Prenatal vitamin D3 supplementation: pharmacology and offspring health outcomes

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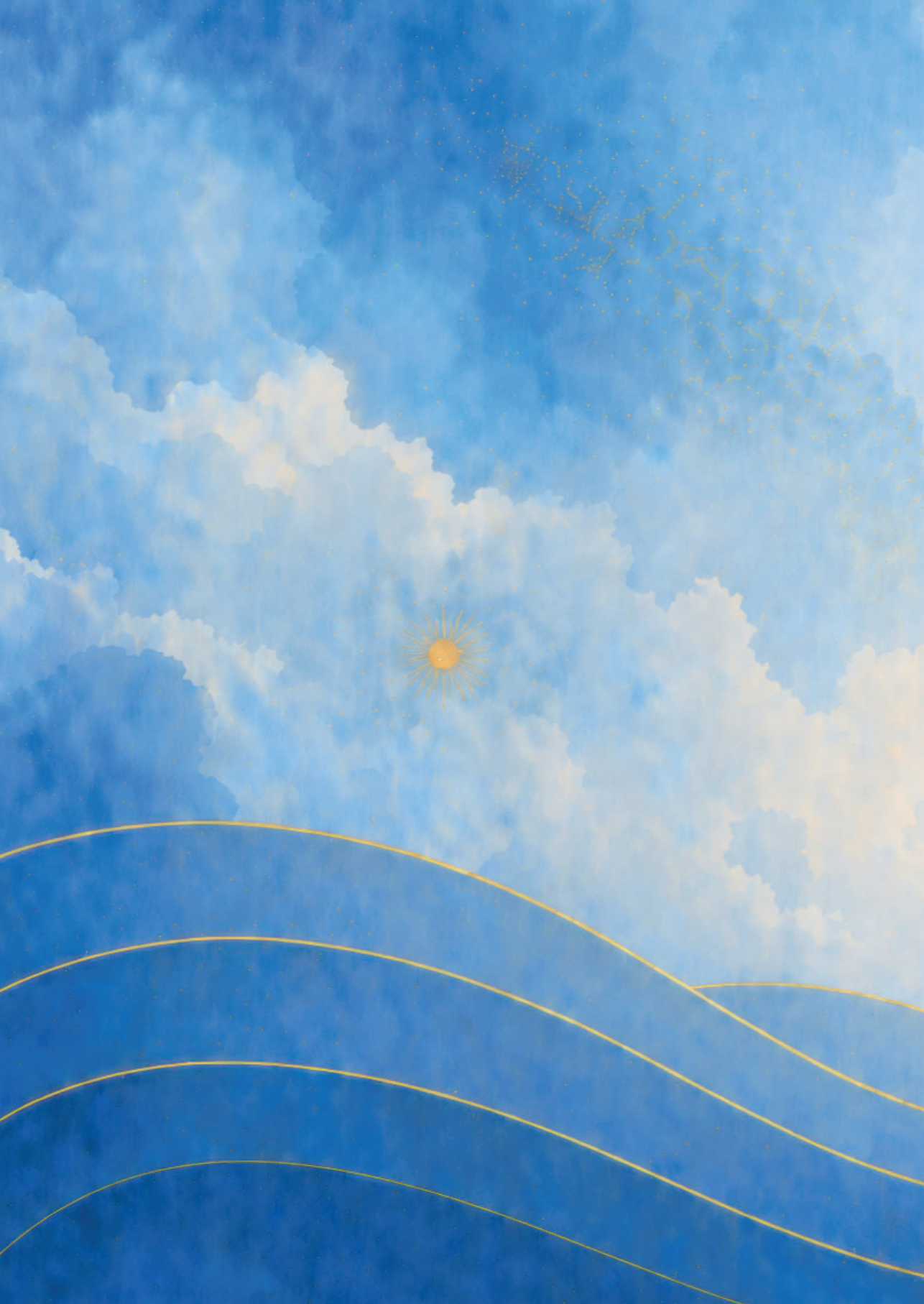

