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Early View

Original article

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The Role of the 17q21 Genotype in the Prevention of Early Childhood Asthma and Recurrent Wheeze by Vitamin D

Subtitle: A combined Secondary Analysis of two Randomized Controlled Trials

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This study strengthens the evidence for the roles of ORMLD3 in the 17q21 locus and vitamin D in asthma risk, while demonstrating for the first time in a human population that sphingolipid biosynthesis may partially underlie these relationships.

ABSTRACT:

BACKGROUND: Evidence suggests vitamin D has preventive potential for asthma, however, not all children benefit from this intervention. This study aims to investigate whether variation in the functional 17q21 SNP; rs12936231 affects the preventive potential of vitamin D against asthma.

METHODS: A combined secondary analysis of two randomized-controlled trials of prenatal vitamin D supplementation for the prevention of asthma in offspring (Vitamin D Antenatal Asthma Reduction Trial (VDAART); and Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$)) was performed stratifying by genotype and integrating metabolite data to explore underlying mechanisms.

RESULTS: The protective effect of vitamin D on asthma/wheeze was evident among children with the low-risk rs12936231 GG-genotype (HR (95%CI) 0.49 (0.26, 0.94), p=0.032), but not the high-risk CC-genotype (HR(95%CI) 1.08 (0.69,1.69), p=0.751). In VDAART, in the GG-genotype vitamin D supplementation was associated with increased plasma levels of sphingolipids, including sphingosine-1-phosphate: (sphingosine-1-phosphate (β (95% CI) 0.022 (0.001, 0.044), p=0.038)); but this was not evident with the CC-genotype, known to be associated with increased expression of *ORMDL3* in bronchial epithelial cells. Sphingolipid levels were associated with decreased risk of asthma/wheeze, and there was evidence of interactions between sphingolipid levels, vitamin D and genotype ($p\text{-interaction}_{vitaminD*genotype*age1:sphingosine\text{-}1\text{-}phosphate}$=0.035). In a cellular model, there was a significant difference in the induction of sphingosine-1-phosphate by vitamin D between a control Human bronchial epithelial cell-line and a cell-line overexpressing *ORMDL3* (p=0.002).

CONCLUSION: Results suggest prenatal vitamin D supplementation may reduce risk of early childhood asthma/wheeze via alterations of sphingolipid metabolism dependent on 17q21 genotype.

Trial Registration: clinicaltrials.gov Identifiers: VDAART NCT00920621, $COPSAC_{2010}$ NCT00856947

Key Words:

Asthma; *ORMDL3*; vitamin D; VDAART; COPSAC; sphingolipid; metabolites; primary prevention; 17q21; rs12936231

Abbreviations:

$COPSAC_{2010}$	Copenhagen Prospective Studies on Asthma in Childhood 2010
HR	Hazard Ratio
MAF	Minor Allele Frequency
QC	Quality control
RCT	Randomized Controlled Trial
SNP	Single nucleotide polymorphism
SPT	Serine palmitoyltransferase
VDAART	Vitamin D Antenatal Asthma Reduction Trial

Introduction:

Asthma is hypothesized to emerge from gene-environment interactions disturbing fetal developmental processes *in utero*. [1-3] Therefore, novel preventative methods targeted at pregnant women may help alleviate the burden of asthma.

We recently conducted two independent randomized clinical trials (RCTs) of prenatal vitamin D supplementation for the reduction of asthma among offspring: the Vitamin D Antenatal Asthma Reduction Trial (VDAART)[4] and the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$).[5] A combined analysis of the two trials demonstrated a significant 25% reduction in risk.[6] However, not all children benefited from the intervention and we hypothesize that the child's underlying genetic susceptibility to asthma may have attenuated the impact of the vitamin D supplementation on asthma development.

Chromosomal region 17q21 is the most replicated childhood asthma locus.[7, 8] The high-risk genotype defines an early-onset asthma phenotype with recurrent wheeze[9], that can be modulated by environmental exposures.[1, 2, 10] Expression of *ORMDL3* at the 17q21 locus is thought to confer an increased risk of asthma, in part, through inhibition of the serine palmitoyltransferase (SPT) enzyme. SPT catalyzes the first step in the synthesis of sphingolipids; the condensation of serine and palmitoyl CoA to produce 3-ketodihydrosphingosine,[11] and is therefore a key regulator of sphingolipid levels , which have been associated with bronchial reactivity and asthma in mechanistic studies.[12-14] However, human studies investigating whether *ORMDL3*-regulated sphingolipid pathways contribute to the pathogenesis of asthma are lacking[15].

In this study, we explore the hypothesis that genetic variation in the functional SNP rs12936231 affected the outcome of our prenatal vitamin D trials, through alterations of sphingolipid metabolism. We focused on this SNP in particular as it has been denoted as the strongest candidate for a functional SNP within 17q21[16]; it is known to influence the expression of *ORMDL3* in multiple cell types [16, 17]; it has been repeatedly associated with asthma[12], and it has links to vitamin D in the literature[18], We conducted this study in the VDAART and $COPSAC_{2010}$ populations, utilizing genotype data, sphingolipid metabolite levels and cell line studies.

Methods:

Study Populations:

The study populations have been described in detail previously [19, 20] and the trial protocols [4-6] are at clinicaltrials.gov; VDAART NCT00920621 and $COPSAC_{2010}$ NCT00856947. In brief, both studies recruited pregnant women (VDAART: 10-18 weeks; $COPSAC_{2010}$: 22-26weeks) and randomized them to daily vitamin D_3 (VDAART: 4,000 IU; $COPSAC_{2010}$: 2,400 IU) or placebo until delivery. All women additionally received a daily multivitamin containing 400 IU vitamin D_3. Written and oral consent, including for secondary analyses, was obtained from both cohorts (full details in **eMethods**).

Data Collection:

In both cohorts, children were followed for incident asthma/recurrent wheeze (defined in **eMethods**) and genotyped for the 17q21 rs12936231 SNP with the Illumina Infinium HumanOmniExpressExome Bead chip. For a subset of children metabolomic profiling at Metabolon, Inc., NC. was performed on plasma samples (VDAART: ages 1 and 3; $COPSAC_{2010}$: age 6 months) (**eMethods**). We extracted the five available metabolites from the sphingolipid biosynthesis pathway; sphingosine-1-phosphate (S1P); shown to be one of the most important sphingolipid metabolites for airway hyperresponsiveness, mast cell activation and inflammation in mechanistic asthma models; [13, 14, 21]; as well as sphinganine; sphinganine-1-phosphate; phosphoethanolamine and sphingosine

Statistics:

Differences between the vitamin D and placebo arms were assessed using the chi-squared test. In each 17q21 genotype strata (GG, GC, CC), the effect of prenatal vitamin D supplementation on asthma/recurrent wheeze from age 0 to 3 years was analyzed by event-time models using cox proportional hazards regression. Analyses were conducted independently in each cohort, then combined using a fixed-effects meta-analysis model, weighting estimates by the inverse of the estimate variance. The heterogeneity between the two studies was tested with a Cochran Q test and quantified using I^2 statistics.

To explore the role of sphingolipid metabolism in the relationship between 17q21 genotype and asthma/recurrent wheeze, we determined the association between the vitamin D intervention and sphingolipid peak intensities at ages 1 and 3 using linear regression models, stratified by 17q21 genotype. In order to account for the 203 subjects with

measures of sphingolipids at both ages, we additionally performed combined analyses using a linear mixed model with a random intercept. We then constructed multivariable regression models to test for interactions between rs12936231 genotype, prenatal vitamin D supplementation and sphingolipid metabolite level in the risk of asthma/recurrent wheeze by age 3 years.

Assessment of sphinosine-1-phosphate concentration in an *ORMDL3* overexpressed cell-line

To determine whether there are functional implications of vitamin D treatment on sphinosine-1-phosphate levels when *ORMDL3* is overexpressed, we established a human bronchial epithelial cell-line, 16HBE cells, with overexpression of *ORMDL3* by lenti-viral based infection. **eFigure1** demonstrates the efficiency of the overexpression of *ORMDL3*, as demonstrated by both Reverse transcription polymerase chain reaction (*RT-PCR*) at the mRNA level and by Western blotting at the protein level. We treated 16HBE cells with or without overexpression of *ORMDL3* with 1α,25-vitamin D3 (Sigma-Aldrich) at varying concentrations (0, 0.1, and 1nM) for 10 hours, then measured cytoplasmic sphinosine-1-phosphate levels by ELISA (MyBioSource). Three biological replicates were performed at each concentration. An unpaired Students t-test was used to compare induced sphinosine-1-phosphate levels, as measured by nanograms per milliliter, in the control cell-line, and in the cell-line overexpressing *ORMDL3*. (**eMethods**).

All statistical analyses were conducted using R v. 3.2.3 and the packages 'survival', 'survminer', meta' and 'AUCRF'. All hypothesis tests were two sided and a confidence level of 95% was employed.

The funding sources played no role in design and conduct of the study; collection, management, and interpretation of the data; preparation, review, or approval of the manuscript.

