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Associations of maternal and fetal 25-hydroxyvitamin D levels with childhood lung function and asthma. The Generation R Study.

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Contribution of authors to the study

TG, HD, VJ, LD contributed to the conception and design, acquisition of data, analyses and interpretation of the data, drafted the article, revised it critically for important intellectual content, and gave final approval of the version to be published.

JM, DE, TB performed the vitamin D assays on maternal and cord blood, revised the drafted manuscript critically for important intellectual content, and gave final approval of the version to be published.

JJ, OF, HT, IR contributed to the conception and design, acquisition of data, revised the drafted manuscript critically for important intellectual content, and gave final approval of the version to be published.

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ABSTRACT

Background Exposure to low levels of vitamin D in fetal life might be a risk factor for childhood asthma.

Objective We examined whether 25-hydroxyvitamin D levels in mid-gestation and at birth were associated with higher airway resistance and inflammation, and increased risks of wheezing and asthma in school-age children.

Methods We performed a population-based prospective cohort study among 3,130 mothers and their children. Maternal blood samples in mid-gestation and umbilical cord blood samples at birth were used to determine 25-hydroxy vitamin D levels. At age 6 years, airway resistance (Rint) was measured by interrupter technique and airway inflammation by Fractional exhaled Nitric Oxide (FENO) using NIOX chemiluminescence analyzer. Wheezing and asthma were prospectively assessed by annual questionnaires until age 6 years.

Results Maternal levels of 25-hydroxy vitamin D in mid-gestation were not associated with Rint, FeNO, wheezing patterns or asthma. Children in the lowest tertile of 25-hydroxy vitamin D levels at birth had a higher Rint (Z-score (95% Confidence Interval[95% CI]): -0.42 (-0.84, -0.01), p-value for trend < 0.05), compared to those in the highest tertile group. The effect estimate attenuated when child's current 25-hydroxy vitamin D level was taken into account (Z-score (95% CI): -0.55 (-1.08, 0.01)).

Conclusion and clinical relevance Low levels of 25-hydroxy vitamin D at birth were associated with a higher airway resistance in childhood. Additional adjustment for child's current 25-hydroxy vitamin D level reduced the effect size of the association. Further studies are needed to replicate these findings and to examine mechanisms underlying the observed association, and the long-term consequences.

INTRODUCTION

Asthma affects 5-10% of school-age children with a considerable public health burden [1].

The highest rate of lung development occurs throughout pregnancy and in early life [2].

Adverse exposures in this critical period, such as low levels of vitamin D, might lead to developmental changes including reduced lung and airway growth and could therefore be of major importance for the development of asthma [1]. In vitro studies showed that human lines of alveolar cells type II have a high capacity for binding 1,25-dihydroxy vitamin D, the active form of 25-hydroxyvitamin D, resulting in surfactant synthesis stimulation and maturation of airway epithelium [3, 4]. Vitamin D is considered to affect the innate and adaptive immune system through lymphocyte activation and proliferation, and T-helper cell differentiation [5]. Studies in animals suggested that introduction of vitamin D in diet after deficiency during pregnancy and in early-life might reduce eosinophilic inflammation, airway remodeling and serum IgE levels [6]. Based on these findings we hypothesized that low levels of vitamin D during pregnancy might influence epithelial airway inflammation, airway resistance and possibly development of childhood asthma. Thus far, previous published studies in humans reported inconsistent results for the associations of maternal vitamin D level during pregnancy with the risk of asthma in their children [7-23]. The difference in results might be explained by different study designs [7-23]. Also, methods by which vitamin D was measured, either through food frequency questionnaires [7-10] or serum levels [14-23], might explain the differences in results. Furthermore, previous studies focused on the association of vitamin D levels during pregnancy or at birth with asthma, asthma-related lung function and airway inflammation measures in childhood, did not explore the mediating role of child's current vitamin D levels [14-16]. The level of 25-hydroxy vitamin D during pregnancy might be correlated with child's current vitamin 25-hydroxy vitamin D level. Also, previous studies considered an association of child's 25-hydroxy vitamin D levels with lung function and asthma [24-27].

Therefore, we examined in a population-based prospective cohort study among 3,130 mothers and their children whether 25-hydroxyvitamin D levels in mid-gestation and at

birth are associated with airway resistance, airway inflammation, wheezing and asthma in school-age children. Secondly, we explored whether any association was mediated by child's current 25-hydroxyvitamin D levels.