Chapter 11

General discussion

Preclinical insights into vitamin D tissue distribution

Although vitamin D's classical endocrine role in mineral homeostasis is well characterized, novel insights in its paracrine and autocrine activity in vitamin D metabolizing tissues have raised additional questions about local concentrations, and how these relate to health outcomes. In **Chapter 2**, we mapped vitamin D concentrations beyond circulation and reported that vitamin D metabolites are differentially distributed across tissues after vitamin D supplementation. We further showed that serum 25(OH)D levels, the clinical marker of vitamin D adequacy, are not fully reflective of tissue stores. Both human data and animal models show a selective retention of vitamin D metabolites in certain organ tissues (e.g., adipose, muscle, lung) with tighter control of active metabolite $1,25(OH)_2D$. Nevertheless, regulatory control of active vitamin D is relaxed during pregnancy, allowing for accumulation in maternal tissues such as the placenta. These insights indicate that reliance on serum 25(OH)D as a sole marker may overlook local deficits in specific organs. From an evolutionary perspective, humans evolved with daily sun exposure, guaranteeing steady-state vitamin D kinetics between blood and tissues. However, modern lifestyles, which include indoor living, clothing habits covering body surface and processed foods, lead to poor vitamin D exposure and potential insufficiency in certain physiological compartments. During pregnancy, vitamin D demands surge, which could create or amplify existing vitamin D deficiency. Therefore, given that pregnant individuals are at higher risk of vitamin D deficiency on top of the modern-day baseline risk, understanding vitamin D tissue distribution during gestation has clinical relevance, particularly considering locally mediated effects of $1,25(OH)_2D$ which may be associated with good pregnancy outcomes.

The placenta

A major gap identified in **Chapter 2** was the lack of knowledge on how altered vitamin D pharmacokinetics during pregnancy affect tissue allocation (a lack of general tissue data beyond pregnancy was also highlighted). **Chapter 3** aimed to address this knowledge gap using a mouse supplementation study, for the first time quantifying maternal-fetal handling of vitamin D metabolites in vivo. Pregnancy induced a shift in the distribution of vitamin D metabolites, showing substantially increased serum and renal levels of $1,25(OH)_2D$ and high placental 25(OH)D accumulation. Interestingly, lung tissue also showed significant retention of 25(OH)D. The relatively high levels in these target compartments compared to other investigated tissues point towards an important role of vitamin D in these organs.

The central role of the placenta in vitamin D metabolism (i.e., sequestration of 25(OH)D, active conversion and dynamic transfer) is also suggested in humans. An ex vivo human placenta model demonstrated that placental tissue is capable of hydroxylating 25(OH)D to both $1,25(OH)_2D$ and $24,25(OH)_2D$ and indicated that these metabolites were released to the maternal and fetal circulation. Furthermore, sampled human placentas after birth showed noticeable levels of 25(OH)D and $1,25(OH)_2D$, and levels of 25(OH)D are significantly decreased in adverse pregnancy outcomes such as preterm birth.[1,2] Taken together, such data is indicative of a role of the placenta in the local production and modulation of vitamin D metabolites to support pregnancy-induced demand, which may relate to placental implantation, overall function, and maternal and fetal immune profiles.[3-6]

Of note, the mouse data suggests that supplemented vitamin D is routed to the relevant compartments during pregnancy. However, at high vitamin D doses, fetal transfer of 25(OH)D was decreased, indicating that the maternal-fetal interface dynamically regulates the availability of vitamin D metabolites to the fetus.

The lungs

Although fetal lung levels of vitamin D metabolites could not be measured in the mice, the high presence in maternal lungs suggests that respiratory tissue is a key vitamin D target. This is intriguing, as the lung is a key immune organ which is rich in macrophages and lymphocytes, and the primary pathological site of infant asthma.

Indeed, in humans, alveolar type 2 cells, airway epithelial cells and smooth muscle cells, alveolar macrophages and fibroblasts express the vitamin D receptor (VDR), with most co-expressing CYP27B1 and CYP24A1 for $1,25(OH)_2D$ synthesis.[7] Furthermore, $1,25(OH)_2D$'s paracrine and autocrine signaling in immune cells is well-documented, as virtually all immune cells express CYP27B1 to locally synthesize $1,25(OH)_2D$ and VDR to induce associated gene modulation. For example, CD46-mediated complement engagement upregulates VDR and CYP27B1 in T helper type 1 (Th1) cells and $1,25(OH)_2D$-VDR signaling then restructures Th1 epigenetic landscape, recruiting several transcription factors (e.g., c-JUN, STAT3 and BACH2) to suppress IFN-gamma and induce IL-10 to shut down the pro-inflammatory Th1 programs.[8] Another method of how $1,25(OH)_2D$ mediates immune tolerance is through inhibition of NF-kB in dendritic cells, suppressing cell maturation signals and T cell-recruiting chemokines and co-stimulating cytokines, thereby reprograming dendritic cells to induce a tolerogenic state.[9] Antigen presentation by these tolerogenic dendritic cells allows for expansion of regulatory $CD4^+$ T cells (Tregs) and downregulation of effector $CD8^+$ T cell. In pregnancy, 25(OH)D serum levels correlate with Treg levels,

and upon incubation with 1,25$(OH)_2$D, isolated $CD4^+$ T cells from pregnant women show increased FOXP3 and downregulated RORγt expression, key transcription factors for Tregs and Th17 cells, respectively.[10] Moreover, CYP27B1 and VDR expression co-localize with key regulators of tolerogenicity, such as HLA-G and HLA-C, in extravillous trophoblasts and, subsequently, low 25(OH)D levels are associated with downregulation of these HLA proteins.[10,11] Particularly HLA-C has been identified as a sensitive vitamin D target gene, with a strong vitamin D response element (VDRE) distal from its transcription start site.[12]

