Prenatal vitamin D3 supplementation: pharmacology and offspring health outcomes

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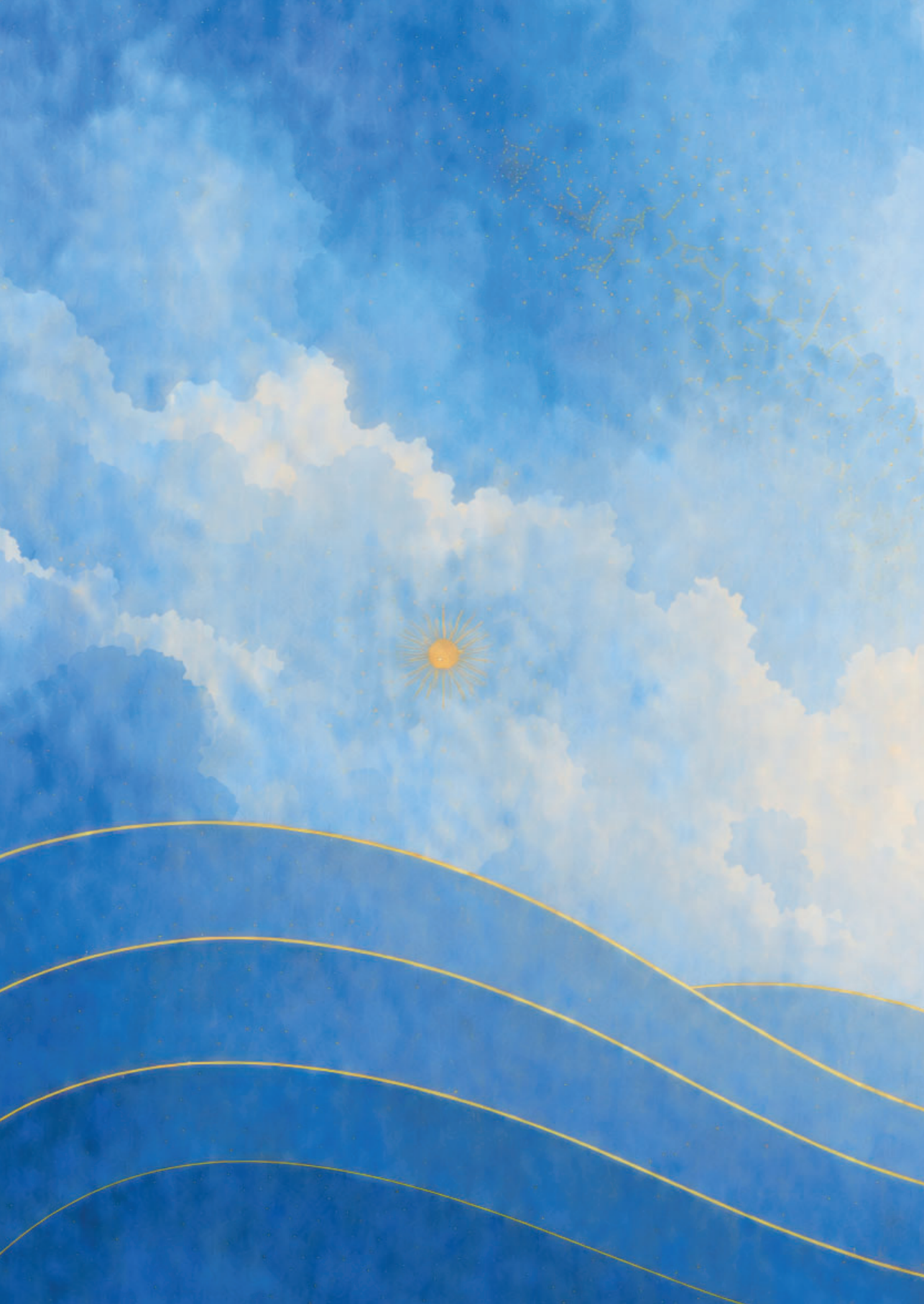
Part II

Clinical pharmacology



Chapter 4

Pharmacokinetic modeling of prenatal vitamin D exposure and the impact on offspring asthma and pulmonary function

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Abstract

Gestational 25-hydroxyvitamin D (25[OH]D) is important in fetal lung development and may influence offspring respiratory outcomes, making accurate exposure assessment essential to understand clinical associations. Therefore, we used the combined data from two large RCTs investigating prenatal vitamin D supplementation, which included early and late prenatal 25(OH)D measurements, to refine a population pharmacokinetic model of vitamin D-25(OH)D and estimate individual area under the curve (AUC) z-scores. The primary outcome was physician-diagnosed offspring asthma/wheezing at ages 3 and 6 years, and lung function, as a secondary outcome, was evaluated by spirometry at the ages 6 and 8 years. In total, 1,319 mother-child pairs were included. We found that clearance of 25(OH)D increased with gestational age and bodyweight, and decreased with higher baseline 25(OH)D levels. Prenatal 25(OH)D AUC z-scores were negatively associated with asthma/wheezing at age 3 years (aOR=0.75, 95% CI=0.64–0.88, p<0.001) and 6 years (aOR=0.83, 95% CI=0.72–0.95, p=0.008). Longitudinal analysis of lung function from age 6 to 8 years showed that AUC z-scores were positively associated with percent-predicted FEV1 (β=1.21%, 95% CI = 0.30-2.11; p = 0.009), FVC (β=0.79%, 95% CI=0.13–1.46; p=0.021), FEV1/FVC ratio (β=0.56%, 95% CI=0.11–1.01; p=0.015) and FEF25-75% (β=2.18%, 95% CI=0.46–3.91; p=0.009). These results together indicate an exposure-outcome relationship where higher gestational 25(OH)D exposure, estimated by AUC, is associated with reduced childhood asthma/recurrent wheeze and improved lung function.

Introduction

Pregnancy triggers the most notable non-pathological adaptation of vitamin D metabolism. Following placental implantation, serum levels of the hormonal form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) start to rise, peaking at approximately twice their normal concentrations by the end of the second trimester.[1,2] Although serum 25-hydroxyvitamin D (25(OH)D) levels modestly increase, they remain relatively stable despite the expanded plasma volume, which is in part due to elevated gestational levels of vitamin D binding protein (DBP).[1,2] Consequently, the correlation between 25(OH)D and $1,25(OH)_2D$ is observed to be stronger during pregnancy than at any other time in life, and $1,25(OH)_2D$ concentrations are more amendable to sun exposure and vitamin D supplementation.[1,3] The mechanisms driving the increase in $1,25(OH)_2D$ are yet to be fully elucidated. Initially, it was suggested that the rise in $1,25(OH)_2D$ served to meet the increased calcium demand of the mother and fetus by promoting intestinal absorption.[4] However, growing evidence additionally points towards an important immunomodulatory function of vitamin D. These include dampening T_H2 mediated allergic responses,[5,6] and possibly T_H1 immune responses,[5,7] which may promote a coordinated and balanced immune environment at the maternal-fetal interface.[8,9] Moreover, the fetal lungs have emerged as key target of $1,25(OH)_2D$.[10,11] Animal studies have shown that prenatal vitamin D deficiency impairs critical lung development processes, such as branching morphogenesis, fibroblast proliferation and alveolarization of the fetal lung,[12,13] and results in structural respiratory deficits, decreased surfactant synthesis, increased neutrophil count and airway resistance.[14-17] Prenatal vitamin D supplementation alleviates these effects.[18] Furthermore, in the clinical setting, prenatal vitamin D supplementation has been linked to improved early respiratory outcomes, including a decrease in asthma and respiratory tract infections and improved lung function in infants.[19-23] Proposed mechanisms relate to modulation of the offspring airway immune and microbiota profiles, inhibition of airway remodeling, maternal metabolomic alteration relating to the sphingomyelin pathway, and enhanced antimicrobial defense.[24,25]