MATERIALS AND METHODS

General design This study was embedded in the Generation R Study [28]. The Generation R Study is a population-based prospective cohort study in Rotterdam, the Netherlands. The inclusion criteria for enrollment of the mothers in the study were being resident in the study area at their delivery date, and having a delivery date from April 2002 until January 2006. The study is designed to identify early environmental and genetic causes of normal and abnormal growth, development and health from fetal life until young adulthood. Data collection in children and their parents include questionnaires, interviews, detailed physical and ultrasound examinations, behavioural observations, Magnetic Resonance Imaging and biological samples. The Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam (MEC-2007-413-NL21545.078) approved the study protocol. The present study was performed in Dutch children of which both parents were born in the Netherlands to prevent confounding or effect modification by heterogeneous non-Dutch groups with different skin types and dietary habits. Written informed consent was obtained from 4,236 Dutch parents of participating children at age 6 years (Supplemental Figure E-1). Twins ($n = 135$), and children of whom we had no data on vitamin D levels in mid-gestation and at birth ($n = 674$) and on lung function tests, wheezing patterns and asthma ($n = 297$) were excluded, leaving a total of 3,130 Dutch children for the current analyses.

25-hydroxy vitamin D We collected maternal venous blood samples in mid-gestation (median 20.5 (range 18.1-24.9) weeks of gestation) and umbilical cord blood samples at birth (median 40.1 (range 27.6-43.4) weeks of gestation). Blood samples were transported to a dedicated laboratory facility of the regional laboratory in Rotterdam, the Netherlands (STAR-MDC) for further processing and storage. Levels of 25-hydroxyvitamin D were

analyzed from stored frozen samples at -80 °C [28]. Measurements of 25-hydroxyvitamin D levels both from maternal and cord blood were conducted at the Eyles Laboratory at the Queensland Brain Institute, University of Queensland, Australia. Total 25-hydroxyvitamin D level was reported as the sum of 25-hydroxyvitamin D₂ and D₃ species measured in plasma using a modification of a method previously described [29]. Briefly, 50 µL milli-Q water and 500 µL of acetonitrile (ACN) containing 6,19,19-[2H₃]-25-hydroxyvitamin D₂ and 6,19,19-[2H₃]-25-hydroxyvitamin D₃ at 10 nmol/L each were added to 3 µL plasma, sonicated, vortexed and centrifuged. The supernatant was filtered using a TiO₂/ZrO₂ filter plate (Glygen, USA) and evaporated to dryness. Samples were derivatised using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and reconstituted in ACN:H₂O (1:3) prior to analysis. Samples were quantified using isotope dilution liquid chromatography-tandem mass spectrometry. The analytical system was comprised of a Shimadzu Nexera UPLC coupled to an AbSciex 5500 QTRAP equipped with an APCI source. Chromatographic separation was achieved using a Kinetex XB-C18 column (50 × 2.1 mm, 1.7 µm; Phenomenex, USA), and 72% acetonitrile/32% aqueous 0.1% formic acid at a flow rate of 0.5 mL/min. Linearity was assessed using matrix-matched calibration standards, with R² values of >0.99 across the calibration range (10–125 nmol/L). Inter-assay inaccuracy and imprecision were assessed at four concentration levels for 25-hydroxyvitamin D₃ (48.3, 49.4, 76.4 and 139.2 nmol/L) and a single level (32.3 nmol/L) for 25-hydroxyvitamin D₂ using certified reference materials purchased from the National Institute of Standards and Technology (NIST) (NIST SRM 972a Levels 1-4), and were excellent at all concentration levels tested. Inter-assay inaccuracy and imprecision were both <10% for 25-hydroxyvitamin D₃ and <17% for 25-hydroxyvitamin D₂. Assay repeatability was assessed via replicate analysis of an independent reference material (NIST SRM1950, 61.9 nmol/L 25-hydroxyvitamin D₃), with inter-assay inaccuracy and imprecision both <11% (n = 343).

In children circulating serum 25-hydroxyvitamin D levels were measured at a median age of 6.0 years (range 5.6-7.9). Non-fasting blood samples were drawn by antecubital venipuncture and stored at -80 °C until analysis [28]. Measurements of 25-hydroxyvitamin D

levels were conducted at the Endocrine Laboratory of the VU University Medical Center, Amsterdam and have been previously described in detail [30]. Child's current 25-hydroxyvitamin D status was available in a subgroup of 1,734 Dutch subjects.

