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25-hydroxyvitamin D3 status is associated with developing adaptive and innate immune responses in the first 6 months of life

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Abstract

Background: Vitamin D (25[OH]D3) status in early life has been linked to the risk of allergic disease in multiple observational studies. While immunomodulating properties are well-recognised there are few longitudinal studies of 25(OH)D3 status, immune function and allergic disease in infants.

Objective: To investigate 25(OH)D3 levels at birth (cord blood [CB]) and at 6 months of age in relation to immune function at 6 months of age, and clinical outcomes up to 30 months of age in infants with a maternal history of atopy.

Methods: In a subset of infants (n=225) enrolled in a RCT (ACTRN12606000281594) 25(OH)D3 levels were assessed in relation to peripheral blood mononuclear cell cytokine responses to house dust mite (HDM), ovalbumin (OVA) and β -Lactoglobulin allergens, or toll-like receptor (TLR) ligands (lipopolysaccharide, lipoteichoic acid, polyinosinic:polycytidylic acid and CpG oligonucleotide) at 6 months of age, in addition to clinical outcomes (eczema, wheeze and allergen sensitisation) up to 30 months of age.

Results: Infants with higher 25(OH)D3 at birth (>75 nmol/L, compared with <50 nmol/L) had lower IL-5 and IL-13 responses to HDM by 6 months ($p<0.001$ and $p=0.003$, respectively). This was also reflected in strong inverse correlations between CB 25(OH)D3 levels and HDM IL-13 ($\rho=-0.57$; $p=0.0002$) and IL-5 ($\rho=-0.59$, $p=0.0001$) responses, with a similar trend for IL-5

($\rho=-0.29$; $p=0.009$) responses to OVA. For innate stimulations, higher 25(OH)D3 levels at 6 months were associated with greater responses to TLR ligands. Additionally, higher CB 25(OH)D3 was associated with reduced risk eczema at 6 months ($p=0.011$) and 12 months ($p=0.034$),

Conclusion: This suggests that improving 25(OH)D3 status in pregnancy or early infancy may reduce the development of allergic disease in high risk infants by inhibiting cytokine profiles associated with allergy. Results of clinical trials are awaited to determine the efficacy of vitamin D supplementation in allergy prevention.

Introduction

The global rise in allergic disease, particularly in very young children [1, 2] has increased the need to identify both the early risk factors and the developmental periods during which the immune system is most vulnerable to these effects. Already, studies of immune function at birth have demonstrated distinct differences in cytokine production profile between allergic and non-allergic individuals, which consolidate in early childhood as the clinical symptoms develop [3, 4]. Early immune function is also modified by a range of early life exposures including maternal microbial exposure in pregnancy (reviewed in [5]), maternal dietary patterns [6, 7] and maternal smoking [8]. Recently, vitamin D status has also been proposed as a potential risk factor for the epidemic of allergic disease so early in life [9].

Low levels of the major circulating vitamin D metabolite, 25-hydroxyvitamin D (25(OH)D), at birth and during infancy have been associated with an increased risk of eczema [10, 11], food allergy [12, 13], respiratory tract infections and wheeze [14, 15] in early childhood, while low levels in older children are reported to be predictive of asthma and atopy [15]. This is consistent with immunological research demonstrating that the active vitamin D metabolite, 1 α ,25-dihydroxyvitamin D (1,25(OH)₂D), which can be synthesised from 25(OH)D by antigen presenting cells (APC), promotes tolerogenic adaptive immunity through the modulation of APC function, induction of regulatory T cells (reviewed in [16]), and altered Th cytokine responses in vitro [17, 18]. However, understanding of the relationship between 25(OH)D₃ status and allergic disease has been limited by the small number of studies with both immunological and clinical outcomes, or of a longitudinal design. The present study aims to address these issues by investigating the association between 25(OH)D₃ status at multiple time points in relation to adaptive and innate immune function and clinical allergic disease in infancy and early childhood.

Methods

Study Group

The study population was a subset of infants recruited to investigate the effects of fish oil supplementation in infancy on allergic outcomes (Australian New Zealand Clinical Trials Registry number 12606000281594), as approved by the Princess Margaret Hospital Human Research Ethics Committee. Full details of the original study have been previously published [19]. Briefly, n=420 high risk infants of non-smoking atopic mothers (with a history of allergic symptoms in combination with a positive skin prick test) were randomly assigned to receive 650mg of fish oil (Ocean Nutrition, Canada Ltd) or placebo (olive oil) daily from birth to 6

months of age. There were no differences in the prevalence of allergic outcomes between the intervention and control group, however some differences in immune response were identified [20]. The subset of participants included in this study was determined by availability of blood for 25(OH)D3 analysis, and matched to cell culture and clinical follow up data. Participant numbers are presented in **Figure 1**. Plasma for 25(OH)D3 analysis at 6 months of age was unavailable for n=9 participants who otherwise had CB 25(OH)D3 and cell culture data.