Results:

Baseline Characteristics

The study populations for each of the conducted analyses are described in **eTable1.** The primary analyses in both studies were based on those with genotype data and complete follow-up for asthma/recurrent wheeze until age three **(Table1)**. In VDAART, the genotype distribution was CC: N=171 (27.7%), GC: N=313 (50.6%), GG: N=134 (21.7%), corresponding to a minor allele frequency (MAF) of 0.47 (G allele) and Hardy-Weinberg equilibrium (HWE), p=0.72. In $COPSAC_{2010}$, it was CC: N=121 (23.4%), GC: N=259 (50.1%), GG: N=137 (26.5%), MAF=0.52, HWE p=0.98. There was no difference in the distribution of genotype, sex, asthma/wheeze status or race between the vitamin D and placebo arm in either VDAART (vitamin D n=311; placebo n=307) or $COPSAC_{2010}$ (vitamin D n=261; placebo n=256) and the relative proportions were representative of the two parent studies.[4, 5] Serum vitamin D levels at age three were available for VDAART only; although the point estimates were higher among the children whose mothers received the intervention, this difference was not significant**.** Similarly, there were no significant differences in serum vitamin D levels in VDAART between the placebo and intervention group when stratified by genotype (p>0.1 for all comparisons) .In COPSAC a proportion of mothers additionally received n-3 long-chain polyunsaturated fatty acids via fish-oil capsules during pregnancy, but we determined there was no significant difference in genotype distribution between these four intervention groups; vitamin D only, Fish Oil only, Vitamin D and Fish-oil, placebo only (**eTable2**).

Table 1: Baseline characteristics of the participants from the VDAART and COPSAC$_{2010}$ prenatal vitamin D trials with genotype data stratified by intervention

Characteristic	VDAART[a] N=618			COPSAC$_{2010}$[b] N=517		
	Placebo (n=307)	Vitamin D (n=311)	p-value	Placebo (n=256)	Vitamin D (n=261)	p-value
rs12936231, N (%)						
CC	78 (25.4)	93 (29.9)	0.454	59 (23)	62 (23.8)	0.820
GC	161 (52.4)	152 (48.9)		126 (49.2)	133 (51)	
GG	68 (22.1)	66 (21.2)		71 (27.7)	66 (25.3)	
Asthma/wheeze, 0-3yrs, N (%)						
No	215 (70)	235 (75.6)	0.146	206 (80.5)	219 (83.9)	0.364
Yes	92 (30)	76 (24.4)		50 (19.5)	42 (16.1)	
Sex, N (%)						
Male	164 (53.4)	157 (50.5)	0.515	128 (50)	141 (54)	0.408
Female	143 (46.6)	154 (49.5)		128 (50)	120 (46)	
Race, N (%)						
Black	146 (47.6)	144 (46.3)	0.359	-	-	-
Caucasian	92 (30)	108 (34.7)		256 (100)	256 (100)	
Other	69 (22.5)	59 (19)		-	-	
Serum vitamin D levels, mean [SD][c]						
Age 3 ng/ml	20.71 [8.19]	21.16 [9.32]	0.630	-	-	

[a] The total VDAART population was 806 children, the dose was 4400 IU daily in the intervention arm versus· 400 IU in the placebo arm and was initiated in weeks 10-18

[b] The total COPSAC$_{2010}$ population was 581 the dose was 2800 IU daily in the intervention arm versus· 400 in the placebo arm and was initiated in weeks 10-18

[c] Serum vitamin D levels were available for VDAART only

Vitamin D, 17q21 Genotype and Development of Asthma/Recurrent Wheeze

The results from both trials suggested that the effect of the prenatal vitamin D supplementation on the development of asthma/recurrent wheeze from ages 0 to 3 years was dependent on the child's 17q21 genotype; with an increasingly protective effect of vitamin D with decreasing number of C risk alleles (**eFigure2**). The effect of the high-dose vitamin D intervention in the rs12936231 genotype strata in VDAART was: CC: HR=1.07 (95% CI: (0.59-1.95), GC: HR=0.75 (95% CI: 0.50-1.11), and GG: HR=0.51 (95% CI: 0.21-1.19). In COPSAC$_{2010}$, the observed pattern was very similar: CC: HR=1.08 (95% CI, 0.55-2.15), GC: HR=0.73 (95% CI: 0.39-1.37), and GG: HR=0.48 (95% CI: 0.18-1.27).

A combined analysis of the two trials confirmed that high-dose vitamin D intervention conferred a 50% reduced risk of developing asthma/recurrent wheeze by age 3 in children with the low-risk GG-genotype: fixed effects HR=0.49 (95% CI: 0.26-0.94), and that this protective effect decreased with increasing number of risk alleles: GC: HR=0.74 (95% CI: 0.53-1.04) and CC: HR=1.08 (95% CI: 0.69-1.69) (**Table2**). These findings also held true when assuming the G allele is dominant (**eTable3**).

Table 2: Effect of prenatal vitamin D supplementation on development of asthma/persistent wheeze from 0 to 3 years in the VDAART and COPSAC$_{2010}$ trials stratified by genotype of the 17q21 functional SNP rs12936231

Vitamin D vs· placebo	**VDAART, N=689**		**COPSAC$_{2010}$, N=517**		**Combined analyses**
rs12936231 strata	total/ cases	HR (95% CI), p-value	total/ cases	HR (95% CI), p-value	HR[a] (95% CI), p-value
GG	134/23	0·51 (0·21-1·19), p=0·119	137/19	0·48 (0·18-1·27), p=0·140	0·49 (0·26-0·94), p=0·032
GC	313/101	0·75 (0·50-1·11), p=0·147	259/40	0·73 (0·39-1·37), p=0·327	0·74 (0·53-1·04), p=0·080
CC	171/44	1·07 (0·59-1·95), p=0·821	121/33	1·08 (0·55-2·15), p=0·822	1·08 (0·69-1·69), p=0·751

Cases refer to number of children with asthma/persistent wheeze at from age 0 to 3 years·
HR=hazard ratio
[a]Fixed Effects Meta-analysis HR combining VDAART and COPSAC$_{2010}$

Vitamin D and Sphingolipid Metabolism

Metabolomic profiling of plasma was available in a subset of the VDAART children in the samples extracted at age 1 (n=413) and age 3 years (n=353, including 203 of the children with an age 1 sample) (**eTable1**). In the linear regression models, there was a consistently positive association between prenatal vitamin D intervention and higher levels of the five sphingolipids among children with the GG genotype. This association reached significance in the mixed models including all samples, for sphinganine-1-phosphate (β (95% CI) 0.040 (0.002, 0.078), p=0.038) and sphingosine-1-phosphate (β (95% CI) 0.022 (0.001, 0.044), p=0.038); and was approaching significance (p<0.1) for sphinganine, sphingosine and phosphoethanolamine. In contrast, there was no observed increase in sphingolipid levels associated with the vitamin D intervention in those children with the high-risk CC-genotype (**Table3**). However, these findings were not recapitulated in the six-month samples from 441 children from COPSAC$_{2010}$ (**eTable4**). Combined analyses with VDAART were not conducted due to differences in sample collection age.

Table 3: Association between blood peak intensity of five key sphingolipid metabolites and vitamin D intervention in VDAART children stratified by 17q21 genotype; results shown for age one, for age three and in all samples combined according to a mixed model

	Age One Samples			Age Three Samples			All Samples		
	Coefficient	**95% CI**	**p value**	**Coefficient**	**95% CI**	**p value**	**Coefficient**	**95% CI**	**p value**
sphinganine-1-phosphate									
CC	-0.016	(-0.073,0.002)	0.065	0.001	(-0.044,0.051)	0.895	-0.015	(-0.048,0.017)	0.350
GC	0.003	(-0.019,0.034)	0.580	0.004	(-0.026,0.043)	0.635	0.007	(-0.016,0.029)	0.548
GG	0.016	(-0.013,0.086)	0.147	0.017	(-0.014,0.094)	0.149	0.040	(0.002,0.078)	0.038*
sphinganine									
CC	-0.004	(-0.072,0.052)	0.752	0.006	(-0.067,0.094)	0.744	0.001	(-0.052, 0.053)	0.982
GC	0.010	(-0.023,0.067)	0.334	-0.007	(-0.073,0.043)	0.608	0.005	(0.032, 0.042)	0.787
GG	0.013	(-0.079,0.141)	0.586	0.041	(-0.002,0.193)	0.059	0.065	(-0.011,0.141)	0.091
sphingosine-1-phosphate									
CC	-0.009	(-0.039,0)	0.054	-0.001	(-0.032,0.027)	0.872	-0.012	(-0.031,0.007)	0.212
GC	-4.6E-04	(-0.017,0.015)	0.898	0.003	(-0.016,0.028)	0.610	0.002	(-0.012, 0.016)	0.776
GG	0.006	(-0.015,0.042)	0.352	0.015	(0.005,0.065)	0.027*	0.022	(0.001, 0.044)	0.038*
sphingosine									
CC	-0.009	(-0.077,0.037)	0.486	0.005	(-0.059,0.084)	0.733	-0.005	(-0.052,0.041)	0.821
GC	0.010	(-0.019,0.065)	0.284	-0.006	(-0.07,0.04)	0.599	0.006	(-0.029,0.041)	0.736
GG	0.009	(-0.077,0.118)	0.679	0.043	(0.015,0.185)	0.024*	0.057	(-0.01,0.124)	0.094
phos-phoethanolamine									
CC	-0.009	(-0.069,0.027)	0.389	0.015	(-0.006,0.074)	0.096	0.003	(-0.032,0.038)	0.870
GC	-0.011	(-0.06,0.008)	0.142	-0.009	(-0.054,0.014)	0.251	-0.021	(-0.047,0.005)	0.120
GG	0.020	(-0.016,0.108)	0.152	0.018	(-0.005,0.086)	0.086	0.038	(-0.003,0.08)	0.071

Age one:CC n=112 (27%); GC n=221 (54%); GG n=80 (19%)
Age three:CC n=106 (30%); GC n=172 (49%); GG n=75 (21%)
All samples:CC n=218 (28%); GC n=393 (51%); GG n=155 (20%)
**Significant at the 95% Confidence interval*

Vitamin D, sphingolipid Metabolism, 17q21 Genotype and Development of Asthma/Recurrent Wheeze

In VDAART, sphingosine-1-phosphate, phosphoethanolamine and Sphinganine-1-phosphate demonstrated a significant ($P<0.05$) or approaching significant ($p<0.1$) three-way interaction between the vitamin D intervention, rs12936231 genotype and metabolite in the risk of asthma/recurrent wheeze at ages one and three. This suggests that sphingolipid metabolites and rs12936231 genotype may jointly influence the effect of vitamin D on asthma risk (**eTable5**). However, significant interactions were not noted in the six-month samples from $COPSAC_{2010}$ (**eTable6**).