Childhood asthma outcomes Children visited the research center at age 6 years and lung function measurements were performed according to European Respiratory and American Thoracic Societies guidelines [31]. Airway resistance (Rint) in kPa/L (MicroRint, MicroMedical, Rochester, Kent, UK) was measured during tidal expiration, with occlusion of the airway at peak expiratory flow. Median values for at least 5 acceptable Rint measurements were calculated and these were used to compute sex- and height-adjusted z-scores [32]. Fractional exhaled nitric oxide (FeNO) was measured using the NIOX chemiluminescence analyzer (Aerocrine AB, Solna, Sweden), and natural log-transformed to obtain normality. Information on wheezing was collected with questions adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) at age 1, 2, 3, 4 and 6 years. Available data on wheezing on at least two time points were used [33] to define the following wheezing patterns: 1) no wheezing, no wheezing episode at any age; 2) early wheezing, at least 1 wheezing episode during the first 3 years of life but no wheezing episode at 4 and 6 years of age; 3) late wheezing, no wheezing episodes during the first 3 years of age but at least 1 wheezing episode at 4 or 6 years of age; and 4) persistent wheezing, at least 1 wheezing episode in the first 3 years of life and 1 episode of wheezing at 4 or 6 years of age. Information on ever physician-diagnosed asthma was obtained from the parents at age 6 years.

Covariates We obtained information on maternal age, body mass index at enrolment, educational level, history of asthma and atopy, maternal psychological distress, parity, smoking during pregnancy and pet keeping from questionnaires during pregnancy. Maternal psychological distress was defined using the Global Severity Index (GSI) [34]. Maternal folate levels were measured in early pregnancy (median 13.2 (6.2-17.9) weeks of gestation)

as previously described [35]. Season of blood sampling was recorded at time of blood sampling for 25-hydroxy vitamin D. We obtained sex, gestational age at birth and birth weight of the child from midwife and hospital records or questionnaires at birth. Information about breastfeeding, lower respiratory tract infections, physician-diagnosed inhalant allergies (pollen, house dust mite, pets)and eczema were collected by a questionnaire at age 6 years.

Statistical analysis We examined 25-hydroxyvitamin D levels both continuously and in tertiles to test for non-linearity and to create power. Mid-gestation tertile cut-offs were <52.4 nmol/L (lowest tertile), ≥52.4 – 80.7 nmol/L (middle tertile), and ≥80.7 (highest tertile), and at birth tertile cut-offs were <30.5 nmol/L (lowest tertile), ≥30.5– 49.0 (middle tertile) and ≥49.0 (highest tertile). ANOVA, Kruskal-Wallis and Chi-square tests were used to examine differences in characteristics between tertile groups of maternal mid-gestation 25-hydroxyvitamin D levels. For clinical interpretation, results based on clinical cut-offs of absolute values are given in the Supplementary Tables (E-7 and E-8). To examine the correlation between selected variables we applied Spearman's correlation test. Multivariate linear, multinomial and logistic regression models were used to examine the associations of mid-gestation and cord blood 25-hydroxyvitamin D levels with Rint and FeNO, and risks of wheezing and asthma, respectively. Models were adjusted for maternal age, body mass index, educational level, history of asthma and atopy, psychological distress, parity, smoking during pregnancy, pet keeping, folate level in 1st trimester, mid-gestational season of blood sampling, child's sex, gestational age at birth, birth weight, breastfeeding, lower respiratory tract infections, inhalant allergies, season of blood sampling at birth, and mutually for mid-gestational and cord blood 25-hydroxyvitamin D levels. Confounders were included in our models based on literature, if they were associated with both the determinant and the outcome, or if they changed the effect estimates with ≥10%. Missing data of covariates were ≤ 20.0 %, except for maternal folate level (22.2%) and season of cord blood sampling (31.6%). To explore the mediating effect of child's current 25-hydroxyvitamin D levels, we additionally adjusted our models for child's current 25-hydroxyvitamin D levels as a

continuous variable. To reduce bias and imprecision, we imputed missing data of the covariates with multiple imputations using chained equations to select the most likely value for a missing response [36]. Ten new datasets were created by imputation and analyzed altogether [37]. Characteristics of the observed and imputed variables in the analysis are given in supplementary Table E-1. We only present the results based on imputed data sets. All measures of association are presented as odds ratios (OR), sympercents (s%) of FeNO [38], or Z-scores of Rint with their 95% confidence intervals (95% CI). Symmperscents of FeNO represent the regression coefficient of $\log$ transformed FeNO*100%, and can be interpret as percentage change without transforming a variable back to its original scale. Statistical analyses were performed using SPSS version 21.0 for Windows (SPSS Inc., Chicago, IL, US).