The potential of prenatal vitamin D supplementation to reduce childhood asthma and wheezing has been explored in two large-scale randomized trials; the Vitamin D Antenatal Asthma Reduction Trial (VDAART) and the Copenhagen Prospective Studies on Asthma in Childhood ($COPSAC_{2010}$).[26,27] Despite the robust trial design, the primary outcome – reduction in offspring asthma or recurrent wheeze by the ages 3 or 6 years – was not achieved in standard intent to treat analyses.[28-31] However, a meta-analysis combining both trials revealed a significant protective effect of the vitamin D intervention by age of 3 years.[32] Post-hoc analyses provided further insight into the inconclusive results, demonstrating

high variability in participant's serum 25(OH)D responses to the different doses of vitamin D supplementation by baseline 25(OH)D status.[33] This variability highlights the challenges of evaluating a prodrug like vitamin D, which is subject to endogenous production and complex regulation, particularly during pregnancy. Thus, despite the body of preclinical and observational data to link prenatal vitamin D exposure and offspring respiratory outcomes, traditional arm-based analyses of RCTs may not fully capture these relationships. For compounds like vitamin D with detectable baseline levels and variable individual responses, an exposure-driven analysis may provide more meaningful insights.[9]

To address these challenges, we employed population pharmacokinetic (popPK) modeling, a computational method that is useful for disentangling the relationship between dosing, pharmacological exposure, and clinical outcomes. This approach leverages population-level estimates (typical values) and inter-individual variability to calculate popPK parameters, making it particularly useful when dealing with sparse observations in subjects,[34] as is often encountered in prenatal vitamin D supplementation trials. Individual parameters, including area under the curve (AUC), can be derived from the final model using the posterior distribution.[34] Therefore, popPK models are ideally suited for transforming opportunistic prenatal serum 25(OH)D measurements into a more meaningful exposure metric that accounts for baseline levels, temporal variations, and other covariates, instead of relying solely on dosage information. Currently, no popPK models describing the PK of prenatal vitamin D supplementation have been developed.

In this post-hoc study, we refined an existing vitamin D popPK model to re-analyze exposure-response data from the two largest clinical trials to date that have investigated prenatal vitamin D supplementation and offspring asthma outcomes. We hypothesized that using a popPK model to evaluate vitamin D exposure would allow us to account for individual variability in responses to supplementation, offering a more nuanced understanding of vitamin D pharmacokinetics across diverse trial populations. By leveraging popPK modeling, we aimed to provide novel insights into the relationship between prenatal vitamin D levels and offspring respiratory outcomes, specifically asthma or recurrent wheeze and spirometric indices. This comprehensive assessment of vitamin D exposure throughout pregnancy could identify significant associations that previous analyses may have overlooked, thereby advancing our understanding of the role of prenatal vitamin D in offspring respiratory health.

Materials and methods

Study populations

The trial design and populations of VDAART (NCT00920631, USA) and $COPSAC_{2010}$ (NCT008566947, Denmark) were approved by the appropriate regulatory and ethical institutes, and have been described in detail in previous publications.[28,30] In VDAART, pregnant women were randomized at 10–18 weeks of gestation, and in $COPSAC_{2010}$ at 22–26 weeks of gestation, to receive high-dose vitamin D_3 supplementation (4,000 IU daily in VDAART and 2,400 IU daily in $COPSAC_{2010}$) or placebo, along with a regular prenatal vitamin containing 400 IU vitamin D_3 for all mothers. VDAART, with an intent-to-treat (ITT) population of 806, was enriched for a parental history of allergic disease (asthma, eczema or allergic rhinitis). $COPSAC_{2010}$, which was population-based, had an ITT population of 581. In both trials, children were followed up for the primary outcome of "asthma or recurrent wheeze" until the age of 3 and 6 years. Additionally, lung function measurements were available for a subset of participants by the age of 8 years in both trials, as part of the trials' long-term follow-up protocols.

25-hydroxyvitamin D measurements

Maternal serum levels of total 25(OH)D were measured at enrollment (10–18 weeks of gestation) and again between 32–38 weeks of gestation in VDAART. In COPSAC, measurements were taken between 22–26 weeks of gestation and again one-week postpartum, constituting an approximately 20-week window in both trials. VDAART used a validated (inter-assay coefficients of variation <6.8%) chemiluminescence immunoassay (DiaSorin Liaison®[35]) for quantitative analysis and COPSAC relied on liquid chromatography-tandem mass spectrometry (LC-MS/MS).[36]

Primary clinical outcome: Asthma or recurrent wheeze

Asthma or recurrent wheeze in the child's first 3 or 6 years of life was the primary outcome in both VDAART and COPSAC, which was evaluated based on predefined criteria in each trial. In VDAART, asthma was defined as parental report of physician-diagnosed asthma as derived from quarterly questionnaires since birth. Recurrent wheeze between year 0–3 was established when one of the following conditions was met: 1) a report of wheeze before the child's second birthday followed by a report of wheeze or use of asthma medication after the second birthday, or 2) two distinct episodes of wheeze or use of asthma medication, or one of each, after the second birthday. For the assessment at age 6 years, recurrent wheeze was defined as reports of wheeze in 2 separate years over the first 6 years.

In COPSAC, asthma or recurrent wheeze between year 0–6 was defined as satisfying all following criteria ascertained from daily symptom diaries since birth: 1) five or more episodes of troublesome lung symptoms within 6 months, each lasting at least 3 consecutive days, or 4 consecutive weeks with symptoms, 2) typical symptoms of asthma, 3) use of intermittent bronchodilator, and 4) response to 3 months of inhaled corticosteroids and relapse upon cessation.

Secondary clinical outcome: Lung function

The quality control protocol for VDAART lung function measurements has been previously published.[29] Lung function was measured by spirometry at the 6 and 8 year visits using the MasterScreen PFT System in combination with the Jaeger Pneumotach (Vyaire Medical, Mettawa, IL), following the American Thoracic Society guidelines.[37] Spirometry was performed standing up with a nose clip, and two or more acceptable recordings. The highest value of each index was used for analysis after adjusting for the Global Lung Function Initiative (GLI) reference values to obtain age-, height-, sex- and race-adjusted percent-predicted values.[38] Recordings that were preceded within 24 hours by inhaled glucocorticoids or leukotriene modifiers or within 8 hours by any bronchodilators were excluded from the analysis.

In the COPSAC trial, lung function was assessed by spirometry at the ages 6 and 8 years.[39] Spirometry was performed using a MasterScope Pneumoscreen spirometer with the Pneumotach plastic PT 36 (Erich Jaeger, Germany). For each session, the highest FEV_1 from at least three assessments was selected, with a maximum within-test variation of 100 mL or 10%. Other indices corresponding to this highest FEV_1 were also recorded. Percent-predicted values were calculated using GLI reference values.[38] To ensure data quality, assessments were excluded if child had experienced lower respiratory symptoms within one week prior or used inhaled β2-agonists within 12 hours before the test.

Pharmacokinetic model development and evaluation

A popPK model was used to estimate the individual dose-exposure curves, using the maternal serum 25(OH)D data. In the COPSAC cohort, it was assumed that 25(OH)D levels at delivery (after an average treatment duration of 109 days) were comparable to those one week postpartum, when actual measurements were taken. This assumption is based on evidence that steady-state 25(OH)D levels during pregnancy are typically achieved after a maximum of 94 days of treatment.[2] The popPK analysis was performed using a non-linear mixed-effect approach implemented in Monolix2024R1 from MonolixSuite2024R1 (Lixoft

SAS, a Simulations Plus company, Antony, France). Model development was performed in 3 steps: 1) testing and selection of structural and statistical models informed by the literature, 2) covariate analysis, 3) internal validation of the model.

PopPK parameters were estimated with the Stochastic Approximation Expectation Maximization (SAEM) algorithm. Due to the sparsity of the available sample data, the model was prone to overfitting. To address this issue, the absorption rate constant and volume of distribution were fixed to literature values, reducing model complexity. Additionally, a fixed residual error of 2.5% was applied. Interindividual variability (IIV) was modeled only for the parameter clearance and inter-occasion variability was considered.