Our finding that murine lung tissue readily takes up vitamin D metabolites, with 25(OH) D serving as a substrate for local conversion to 1,25$(OH)_2$D, may suggest a plausible mechanism by which adequate maternal vitamin D metabolite exposure could prime the respiratory-immune milieu (if conserved in humans). The shift away from pro-inflammatory Th1/Th17 (and potentially Th2) responses could explain why adequate prenatal vitamin D exposure may reduce early-life wheeze by programming a less hyperresponsive immune environment in developing airways, assuming fetal lung tissue has similar uptake. Additionally, the enhanced regulatory T cell function and IL-10 secretion associated with sufficient vitamin D levels suggests a potential mechanism by which prenatal vitamin D supplementation might reduce the excessive inflammatory responses that characterize asthmatic airways in offspring. Moreover, 1,25$(OH)_2$D substrate availability could enhance maternal immune readiness and balanced placental and embryonic development, inducing a more tolerogenic immune environment at the maternal-fetal interface, which could aid pulmonary development in utero.

In support of these roles are various animal experiments that show that prenatal vitamin D deficiency has noticeable effects on lung development. These studies, across different models, report that diet-induced vitamin D restriction during gestation yields offspring with smaller lungs and decreased lung function, larger and fewer alveoli, reduced expression of surfactant proteins, increased airway smooth muscle mass and pro-inflammatory immune profiles,[13-17] with dose-response protection of vitamin D supplementation against these outcomes,[18] potentially through improved epithelial repair and mesenchymal-epithelial signaling.[19] In adult VDR-knockout mice, a phenotype including altered tight junction expression in the lungs was observed, resulting in weaker airway more prone to hyperresponsiveness.[20] Mechanistically, the studies suggest that vitamin D deficient dams produce fetuses with downregulated surfactant-regulating transcription factors (e.g., TTF-1 and FoxA), impaired lipofibroblast support mediated by PPAR-γ, and altered VEGF and Wnt/β-catenin pathways (crucial for embryonic development), which collectively compromises lung structure and function. Notably, simplified alveolar architecture and thicker and potentially

more reactive airways touch upon several key elements of asthma, possibly predisposing neonates to the airway hyperresponsive, defective epithelial integrity and enhanced inflammatory responses underlying asthma pathogenesis.

Overall, the preclinical findings from **Chapter 2 and 3** provide mechanistic information on how prenatal vitamin D supplementation is distributed in the maternal-fetal unit, highlighting the placenta as an active buffer that directs vitamin D metabolites to areas of need and the lungs as an important target. The preclinical data generated by other investigators using rodent models of prenatal vitamin D deficiency provide preliminary experimental evidence suggesting that severe vitamin D deficits are detrimental for normal fetal lung development, which may increase the risk of associated respiratory morbidities such as asthma.

Clinical evidence for prenatal vitamin D supplementation during pregnancy to prevent childhood asthma

A recent Cochrane review including 16 RCTs (over 10,000 participants) evaluated the efficacy of prenatal high dose vitamin D supplementation to prevent childhood asthma, wheeze and related outcomes versus low/standard dose or placebo/no supplementation in pregnant women and children.[21] Regarding prenatal supplementation, the totality of evidence suggests that high dose vitamin D supplementation during pregnancy reduces the risk of early life wheeze, with a smaller and more uncertain effect on asthma itself. Compared to a low dose or standard regimen, high dose supplementation had no consistent influence on other allergic or infectious respiratory outcomes. The review concluded that the most consistent signal favoring high dose supplementation is observed in the prevention of wheeze, and that evidence for its effects on childhood asthma is less certain. Across the trials, differences in doses and dosing regimens across the trials and large numbers of enrolled women with sufficient serum 25(OH)D may inflate variability around effect estimates. Given the observations that supplementation may be most efficacious when pregnant women reach sufficiency early enough to cover key windows of fetal lung (and immune) development, future trials may benefit from additional trial refinements, ensuring target exposure are reached and maintained and optimizing trial population selection, biomarker strategies and timing of supplementation initiation. Together these steps may help to triangulate the population most likely to benefit from prenatal vitamin D supplementation and clarify the potential causal effects on childhood respiratory outcomes.

