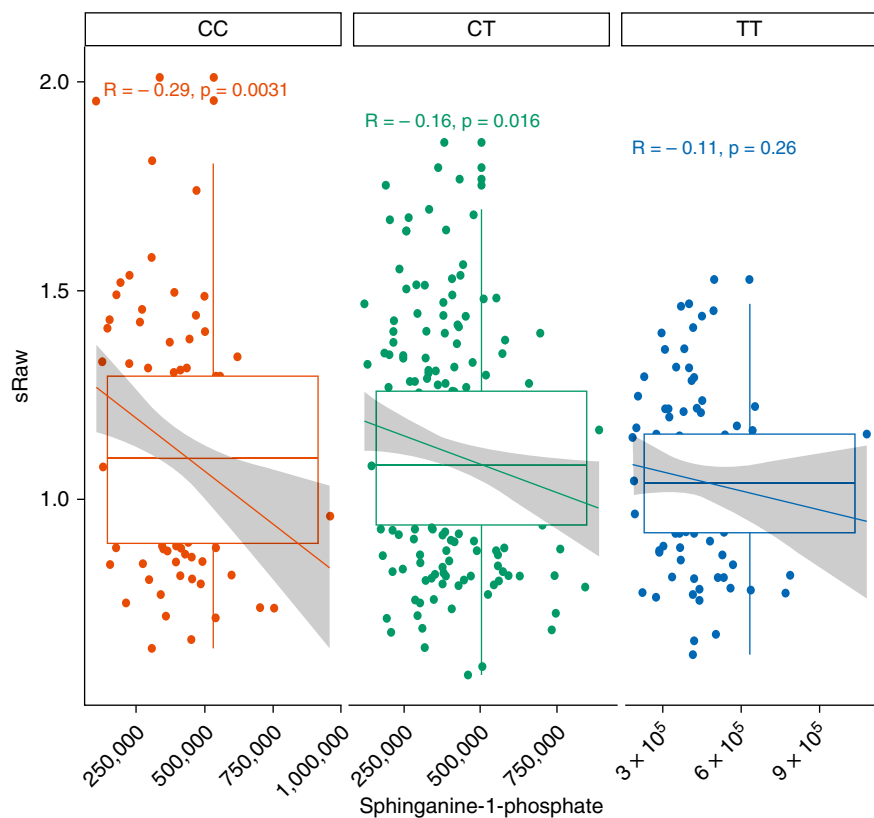


Figure 3. Association between sphinganine-1-phosphate and sRaw stratified by rs1293623 genotype (i.e., CC, CT, and TT). Each box represents the median with a 95% confidence interval. The line and the text above each group represents the Spearman correlation value. sRaw = specific airway resistance.

those investigating the role of the phosphosphingolipid sphingosine-1-phosphate, have shown diverging results. A study in mice showed that increasing amounts of sphingosine-1-phosphate by systemic administration was linked to increasing airway hyperresponsiveness (32), and another study using a mast cell-dependent murine model of allergic asthma showed that lowering the concentration of sphingosine-1-phosphate by using a sphingosine kinase 1 inhibitor attenuated airway hyperresponsiveness and inflammation (33) (i.e., better asthma outcomes in mice with lower phosphosphingolipid concentrations). A study by Ammit and colleagues showed that increasing the amount of sphingosine-1-phosphate in the BAL fluid of allergen-challenged individuals with asthma modulated airway smooth muscle cell functions that promote inflammation and airway remodeling (34). Furthermore, decreased sphingolipid synthesis, including in the phosphosphingolipids sphinganine-1-phosphate and sphingosine-1-phosphate, has been observed in mice overexpressing

ORMDL3 (35) as well as in children with 17q21 asthma risk genotypes (15), which was associated with asthma and airway remodeling (32) (i.e., worse asthma outcomes with lower phosphosphingolipid concentrations in genetically asthma-susceptible mice and children).

A lower amount of sphingolipid formation has previously been linked to the homeostatic regulation of *ORMDL3* on the SPT enzyme, as first demonstrated in yeast (36) and then in mice (37) as well as human cell lines (38, 39). The SNPs in the 17q21 region, which are associated with development of childhood asthma, have been shown to be strongly associated with increased expression of the *ORMDL3* gene (25, 40). Furthermore, in mouse models and human cell line studies, the 17q21 risk allele increases the transcription of the *ORMDL3* protein, which blocks the SPT enzyme, leading to lower amounts of sphingolipid formation (14). Our exploratory study shows associations that support the existence of such mechanisms in the inception of a particular childhood asthma endotype with early onset.