Sensitivity analysis

Race: Sensitivity analyses were run to account for the potential influence of race in VDAART. Genotype frequencies were very similar in the African-American (n=290 children) and Caucasian (n=200) populations, although they deviated slightly in the "Other" category which included Asian and Native Hawaiian children (n=128) (**eTable7**). Cox proportional regression models were rerun, stratified by race, then results combined using a meta-analysis model. Due to small numbers, the "Other" and "Caucasian" categories were combined. The combined results were supportive of the conclusion that the effect of prenatal vitamin D supplementation on the development of asthma/recurrent wheeze was modified by 17q21 genotype **(eTable8)**. There was no protective effect of the vitamin D intervention in children with the high-risk CC-genotype: HR=1.05 (95% CI: 0.58-1.92), while there was a trend toward an increasingly protective effect with a decreasing number of risk alleles: GC: HR=0.76 (95% CI: 0.51-1.14) and GG: HR=0.53 (95% CI: 0.22-1.29). However it should be noted that these results appeared to be largely driven by the African-American Children.

We additionally reran the sphingolipid analyses; the regression models and the interactions models stratifying by race category. While the overall pattern of results in terms of genotype and direction of effect held true across all populations for both the vitamin D supplementation~metabolite regression model (**eTable9)**, and the multivariable interaction model (**eTable10)**, there was some evidence that these results were being driven by children in the 'African American' and 'Other' populations.

Fish-oil Supplementation: To reflect the fact that a proportion of the included mothers in COPSAC were also receiving fish-oil supplementation throughout pregnancy, we reran the analyses excluding the 256 children of these

women. When comparing the 128 children whose mothers received only vitamin D with the 133 whose mothers received placebo only, there remained evidence of trend of an increasing protective effect of vitamin D supplementation with decreasing number of C alleles. Furthermore, when combined with the VDAART population, the decreased risk in the GG strata was borderline significant with a similar magnitude to when the full COPSAC population was included (HR: 0·49 (0·24-1.02), p=0·055); while there was no evidence of a protective effect in the CC strata (HR: 1·00 (0·60-1·66), p=0·999) (**eTable11**).

We reran the sphingolipid analyses excluding the children's whose mothers had received fish-oil supplementation throughout pregnancy. The results were largely unchanged for the regression models (**eTable12**) in terms of direction of effect and significance level. Similarly, the results of the three-way interaction population were consistent when the fish-oil supplementation subgroup was removed (**eTable13**). Taken together these results suggest that concurrent fish-oil supplementation was not affecting the results in the $COPSAC_{2010}$ population with regards to the genotype-specific protective effect of vitamin D supplementation and its relationship with sphingolipids.

Safety

There was no significant difference in the rates of severe adverse events between the trial arms in either study.[4, 5]

Assessment of sphinosine-1-phosphate concentration in human bronchial epithelial cells

Given the observed interaction between vitamin D treatment, sphingosine-1-phosphate levels and rs12936231 genotype in human subjects, we then explored the relationship between *ORMDL3* expression and levels of sphingosine-1-phosphate in response to vitamin D treatment in cellular models. We performed three biological replicates at three concentrations of 1α, 25-vitamin D3; 0nM, 0.1nM and 1nM. We then compared the mean level of induced sphingosine-1-phosphate (as measured by nanograms per milliliter) across the three replicates between the control and the *ORMDL3* overexpression cell-line, for each concentration. We found no significant difference in the levels of induced sphingosine-1-phosphate between the control and the *ORMDL3* overexpression cell-line, when treated with 0nM and 0.1nM 1α, 25-vitamin D3. However, there was a significant (p=0.002), difference between the control (mean of 3 replicates; 62.3ng/ml) and the *ORMDL3* line (mean of 3 replicates; 53.0ng/ml) when treated with 1nM (**Figure1**). This suggests that higher expression of *ORMDL3*, generally associated with risk CC-genotype at

rs12936231[17], leads to reduced induction of sphingosine-1-phosphate by Vitamin D in human bronchial epithelial cells.

Discussion:

An increasing body of evidence now supports the preventive and protective potential of vitamin D on asthma and its symptoms.[22] This study suggests that the preventive actions of prenatal vitamin D supplementation on early childhood asthma/wheeze during the first 3 years of life may be modified by genetic variants in 17q21; a crucial asthma GWAS locus. Children with the low risk GG-genotype in rs12936231 whose mothers were supplemented with vitamin D had a 50% reduced risk of asthma, whereas there was no protective effect in children with the high-risk CC-genotype. Based on our exploratory sphingolipid and functional work, we hypothesize that for a child with the non-risk GG-genotype, prenatal vitamin D supplementation increases sphingolipid metabolism, and subsequently the levels of downstream sphingolipids that are protective against asthma/recurrent wheeze. Conversely, for a child with the high-risk CC-genotype, increased expression of *ORMDL3*, known to inhibit SPT - a rate-limiting enzyme for *de novo* sphingolipid biosynthesis [23] – modifies the relationship between vitamin D and sphingolipid synthesis, and thus may render the vitamin D intervention ineffective at reducing asthma/recurrent wheeze risk (**Figure 2**).

17q21 was first discovered as a childhood asthma susceptibility locus in 2007, when genetic variants were shown to regulate transcription of the *ORMDL3* gene in lymphoblastoid cell-lines.[24] For this study we focused on a specific SNP in this region; rs1293623, a functional variant located in intron 4 of *ZPBP2* which independently controls *ORMDL3* expression.[12] rs12936231 alters the CTCF binding motif with a G to C change, to the point where it is almost completely lost in the *ZPBP2* intronic region for subjects carrying the asthma-risk C allele [12][25]. Instead, it switches the binding site of CTCF to the *ORMDL3* intronic region, which is hypothesized to alter the 3D architecture of the 17q21 locus favoring enhanced transcription of *ORMDL3* [16][12] The asthma-associated C allele of this SNP is therefore associated with a downregulation of sphingolipid metabolism via increased expression of *ORMDL3* and inhibition of SPT [25]. The link between sphingolipid metabolism and asthma is further supported by mouse-studies showing that decreased sphingolipid synthesis in lung epithelial tissue [14] and SPT knockouts [21] both associate with increased airway hyperresponsiveness and inflammation [13], and that

sphingolipids play an important role in the maturing postnatal lung [26]. Interestingly, experimental studies have shown that vitamin D metabolites are capable of activating the sphingolipid pathway,[27, 28] and vitamin D has been shown to alter the recruitment of CTCF,[18] making rs12936231 a particular SNP of interest for this study. rs12936231 is also unique in that it has been shown to impact the function of, not only immune cells, but also multiple other cell types that are potentially involved in asthma pathogenesis.[16]Taken together, it is biologically plausible that prenatal vitamin D supplementation acts to reduce the risk of childhood asthma in part through 17q21 dependent sphingolipid metabolism, and that rs12936231 is a particularly informative SNP to study in this regard.

We observed a clear allele-additive modifying effect, in two independent cohorts, of rs12936231, with a decreasing effect of the vitamin D intervention on asthma/recurrent wheeze with an increasing number of risk alleles. In VDAART, we then determined that prenatal vitamin D supplementation resulted in increased levels of key sphingolipids in offspring with the rs12936231 GG or GC-genotype, but not those with the CC risk genotype. Furthermore, we present some evidence of significant interactions between prenatal vitamin D intervention, with offspring genotype, and sphingolipid metabolites on the risk of asthma/recurrent wheeze, again suggesting that the effect of prenatal supplementation may be modified by these factors. Additional supporting evidence came from cellular models. Genetic overexpression of *ORMDL3*, led to decreased induction of sphingosine-1-phosphate in response to vitamin D treatment. This suggests that *ORMDL3* may modify the vitamin D-sphingosine-1-phosphate relationship. Nevertheless, we recognize that no cellular model can completely recapitulate the effects of the prenatal vitamin D supplementation on the risk of asthma in offspring. Furthermore, it should be noted that in addition to human bronchial epithelial cells there are other sources of sphingosine-1-phosphate production.

Differences in the trial design and populations between the two cohorts represent one of the biggest limitations of this study; VDAART is a high-risk cohort recruiting parents with asthma/allergy, and included a large group of African-Americans in the trial, who have a higher risk of developing asthma and allergy, [29]. In contrast, $COPSAC_{2010}$ is a population-based cohort primarily which only genotyped Caucasian individuals. This is important as the 17q21-associated increased risk of childhood asthma and 17q21 MAFs vary on a SNP-level basis across ethnicities. [25] However, we observed very similar MAFs in VDAART and $COPSAC_{2010}$ and sensitivity analyses demonstrated that the genotype results were not confounded by race. Despite the heterogeneity between the trials, we observed a nearly identical pattern of an increased preventive effect of vitamin D by decreasing number of 17q21

risk alleles in both trials. It should also be noted that unlike VDAART, $COPSAC_{2010}$ had a randomization within a cohort design, however it has been stated that the findings from such studies are as valid as those from conventional RCT, [30] therefore we are not concerned that this affected our results and conclusions regarding the influence of rs12936231.