Target exposure

On the basis of the preclinical data, two of the largest trials to date, VDAART and COPSAC, were launched to investigate whether prenatal vitamin D supplementation prevents childhood asthma or recurrent wheeze. VDAART showed a linear trend towards lower asthma/wheeze incidence by age 3 years, which became less pronounced by age 6, and COPSAC revealed a smaller, but directionally consistent, effect.[22,23] A subsequent meta-analysis of the two trials suggested a protective benefit of prenatal vitamin D supplementation.[24] However, heterogeneity in trial design and patient characteristics complicates this meta-analysis, as well as others on prenatal vitamin D. Key issues are variations in baseline 25(OH)D levels, with a proportion of individuals having close to sufficient or more than sufficient vitamin D status at enrollment, differences in the doses in the intervention arm and in the alternative vitamin D input (solar and dietary), variability in the serum 25(OH) D responses to the supplementation, and the placebo group receiving an ethical 400 IU dose of vitamin D daily. All these elements may have diluted the true effect in the RCTs. These challenges highlight the need for a more targeted trial design, incorporating total vitamin D exposure, and better understanding of the pharmacokinetics (PK) of vitamin D in pregnancy, which is why we established a first population PK model of maternal vitamin D supplementation and 25(OH)D exposure in **Chapter 4**.

Our pop-PK compartmental model indicated a higher clearance of serum 25(OH)D as pregnancy advances and with increasing maternal body weight. This aligns with the observation that serum 25(OH)D levels reach steady state during the last trimester, and lower observed circulatory levels in obese mothers. They also reflect our preclinical findings suggesting increased placental uptake in late gestation and sequestration of vitamin D under high-adiposity conditions. Maternal characteristics and timing are therefore anticipated to be critical in dosing strategies. In addition, the model showed that individuals with higher baseline 25(OH)D levels had slightly lower clearance, likely due to overall higher baseline vitamin D stores. This is in line with the common nutritional paradigm that deficient patients benefit the most from repletion and the data from VDAART demonstrating that deficient mothers had the greatest absolute increase in serum 25(OH)D after supplementation, which was linked to the greatest reduction in the incidence of offspring asthma (**Chapter 5**).

Estimating total exposure to gestational 25(OH)D with compartmental and noncompartmental models yielded a clear exposure-outcome relationship with offspring asthma and a modest but statistically significant improvement lung function (**Chapter 4**). Notably, a linear association fit the data better than a nonlinear one, indicating a benefit across the analyzed spectrum of maternal 25(OH)D levels, from approximately 12 ng/mL to around 60

ng/mL. In other words, offspring born to mothers with severely deficient vitamin D status had the highest odds of adverse respiratory outcomes, while the odds were progressively lower as maternal vitamin D exposure improved, even into the high-normal range. Although such post-hoc analyses break randomization and can therefore never be used to make inferences on causality, better outcomes in individuals with higher exposure are in line with the hypothesis that prenatal supplementation is beneficial. In addition, the randomization arm was the strongest predictor of exposure. These findings provide an initial indication that maintaining maternal 25(OH)D levels above the conventional skeletal sufficiency thresholds of 20–30 ng/mL may have additional non-skeletal benefits. Based on tissue-level difference in storage patterns of vitamin D metabolites, as outlined in **Chapter 2 and 3**, these outcome-specific thresholds for different organs would be expected and are worth exploring in future trials.

Timing of supplementation initiation

The 2012 WHO guideline on gestational vitamin D supplementation identified the timing of initiation as a major evidence gap. The 2020 guidance reiterates this point, noting limited progress toward a definitive recommendation.[25] **Chapter 5** further explored how timing of supplementation initiation modified maternal and offspring outcomes in VDAART. Starting supplementation in the first trimester was associated with a greater reduction in the odds of asthma, which is when CYP27B1 expression is at its highest level.[26] It would be consistent with a role of vitamin D in lung development as early as the pseudoglandular stage, when critical airway structures are formed. It may also reflect a slower or more variable ramp to sufficiency, giving mothers more time to achieve higher serum 25(OH)D levels later in the pregnancy. This coincides with the saccular and alveolar stages of fetal lung development, which is when vitamin D may support surfactant production and alveolar formation as observed in animal models.

It is worth noting that VDAART enrolled a high percentage of African American individuals. Individuals of African ancestry have higher rates of vitamin D deficiency frequently associated with a darker skin tone, as well as higher rates of childhood asthma in the US, and implications of effect modification for the general population should be considered with care.