Using nasal-brushing transcriptomic data, we assessed the role of the SPT enzyme and 17q21 risk variants in plasma phosphosphingolipid concentrations. The SPT enzyme is encoded by the *SPTLC1*, *SPTLC2* (41), and *SPTLC3* (42) genes and by the two small subunits *SPTSSA* and *SPTSSB*. SPT1 and SPT2 are the main active forms of the enzyme, but either the *SPTSSA* or the *SPTSSB* protein confers full enzyme activity (43). We hypothesize that a significant association with one or more of the subunits expressing the SPT enzyme might be sufficient to affect *de novo* sphingolipid production, as they are all involved in full enzyme activity. Therefore, we performed our analysis using all five subunits and thereby identified an inverse association between lower amounts of phosphosphingolipids and increasing sRaw by the age of 6 years. This relationship was significantly affected by the 17q21 genotype, which interacted with the expression of the SPT enzyme and decreased the phosphosphingolipid concentrations (e.g., significant for sphingosine-1-phosphate when interacting with *SPTSSB*).

We did not observe a significant interaction between 17q21 genotypes and the *ORMDL3* expression in relation to sRaw, but in children without any risk alleles, there was a significant negative association for rs2305480 and a positive association between sRaw and *SPTLC1*. Both phenomena are plausible in children with a wild-type genotype, due to other biological conditions, which could affect the expression of *ORMDL3*, translating to a suboptimal protein amount and then leading to worse lung function. Similar hypotheses could also explain the increased SPT expression also affecting the phosphosphingolipid production in a nonoptimal way. The significant interaction between *ORMDL3* and the SNPs rs2305480 and rs7216389 was not present in the stratified model, which could indicate a spurious finding.

Another important point to consider is *ORMDL3*/SPT stoichiometry playing a role in the regulation of the SPT enzyme activity. In fact, it has been demonstrated that overexpression of *ORMDL3* in the lung epithelium increased ceramides concentrations, potentially with a feedback mechanism from the salvage pathway (44). On the other hand, it was also shown that a small increase in *ORMDL3* expression decreased ceramides concentrations (44), which might explain our findings.

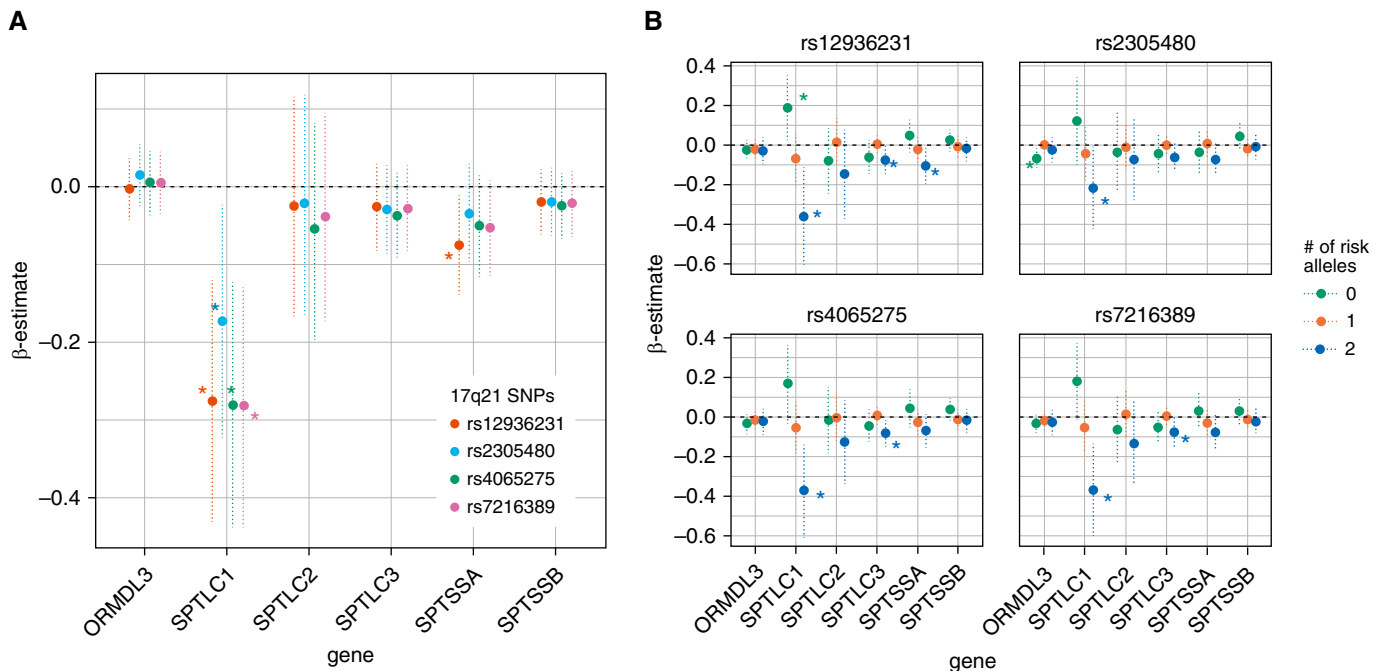


Figure 4. (A) Interaction model between nasal SPT (serine palmitoyl-CoA transferase) and *ORMDL3* gene expression (x-axes) and 17q21 genotypes in relation to specific airway resistance (sRaw). (B) Association between nasal SPT and *ORMDL3* gene expression (x-axes) and sRaw stratified by 17q21 genotypes. Each point represents the β -estimate of the association, and the dotted vertical lines represent the confidence intervals. * $P \leq 0.05$.