We were not able to validate our exploratory sphingolipid findings from VDAART in the $COPSAC_{2010}$ population, which we believe may be due to underlying differences between the populations. One of the potentially most important of these differences was the factorial design of $COPSAC_{2010,}$ whereby a proportion of mothers were additionally receiving daily prenatal supplementation of n-3 long-chain polyunsaturated fatty acids via fish-oil capsules.[31] There is some evidence that fish-oil can alter sphingolipid metabolism and sphingolipid levels,[32, 33] which may explain the lack of difference in sphingolipid levels between the vitamin D and placebo groups. Therefore, we ran additional sensitivity analyses excluding the children of mothers who received fish-oil throughout pregnancy. These analyses did not support our hypotheses that the lack of replication was due to the fish-oil, however we were somewhat limited by power in these analyses and we can not discount that residual confounding from this additional supplementation may have been influencing our COPSAC findings . Importantly, there were also substantial dietary differences between the two populations, with lower baseline levels of vitamin D and more vitamin D deficient mothers in the VDAART trial[6]which likely resulted in differing baseline sphingolipid levels. Finally, the timing of the blood sampling, the onset of supplementation and the daily dose of vitamin D differed between the two cohorts, which may again explain the lack of replication.

Based on our race stratified sphingolipid findings in the VDAART population, in which we found that the significant results of interest were primarily driven by the non-Caucasian children, we suggest that race may have played a role in our inability to replicate the sphingolipid findings in $COPSAC_{2010}$. We are not away of evidence in the literature for a difference in sphingolipid metabolism by race; however there is evidence to suggest that vitamin D metabolism may differ by race[34], which may help to explain the results of our race-stratified analyses. Consequently, although the genetic findings were of similar magnitude and reach statistical significance when

combined, and we demonstrated that the COPSAC genetic findings were not driven by the fish-oil supplementation, further work is needed to explore the wider generalizability of the sphingolipid analyses.

We also note that we were limited by power in some of the cohort-specific stratified analyses, particularly with respect to the number of cases in the GG genotype strata. We note that, for both cohorts the trend was that same, but it was only in the combined meta-analysis that the results reached significance. Furthermore, due to the secondary and exploratory nature of these analyses, they should be considered hypothesis generating and a nominal p-value threshold was used to denote significance. Similarly, we note we did not account for multiple testing in our sphingolipid analysis, however due to the highly correlated nature of these metabolites and their existence within a single coordinated metabolic pathway, application of classical multiple testing corrections such as Bonferroni would be too stringent for such data. We also recognize that given the complexity of the 17q21 locus there are possibly other causal variants or genes that may influence risk. However, given the demonstrated independent functional nature of rs12936231 *ORMDL3* expression [12, 16], and the role of *ORMDL3* in sphingolipid biosynthesis [35], we consider these optimal for investigation.

Despite the limitations, this study was unique in our access to data from two independent prenatal vitamin D RCTs, which were aligned with respect to the predetermined endpoint of asthma/recurrent wheeze by age three and had available genotyping. This enabled discovery and a replication of a 17q21 genotype dependent effect of the prenatal vitamin D intervention on asthma/recurrent wheeze. The sphingolipid data is a major advantage of this study and serves as an example of how this metabolomics, which provides a physiological 'snapshot' of a biological system, may be useful to increase the understanding of gene-environment interactions in health and disease. This approach enabled us to generate hypotheses regarding plausible underlying biological mechanisms,[23] which we then explored in a cellular model.

We are unable to determine, within the confines of this study, whether the lack of preventive effect of vitamin D in children carrying the high-risk 17q21 genotype is due to our use of a dosage of vitamin D that was too low or was initiated too late in pregnancy or whether these children have a complete breakdown of the sphingolipid pathway

rendering even high doses of vitamin D ineffective. We also recognize that vitamin D likely has other effects on fetal programming very early in pregnancy and may also act to reduce the risk of asthma through immune modulations [36] and effects on lung organogenesis from branching morphogenesis throughout the alveolar stage of lung development.[37, 38] Furthermore, it has been previously noted that the effect of *ORMLD3* overexpression on sphingolipid metabolism is dependent on both the extent of overexpression and the underlying physiologic conditions.[39] Finally, we acknowledge that race may be influencing our findings, particularly with regard to the sphingolipid results. Additional work is required to explore these issues, specifically within study with adequate sample size and power for the strata of interest.

Conclusion:

This is the first study to demonstrate a link between vitamin D and the 17q21 locus in the development of childhood asthma and recurrent wheeze. Based on our exploratory analyses, we suggest that vitamin D acts to reduce the risk of asthma through an increase in sphingolipid metabolism, but that this pathway may be attenuated in those with key genetic variants in 17q21 that influence the expression of *ORMDL3*. These findings provide additional insights into the pathogenesis of childhood asthma and in particular the role of *ORMDL3*-regulated sphingolipid pathways[15].

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Authors Contributions:

RK and BC performed the statistical analysis and wrote the first draft of the manuscript. Functional work was performed by FG and XZ, with support from BR and BD. AL and ST are the PIs of VDAART and HB is the PI of COPSAC, and as such contributed to the overall concept and study design together with JL-S. All four provided funding support. KB, DR, JS, and KB provided analytical support. All authors assisted with the editing of the final draft of the manuscript and provide their approval for its submission. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication and takes full responsibility for the integrity of the data and the accuracy of the data analysis. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

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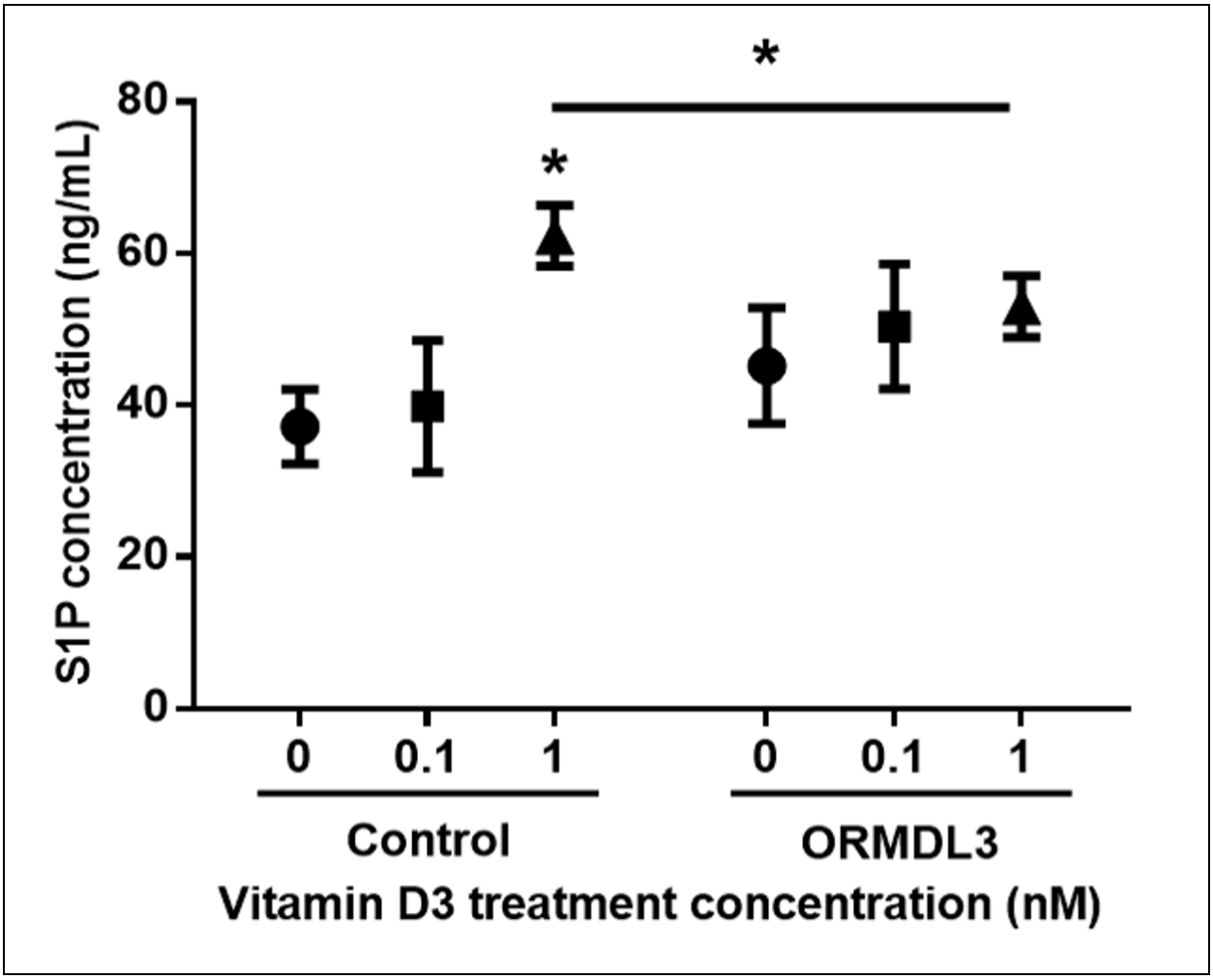

Figure 1: Measurements of sphingosine-1-phosphate levels in 16HBE cells after vitamin D3 treatment. Human bronchial epithelial cells, 16 HBE cells and ORMDL3 overexpressing stable 16 HBE cells treated with Vitamin D3 at three concentrations (0, 0.1 and 1nM) for 10 hours. Levels of Sphinosine-1-phosphate in cells were measured by ELISA. Mean± SD shown for 3 independent experiments in ELISA assay. * indicates $p < 0.05$, according to the unpaired Student's t test