Model development was guided by a reduction in -2 times the log-likelihood (-2LL, also referred to as the objective function value [OFV]) and the corrected Bayesian Information Criterion (BICc) for nested models. A significance level of $p<0.01$ was used (-6.6 drop in OFV per Chi-Square distribution) was used for hypothesis testing, combined with a decrease in BICc to select the most parsimonious model. Additionally, parameter precision was evaluated based on relative standard errors (RSE) around parameter estimates, with RSE <30% considered acceptable. Model evaluation included: (1) goodness-of-fit (GOF) plots, (2) prediction-corrected Visual Predictive Checks (pcVPC), and (3) Normalized Prediction Distribution Errors (NPDE) based on 1,000 simulations of the final model.

Covariates were selected for testing based on visual examination of IIV on clearance versus covariates. These included maternal age, baseline 25(OH)D levels, body weight, gestational age, maternal race and vitamin D treatment arm. A non-linear effect of treatment arm on bioavailability was evaluated based on existing literature.[40,41] For body weight, linear interpolation between available measurements was performed to obtain a time-varying covariate. Preliminary covariate selection was performed using COnditional Sampling use for Stepwise Approach based on Correlation tests (COSSAC) within Monolix, with $p<0.01$ for forward inclusion and backward elimination.[42] This method, while 2–20 times faster than standard stepwise covariate modelling (SCM), yields almost identical outcomes by relying on information in the current model to decide the next step.[42] After preliminary selection, we performed an SCM procedure to determine the final covariate-parameter relationships.

The partial AUC of 25(OH)D for each participant was calculated using the Monolix software, from the time of the baseline measurement ($t=0$) to the final measurement ($t\approx20$ weeks). Individual PK estimates were derived using SAEM. To ensure consistency, these estimates were cross-validated with the mode (empirical Bayes estimates) and the mean of each individual's posterior distribution.[43]

Statistics

Demographic differences between groups were compared using Welch's t-test, Fisher's exact test, and chi-square tests as appropriate. Pharmacodynamic outcomes were analyzed in R Version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria) with statistical significance set at $p<0.05$. AUC values were standardized into z-scores for use in the logistic regression modeling of "asthma or recurrent wheeze", the same models used in the primary publications of the VDAART and COPSAC trials. Outliers beyond the AUC z-score [-3,3] range were excluded. Results were expressed as adjusted odds ratios (aOR) with 95% confidence intervals (95% CI). The models were adjusted for following covariates: study cohort, vitamin D treatment arm, maternal factors (asthma, age, BMI at enrollment, and education), household factors (income and prenatal or early life tobacco exposure), paternal asthma, season of first blood draw, and child factors (premature birth, sex, and race). Longitudinal lung function trajectories from age of 6 to 8 years were assessed using linear mixed-effect models, with subject ID as random effect. All lung function analyses were adjusted for study cohort, vitamin D treatment arm, maternal education, child's age, height, sex, weight, race, household income and tobacco exposure, and childhood asthma status. To account for the potential impact of asthma development on lung function, childhood asthma status was further classified into subphenotypes in both trials. These subphenotypes, used to adjust secondary outcome analyses of spirometry, were defined as: 1. Early transient asthma (asthma diagnosed within the first 3 years of life, but resolved by age 6 years). 2. Persistent asthma (asthma diagnosed within the first 3 years of life and still present at age 6 years). 3. Asymptomatic (no asthma diagnosis by age 3 or 6 years). The package 'Effects' was used to visualize the marginal effects of AUC on the outcomes.[44]

Sensitivity analyses

We conducted three sensitivity analyses to ensure robustness of the model, the model-derived exposure variable AUC, and its potential associations with the outcomes. (1) Impact of the fixed volume of distribution in the PK model: we systematically varied the fixed value for volume of distribution in Monolix by ±25% (i.e., 292+25% and 292-25%) and assessed its impact on the PK model outputs. (2) Noncompartmental analysis (NCA): we used the trapezoidal rule ($AUC = (t_2-t_1)(C_2+C_1)/2$) to estimate individual AUC z-scores for maternal 25(OH)D. These AUC z-scores were applied to the same outcome models used in the primary analysis. Then, we compared the exposure-outcome associations between methods.[45] (3) Cohort-specific analysis: we calculated the AUC z-scores separately for each cohort, instead of the total dataset. Next, we applied the same outcome models as the

primary analysis, stratified by cohort, and then meta-analyzed the results using the 'Meta' package. We compared these results to those from the combined model.[46]

Results

Baseline characteristics

Of the 806 mother-child pairs in VDAART and 581 in COPSAC, 764 (94.8%) and 569 (97.9%), respectively, were included in the current study. These pairs had complete data on maternal 25(OH)D levels and childhood asthma or recurrent wheeze by age 3 and 6 years (**Table 4.1**). Baseline characteristics between included and excluded subjects were similar in both cohorts, except for a higher prevalence of premature birth among the excluded subjects compared to the included ones in VDAART (6.7% vs 47.6%, **Online Tables 1 and 2**). This discrepancy was primarily due to mothers who gave birth prematurely (<37 gestational weeks) being more likely to have missed their third trimester 25(OH)D sample scheduled at 32–38 weeks. In VDAART, data on at least one successful spirometry measurement at the age of 6 or 8 years was available for 436 (57.1%) of the 764 children. A total of 223 (29.2%) children had successful measurements at both visits. In the COPSAC cohort, 520 (91.4%) of the 569 children had at least one successful measurement at age 6 or 8 years, and 426 (74.9%) children had successful measurement on both visits. The trials flowcharts and spirometry completion rates are illustrated in **Figure 4.1**.

Population pharmacokinetic analysis and model evaluation

We evaluated three popPK vitamin D-25(OH)D models from the literature.[47-49] Serum 25(OH)D concentrations were best described by the 1-compartment model of Wan et al. (based on RSE% on parameters and inspection of goodness of fit plots) with IIV on clearance and a fixed proportional error to describe the residual variability, representing the lowest proportional error reported across the models.[47] Inclusion of interoccasion variability did not improve the model performance and was dropped. Model fit was further improved by incorporating a non-linear dose-dependent bioavailability, using a sigmoid E_{max} (Hill equation) model ($p<0.001$, $\Delta OFV = -6114$]). Using COSSAC, we identified baseline 25(OH)D ($p<0.001$, $\Delta OFV=-175$), gestational age ($p<0.001$, $\Delta OFV=-10$) and body weight ($p<0.001$, $\Delta OFV=-14$) as most predictive covariates for clearance. While inclusion of maternal race improved model fit ($p<0.001$, $\Delta OFV=-10$), it disproportionally increased the model's complexity ($\Delta BICc = +4.9$) and was therefore excluded. The final covariate model, determined through stepwise covariate analysis, included an exponential influence of gestational age on clearance, and a power function for body weight and baseline 25(OH)D. Additional details on the stepwise covariate analysis can be found in

Online Table 3. Plots of the IIV around clearance (η) versus covariates showed no further trends across covariates, indicating appropriate covariate implementation (**Online Figure 1**). Parameter estimates of the final model demonstrated good precision (RSE<10%, **Table 4.2**).