RESULTS

Subject characteristics Median value of 25-hydroxy vitamin D in mid-gestation was 62.8 nmol/L (range 2.3-193.2nmol/L), at birth 40.2 nmol/L (range 0.1-144.9 nmol/L), and at age 6 years 64.0 nmol/L (range 4.1-211.0nmol/L). Maternal and child characteristics are presented in Table 1. Mothers in the lowest tertile of 25-hydroxyvitamin D levels had a higher body mass index at enrolment, were more often multi-parous, had lower folate levels, had more often mid-gestational blood sampling in spring and winter, and less often breastfed their child (p-values<0.05). Non-response analysis mainly showed that compared with those included in the study, mothers who were lost to follow-up were younger, had a higher body mass index at enrolment, has a lower educational level, were more often multiparous, more often had pets and lower folate levels. Also, children of these mothers more often had a lower gestational age and size at birth and were less often breastfed (Supplementary Table E-2).

25-hydroxy vitamin D levels, lung function, wheezing and asthma The level of 25-hydroxyvitamin D in mid-gestation correlated with the levels of 25-hydroxyvitamin D at birth and at age 6 years (Spearman's rho: 0.337; p<0.001 and Spearman's rho: 0.137, p<0.001,

respectively). Similarly, the level of 25-hydroxyvitamin D at birth correlated with levels of 25-hydroxyvitamin D measured in children at age 6 years (Spearman's rho: 0.289; $p < 0.001$). Unadjusted models are presented in the Supplementary tables E-3 and E-4. In adjusted models, we observed that 25-hydroxy vitamin D levels in mid-gestation were not associated with childhood Rint or FeNO (Table 2). Children in the lowest tertile of 25-hydroxy vitamin D levels at birth, had a higher Rint (Z-score (95% CI): -0.42 (-0.84, -0.01), p -value for trend < 0.05), compared with those in the highest tertile group (Table 2). The results did not change after additional adjustment for child's current 25-hydroxy vitamin D levels (Z-score (95% CI): -0.55 (-1.08, 0.01)) (Supplementary Table E-5). Levels of 25-hydroxy vitamin D at birth were not associated with FeNO. Both 25-hydroxy vitamin D levels in mid-gestation and at birth were not associated with wheezing patterns or asthma (Table 3). When clinical cut-offs were used, we observed similar results (Supplementary tables E-7 and E-8).

DISCUSSION

The results of this population-based prospective cohort study suggest that low levels of 25-hydroxy vitamin D at birth are associated with a higher Rint in childhood. Additional adjustment for childhood vitamin D status reduced the effect size of the association. We did not observe associations of 25-hydroxy vitamin D levels at birth with FeNO, wheezing patterns or asthma. Also, no associations of 25-hydroxy vitamin D in mid-gestation with Rint, FeNO, wheezing patterns or asthma were observed.

Comparison of main findings with other studies Prospective cohort studies that examined associations of maternal 25-hydroxy vitamin D levels during pregnancy with lung function are scarce [14-16]. These studies used spirometry as lung function outcome and reported no association of maternal 25-hydroxyvitamin D levels in late pregnancy with either forced expiratory volume in 1 second (FEV1) or forced vital capacity (FVC) [14, 15]. However, one study observed that higher maternal 25-hydroxyvitamin D levels in mid-pregnancy was associated with a higher FEV1 among girls, and a higher FVC among girls and boys at age 6 years (16). We observed no association of 25-hydroxyvitamin D levels in