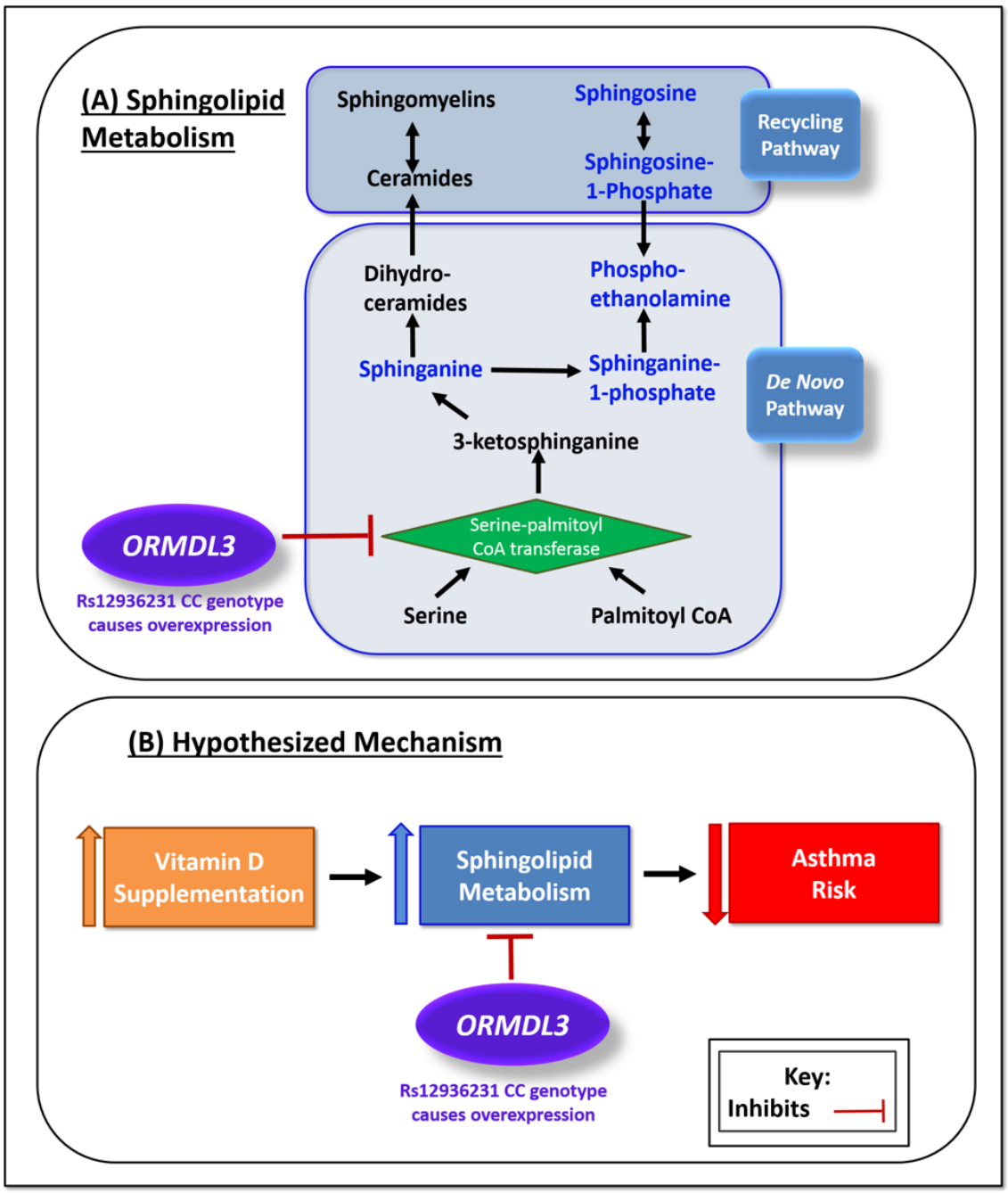

Figure 2: Hypothesised Mechanism
(A) Sphingolipid Metabolism: Serine-Palmitoyl transferase catalyzes a crucial reaction in the production of sphingolipids. ORMDL3 inhibits the action of serine-palmitoyl transferase and therefore the de novo pathway production of sphingolipids. Expression of ORMDL3 is increased by the CC-genotype at rs12936231; leading to increased inhibition of sphingolipid production. (B) Hypothesized Mechanism: Prenatal vitamin D supplementation increases the production of sphingolipids in offspring via the sphingolipid metabolism pathway, resulting in a decreased risk of asthma. When ORMDL3 is overexpressed the sphingolipid metabolism pathway is inhibited, sphingolipid production is not increased and there is no protective effect of vitamin D on asthma risk.

Prevention of Early Childhood Asthma and Recurrent Wheeze by Vitamin D and the Role of the 17q21 Genotype: Secondary Analysis of two Randomized Clinical Trials

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eTable 6: Results of multivariable interaction models exploring the relationship between a diagnosis of asthma/recurrent wheeze by age 3 years, the vitamin D intervention, 17q21 genotype and five key sphingolipids measured at six months in 441 children from $COPSAC_{2020}$· The table shows Beta estimate (p-values) for the individual variables/terms for a generic model: *asthma/recurrent wheeze~sphingolipid metabolite* vitamin D intervention* genotype*

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eTable 13: Results of multivariable interaction models exploring the relationship between a diagnosis of asthma/recurrent wheeze by age 3 years, the vitamin D intervention, 17q21 genotype and five key sphingolipids in VDAART· The table shows Beta estimate (p-values) for the multiinteraction term: *asthma/recurrent wheeze~sphingolipid metabolite* vitamin D intervention* genotype*; with additional adjustment for, or stratification by, race

References

eMethods

Study Populations

VDAART: Pregnant non-smoking women between 10 to 18 weeks of gestation with a history of asthma, eczema, or allergic rhinitis, or women who conceived the child with a man with a history of such diseases were recruited from three sites across the USA between October 2009 and July 2011; Boston, San Diego and St Louis.[1] Women were randomized 1:1 to a daily dose of 4,000 IU vitamin D_3 or a placebo tablet until delivery. All women additionally received a daily multivitamin containing 400 IU vitamin D_3. Randomization was performed using an automated system taking study-site and race into account, and the study was double-blinded. The families were followed post-delivery by quarterly telephone interviews and yearly in-clinic visits. VDAART was approved by the Institutional Review Boards (IRB) of the participating Clinical Centers and the Data Coordinating Center, with pregnant women signing informed consent at the enrollment visit covering both primary and secondary data analyses.

$COPSAC_{2010}$: Pregnant women between 22 to 26 weeks of gestation were recruited from across Zealand, Denmark between March 2009 and November 2010, and were randomized 1:1 to a daily dose of 2,400 IU vitamin D_3 or a placebo until 1 week postpartum.[2] All women also received a multivitamin containing 400 IU vitamin D_3. Randomization was performed using a computer-generated list of random numbers, supplied by an external investigator, and the study was double-blinded. The children were followed at the COPSAC research unit with nine scheduled clinic visits between the ages of 0 to 3 years, *ad hoc* visits for respiratory symptoms, and by daily diary-cards. The $COPSAC_{2010}$ study was approved by the Local Ethics Committee (H-B-2008-093; H-B-2009-014), the Danish Data Protection Agency (2008-41-2599), and the Danish Health and Medicines Authority (2612-3959). Written and oral informed consent for primary and secondary analyses was obtained at enrollment.

Asthma/Recurrent Wheeze Diagnosis from 0 to 3 Years:

VDAART: the children were diagnosed with asthma/recurrent wheeze using parental reporting of a physician diagnosis which was based on the following: (1) wheeze after the child's second birthday, preceded by at least one report of wheeze prior to the second birthday; (2) use of asthma controller medication after the second birthday, preceded by wheeze before the second birthday; (3) two or more reports of wheeze after the second birthday; (4) at least one report of wheeze and use of asthma controller medications at distinct visits after the second birthday; or (5) two distinct reports of use of asthma controller medications after the second birthday.[1]

$COPSAC_{2010}$: the children were diagnosed with asthma/wheeze by the COPSAC pediatricians based on the clinic visits and the diary cards, according to a previously validated quantitative symptom algorithm requiring occurrence of the following: (1) five episodes of troublesome lung symptoms within six months, each lasting at least three consecutive days; (2) typical asthma symptomatology, including exercise induced symptoms, prolonged nocturnal cough, and persistent cough outside common cold; (3) need for intermittent use of inhaled β2-agonist; and (4) response to a 3-month course of inhaled corticosteroids and relapse upon the termination of treatment. [3; 4]

Genotyping:

VDAART: Genotyping was performed using the Illumina Infinium HumanOmniExpressExome Bead chip at Illumina, San Diego, CA. Genotypes were called with Illumina Genome Studio software. The 17q21 rs12936231 SNP was genotyped on this array. We excluded individuals with an individual genotyping call rate < 0.95 or a sex mismatch.

$COPSAC_{2010}$: Genotyping was performed using the Illumina Infinium HumanOmniExpressExome Bead chip at the AROS Applied Biotechnology AS center, Aarhus, Denmark. Genotypes were called with Illumina Genome Studio

software and rs12936231 was genotyped on this array. We excluded individuals with individual genotyping call rate < 0.95, sex mismatch, genetic duplicates, outlying heterozygosity > 0.27 and < 0.037, and those individuals not clustering with the CEU individuals (Utah residents with ancestry from northern and Western Europe) through a multi-dimensional clustering analyses (MDS) seeded with individuals from the International Hap Map Phase 3.

Sphingolipid Metabolism:

VDAART: Blood was sampled at ages 1 and 3 years at each clinical site and shipped to the Data Coordinating Center in Boston, where processing and aliquoting was done. Plasma was separated and immediately stored at -80^{o}C until global metabolomic profiling at Metabolon, Inc., NC.
COPSAC$_{2010}$: Blood was sampled at 6 months at the clinical research unit, where plasma was separated, aliquoted and then immediately stored at -80^{o}C at the Danish National Biobank until global metabolomic profiling at Metabolon, Inc., NC.