Table 4.1: Baseline characteristics of subjects in VDAART and COPSAC

Variable*	VDAART (n=764)	COPSAC (n=569)
Vitamin D treatment arm, n (%)		
400 IU	385 (50.4)	280 (49.2)
2,800 IU	0 (0.0)	289 (50.8)
4,400 IU	379 (49.6)	0 (0.0)
Baseline 25(OH)D level in ng/mL	23.01 (10.21)	30.58 (10.16)
Post-intervention 25(OH)D level in ng/mL	33.02 (14.64)	36.02 (15.03)
Time between baseline and post-intervention measurement in weeks	19.69 (2.97)	15.60 (1.77)
Maternal age in years	27.41 (5.50)	32.27 (4.35)
Maternal education, n (%)		
Secondary or less	501 (65.6)	41 (7.2)
Post-secondary	263 (34.4)	528 (92.8)
Household income, n (%)		
<$100,000	492 (64.4)	150 (26.4)
≥$100,000	87 (11.4)	419 (73.6)
Refused to answer or does not know	185 (24.2)	0 (0.0)
Environmental tobacco exposure, n (%)	118 (15.4)	233 (40.9)
Asthmatic history of mother, n (%)	304 (39.8)	155 (27.2)
Asthmatic history of father, n (%)	177 (23.2)	119 (20.9)
Maternal BMI at first doctor's appointment	28.81 (7.53)	24.63 (4.49)
Season of first blood drawn, n (%)		
Spring	250 (32.7)	136 (23.9)
Summer	179 (23.4)	158 (27.8)
Autumn	153 (20.0)	187 (32.9)
Winter	182 (23.8)	88 (15.5)
Maternal race, n (%)		
Black/African American	329 (43.1)	0 (0.0)
White	311 (40.7)	543 (95.4)
Other	124 (16.2)	26 (4.6)
Gestational age (GA) at enrollment in weeks	14.15 (2.72)	24.31 (0.82)
Premature birth (<37 wks GA), n (%)	51 (6.7)	20 (3.5)
Child's sex, n (%)		
Female	369 (48.3)	279 (49.0)
Male	395 (51.7)	290 (51.0)
Child's race, n (%)		
Black/African American	367 (48.0)	0 (0.0)
White	254 (33.2)	543 (95.4)
Other	143 (18.7)	26 (4.6)
Child's asthma phenotype, n (%)		
Asymptomatic	448 (58.6)	430 (75.6)
Early transient	102 (13.4)	68 (12.0)
Active asthma	121 (15.8)	46 (8.1)
Missing, n (%)	93 (12.2)	25 (4.4)

* Characteristics are reported as mean (SD) unless otherwise indicated.

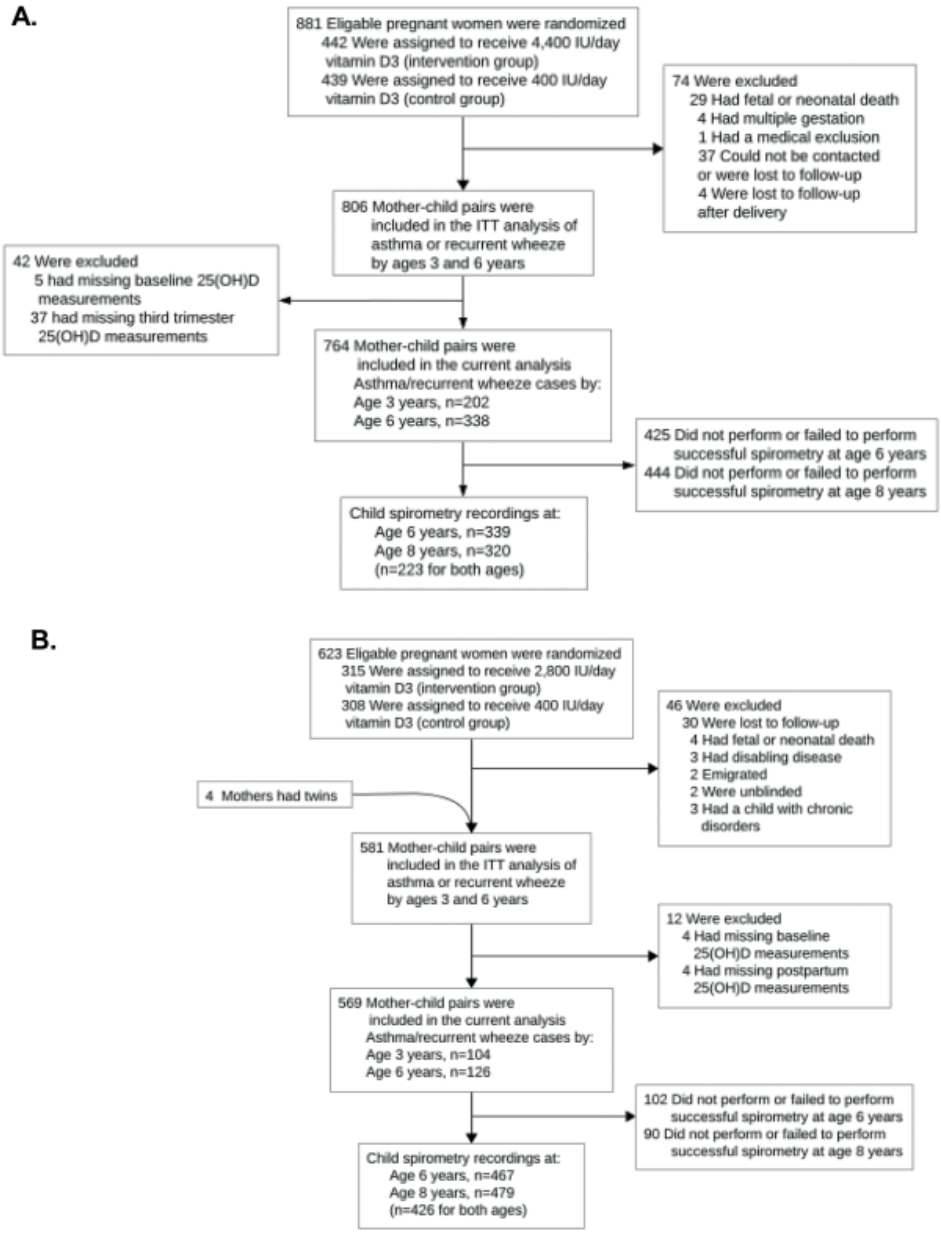

Figure 4.1: Flowchart of the (A) VDAART and (B) COPSAC cohort.

The goodness-of-fit diagnostic plots of the final model revealed no remaining trends in the population and individual predictions, or in the residuals over time, suggesting the absence of significant misspecifications in the structural model and no time-dependent biases (**Figure 4.2**). The NPDE analysis (**Online Figure 2**) showed no major deviations from

Table 4.2: Parameter estimates of the final model

Parameter	Final model estimate (RSE %) [shrinkage %]
k_a (-h)	0.323 (fixed)
V_{pop} (L)	292 (fixed)
$F_{app} = \frac{E_{\max} * Dose^n}{(E_{50}{}^n + Dose^n)}$	
E_{max}	18 (2)
E_{50} (IU)	75 (3)
n	-1.58 (2)
$CL_{ind} = CL_{pop} * e^{(GA-27.64)*\theta_{GA}} * \left(\frac{WT}{76.82}\right)^{\theta_{WT}} * \left(\frac{BASE}{63.4}\right)^{\theta_{BASE}}$	
CL_{pop} (L/h)	0.23 (1)
θ_{GA}	0.016 (9)
θ_{WT}	0.176 (9)
θ_{BASE}	-0.408 (2)
Interindividual variability (IIV)	
IIV on CL (%)	44 (2) [1]
Residual variability	
Proportional error (%)	2.5 (fixed)

RSE%: relative standard error; k_a: absorption rate constant; V: volume of distribution of 25(OH)D; pop: population mean value of a parameter for an individual with a GA of 27.6 weeks, current weight of 76.8 kg and baseline 25(OH)D of 63.4 nmol/L; GA: individual gestational age in weeks; WT: individual current body weight in kilograms; BASE: individual baseline serum 25(OH)D in nmol/L; CL: clearance of 25(OH)D; θ_{GA}: exponent for influence of GA on CL; θ_{WT}: exponent for influence of WT on CL; θ_{BASE}: exponent for influence of BASE on CL.

normality in the prediction errors. Visual confirmation of the final model fit was obtained from the PcVPCs of the serum 25(OH)D versus time, presented in **Online Figure 3**.