Potential modifiers of childhood asthma and prenatal vitamin D supplementation efficacy

Follow-up studies in **Chapter 6 and 7** examined other modifiers of childhood asthma, which have also been related to prenatal vitamin D status; early life growth patterns. In **Chapter 6**, we describe how increasing weight gain during infancy was related to higher odds of asthma or recurrent wheeze. Interestingly, the association differed by gestational age category. Among children born on term, a higher weight-for-age z-score was linked to increased asthma and wheeze, in line with reports showing that excessive weight gain or adiposity in early life can promote airway dysfunction or a pro-inflammatory phenotype, thus increasing asthma risk.[27] However, in preterm neonates, catch-up weight gain (towards normal weight-for-age percentiles in infancy) lowered the odds of asthma and wheeze. This context-dependent impact of weight gain may suggest that accelerated growth allows for better recovery and lung development of premature neonates but might be adverse when neonates are already at normal weight-for-age. Prenatal vitamin D supplementation may modify asthma risk in this scenario as pooled data from RCTs suggests that supplementation reduces the likelihood of low birthweight.[28] Given the strong correlation between maternal weight, gestational weight gain and offspring weight is shown in the same VDAART population in **Chapter 7**, an important role of maternal and neonatal weight (gain) in childhood asthma emerges. This is particularly relevant given the global obesity epidemic, with estimates of over 1 billion obese adults in 2030, disproportionally affecting women (20% of adult women projected to be obese).[29] Therefore, a continued trend in excessive weight and adiposity, also during pregnancy and at birth, could be a driver of an increased incidence of childhood asthma over the coming years.

There is well-documented link between vitamin D supplementation efficacy and maternal adiposity. We previously described how prenatal vitamin D PK are altered by adiposity of the mother and evidence from large trials (e.g., the VITAL trial[30]) similarly suggests that higher BMI or adiposity could modify the PK/PD response to supplemented vitamin D. Thus, greater maternal weight and adiposity may attenuate the potential benefits of prenatal vitamin D on early life respiratory outcomes and on favorable neonatal weight profiles by affecting the PK/PD of supplementation, while independently increasing the risk of childhood asthma through higher offspring weight-for-age, leading to risk stacking. Our analyses indicate preterm neonates in particular may be at risk of childhood asthma with decreasing weight-for-age (**Chapter 6**), suggesting that the effect of vitamin D on increasing birth weight may be one potential pathway via which supplementation provides a more supportive basis postnatally for lung maturation in underdeveloped neonates.

Beyond weight status and pulmonary health, other pregnancy outcomes may be associated with prenatal vitamin D status and supplementation, which subsequently affect offspring health. These include preeclampsia (**Chapter 8**), the microbiome (**Chapter 9**) and markers of immuno-inflammation (**Chapter 10**).

Preeclampsia

The mechanistic pathways linking vitamin D to preeclampsia are multifactorial. Bioactive vitamin D aids placental development by regulating angiogenesis and trophoblast invasion. In vascular smooth muscle cells, $1,25(OH)_2D$ induces vascular endothelial growth factor (VEGF) by binding to two VDREs in the VEGF promoter, stimulating angiogenesis.[31] Also, in human umbilical vein endothelial cells, $1,25(OH)_2D$ has been demonstrated to activate nuclear factor erythroid 2-related factor 2 (Nrf2) in an ERK1/2-dependent manner.[32] Studies in PE rats and hypoxia-cultured placental trophoblast cells are consistent with these pathways, demonstrating that $1,25(OH)_2D$ alleviates placental inflammation through Nrf2 signaling, which reduces NLRP3 inflammasome activation and NLRP3-mediated IL-1β release.[33] In humans, preeclampsia is associated with disrupted vitamin D metabolism and altered placental expression of CYP27B1 and CYP24A1.[34] Notably, autocrine action of $1,25(OH)_2D$ in syncytiotrophoblasts stimulates the secretion of human chorionic gonadotropin (hCG), which is crucial for trophoblast growth, placental development and maternal-fetal immunomodulation.[35] Genetic analyses of the VDAART cohort have demonstrated that women who went on to develop PE had distinct immune responses early in pregnancy characterized by differentially expressed vitamin D-associated transcriptomes related to systemic immuno-inflammation.[36] These observations implicate vitamin D metabolite signaling in extravillous trophoblast invasion and placental vascularization and inflammation during pregnancy. Hence, reduced local availability of bioactive vitamin D, due to dysregulated expression of activating/inactivating enzymes, might contribute to PE.

In the clinic, large meta-analyses suggest that prenatal vitamin D supplementation probably reduces preeclampsia versus no supplementation,[37] which becomes a smaller and more uncertain benefit when the comparator arm includes low dose vitamin D supplementation.[38] Of note, the certainty of the evidence remains moderate, and the authors of the Cochrane review more recently updated the analysis, putting 20 out of the 30 previously included studies in the 'awaiting classification' to re-evaluate after using a novel trustworthiness framework.[39]

Our work in **Chapter 8** sheds more light on the potential long-term implications of preeclampsia for the offspring. It adds another dimension by demonstrating that preeclampsia

may be associated with specific DNA methylation changes in cord blood, enriched for cardiovascular pathways, such as the apelin pathway. These observations could suggest that certain adverse pregnancy outcomes can epigenetically reprogram neonates, predisposing them to comorbidities later in life. Therefore, it offers another mechanistic pathway through which improving maternal health and overall pregnancy success may provide longer-term benefit to offspring. In case such adverse pregnancy outcomes can be reduced, even marginally, by achieving sufficient maternal vitamin D status during gestation, it could confer life-long benefit for offspring.