mid-gestation with lung function as measured with childhood Rint, but did observe that low levels of 25-hydroxy vitamin D at birth were related to a higher Rint in childhood. The difference with the previous studies might be attributable to the specific period during pregnancy of 25-hydroxy vitamin D sampling [15] besides the different methods of lung function assessment [14-16]. Additionally, we showed that the effect of 25-hydroxy vitamin D levels at birth on Rint was reduced when adjusted for child's current 25-hydroxy vitamin D levels. This might be explained by either a mediating effect of child's current 25-hydroxy vitamin D levels for the association of 25-hydroxy vitamin D levels in mid-gestation and at birth with asthma, or overadjustment due to the high correlations of 25-hydroxy vitamin D levels in mid-gestation and at birth with child's current 25-hydroxy vitamin D levels. Also, we might have lacked power to remain a positive association of 25-hydroxy vitamin D level at birth and Rint when we additionally adjusted for child's current 25-hydroxy vitamin D level. Thus, further studies are needed to explore whether 25-hydroxy vitamin D levels in pregnancy, at birth and in childhood are associated with changes in airway development leading to lower lung function and higher airway inflammation, and subsequently increased risk of wheezing and asthma in later life. Levels of 25-hydroxy vitamin D at birth, mainly arisen through mother's 25-hydroxy vitamin D levels which is suggested to cross the placenta, and in childhood might be mostly alike due to similar lifestyle and dietary habits of mother and child. As a result, inclusion of current 25-hydroxy vitamin D levels in the analysis should be interpreted carefully. Associations of 25-hydroxy vitamin D levels in pregnancy or at birth with lung function in later childhood and adulthood are suggested to be negative [39], but further studies are warranted.

Little is known about the association between maternal levels of 25-hydroxy vitamin D during pregnancy and FeNO in childhood. We observed no associations between both 25-hydroxy vitamin D levels in mid-gestation and at birth with childhood FeNO. Our results are in line with previous published studies that observed that vitamin D status, measured as intake by food frequency questionnaire during pregnancy, or by blood levels of 25-hydroxy vitamin D in late gestation were not associated with change in FeNO level in

childhood [7, 14]. A recent double-blind, randomized, placebo-controlled trial in adults indicated that vitamin D supplementation reduces eosinophilic airway inflammation in patients who have non-atopic asthma with severe eosinophilic airway inflammation [40], but these effects in children are unclear. Thus, the association of 25-hydroxy vitamin D levels, or the potential beneficial effect of vitamin D supplements, in pregnancy, childhood or adulthood with FeNO levels in later life need to be further explored, as well as the association of 25-hydroxy vitamin D levels with other types of epithelial inflammation.

Our results concerning the association of maternal 25-hydroxy vitamin D levels during pregnancy with childhood wheezing are in line with the majority of previous published studies, which observed no associations [17, 18]. Only a small sample-sized study observed that a higher levels of 25-hydroxy vitamin D at birth was associated with a 33% decrease in risk of wheezing, but not with asthma at age 5 years [23]. However, current vitamin D status of the child was not taken into account, in contrast with our study. Similar as for wheezing, the majority of previously published studies observed no association of 25-hydroxy vitamin D levels during pregnancy with childhood asthma [15, 17, 18]. Only one study reported that compared with the lowest quartile, the highest quartile of maternal 25-hydroxy vitamin D during mid-pregnancy was associated with increased risk of asthma at age 9 years [19]. Differences in results with our study might be explained by the study sample sizes [10]. Similar to our study, previous studies did not observe associations of 25-hydroxy vitamin D levels at birth with childhood asthma [21-23, 41].

Interpretation of results The optimal 25-hydroxyvitamin D level during pregnancy is not known, however most researchers consider 25-hydroxyvitamin D levels of pregnant women similar as of non-pregnant women which subsequently leads to adequate 25-hydroxyvitamin D levels of their newborns [42]. The association of lower levels of vitamin D at birth with a higher airway resistance in childhood might be explained by a role of vitamin D in promoting immune regulation [43]. In animal models, exposure to vitamin D deficiency in utero and in early life was associated with an increase of Th2 lymphocyte cells, and a reduction in T