The 25OHD AUC z-scores were derived from the popPK model using the previously described SAEM algorithm. As confirmed in our cross-validation process, alternative mathematical methods (mode and mean of the individual parameter estimates) produced nearly identical AUC z-scores (**Online Figure 4**). AUC z-scores of 25(OH)D were significantly lower in the COPSAC cohort compared to VDAART (mean difference = 0.29σ, p<0.001). AUC z-scores of 25(OH)D varied substantially according to baseline 25(OH)D group and vitamin D dose groups. **Figure 4.3A** demonstrates a clear trend where higher baseline 25(OH)D levels and higher vitamin D doses are associated with increased AUC z-scores, highlighting the combined influence of initial 25(OH)D status and administered dose on overall prenatal

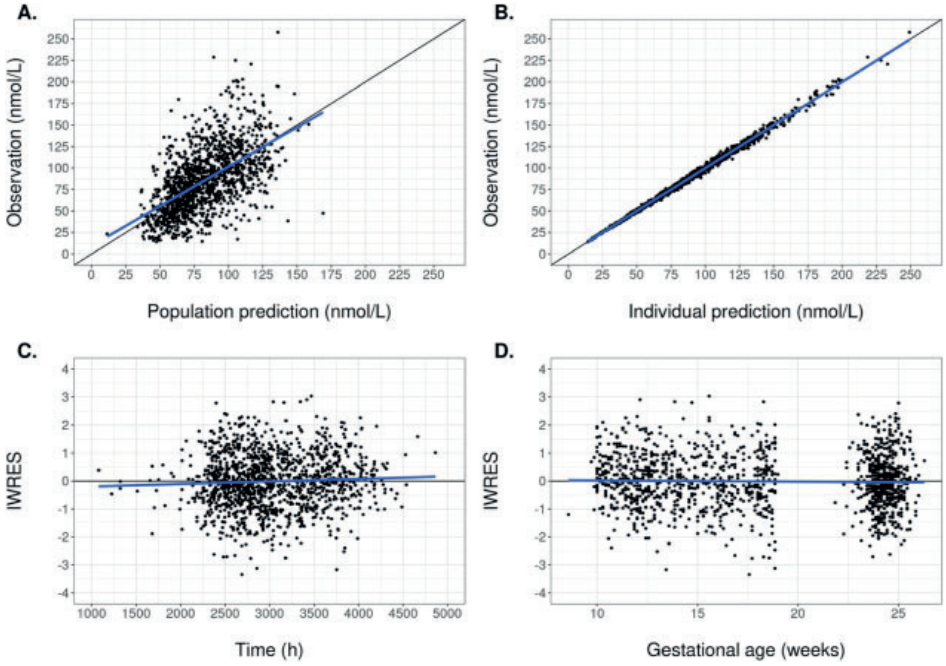

Figure 4.2: Goodness of fit of the final model and residuals over time. (A) Population predicted concentrations of serum 25(OH)D versus observations. **(B)** Individual predicted concentrations of serum 25(OH)D versus observations. **(C)** Individual weighted residuals (IWRES) at the time of the post-interventional 25(OH)D measurement. **(D)** Individual weighted residuals (IWRES) at the gestational age at trial enrollment. Black dots represent data per study subject, black lines represent the line of unity, and blue lines depict linear trends.

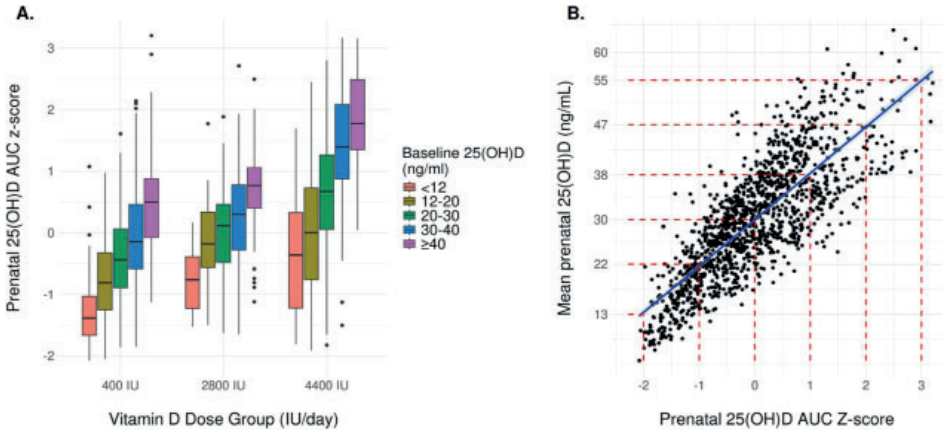

Figure 4.3: Associations of Area under the curve z-scores of prenatal 25(OH)D and dose, baseline levels and mean prenatal 25(OH)D. (A) Boxplots of 25(OH)D AUC z-scores per vitamin D supplementation group and stratified for maternal baseline 25(OH)D category. VDAART participants were randomized to a dosing regimen of 4,400 IU vitamin D daily (intervention) or 400 IU vitamin D daily (placebo) for a mean 19.6 weeks. COPSAC participants were randomized to either 2,800 IU (intervention) or 400 IU (placebo) vitamin D daily for a mean 15.6 weeks. **(B)** Scatterplot of 25(OH)D AUC z-scores versus mean prenatal 25(OH)D levels in ng/mL. Blue lines and shading represent the linear trend line and 95% confidence interval, respectively. Dashed red lines show the mean prenatal 25(OH)D levels corresponding with the 25(OH)D AUC z-scores. To convert ng/ml 25(OH)D to nmol/L multiply by 2.496.

vitamin D exposure. Interestingly, the impact of dose appears more pronounced in groups with lower baseline 25(OH)D levels. Prenatal 25(OH)D AUC z-scores correlated well with mean (early and late averaged) prenatal 25(OH)D serum levels (**Figure 4.3B**). The range of AUC z-scores from -2 to 3 corresponded with a mean prenatal 25(OH)D serum level of 13 to 55 ng/mL, with an AUC z-score of 0 relating to a level of 30 ng/mL (**Figure 4.3B**). More variability was observed at higher levels, which may be due to a greater influence of varying times spent on treatment. Notably, AUC z-scores of 25(OH)D revealed suboptimal separation between the original trial randomization arms in both cohorts in terms of vitamin D exposure (**Online Figure 5**). The association between the covariates used in the analyses and AUC z-scores are presented in **Online Figure 6**.

Primary clinical outcome

After excluding 14 subjects (1.1%) with outlier AUC z-scores (<-3 and >3), 1319 (VDAART: n=750, COPSAC: n=569) mother-child pairs were included in the analysis of the primary outcome. In total, 306 (23%) of the studied children had developed asthma or recurrent wheeze by the age of 3 years, with 464 (35%) cases by the age of 6 years. In the adjusted logistic model, increased prenatal 25(OH)D AUC z-scores were associated with a decrease in the odds of offspring asthma or recurrent wheeze by age 3 years (aOR=0.75, 95% CI: 0.64–0.88, p<0.001) and 6 years (aOR=0.83, 95% CI: 0.72–0.95, p=0.008, **Table 4.3**).

Table 4.3: Prenatal 25(OH)D area under the curve (AUC) z-scores and childhood asthma or recurrent wheeze by offspring age 3 and 6 years

Exposure	Asthma or recurrent wheeze			
	By age 3 years		By age 6 years	
	aOR (95% CI)	p-value	aOR (95% CI)	p-value
25(OH)D AUC z-score	0.75 (0.64–0.88)	<0.001	0.83 (0.72–0.95)	0.008

Data is presented as adjusted odds ratios (aOR) with 95% confidence intervals (95% CI) and p-values for the difference in asthma or recurrent wheeze incidence according to a unit increase in prenatal 25(OH)D AUC z-score in the logistic regression models. Covariates included study cohort, vitamin D treatment arm, maternal asthma, maternal age, maternal BMI at enrollment, maternal education, household income, season of first blood draw, paternal asthma, premature birth, child's sex, child's race and any household tobacco exposure prenatally or during early life. P-values less than 0.05 are in boldface.