Microbiome

Data from VDAART allowed for exploration of the offspring gut microbiome and how prenatal factors help shape it, which in turn may influence disease risk. Emerging microbiome research indicates a crucial role for the prenatal and early life gut microbiome in educating the offspring immune system, maintaining barrier defenses and developing oral and respiratory tolerance.[40] Via these pathways, the early life microbiome, including the maternal microbiome and associated prenatal factors, may modify the risk of several immune-mediated, inflammatory diseases such as asthma.[41]

We investigated if and how the adverse pregnancy outcomes PE and spontaneous preterm birth (sPTB) affect the developmental trajectory of the early life microbiome (**Chapter G**). After a 5-year longitudinal follow-up, we found distinct differences in microbial composition in the gut from children born from PE or sPTB pregnancies.

Several taxa implicated in inflammatory processes were enriched in the PE and sPTB offspring, suggesting a potentially lasting influence of the in utero circumstances on the childhood microbiome. However, broad maturation, in terms of alpha and beta diversity, showed similar trajectories between the affected and control groups. These findings highlight the complexity of the microbiome. While overall development could appear unremarkable at first sight, higher resolution analyses may demonstrate an impact of prenatal adverse events on particular microbial genera.

In the VDAART dataset, PE was an independent risk factor of offspring asthma or recurrent wheeze. It could be hypothesized that changes to the offspring microbiome due to PE are one of the pathways by which PE modulates childhood asthma risk.

Regarding vitamin D, the question that further arises is: does maternal vitamin D status and supplementation alter the maternal and offspring's microbiome and could it meditate the association between the prenatal microbiome and early life diseases?

Research in this context is early but starting to emerge. Certain observational studies link maternal 25(OH)D levels to changes in the relative abundances of maternal gut and vaginal microbes.[42-44] In VDAART, a higher maternal baseline vitamin D status was associated with increased maternal microbiome beta diversity (composition), but no shift in diversity due to subsequent vitamin D supplementation was observed.[42] This may reflect how the maternal microbiome, shaped by diet, lifestyle factors and as a marker of overall health, affects the mother's ability to process vitamin D. Furthermore, an association between prenatal vitamin D status and offspring gut microbiome between 3–6 months of age was found, which is to be expected given that the instrumental role of the maternal microbiome in colonizing the neonatal gut microbiome.[45] Therefore, vitamin D could be a mediator in the relationship between the maternal microbiome, the offspring microbiome and disease risk. Indeed, a follow-up analysis including both the maternal and offspring microbiome found early evidence of effect modification by vitamin D treatment on offspring microbiome-asthma associations.[46] Interestingly, the COPSAC trial similarly found that vitamin D (and fish oil) supplementation did not alter the maternal vaginal or offspring microbiome, but showed that supplementation did affect the microbial composition of the offspring's airways.[47] These shifts in beta diversity associated with changes in immune mediator profiles, suggesting nutrient-induced immunological programming of the offspring.

Also, decreased airway microbiome maturation (an immature airway microbiome has been linked to increased asthma risk[48]) and specific bacterial genera related to inflammatory diseases were observed. In the mediation analysis, the changes in airway microbiome were a modest mediator of the association between the dietary intervention and reduced risk of asthma, which also reflects the smaller overall effect size of the intervention reported in COPSAC. Given the pleiotropic actions of vitamin D and the equally widespread influences of the microbiome, many different mechanistic pathways are probably involved. Although initial evidence indicates that vitamin D and the microbiome are entangled and may jointly modify offspring asthma risk, further research is required to understand which specific microbial taxa or metabolites are involved.

Finally, outside of pregnancy, a more favorable gut microbiome diversity was associated specifically with increased levels of the active vitamin D metabolite $1,25(OH)_2D$ and a higher activation ratio [$1,25(OH)_2D/25(OH)D$], whereas no association with 25(OH)D itself was identified.[49] This notable finding emphasizes the relevance of local concentrations of vitamin D metabolites, beyond serum 25(OH)D, and vitamin D metabolite ratios as unique biomarkers. As shown in **Chapter 2 and 3**, pregnancy alters vitamin D metabolism and local

metabolite tissue concentrations. Therefore, such local concentrations and ratios should be further investigated to better characterize their role during gestation.