regulatory cells and production of interleukin-10 [6], which subsequently may activate pro-inflammatory cytokine production through macrophages and dendritic cells [44]. This process might lead to an increased pro-inflammatory response of the airway epithelium and subsequent airway obstruction. Also, animal models indicated that low 25-hydroxy vitamin D levels in diet affected airway hyperresponsiveness and structural changes of the airway walls, and airway remodeling [43, 45]. Airway remodeling includes myocyte proliferation resulting in increased airway smooth muscle mass [43, 46], which, in humans, is correlated with a lower FEV1 [47]. We hypothesize that subtle changes such as subepithelial basement membrane thickening and epithelial cell disruption lead to airway obstruction and increased airway resistance and might already be present before respiratory symptoms occur. Although in vitro and animal studies suggest that low levels of 25-hydroxy vitamin D during pregnancy have a role in generating inflammatory response of the airways, and airway remodeling leading to higher airway resistance, the effects in humans are not yet clear. Thus, further studies are needed to explore whether 25-hydroxy vitamin D levels in pregnancy, at birth and in childhood are associated with changes in airway development leading to lower lung function and higher airway inflammation, and subsequently increased risk of wheezing and asthma in later life.

The observed association of low levels of 25-hydroxy vitamin D at birth with a higher airway resistance in childhood is of importance from an etiological perspective, and may be important on a population-level. The results strengthen the hypothesis that low levels of vitamin D lead to developmental changes of lung and airway growth leading to obstruction-related higher airway resistance, and potential risk of asthma. However, the effect estimates could be considered small and without clinical relevance. Therefore, further replication and randomized-controlled trial studies are needed before any preventive strategies for lung function changes and asthma could be developed.

The highest rate of lung and airway development occurs in pregnancy and infancy [48]. We only observed an association of low 25-hydroxy vitamin D level at birth with higher Rint, and this association weakened after additionally adjustment for child's current 25-

hydroxy vitamin D level. This suggest that adverse exposures, such as low levels of 25-hydroxy vitamin D at birth, might affect early airway and lung growth leading to obstruction related airway resistance at a later age. It has been observed in animal models that a lack of perinatal 25-hydroxyvitamin D3 supplementation in diet increased tracheal contractility, and consequently increased airway resistance[49]. Therefore, fetal lung maturation and airway smooth muscle cell proliferation and differentiation may be affected by the 25-hydroxyvitamin D levels at birth.

Strengths and limitations The strength of our study is its population-based prospective cohort design studying participants from fetal life onwards with a large number of potential confounding factors, the objective measurements of 25-hydroxy vitamin D levels in blood in mid-pregnancy and at birth, and the detailed lung function and asthma outcome measurements. Still, some limitations need to be discussed. First, selection bias in cohort studies mainly arises due to loss to follow-up [50]. In our study population this was 26.1%, and subjects not included in the study were less affluent and had less favorable socio-economic status, life style habits and health outcomes than those included in the study. In our study population this was 26.1%, and could have led to biased effect estimates if associations of 25-hydroxy vitamin D levels in mid-pregnancy and at birth with Rint, FeNO, wheezing patterns or asthma would be different between children included and not included in the analyses. This seems unlikely but cannot be totally excluded. Second, we were not able to perform analyses with commonly used clinical cut-offs of vitamin D (insufficient <50nmol/L, sufficient 50-75 nmol/L and optimal >75nmol/L) and our respiratory outcomes due to lack of power. Third, we used Rint and not spirometry. Spirometry is the preferred method to measure lung function related to asthma. However, spirometry measurements seem not successful in approximately 50% of children aged 6 years [51]. Instead, the Rint technique demonstrates high feasibility in this age group, and detects small changes of the airways with adequate within- and between-occasion reproducibility [52]. Changes in Rint are related with clinically relevant outcomes such asthma and wheezing, and differentiates

children with and without symptoms of impaired lung function [53]. The Rint technique is known to have a high feasibility at age 6 years, detects small changes in proximal and more distal airway function with good within- and between-occasion reproducibility [52], and is associated with clinically relevant end points such as wheezing and asthma. Fourth, wheezing patterns and asthma were self-reported, which might have led to reporting bias despite the use of recommended and commonly used ISAAC questions. Fifth, we did not have data on asthma medication which might partly have diluted our effect estimates. Data on allergic sensitization measured by either specific immunoglobulin E in blood samples or skin prick tests were not available. As a proxy, we used data on physician-diagnosed inhalant allergies based on self-reports. This might have led to under- or overreporting of allergies, subsequently affecting the size of the effect estimates. Sixth, although we have performed the analysis restricted to subjects classified as Dutch to reduce variations in diet and skin color effects, their skin color, or for example second generation immigrants, may not have been identical. This might have diluted our effect estimates. Last, we cannot exclude residual confounding such as other vitamin or supplement use, as in any prospective cohort study.