Compared to mothers in the lowest quartile (AUC z-score -2.1 to -0.7, corresponding to a mean gestational 25(OH)D of 12–24 ng/mL), those in the highest 25(OH)D AUC z-score quartile (AUC z-score 0.7 to 3.0, corresponding to mean gestational 25(OH)D of 36–55 ng/mL) had 56% lower odds of offspring asthma or recurrent wheeze by age 3 years (aOR=0.44,

95% CI: 0.28–0.68, p<0.001) and 41% (aOR=0.59, 95% CI: 0.40–0.88, p=0.009) by age 6 years. The marginal effects of 25(OH)D AUC z-score on asthma or wheeze by age 3 and 6 years are illustrated in **Figure 4.4**, showing a continuous decrease in the incidence of asthma or recurrent wheeze until an AUC z-score of 2, which relates to a mean 25(OH)D serum level of about 47 ng/mL. No interactions were found between vitamin D exposure and any of the following factors: maternal race, maternal BMI at enrollment, maternal baseline 25(OH)D or child's sex, on the outcome at either of these timepoint.

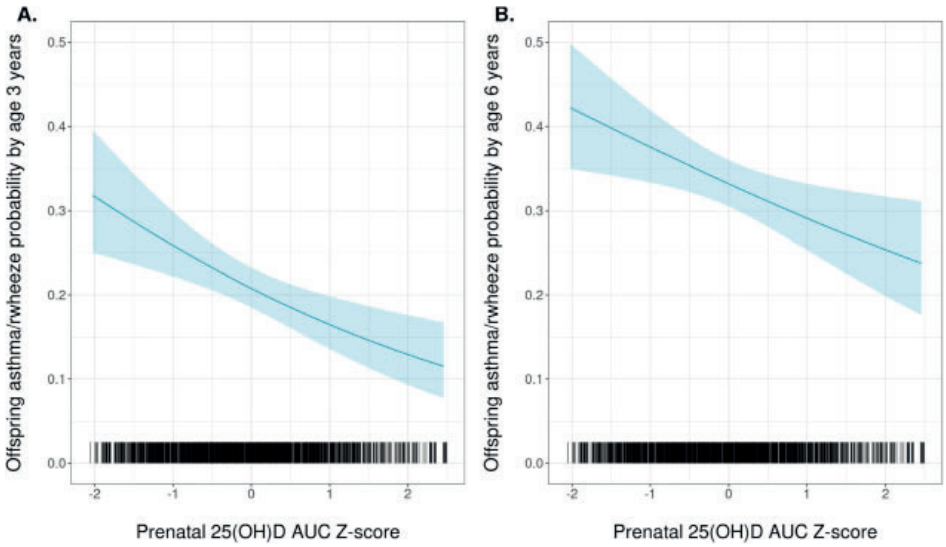

Figure 4.4: Marginal effects of 25(OH)D area under the curve z-scores on asthma or recurrent wheeze by offspring age 3 and 6 years. Probability of offspring asthma or recurrent wheeze by **(A)** age 3 years, and **(B)** age 6 years. The mean effect of prenatal 25(OH)D AUC z-scores (solid blue line) and 95% confidence interval (blue shading) are shown. Inwards ticks denote observations. Covariates included study cohort, vitamin D treatment arm, maternal asthma, maternal age, maternal BMI at enrollment, maternal education, household income, season of first blood draw, paternal asthma, premature birth, child's sex, child's race and any household tobacco exposure prenatally or during early life.

Secondary clinical outcome

In adjusted linear mixed-effect models, prenatal 25(OH)D AUC z-scores showed a positive association with children's longitudinal trajectories of percent-predicted FEV_1, FVC, FEV_1/FVC ratio and $FEF_{25-75\%}$ between the ages 6 and 8 years. The most pronounced difference was observed for $FEF_{25-75\%}$, with offspring in the highest prenatal AUC z-score quartile showing 4.8% higher $FEF_{25-75\%}$ compared to the lowest quartile from age 6 to 8 years (89 mL, 1.86 L vs. 1.94 L, p=0.038). Both FEV_1 and FVC showed positive association with prenatal 25(OH)D AUC, with a more marked increased in FEV_1. This led to an elevated FEV_1/FVC ratio, suggesting a stronger improvement in airway function relative to lung volume

Table 4.4: Prenatal 25(OH)D area under the curve (AUC) z-scores and offspring lung function trajectories from age 6 to 8 years

Exposure	Spirometry							
	FEV_1		FVC		FEV_1/FVC ratio		$FEF_{25–75\%}$	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
25(OH)D AUC z-score	1.21 (0.30–2.11)	**0.009**	0.79 (0.13–1.46)	**0.021**	0.56 (0.11–1.01)	**0.015**	2.18 (0.46–3.91)	**0.014**

Data is presented as beta estimates (β) with 95% confidence intervals (95% CI) and p-values for the difference in percent-predicted forced expiratory volume in one second (FEV1), forced vital capacity (FVC), the ratio of FEV_1 and FVC (FEV_1/FVC) and forced midexpiratory flow between 25% and 75% of FVC ($FEF_{25–75\%}$) according to a unit increase in prenatal 25(OH)D AUC z-score in the mixed-effects models. Fixed covariates included study cohort, vitamin D treatment arm, maternal education, household income, child's sex and race, household tobacco exposure and childhood asthma phenotype. Time-dependent covariates included child's age, height and weight. Random effects included study subject identifiers. P-values less than 0.05 are in boldface.

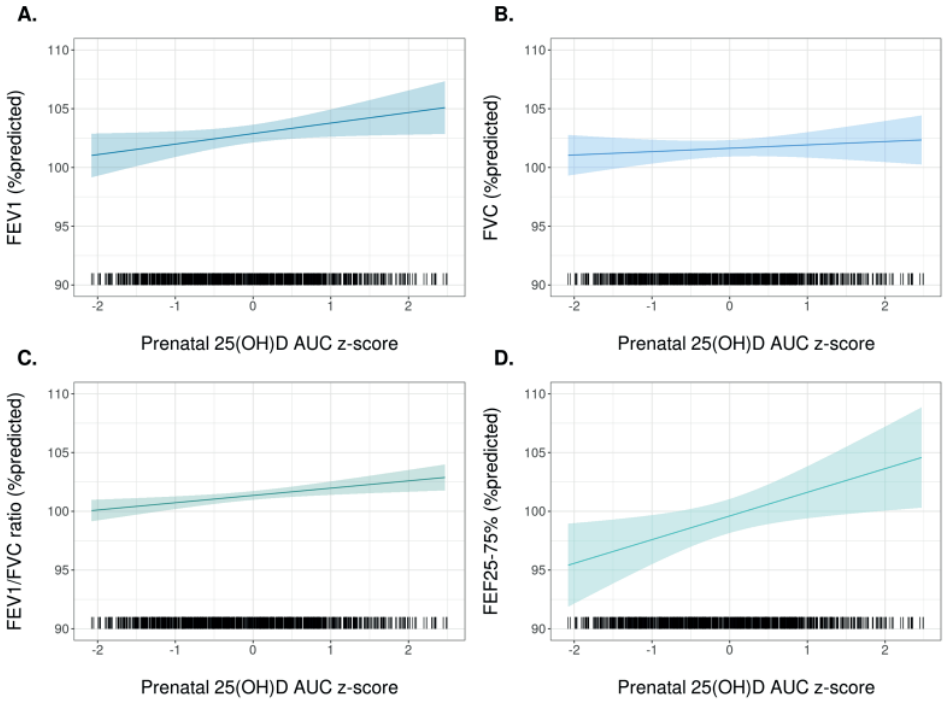

Figure 4.5: Marginal effects of 25(OH)D area under the curve z-scores on lung function indices trajectories from offspring age 6 to 8 years. The increase in the longitudinal development of percent-predicted **(A)** forced expiratory volume in one second (FEV_1), **(B)** forced vital capacity (FVC), **(C)** the ratio of FEV_1 and FVC (FEV_1/FVC) and **(D)** forced midexpiratory flow between 25% and 75% of FVC ($FEF_{25–75\%}$) between offspring age 6 and 8 in relation to AUC z-scores. The mean effect of prenatal 25(OH)D AUC z-scores (solid line) and 95% confidence interval (shading) are shown. Inwards ticks denote observations. Fixed covariates included study cohort, vitamin D treatment arm, maternal education, household income, child's sex and race, household tobacco exposure and childhood asthma phenotype. Time-dependent covariates included child's age, height and weight. Random effects included study subject identifiers.