Our study is an exploratory study, which deals with the intrinsic data collinearity that is present in all untargeted metabolomic data sets (45). This collinearity

is due to biological factors (i.e., the fact that similar metabolites behave similarly, particularly in the same pathway, as in the sphingolipids) but is also due to analytical

factors (e.g., chromatographic coelution, charge competition, etc.). We chose a setup with a discovery and replication cohort, which showed similar findings for the early

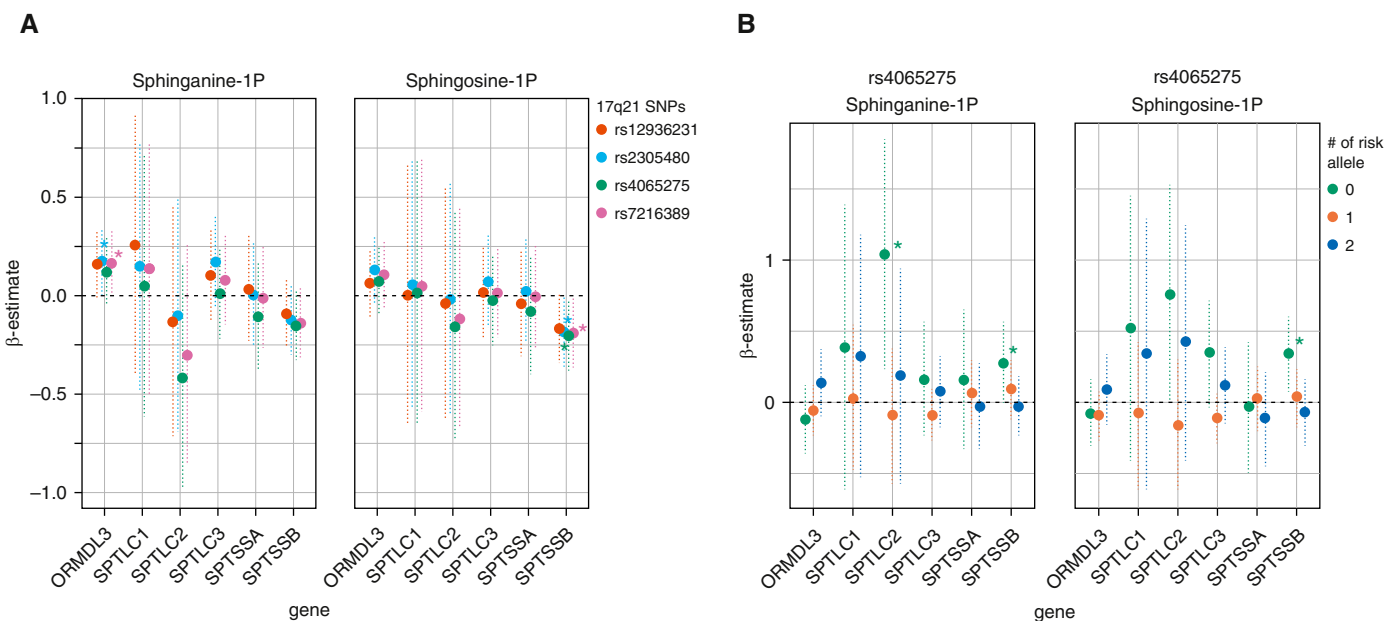


Figure 5. (A) Interaction model between nasal SPT (serine palmitoyl-CoA transferase) and *ORMDL3* gene expression (x-axes) and 17q21 genotypes in relation to plasma phosphosphingolipid levels. (B) Association between nasal SPT and *ORMDL3* gene expression (x-axes) and phosphosphingolipid levels stratified by 17q21 genotypes. Each point represents the β -estimate of the association, the dotted vertical lines represent the confidence intervals, and each color indicates the genotype stratum expressed as the number of risk alleles. * $P \leq 0.05$.

metabolomic time point in relation to early-onset asthma/wheezing, but the results did not survive multiple-testing correction. As we had no replication for the metabolomic data at the age of 6 years, we validated the univariate findings in a multivariate PLS-DA model, which showed similar results.

Conclusions

This exploratory study suggests the existence of a childhood asthma endotype with early onset of symptoms and increased airway resistance, which is characterized by reduced sphingolipid concentrations that are associated with 17q21 genetic variants and expression of the SPT enzyme. ■

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

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