For both the VDAART and the COPSAC$_{2010}$ sample profiling was performed using ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and four platforms covering a broad range of the metabolome: (1) The first aliquot was analyzed using positive ion mode, chromatographically optimized for more hydrophilic compounds. The extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA); (2) The second aliquot was also analyzed using positive ion mode, however it was chromatographically optimized for more hydrophobic compounds. The extract was gradient eluted from the same C18 column as above using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA operated at an overall higher organic content; (3) The third aliquot was analyzed using negative ion mode with a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water with 6.5mM ammonium bicarbonate at pH 8; (4) The fourth aliquot was analyzed via negative ionization following elution from HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate, pH 10.8. Metabolites were analyzed as measured LC-MS peak areas. In VDAART, data were processed in two batches sent six months apart (batch one n=245; batch two n=688) sent six months apart then merged and scaled together based on equivalence of the control groups. If a metabolite had a missingness of 50% or greater in either dataset it was excluded from further analysis. The COPSAC$_{2010}$ data underwent the standard metabolomic QC pipeline[3,66-68]; metabolites with a signal-to-noise ratio <10 were considered unquantifiable and excluded, as were metabolites with undetectable/missing levels for >10% of the samples. All remaining missing values were imputed with the half the minimum peak intensity for that metabolite across the whole population, then data were pareto scaled to account for the differences in the scales of measurements across the metabolome. In both datasets metabolites were log-transformed to create approximately Gaussian distributions and to stabilize variance.

Metabolites were identified by their mass-to-charge ratio (m/z), retention time (rt), and through a comparison to library entries of purified known standards. From the metabolomic profiles, we extracted five metabolites from the sphingolipid metabolism pathway which were measured and passed QC in our metabolomic dataset. Sphingosine-1-phosphate (HMDB00277) has been shown to be one of the most important sphingolipid metabolites for airway hyperresponsiveness, mast cell activation and inflammation in mechanistic asthma models.[5-7] To further characterize the sphingolipid metabolism pathway downstream of the *ORMDL3*-regulated rate limiting SPT enzyme, we also analyzed levels of Sphinganine (HMDB00269), Sphinganine-1-phosphate (HMDB01383), Phosphoethanolamine (HMDB00224) and Sphingosine (HMDB00252).

Establishment of ORMDL3-overexpressing stable line

Experiments were conducted in 16HBE cells purchased from ATCC. *ORMDL3* was stably overexpressed in 16HBE cells using a lenti-viral system. An *ORMDL3* Human Tagged open reading frame (ORF) clone in lentiviral particles was obtained from Origene (RC202279L3V). For lenti-viral transduction, 5×10^4 cells were seeded in 48-well plates, and lenti-virus was added to the cells in the presence of 8 µg/ml polybrene (Millipore, TR-1003-G) overnight. After puromycin selection, the overexpression of *ORMDL3* was determined by qPCR and Western blot analysis (**eFigure 1**).

Measurement of Sphinosine-1-phosphate levels in a ORMDL3 overexpressing cell line:

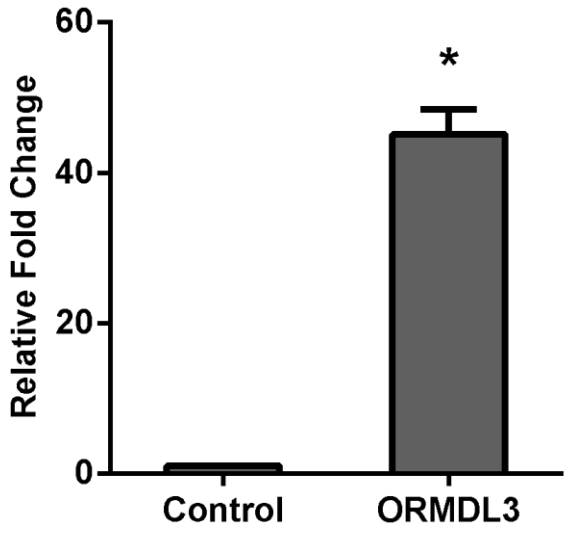
In order to determine whether there are functional implications of vitamin D treatment and *ORMDL3* expression on the levels of Sphinosine-1-phosphate, we treated 16HBE cells with or without overexpression of *ORMDL3*.

To quanify sphingosine-1-phosphate the cells were plated into 12-well plates at 4×10^5 cells/well. After 24 hours, the cells were starved overnight, then treated with 1α,25-vitamin D3 (Sigma-Aldrich) at three diffferent concentrations (0nM, 0.1nM and 1nM) for 10 hours. The levels of sphingosine-1-phosphate were subsequently quantitated by ELISA (MyBioSource). Independent ELISA were performed three times. The concentrations in the *ORMDL3* overexpressed cell line and the controls were compared to determine whether overexpression of *ORMDL3* reduced the ability of vitamin D to generate increased sphingolipid levels. An unpaired Students t-test was used to compare levels of Sphingosine-1-phosphate between the control and the ORMDL3 overespressed lines, at the three different vitamin d treament levels 0nM, 0.1nM and 1nM.

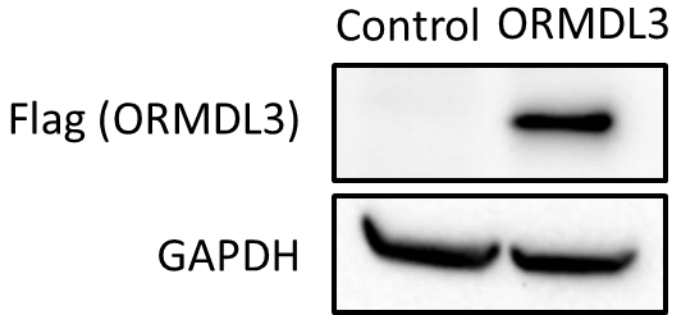
eFigures:

eFigure1: ORMDL3 overexpression level detection. ORMDL3 overexpression level was deteced by (A) mRNA level (RT-PCR) (Mean and SD was shown), and (B) protein level (western blot). * indicates $p < 0.05$, according to the unpaired Student's *t* test.

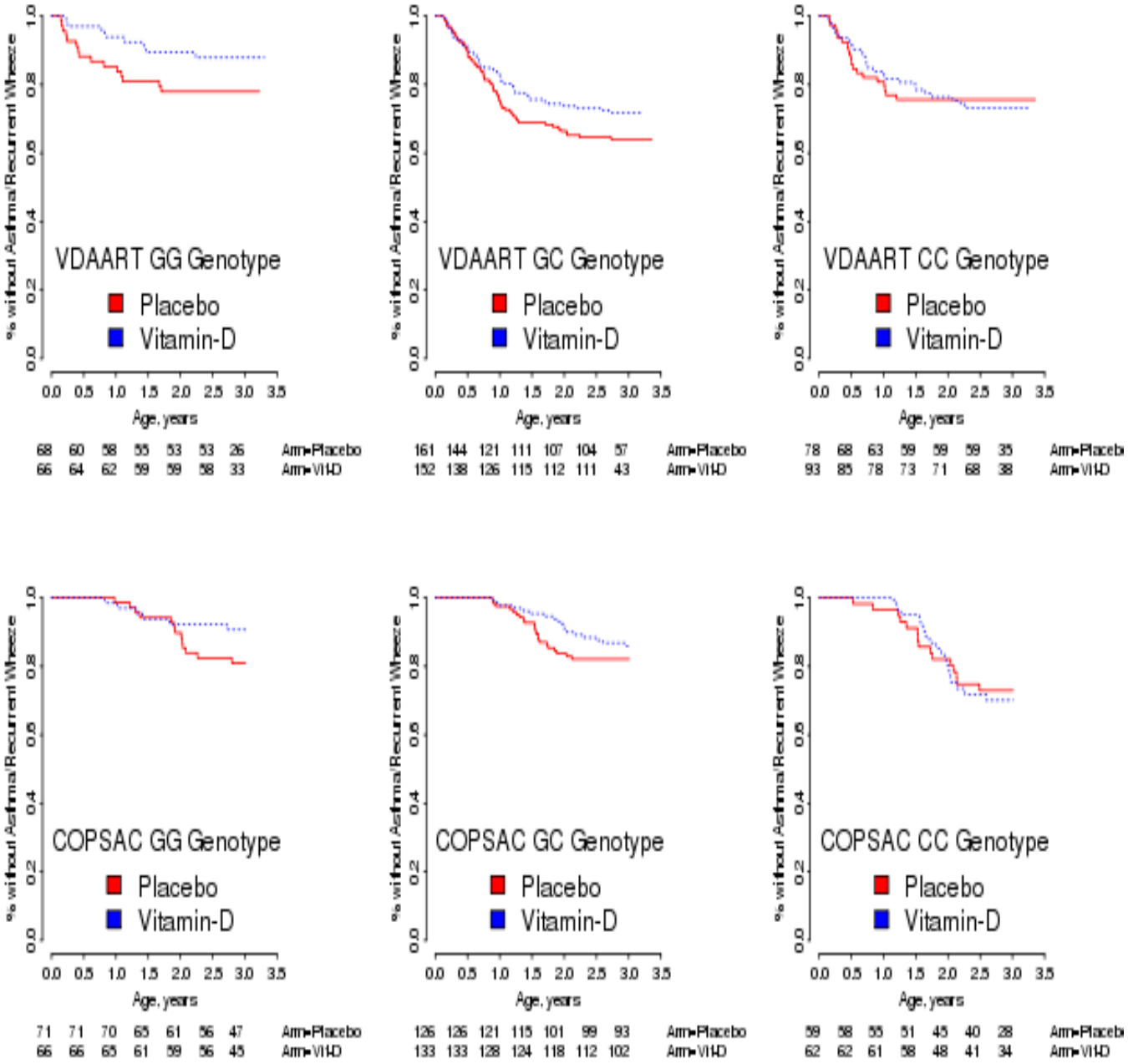
A



B



eFigure 2: Kaplan-Meier survival curves showing the effect of the prenatal vitamin D supplement on the offspring's risk of developing asthma/persistent wheeze at age 0 to 3 years in different genotype strata of the 17q21 functional SNP rs12936231 in the COPSAC$_{2010}$ and VDAART trials