(**Table 4.4**). **Figure 4.5** illustrates the marginal effect of AUC z-score on the lung function indices. Notably, no significant interactions between 25(OH)D AUC z-scores and child lung function were identified by maternal race, maternal BMI at enrollment, maternal baseline 25(OH)D, or child's sex.

Sensitivity analyses

(1) Volume of distribution

A ±25% change in the fixed volume of distribution minimally influenced model parameter estimates, with the largest deviation for clearance being -1%. AUC estimates remained consistent (max. +2%) across all tested volume values, demonstrating model robustness (**Online Table 4**).

(2) Noncompartmental analysis (NCA)

The NCA yielded lower 25(OH)D AUC values than the main compartmental analysis, consistent with the use of linear versus non-linear kinetics (**Online Figure 7**). Compared to the compartmental model, the NCA-derived model coefficients (β) for the offspring asthma or recurrent wheeze outcomes were slightly lower (β_{NCA}=-0.16, 95% CI: -0.30–-0.02; vs. β_{comp}=-0.19, 95% CI: -0.33–-0.05, by age 6 years). Conversely, the association between 25(OH)D AUC z-scores and lung function indices was estimated to be stronger (FVC: β_{NCA}=0.91, 95% CI: 0.27–1.55; vs. β_{comp}=0.79, 95% CI: 0.13–2.11). Although minor differences were observed between the models, the adjusted AUC z-score coefficients from the NCA aligned well with those from the compartmental model, suggesting that the main model was not overparametrized (**Online Table 5**).

(3) Cohort-specific analysis

To account for cohort-specific factors potentially affecting the prenatal 25(OH)D exposure, such as baseline levels or gestational age at treatment initiation, AUC z-scores were calculated separately for each cohort and subsequently meta-analyzed. No significant heterogeneity was observed between the trials for both the primary and secondary outcomes (Chi-squared $p>0.05$, $I^2=0\%$). The results of the meta-analysis were in close accordance with the results of the combined analysis, showing consistent common effects across all measures (**Online Figure 8**). However, the association with FVC did not reach statistical significance ($p=0.17$).

Discussion

Understanding the relationship between prenatal vitamin D exposure and offspring respiratory health has been challenging due to the complex regulation of vitamin D metabolism during pregnancy and significant variability in individual responses to supplementation. Previous research, including large randomized clinical trials, has yielded inconsistent results, potentially due to reliance on treatment allocation or single time-point measurements rather than comprehensive exposure assessment. In this study, we addressed these limitations by applying popPK modeling to estimate total 25(OH)D exposure in participants from the two largest clinical trials to date examining prenatal vitamin D supplementation and offspring respiratory outcomes. This approach allowed us to account for individual variability in vitamin D metabolism and provide a more comprehensive measure of vitamin D exposure throughout pregnancy. Our analysis revealed two key findings that advance our understanding of the relationship between prenatal vitamin D and offspring respiratory health: 1) Higher prenatal 25(OH)D exposure was independently associated with a decrease in the incidence of childhood asthma or recurrent wheeze by ages 3 and 6 years. 2) Increased exposure was associated with improved spirometry indices between ages 6 and 8 years. The observed exposure-response relationship between serum 25(OH) D and respiratory outcomes, strengthens the evidence for a potential causal association and reduces the likelihood of chance findings. These results suggest that achieving and maintaining adequate vitamin D levels throughout pregnancy may be more important than previously recognized for optimal respiratory outcomes in offspring.

Two prior studies have evaluated 25(OH)D AUC during pregnancy after oral vitamin D supplementation.[2,50] In the first study, a single oral dose 70,000 IU vitamin D was administered.[50] However, due to non-linear kinetics of vitamin D at high dosages, this study is not well-suited for comparison to our results. The second study administered a lower single dose of 1,000 IU trideuterated vitamin D, offering greater accuracy and higher likelihood of demonstrating linear kinetics, as evidenced by the PK curves in Best et al. (**Figure 4.2 and 4.3**).[2] The authors reported an AUC0-456h of 318 ng/mL*h. In our study, the average administered dose had twice the potency. Assuming linearity, we calculated an equivalent AUC of 390 ng/mL*h by halving our AUC and dividing by the average number of administrations. The minor quantitative differences between studies can be attributed to our inclusion of baseline 25(OH)D levels in the AUC calculation, which was not included in the isotope study of Best et al., as well as the different dosage regimens employed. Of note, Best et al. identified a strong correlation between serum DBP levels and AUC. While this finding suggests the potential impact of DBP on vitamin D pharmacokinetics, DBP levels were not available for the current analysis.

Large meta-analyses of RCTs on vitamin D supplementation, including data on pregnant women, show non-linear decreasing increments of 25(OH)D with increasing dose of supplemented vitamin D.[41,51] In line with these findings, our model's fit was improved with a dose-dependent apparent bioavailability. This concept encompassed the conversion of the supplemented vitamin D to the serum 25(OH)D and accounted for all external and endogenous sources of vitamin D, making it challenging to interpret specific parameters such as E_{max} and E50. The apparent bioavailability of 25(OH)D for the 400, 2,800 and 4,400 IU vitamin D dosages was 15.1, 3.6, and 1.9, respectively. These values indicate a major influence of sources other than supplementation and underscores how comparatively small the recommended prenatal dose of 400 IU appears to be. UVB-induced production from sunlight is likely the largest (non-supplement) contributor, as one minimum erythema dose (MED) of total-body radiation has been suggested to be equivalent to a dose of 10,000 IU vitamin D.[52,53] An inverse relationship between baseline 25(OH)D levels and serum 25(OH)D increment has been reported during pregnancy and non-pregnancy,[41] and could mediate our observed dose-dependent bioavailability via increased induction of metabolic enzymes.[54] Interestingly, we found that baseline 25(OH)D levels were negatively associated with clearance, a finding also observed by Hsu et al. in a PK study of IV deuterated 25(OH) D in non-pregnant subjects.[55] This may indicate that vitamin D-deficient subjects require relatively higher dosages during pregnancy to offset their higher clearance. Additionally, our finding may imply that the observed lower 25(OH)D serum increments at higher baseline levels are not primarily due to increased clearance of 25(OH)D, but rather might be attributed to reduced absorption or increased unchanged storage of vitamin D.[56] These aspects may be further amplified during pregnancy by altered intestinal absorption, changes in the gut microbiome, and increased volume of distribution due to expanding plasma volume and fat reserves.[57,58] However, significantly more data around the absorption phase is required to make definitive statements on bioavailability, as we as we fixed the absorption rate constant and could therefore not deduce bioavailability adjusted clearance (CL/F; apparent clearance) but only actual clearance.