Conclusion

Our results suggest that low levels of 25-hydroxy vitamin D in cord blood but not in mid-gestation are associated with a higher Rint among Dutch school-age children. However, additional adjustment for childhood 25-hydroxy vitamin D levels reduced the effect size of the association. No associations of 25-hydroxy vitamin D levels in mid-gestation or at birth with FeNO, wheezing patterns or asthma were observed. Further studies are needed to replicate our findings, to examine mechanisms underlying the observed association, and the long-term consequences.

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Table 1. Characteristics of mothers and their children according to maternal mid-gestational 25-hydroxyvitamin D tertiles

	1 st tertile	2 nd tertile	3 rd tertile	p-value for difference
	n = 959	n = 948	n = 951	
Maternal characteristics				
Age, years	31.4 (4.6)	31.8 (3.9)	31.8 (3.9)	0.220
Body mass index at enrolment (kg/m ²)	24.6 (4.4)	24.3 (3.9)	23.6 (3.6)	0.001
Educational level, higher (%)	60.2 (578)	62.4 (592)	65.3 (621)	0.062
History of asthma or atopy, yes (%)	37.6 (361)	34.8 (330)	36.1 (343)	0.291
Psychological distress	0.2 (0.2)	0.2 (0.2)	0.2 (0.2)	0.170
Parity, ≥1 (%)	41.4 (397)	38.3 (363)	35.6 (339)	0.035
Smoking during pregnancy, yes (%)	25.1 (241)	25.1 (238)	22.0 (209)	0.241
Pet keeping, yes (%)	40.9 (392)	43.6 (413)	40.3 (383)	0.247
Folate level in 1 st trimester, nmol/L	17.9 (8.6)	21.0 (8.4)	21.9 (8.8)	0.001
Mid-gestational blood sampling season				
Spring	31.0 (298)	30.0 (284)	24.4 (232)	0.001
Summer	7.4 (71)	20.0 (190)	42.5 (404)	
Autumn	24.3 (233)	26.9 (255)	20.9 (199)	
Winter	37.2 (357)	23.1 (219)	12.2 (116)	
Child characteristics				
Female sex (%)	49.6 (476)	51.0 (484)	49.5 (471)	0.759
Gestational age at birth (weeks)	39.9 (27.6,43.1)	39.9 (26.7, 43.4)	39.8 (27.6, 43.1)	0.332
Birth weight (grams)	3,475 (595)	3,516 (545)	3,500 (513)	0.272
Ever breastfeeding, yes (%)	90.3 (866)	91.2 (865)	89.6 (852)	0.017
Lower respiratory infections, yes (%)	3.6 (35)	3.7 (35)	4.8 (46)	0.283
Inhalant allergies, yes (%)	8.4 (81)	8.1 (77)	9.8 (93)	0.423
Eczema	15.2 (146)	17.2 (163)	16.9 (161)	0.774
At birth blood sampling season (%)				
Spring	33.9 (325)	26.4 (250)	14.7 (140)	0.001
Summer	36.6 (351)	26.2 (249)	14.8 (141)	
Autumn	18.6 (178)	26.6 (252)	40.1 (381)	
Winter	10.9 (105)	20.8 (197)	30.4 (289)	

Asthma, yes (%)	6.3 (51)	5.6 (45)	6.3 (51)	0.729
Wheezing patterns (%)*				
Early	29.2 (192)	30.6 (207)	23.8 (163)	0.117
Late	3.6 (24)	3.4 (23)	3.4 (24)	
Persistent	10.9 (72)	12.1 (82)	12.1 (83)	

*not imputed

Values are means (SD), medians (total range) or percentages (absolute numbers) based on imputed data of the covariates excluding exposure or outcome variables (n = 3,130). P-value for difference was calculated using ANOVA for continuous normally distributed, Kruskal-Wallis for continuous non-normally distributed and Chi square for categorical variables

Table 2. Associations of 25-hydroxyvitamin D levels with Rint and FeNO in children at age 6 years