Immuno-inflammation

A well-developed gut and airway microbiome of the offspring provides training for immune tolerance in early life and vitamin D may support this process. As discussed, vitamin D is a recognized immunomodulator and sufficient prenatal vitamin D status may calibrate the maternal and offspring immune responses to be more tolerogenic and less disposed to aberrant inflammation. Prenatal vitamin D exposure and supplementation have been suggested to influence the immune system of the neonate. Maternal supplementation may enhance innate and adaptive cytokine responses in cord blood mononuclear cells, including increased IL-17A and IL-10 production and higher Toll-like receptor expression.[50] These stimulating effects appear balanced between Th1 and Th2 cytokines, with genes regulating development, proliferation and differentiation of T and B cells being turned down.[51] Interestingly, a lower antenatal cytokine production has been associated with an increased risk of asthma in early life.[52] Prenatal vitamin D deficiency has also been linked to adjusted immune cell proportions, similarly pointing to a role of gestational vitamin D in shaping the neonatal immune function.[53] In **Chapter 10** we have explored other maternal variables related to markers of immuno-inflammation during pregnancy. Particularly CRP, but also IL-8, exhibited difference in concentration by fetal sex and maternal factors such as BMI and race/ethnicity, variables known to intersect with vitamin D status. It should be noted that race/ethnicity was self-reported and does not represent biological principles but may overlap with racial health disparities.

In summary, **Chapter 6–10** discussed additional factors that may be confounders, effect modifiers or upstream causal factors in the relationship between prenatal vitamin D status and offspring asthma or recurrent wheeze, as well as PE and sPTB. Many of these factors are interconnected, for example, an optimal microbiome could modulate excessive weight gain and adiposity which in turn affects inflammation and modifies childhood disease risk. Together, they integrate in the general concept of developmental origins of health and disease, where prenatal nutrition can have lasting impact on perinatal physiology and disease susceptibility. Vitamin D appears to be a piece of a complex puzzle that lays the foundation during this critical window of life.

Study limitations and interpretive considerations

There are several limitations that should be acknowledged when interpreting these findings. Although mechanistically informative, the mouse tissue distribution studies may not fully translate to a human pregnancy due to species-specific differences in placental development, structure and vitamin D metabolism. Also, fetal lung concentrations could not be measured directly, which limits direct confirmation of fetal lung tissue exposure to vitamin D, while we did confirm overall transfer of vitamin D metabolites. In the clinical analyses, the observational nature of exposure-response relationships does not enable causal inference as it breaks randomization and residual confounders in the form of unmeasured lifestyle or genetic factors cannot be excluded. The population pharmacokinetic model was developed based on two specific trial populations (VDAART and COPSAC), both with their own particular demographic characteristics associated with the respective countries, potentially limiting generalizability to broader populations. External validation is needed in other care settings and from diverse ancestries. Additionally, our biomarker approach still primarily relied on serum 25(OH)D, which may not fully capture tissue-level vitamin D biology or individual differences in vitamin D activation and sensitivity.

Recommendations and future directions

The current work provides preliminary evidence indicating additional roles of gestational vitamin D and suggestions to fully capture a potential signal during clinical trials. Based on conducted studies, several recommendations can be made to aid future vitamin D research and clinical trials during pregnancy, as outlined below.

1) Enroll a population in need of supplementation

Enrolled individuals should be vitamin D deficient (<20 ng/mL serum 25(OH)D) or severely deficient (<12 ng/mL) at baseline and present with deficiency risk factors (e.g., obesity, dark skin, lifestyle factors) to isolate the proposed effect of vitamin D supplementation. It is not to be expected that pregnant women with sufficiently high vitamin D levels have any benefit from additional supplementation. However, once a benefit has been confirmed in an RCT of deficient subjects, follow-on studies or post-hoc analyses can be used to triangulate what the optimal level for sufficiency level is according to the desired health outcome, as it may be higher or lower than the proposed sufficiency threshold of 20–30 ng/mL for skeletal outcomes.

2) Achieve the target exposure

A trial should set ideally set a target exposure measured over several timepoints during the pregnancy. Given the increased demand for vitamin D during gestation a sufficiency threshold of 30 ng/mL serum 25(OH)D seems warranted. Instead of a fixed dose, subjects could be randomized to treat-to-target strategy using a blinded, algorithm-based dose-adjustment strategy that aims to achieve target levels at fixed timepoints in the intervention arm versus a fixed ethical minimum in the placebo arm with sham adjustments.

3) Characterize vitamin D's PK/PD during pregnancy

Vitamin D PK during pregnancy remains poorly characterized, despite PK data being key information for every substance that is dosed therapeutically. In addition, variability in achieved serum 25(OH)D is notoriously high in vitamin D research and the PD effects may be altered by factors such as adiposity or kidney function. Future efforts should focus on further defining vitamin D metabolite PK after supplementation during gestation, in particular regarding $1,25(OH)_2D$ and $24,25(OH)_2D$, and identifying influential variables to clarify the PK/PD relationship for prenatal vitamin D supplementation. Ultimately, fixed one-size-fits-all strategies should be replaced by personalized dosing to ensure that more women reach target levels recommended by guidelines.