Prenatal vitamin D deficiency has been shown to impair several aspects of fetal lung development in rodents. These include alveolar type II cell proliferation and differentiation, surfactant synthesis, structural (epithelial) integrity and increased airway hyperreactivity. These effect are mediated by a paracrine cascade involving similarly affected lung cells, such as mesenchymal stem cells.[59-62] In humans, poor gestational 25(OH)D status has been linked to higher risk of respiratory disease, while prenatal vitamin D supplementation has been associated with reduced incidence.[21,63] A potential mechanistic link between prenatal

vitamin D supplementation and offspring asthma or recurrent wheeze may be provided by the sphingolipid metabolic pathway. Single nucleotide polymorphisms (SNPs) in the chromosomal region 17q12-21, the most replicated childhood asthma susceptibility locus, alter ORMDL3 expression on primarily $CD4^+$ T cells.[64,65] Increased ORMDL3 expression is thought to increase asthma risk by reducing sphingolipid synthesis, as sphingolipid levels have been mechanistically linked to bronchial reactivity and asthma.[39,66,67] Vitamin D metabolites similarly modulate the sphingolipid pathway by increasing sphingolipid production.[68,69] Increasing gestational 25(OH)D is characterized by a marked enrichment of sphingomyelins in the maternal metabolic profile, with greater enrichment reducing the risk of offspring asthma or recurrent wheezing in both the VDAART and COPSAC trials.[70] Moreover, an interaction has been shown between 17q21 SNPs risk alleles, vitamin D supplementation and sphingolipid levels on reducing the risk of asthma or recurrent wheeze.[71,72] Bioinformatic analysis of human genetics has revealed an intriguing connection between vitamin D and asthma-related genes. Vitamin D receptor (VDR) binding sites overlap with key enhancer regions on 17q21 loci that regulate ORMDL3 and IKZF3 expression on $CD4^+$ T_H2 cells.[6] This overlap suggests a direct interaction between vitamin D signaling and genes associated with asthma risk. Specifically, VDR binding induces IKZF3 expression, leading to suppression of IL-2, IL-5 and IL-13 production through inhibition of the IL-2/Stat5 pathway. Supporting these findings, subsequent mouse models demonstrated that vitamin D deficiency exacerbates T_H2-driven allergic responses, while supplementation alleviates these responses.[6] The studies together provide experimental evidence for a possible molecular mechanism underlying vitamin D's protective role in allergic lung inflammation. Given these molecular insights, an important direction for future research would be to assess how prenatal 25(OH)D exposure (measured as AUC) differentially affects offspring respiratory outcomes based on 17q21 SNP genotypes and by sphingolipid metabolic profiles. This could provide further insights into the complex interplay between vitamin D, genetics, and metabolic factors in the development of childhood respiratory conditions.

Previous research from the VDAART study has shown that a prenatal 25(OH)D status above 30 ng/ml during early and late pregnancy is associated with a reduced progression to active asthma in offspring at age 6 years.[73] Similarly, across mean gestational 25(OH)D quartiles, Knihtilä et al. observed an increase of 10.7% (121 mL) in offspring FEV_1 between the ages 4–6 years, as well as improvements in other lung function measures.[20] Our current study builds upon these findings by utilizing a substantially larger dataset that includes an additional trial cohort (COPSAC), incorporating a continuous and more accurate 25(OH)D exposure metric, and providing greater statistical power. This analysis demonstrates a

clinically relevant reduction in offspring asthma or recurrent wheeze at two timepoints and improvements in lung function indices from age 6 to 8 years with increasing 25(OH)D AUC z-scores. The highest z-score related to a mean prenatal 25(OH)D level of about 55 ng/ml. More specifically, we observed a reduction in asthma or recurrent wheeze odds of 41% by age 6 years when comparing the highest AUC z-score quartile (0.7 to 3.0, corresponding to mean prenatal 25(OH)D levels of approximately 36–55 ng/mL) to the lowest quartile (-2.1 to -0.7, corresponding to mean prenatal 25(OH)D levels of approximately 12–24 ng/mL). Additionally, we found a 4.8% difference in $FEF_{25-75\%}$ between lowest and highest 25(OH)D AUC quartiles. $FEF_{25-75\%}$ may offer advantages over FEV_1 when measuring small airway function in adult asthma and could be better correlated with bronchodilator responsiveness in asthmatic children with normal FEV_1.[74-76] Currently, a minimum clinically important difference (MCID) has not been precisely established for spirometric variables in asthmatic children. Data from asthma and trials on pharmacological interventions suggests an increase of 5–10% or 100–200 mL in FEV_1 from baseline is commonly adhered to by regulators, because it is noticeable for patients.[77-80] In our study, the increase in FEV_1 associated with prenatal 25(OH)D observed in offspring did not reach the MCID threshold between the ages 6 and 8 years (+3.1%, 52 mL). However, while our AUC-based measure of vitamin D exposure is not directly comparable to the mean gestational 25(OH)D concentrations used in Knihtilä et al.'s study,[20] both approaches aim to capture overall prenatal vitamin D status. Hence, despite the methodological differences, the studies together suggest that the effects of prenatal vitamin D on lung function may be more pronounced in younger children and might persist, albeit to a lesser degree, into later childhood. This potential long-term impact underscores that adequate prenatal vitamin D may have important health benefits for children's respiratory development over several years, extending beyond the immediate postnatal period. Importantly, the safety profile of high dose prenatal vitamin D supplementation supports its consideration as a potential preventive strategy. Treatment-related adverse events have been exceedingly rare, if not absent, in trials administering up to 4,400 IU/day.[1,29,53] Given these safety considerations and the potential for improved respiratory outcomes, ensuring adequate vitamin D status during pregnancy through appropriate supplementation, as early as possible, could be a valuable approach, particularly in women at risk of vitamin D deficiency.

Several limitations of the present analysis should be noted. Due to the sparse sampling per individual, we opted for a simple model structure based on the literature. The model was optimized for internal AUC estimation based on our specific dataset. While this approach was suitable for the model's primary aim, evaluating individual 25(OH)D exposure, it may

limit the model's generalizability to other external datasets. Further validation studies would be beneficial to assess its performance across diverse populations. Moreover, the selected model from Wan et al. was originally developed based on data from chronic kidney disease patients,[47] which might affect vitamin D kinetics differently than pregnancy. However, because Wan et al. obtained the absorption rate constant from the literature,[49] we primarily relied on their estimated volume of distribution. Our sensitivity analysis confirmed that using these estimates did not destabilize our results. This approach allowed us to leverage existing modeling work while tailoring it to our specific research context. However, future studies might benefit from a well-designed vitamin D PK study in pregnant women specifically aimed at model development. The generalizability of our model is improved by the combination of both a high-risk cohort for offspring allergic disease and non-atopic asthma (VDAART) and an unselected cohort (COPSAC). It's important to acknowledge the demographic differences between the cohorts. VDAART included a high percentage of Black or African American participants, even when compared to the general U.S. population, where COPSAC participants were predominantly of White European decent, reflecting Danish demographics. VDAART also included women at an earlier gestational age and COPSAC had higher baseline 25(OH) D levels. Nonetheless, the meta-analysis showed that cohort-specific characteristics had minimal influence on the overall findings. Finally, despite the inclusion of known covariates, residual confounding factors should be considered. This is important as the potential mechanistic links between vitamin D and fetal lung development remain an active area of research.

Conclusions

Our study demonstrates that higher prenatal exposure to 25(OH)D, as reflected by AUC, is associated with decreased incidence of asthma or recurrent wheeze in offspring by ages 3 and 6 years, and improved lung function between ages 6 and 8 years. These findings persisted after controlling for various sociodemographic and socioeconomic factors. Improvements in outcomes were associated with increasing 25(OH)D AUC z-scores, corresponding to mean prenatal 25(OH)D levels from approximately 12 ng/mL up to 60 ng/mL. The use of AUC as a vitamin D exposure metric provides a more comprehensive measure of vitamin D status throughout pregnancy, capturing both the magnitude and duration of exposure. This approach revealed nuanced relationships that might be overlooked when relying solely on dosage information, single time-point measurements or simple averages. Future studies may benefit from using AUC as an exposure metric to further explore optimal target gestational 25(OH)D concentrations, as well as the ideal timing needed to achieve these levels for favorable respiratory outcomes in children.

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CRediT authorship contribution statement

Iskander L.C. Shadid: Data curation, Conceptualization, Investigation, Formal analysis, Visualization, Methodology Writing – original draft. **Nicklas Brustad:** Data curation, Conceptualization, Investigation, Formal analysis, Visualization, Writing – review & editing. **Bo L. Chawes:** Conceptualization, Visualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Dirk Jan A.R. Moes:** Investigation, Visualization, Methodology, Writing – review & editing, Supervision. **Scott T. Weiss:** Conceptualization, Visualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Henk-Jan Guchelaar:** Conceptualization, Visualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Hooman Mirzakhani:** Data curation, Conceptualization, Investigation, Visualization, Methodology Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing financial interests that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available upon reasonable request as judged by the principal investigators of VDAART and COPSAC.

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