	Rint	FeNO
	Z-score	Symmpercent
	(95% CI)	(95% CI)
	n = 1,929	n = 1,857
25-hydroxy vitamin D in mid-gestation		
1 st tertile, n = 1,083	0.40 (-0.00, 0.80)	-1.4 (-9.8, 7.1)
2.3 – 52.4 nmol/L	597/1,752	612/1,706
2 nd tertile, n = 1,081	-0.06 (-0.44, 0.32)	2.3 (-5.8, 10.4)
52.5 – 80.7 nmol/L	584/1,752	549/1,706
3 rd tertile, n = 1,079	Reference	Reference
80.9 – 193.2 nmol/L	571/1,752	551/1,706

<i>p-value for trend</i>	<i>0.28</i>	<i>0.94</i>
25-hydroxy vitamin D at birth		
1 st tertile, n = 813	-0.42 (-0.84, -0.01)*	-3.0 (-11.5, 5.6)
0.1 – 30.5 nmol/L	449/1,326	439/1,238
2 nd tertile, n = 793	0.10 (-0.29, 0.49)	2.7 (-5.4, 10.9)
30.6 – 49.0 nmol/L	415/1,326	389/1,238
3 rd tertile, n = 801	Reference	Reference
49.2- 144.9 nmol/L	462/1,326	410/1,238
<i>p-value for trend</i>	0.01	<i>0.93</i>

*P < 0.05. Values are changes in Z-score or sympercent (95% confidence interval) from linear regression models. “n =” represents number of cases per total group. Models were adjusted for maternal age, body mass index at enrolment, educational level, history of asthma/allergy, maternal distress, parity, smoking during pregnancy, pet keeping, folate level, mid-gestational season of blood sampling, child’s sex, gestational age at birth, birth weight, breastfeeding, child’s lower respiratory tract infections, inhalant allergies,eczema, at birth season of blood sampling, and mutually for mid-gestational and cord blood 25-hydroxyvitamin D.

Table 3. Associations of 25-hydroxyvitamin D levels with wheezing patterns and asthma of children until age 6 years

	Early Wheezing	Late Wheezing	Persistent Wheezing	Asthma
	Odds Ratio	Odds Ratio	Odds Ratio	Odds Ratio
	(95% CI)	(95% CI)	(95% CI)	(95% CI)
	n = 604	n = 77	n = 255	n = 158
25-hydroxy vitamin D in mid-gestation				
1 st tertile, n = 1,083	1.20 (0.82, 1.75)	0.79 (0.30, 2.10)	1.10 (0.62, 1.96)	1.37 (0.76, 2.48)
2.3 – 52.4 nmol/L	192/657	24/657	72/657	51/782
2 nd tertile, n = 1,081	1.23 (0.88, 1.72)	1.03 (0.44, 2.43)	1.45 (0.89, 2.36)	1.05 (0.59, 1.86)
52.5 – 80.7 nmol/L	207/676	23/676	82/676	45/800
3 rd tertile, n = 1,079	Reference	Reference	Reference	Reference
80.9 – 193.2 nmol/L	163/686	24/686	83/686	51/801
<i>p-value for trend</i>	0.16	0.65	0.93	0.63
25-hydroxy vitamin D at birth				
1 st tertile, n = 813	0.87 (0.58, 1.31)	1.83 (0.67, 5.058)	0.96 (0.53, 1.76)	0.85 (0.46, 1.57)
0.1 – 30.5 nmol/L	130/492	21/492	59/492	37/591
2 nd tertile, n = 793	1.07 (0.76, 1.52)	1.09 (0.43, 2.76)	1.04 (0.62, 1.74)	0.94 (0.54, 1.64)
30.6 – 49.0 nmol/L	144/506	12/506	56/506	36/597
3 rd tertile, n = 801	Reference	Reference	Reference	Reference
49.2- 144.9 nmol/L	134/525	21/525	66/525	41/599
<i>p-value for trend</i>	0.47	0.07	0.69	0.42

Values are odds ratios (95% confidence interval) from polynomial regression and logistic regression models. “n =” represents number of cases per total group. Models were adjusted for maternal age, body mass index at enrolment, educational level, history of asthma/allergy, maternal distress, parity, smoking during pregnancy, pet keeping, folate level, mid-gestational season of blood sampling, child’s sex, gestational age at birth, birth weight, breastfeeding, child’s lower respiratory tract infections, inhalant allergies,eczema,at birth season of blood sampling, and mutually for mid-gestational and cord blood 25-hydroxyvitamin D.