4) A more holistic approach to biomarkers is needed

In line with PK/PD characterization, more research needs to be aimed at testing different biomarkers of total vitamin D storage and/or effects. Besides serum 25(OH)D, vitamin D metabolite ratios (VDMRs) such as $24,25(OH)_2D$:25(OH)D may capture a more holistic view of vitamin D storages in both serum and tissues by better reflecting input and output dynamics around bioactive vitamin D.

5) Start supplementation early

Optimal timing of vitamin D supplementation initiation is a recurring priority research question in the WHO guidelines.[25] Preliminary evidence suggests early initiation may be beneficial for respiratory outcomes and could support placental formation and function. In RCTs of prenatal vitamin D supplementation, it has been shown that a significant increase in serum 25(OH)D due to supplementation can take weeks to months. Therefore, it may be prudent to start supplementation in trials at the earliest convenience to maximize the pleiotropic action and harness the potential advantages of prenatal vitamin D.

In general, harmonization of different trials in terms of the administered form of vitamin D, dosing schedules (preferably daily) and analytical techniques are important to reduce heterogeneity and risk of bias. Large, well designed RCTs should be conducted to allow for quality meta-analyses with high confidence in the outcome, serving as a robust basis for any recommendations regarding prenatal supplementation. To this aim, the COPSAC group has recently initiated the 'Vitamin D in pregnancy for prevention of early childhood asthma' (VICTORY) trial.[54] VICTORY will enroll 2,000 pregnant women, randomized to either high-dose (3,200 IU/day) vitamin D or placebo added on top of the recommended ethical minimum of 400 IU/day. Women are randomized in gestational week 24, which may raise 25(OH)D concentrations too late into pregnancy (**Chapter 5**). Offspring will be followed for 3 years with registration of parent-reported symptoms, hospitalizations, physician diagnoses and medication use. The primary outcome is asthma or persistent wheeze by age 3 years, including pre-defined analyses of differential effects by maternal genotypes. Secondary outcomes include lower respiratory tract infections, croup, troublesome lung symptoms, gastrointestinal infections, eczema, allergy, bone fractures, growth, developmental milestones, mental health and cognition. After unblinding at age 3 years, offspring follow-up on both the primary and secondary outcome measures is planned from age 3–6 years. Such a trial will be able to clearly distinguish a potential protective effect of prenatal vitamin D supplementation to prevent childhood asthma and improve respiratory outcomes.

Conclusion

Prenatal vitamin D supplementation remains a promising, although not yet definitively proven, strategy to lower the risk of early-life wheeze and potentially prevent a subset of childhood asthma. Evidence from landmark RCTs and meta-analyses suggest respiratory benefits in offspring when maternal serum 25(OH)D is maintained within a sufficient range during pregnancy, supported by biological rationale and preclinical data: vitamin D metabolites support fetal lung development, may mediate maternal-fetal immunity, and are likely acting locally in the placenta and respiratory tissues via paracrine and autocrine pathways. Nevertheless, benefits are clearest for early wheeze and currently less consistent for persistent childhood asthma, underscoring that heterogeneity in baseline status, timing, dose, genetics, microbiome, adiposity, and outcome definitions matters.

Current public health guidelines (400–600 IU/day) often fail to attain sufficient vitamin D status in many of the deficient pregnant women, even high dose vitamin D supplementation does not guarantee sufficiency for all, and compliance is low. This emphasizes the need

for PK/PD characterization of gestational vitamin D supplementation and treat-to-target, personalized dosing, started early (ideally first trimester) to reliably achieve and sustain adequate exposure. Because pregnancy alters vitamin D metabolism and tissue distribution, skeletal sufficiency thresholds outside of pregnancy may not translate and broader biomarker strategies (such as metabolite ratios) may better reflect biologically relevant exposure.

Given the low safety and tolerability risks of vitamin D supplementation, low associated costs, high prevalence of deficiency, and possibly extra-pulmonary benefits (e.g., less adverse pregnancy outcomes and improved neonatal weight or growth), ensuring vitamin D sufficiency in pregnancy should be prioritized. The key remaining question is not whether to supplement, but how to dose and when to start to optimize respiratory outcomes in offspring. In the future, trials should focus on enrolling populations in need of supplementation, use treat-to-target strategies, stratify by modifiers such as adiposity and genotype, and redefine biomarkers. When these advances are implemented in rigorous, well-powered trials, prenatal vitamin D supplementation could be shown, beyond any doubt, to be a useful tool in the prevention of offspring asthma or asthmatic symptoms, exemplifying how an optimized fetal environment can help to reshape lifelong respiratory health.

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