Numbers at risk in the Placebo and Vitamin D arm are shown underneath the x-axis

eTables:

eTable 1: Total number of children in VDAART and in COPSAC$_{2010}$ and the number included in each of the analyses, together with their baseline characterstics

Characteristic	VDAART								COPSAC					
	Total Population (n=806)		Genotype Population (n=618)		Age One Yr Metabolite Population (n=413)		Age Three Yrs Metabolite Population (n=353)		Total Population (n=581)		Genotype Population (n=517)		Age Six Month Metabolite Population (n=441)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
rs12936231, N (%)														
CC	188	23.3%	171	27.7%	112	27.1%	106	30.0%	121	20.8%	121	23.4%	97	22.0%
GC	350	43.4%	313	50.6%	221	53.5%	172	48.7%	259	44.6%	259	50.1%	229	51.9%
GG	151	18.7%	134	21.7%	80	19.4%	75	21.2%	137	23.6%	137	26.5%	115	26.1%
missing	117	14.5%	0	0.0%	0	0.0%	0	0.0%	64	11.0%	0	0.0%	0	0.0%
Study Arm, N (%)														
Placebo	401	49.8%	307	49.7%	211	51.1%	174	49.3%	286	49.2%	256	49.5%	213	48.3%
Vitamin D	405	50.2%	311	50.3%	202	48.9%	179	50.7%	295	50.8%	261	50.5%	228	51.7%
Asthma/wheeze, 0-3yrs, N (%)														
No	530	65.8%	450	72.8%	287	69.5%	264	74.8%	477	82.1%	425	82.2%	358	81.2%
Yes	218	27.0%	168	27.2%	126	30.5%	89	25.2%	104	17.9%	92	17.8%	83	18.8%
missing	58	7.2%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Sex, N (%)														
Male	421	52.2%	321	51.9%	218	52.8%	187	53.0%	298	51.3%	269	52.0%	227	51.5%
Female	385	47.8%	297	48.1%	195	47.2%	166	47.0%	283	48.7%	248	48.0%	214	48.5%
Race, N (%)														
Black	390	48.4%	290	46.9%	195	47.2%	168	47.6%	0	0.0%	0	0.0%	0	0.0%
Caucasian	265	32.9%	200	32.4%	134	32.4%	113	32.0%	581	100.0%	517	100.0%	441	100.0%
Other	151	18.7%	128	20.7%	84	20.3%	72	20.4%	0	0.0%	0	0.0%	0	0.0%

eTable 2: Baseline characteristics of the participants from COPSAC$_{2010}$ stratified by vitamin D and fish-oil intervention

Characteristic	Vitamin D Only (n=128)	Fish Oil Only (n=123)	Vitamin D + Fish Oil (n=133)	Placebo Only (n=133)	p-value
rs12936231, N (%)					
CC	24 (18.8)	26 (21.1)	38 (28.6)	33 (24.8)	0.411
GC	71 (55.5)	58 (47.2)	62 (46.6)	68 (51.1)	
GG	33 (25.8)	39 (31.7)	33 (24.8)	32 (24.1)	
Sex, N (%)					
Male	74 (57.8)	62 (50.4)	67 (50.4)	66 (49.6)	0.513
Female	54 (42.2)	61 (49.6)	66 (49.6)	67 (50.4)	

eTable 3: Effect of prenatal vitamin D supplementation on development of asthma/persistent wheeze from 0 to 3 years in the VDAART and COPSAC$_{2010}$ trials stratified by genotype of the 17q21 functional SNP rs12936231; assuming dominance of the G allele

Vitamin D vs· placebo	VDAART N=618		COPSAC$_{2010}$ N=517		Combined analysis
rs12936231	total/ cases	HR (95% CI), p-value	total/ cases	HR (95% CI), p-value	HR (95% CI), p-value
GG/GC	447/124	0·69 (0·48-0·98), p=0·041	396/59	0·65 (0·39-1·09), p=0·103	0·68 (0·50-0·91), p=0·009
CC	171/44	1·07 (0·59-1·95), p=0·821	121/33	1·08 (0·55-2·15), p=0·822	1·08 (0·69-1·69), p=0·751

eTable 4: Mean of blood peak intensity of five key sphingolipid metabolites measured at age six months in 441 children from COPSAC$_{2020}$ in the placebo and vitamin D groups stratified by 17q21 genotype. C is the risk allele

	Six Month Samples		
	Coefficient	**95% CI**	**p value**
sphinganine-1-phosphate			
CC	0.031	(-0.117,0.180)	0.679
GC	-0.115	(-0.224,-0.006)	0.039*
GG	0.005	(-0.164,0.173)	0.958
sphinganine			
CC	0.056	(-0.173,0.286)	0.630
GC	0.009	(-0.123,0.142)	0.893
GG	0.07	(-0.137,0.278)	0.508
sphingosine-1-phosphate			
CC	0.023	(-0.118,0.165)	0.746
GC	-0.104	(-0.204,-0.004)	0.043*
GG	-0.04	(-0.19,0.11)	0.601
sphingosine			
CC	-0.017	(-0.239,0.205)	0.882
GC	-0.012	(-0.165,0.141)	0.873
GG	0.018	(-0.191,0.226)	0.869
phosphoethanolamine			
CC	0.105	(-0.053,0.264)	0.196
GC	-0.04	(-0.151,0.07)	0.476
GG	0.058	(-0.099,0.215)	0.471

CC n=97 (22%); GC n=229(52%); GG n=115 (26%)
**Significant at the 95% Confidence interval*

eTable 5: Results of multivariable interaction models exploring the relationship between a diagnosis of asthma/recurrent wheeze by age 3 years, the vitamin D intervention, 17q21 genotype and five key sphingolipids in VDAART· The table shows Beta estimate (p-values) for the individual variables/terms for a generic model: *asthma/recurrent wheeze~sphingolipid metabolite* vitamin D intervention* genotype*

	sphinganine-1-phosphate	**sphinganine**	**phosphoethanolamine**	**sphingosine**	**sphingosine-1-phosphate**
AGE ONE SAMPLES					
Sphingolipid Metabolite	-9·26 (0·009*)	0·16 (0·893)	-4·04 (0·082)	0·04 (0·976)	-14·02 (0·019*)
Vitamin D Intervention	-3·92 (0·005*)	-0·77 (0·314)	-3·20 (0·005*)	-0·79 (0·369)	-5·52 (0·017*)
rs12936231 genotype	-1·65 (0·040*)	0·10 (0·803)	-0·36 (0·554)	0·13 (0·773)	-2·62 (0·076·)
Vitamin D Intervention*sphingolipid metabolite	11·43 (0·008*)	1·12 (0·541)	8·40 (0·008*)	1·15 (0·581)	16·64 (0·023*)
Vitamin D Intervention*rs12936231 genotype	2·70 (0·013*)	0·56 (0·377)	2·03 (0·024*)	0·40 (0·581)	3·97 (0·034*)
rs12936231 genotype*Sphingolipid metabolite	5·66 (0·020*)	0·02 (0·987)	1·60 (0·364)	-0·05 (0·960)	8·75 (0·059·)
Vitamin D Intervention*rs12936231 genotype*sphingolipid	**-8·15 (0·011*)**	**-1·27 (0·425)**	**-5·72 (0·022*)**	**-0·74 (0·675)**	**-12·35 (0·035*)**
AGE THREE SAMPLES					
Sphingolipid Metabolite	-4·57 (0·133)	-1·13 (0·501)	0·06 (0·986)	-0·24 (0·898)	-3·16 (0·483)
Vitamin D Intervention	-2·89 (0·049*)	-1·74 (0·092)	-1·81 (0·247)	-1·47 (0·185)	-3·79 (0·078)
rs12936231 genotype	-0·63 (0·359)	-0·29 (0·563)	-0·05 (0·948)	-0·12 (0·830)	-0·34 (0·721)
Vitamin D Intervention*sphingolipid metabolite	6·55 (0·126)	2·37 (0·304)	2·79 (0·565)	1·64 (0·522)	9·68 (0·153)
Vitamin D Intervention*rs12936231 genotype	2·33 (0·031*)	1·73 (0·024*)	2·51 (0·039*)	1·55 (0·060)	3·06 (0·050*)
rs12936231 genotype*sphingolipid metabolite	2·58 (0·216)	1·20 (0·320)	0·68 (0·789)	0·72 (0·589)	1·73 (0·586)
Vitamin D Intervention*rs12936231 genotype*sphingolipid	**-6·12 (0·050*)**	**-3·66 (0·040*)**	**-6·84 (0·074)**	**-3·19 (0·106)**	**-8·84 (0·076)**

**Significant at the 95% Confidence interval*

eTable 6: Results of multivariable interaction models exploring the relationship between a diagnosis of asthma/recurrent wheeze by age 3 years, the vitamin D intervention, 17q21 genotype and five key sphingolipids measured at six months in 441 children from COPSAC$_{2020}$. The table shows Beta estimate (p-values) for the individual variables/terms for a generic model: *asthma/recurrent wheeze~sphingolipid metabolite* vitamin D intervention* genotype*

	Sphinganine-1-phosphate	Sphinganine	Phosphoethanolamine	Sphingosine	Sphingosine-1-phosphate
Sphingolipid Metabolite	0·12 (0·867)	-0·27 (0·573)	0·62 (0·419)	0·44 (0·372)	0·48 (0·539)
Vitamin D Intervention	-1·13 (0·019*)	-1·14 (0·020*)	-1·14 (0·018*)	-1·14 (0·018*)	-1·12 (0·021*)
rs12936231 genotype	0·10 (0·684)	0·09 (0·716)	0·11 (0·659)	0·06 (0·793)	0·10 (0·674)
Vitamin D Intervention*Sphingolipid metabolite	0·03 (0·981)	-0·03 (0·973)	-0·22 (0·852)	-0·27 (0·758)	-0·07 (0·955)
Vitamin D Intervention*rs12936231 genotype	0·72 (0·051)	0·74 (0·047*)	0·72 (0·052)	0·75 (0·044*)	0·72 (0·053)
rs12936231 genotype*Sphingolipid metabolite	0·09 (0·878)	0·57 (0·171)	-0·28 (0·670)	0·07 (0·860)	-0·10 (0·873)
Vitamin D Intervention*rs12936231 genotype*Sphingolipid metabolite	-0·33 (0·717)	-0·76 (0·244)	-0·30 (0·750)	-0·29 (0·660)	-0·31 (0·756)

**Significant at the 95% Confidence interval*

eTable 7: rs12936231 genotype and asthma status by race in VDAART

		No Asthma/wheeze, 0-3yrs	Asthma/wheeze, 0-3yrs	Total
		N (%)	N (%)	N (%)
Black (n=290)	**CC**	51 (26·7)	22 (22·2)	73 (25·2)
	GC	81 (42·4)	64 (64·6)	145 (50·0)
	GG	59 (30·9)	13 (13·1)	72 (24·8)
Caucasian (n=200)	**CC**	35 (22·9)	14 (29·8)	49 (24·5)
	GC	82 (53·6)	24 (51·1)	106 (53·0)
	GG	36 (23·5)	9 (19·1)	45 (22·5)
Other (n=128)	**CC**	41 (38·7)	8 (36·4)	49 (38·3)
	GC	49 (46·2)	13 (59·1)	62 (48·4)
	GG	16 (15·1)	1 (4·5)	17 (13·3)

eTable 8: Combined analysis of effect of prenatal vitamin D supplementation on development of asthma/persistent wheeze from 0 to 3 years stratified by genotype of the 17q21 functional SNP rs12936231 in Black and in Caucasian/Other subjects from VDAART

Vitamin D vs· placebo	**African American N=290**		**Caucasian+Other N=328**		**Combined analysis**
rs12936231 strata	total/ cases	HR (95% CI), p-value	total/ cases	HR (95% CI), p-value	HR (95% CI), p-value
GG	72/13	0·29 (0·0·8-1·07), p=0·063	62/10	0·90 (0·26-3·14), p=0·880	0·53 (0·22-1·29), p=0·163
GC	145/64	0·83 (0·50-1·35), p=0·449	168/37	0·66 (0·34-1·28), p=0·219	0·76 (0·51-1·14), p=0·179
CC	73/22	1·29 (0·54-3·09), p=0·560	98/22	0·87 (0·38-2·00), p=0·739	1·05 (0·58-1·92), p=0·870

eTable 9: Association between blood peak intensity of five key sphingolipid metabolites and vitamin D intervention in VDAART children stratified by 17q21 genotype and race; results shown for ages one and three combined according to a mixed model

	African American, Black		Caucasian+Other	
	Coefficient	**P-value**	**Coefficient**	**P-value**
sphinganine-1-phosphate				
CC	-0.012	0.591	-0.015	0.519
GC	0.024	0.159	-0.009	0.558
GG	0.035	0.136	0.053	0.120
sphinganine				
CC	-0.012	0.791	0.012	0.718
GC	0.018	0.543	-0.006	0.798
GG	0.073	0.193	0.067	0.212
sphingosine-1-phosphate				
CC	-0.011	0.482	-0.012	0.316
GC	0.008	0.454	-0.003	0.720
GG	0.025	0.056	0.022	0.257
sphingosine				
CC	-0.006	0.889	-0.006	0.852
GC	0.007	0.821	0.006	0.779
GG	0.061	0.228	0.058	0.190
phos-phoethanolamine				
CC	-0.011	0.646	0.020	0.427
GC	-0.001	0.972	-0.040	0.037
GG	0.007	0.792	0.069	0.033

eTable 10: Results of multivariable interaction models exploring the relationship between a diagnosis of asthma/recurrent wheeze by age 3 years, the vitamin D intervention, 17q21 genotype and five key sphingolipids in VDAART· The table shows Beta estimate (p-values) for the multiinteraction term: *asthma/recurrent wheeze~sphingolipid metabolite* vitamin D intervention* genotype*; with additional adjustment for, or stratification by, race

		Vitamin D Intervention*rs12936231 genotype*sphingolipid				
		sphinganine-1-phosphate	sphinganine	phospho-ethanolamine	sphingosine	sphingosine-1-phosphate
Adjusting for Race Category	YEAR ONE	-8.5 (0.012)	-1.39 (0.402)	-6.33 (0.015)	-0.95 (0.605)	-12.95 (0.034)
	YEAR THREE	-6.47 (0.039)	-4.08 (0.025)	-7.31 (0.058)	-3.69 (0.065)	-9.92 (0.05)
YEAR ONE	African American, Black	-4.17 (0.439)	0.22 (0.926)	-4.2 (0.304)	0.67 (0.785)	-8.04 (0.377)
	Caucasian+Other	-9.56 (0.036)	-3.13 (0.258)	-5.67 (0.117)	-2.97 (0.371)	-13.8 (0.103)
YEAR THREE	African American, Black	-6.24 (0.165)	-5.35 (0.036)	-10.13 (0.065)	-4.22 (0.121)	-7.95 (0.247)
	Caucasian+Other	-4.58 (0.331)	-4.3 (0.152)	-2.76 (0.644)	-4.92 (0.138)	-10.14 (0.179)

eTable11: Effect of prenatal vitamin D supplementation on development of asthma/persistent wheeze from 0 to 3 years in the VDAART and COPSAC$_{2010}$ trials stratified by genotype of the 17q21 functional SNP rs12936231; Excluding the children of 256 mothers who additionally received fish-oil supplementation during pregnancy

Vitamin D vs· placebo	**COPSAC$_{2010}$, N=261**		**Combined analysis with VDAART**
rs12936231 strata	total/ cases	HR (95% CI), p-value	HR[a] (95% CI), p-value
GG	65/9	0·45 (0·11-1·81), p=0·262	0·49 (0·24-1.02), p=0·055
GC	139/23	0·56 (0·24-1·30), p=0·178	0·71 (0·50-1·01), p=0·060
CC	57/17	0.84 (0·31-2·20), p=0·715	1·00 (0·60-1·66), p=0·999

eTable 12: Association between blood peak intensity of five key sphingolipid metabolites and vitamin D intervention in VDAART children stratified by 17q21 genotype Excluding the children of 256 mothers who additionally received fish-oil supplementation during pregnancy; results shown for ages one and three combined according to a mixed model

	Excluding all those whose mothers received fish-oil (n=222 included participants)		
	Coefficient	**95% CI**	**p value**
sphinganine-1-phosphate			
CC	0.058	(-0.152,0.269)	0.59
GC	-0.142	(-0.292,0.008)	0.066
GG	8.8x10-5	(-0.215,0.215)	0.999
sphinganine			
CC	-0.012	(-0.4,0.376)	0.952
GC	0.004	(-0.185,0.193)	0.965
GG	0.098	(-0.239,0.434)	0.572
sphingosine-1-phosphate			
CC	0.034	(-0.183,0.251)	0.76
GC	-0.131	(-0.279,0.017)	0.086
GG	-0.062	(-0.259,0.136)	0.543
sphingosine			
CC	-0.095	(-0.416,0.226)	0.564
GC	-0.005	(-0.229,0.22)	0.968
GG	-0.082	(-0.374,0.21)	0.584
phosphoethanolamine			
CC	0.172	(-0.08,0.424)	0.188
GC	-0.04	(-0.19,0.109)	0.599
GG	0.028	(-0.206,0.262)	0.816

eTable 13: Results of multivariable interaction models exploring the relationship between a diagnosis of asthma/recurrent wheeze by age 3 years, the vitamin D intervention, 17q21 genotype and five key sphingolipids in VDAART· The table shows Beta estimate (p-values) for the multiinteraction term: *asthma/recurrent wheeze~sphingolipid metabolite* vitamin D intervention* genotype*; with additional adjustment for, or stratification by, race

	Sphinganine-1-phosphate	**Sphinganine**	**Phosphoethanolamine**	**Sphingosine**	**Sphingosine-1-phosphate**
Sphingolipid Metabolite	-0.23 (0.82)	-0.34 (0.565)	0.4 (0.76)	0.25 (0.704)	-0.08 (0.946)
Vitamin D Intervention	-1.13 (0.086)	-1.06 (0.126)	-1.16 (0.081)	-1.11 (0.096)	-1.15 (0.081)
rs12936231 genotype	0.14 (0.673)	0.16 (0.635)	0.12 (0.73)	0.1 (0.755)	0.13 (0.691)
Vitamin D Intervention*Sphingolipid metabolite	0.17 (0.92)	-0.18 (0.867)	-0.27 (0.873)	-0.16 (0.902)	0.59 (0.746)
Vitamin D Intervention*rs12936231 genotype	0.54 (0.291)	0.42 (0.453)	0.52 (0.333)	0.49 (0.356)	0.55 (0.288)
rs12936231 genotype*Sphingolipid metabolite	0.13 (0.876)	0.61 (0.235)	-0.22 (0.818)	0.09 (0.869)	0 (0.997)
Vitamin D Intervention*rs12936231 genotype*Sphingolipid metabolite	-0.15 (0.917)	-1.22 (0.151)	-0.88 (0.518)	-0.97 (0.344)	-0.85 (0.579)

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