Blood collection and processing

Cord blood was collected from the umbilical artery or vein at delivery into a serum clot activator tube and processed within 8 hours of collection. Peripheral blood from 6 month old infants was collected by venepuncture into lithium heparin tubes and processed immediately after the clinic appointment. Plasma was collected after the first centrifugation cycle, following which peripheral blood mononuclear cells (PBMC) were separated using density centrifugation. Aliquots of serum and plasma were stored at -80°C, and PBMC were stored in liquid nitrogen (in 7.5% dimethyl sulfoxide) at a concentration of no more than 15×10^6 .

Assessment of 25(OH)D3

Plasma and serum samples were analysed using isotope-dilution liquid chromatography-tandem mass spectrometry (RDDT, Melbourne, Victoria) according to published techniques [21]. Sample type (plasma or serum) does not affect measured 25(OH)D concentration [22], making the two collection types comparable.

PBMC culture

Blood collection volume at the 6 month of age appointment determined the quantity of PBMC available for cell culture. Where the quantity of PBMC was insufficient to conduct all stimulations, cultures were conducted in an order of priority. The number per stimuli with corresponding CB and 6 month of age 25(OH)D3 measures is indicated below.

Assessment of innate immune function

PBMC were cultured at 1×10^6 cells/ml either alone (control) or with optimised concentrations of Toll-like receptor (TLR) ligands for a) TLR3 (Polyinosinic:polycytidylic acid, 50 μ g/ml, paired with CB 25(OH)D3 n=60, paired with 6 month 25(OH)D3 n=73), b) TLR2 (Lipoteichoic acid, 20 μ g/ml, paired with CB 25(OH)D3 n=57, paired with 6 month 25(OH)D3 n=68), c) TLR9 (CpG oligonucleotide, 3 μ g/ml, paired with CB 25(OH)D3 n=46, paired with 6 month 25(OH)D3 n=56), or d) TLR4 (Lipopolysaccharide, 1 ng/ml, paired with CB 25(OH)D3 n=17, paired with 6 month 25(OH)D3 n=19), (all purchased from Invivogen, San Diego, CA, USA). PBMC were cultured for 24 h in RPMI with 10% fetal calf serum (Australian Biosearch, Karrinyup, Australia), at 37°C in 5% CO2 incubators before supernatants were collected and stored at -20°C for batch cytokine analysis.

Assessment of adaptive immune function

To assess adaptive immune function at 6 months of age PBMC were cultured in with AIM V tissue culture media supplemented with 2-mercaptoethanol (ME) for stimulation with the allergens a) house dust mite (HDM) *D. pteronyssinus* (20 μ g/ml, 2×10^6 cells/ml, paired with CB 25(OH)D3 n=37, paired with 6 month 25(OH)D3 n=41, Department of Cell Biology,

Institute for Child Health Research, Perth, Western Australia), b) ovalbumin (OVA 100 µg/ml, 2×10^6 cells/ml, paired with CB 25(OH)D3 n=82, paired with 6 month 25(OH)D3 n=93), and c) betalactoglobulin (BLG, 100 µg/ml, 2×10^6 cells/ml, paired with CB 25(OH)D3 n=78, paired with 6 month 25(OH)D3 n=90) (both Sigma-Aldrich, St Louis, MO, USA). Supernatants were collected after 48 h and stored at -20°C. HDM stimulations were performed in lower number of individuals than remaining stimulations due to technical difficulties with a batch-change of HDM allergen.

Cytokine measurements

Cytokines in PBMC culture supernatants were quantified using Luminex Xmap multiplex technology (Luminex Corp, Austin, TX, USA). IL-6, IL-10, IL-12, IL-1β, TNFα and IFNγ were measured to innate stimuli, and IL-5, IL-10, IL-13, IL-17A, TNFα and IFNγ were measured to allergen stimulation. Cytokine levels are expressed as difference between control and stimulated cells.

Clinical outcomes

Clinical examinations and questionnaires were conducted at 6, 12 and 30 months of age. Parents were asked if their infant had shown typical signs of eczema (dry, red, itchy skin) [23] at all three time points, and SCORAD (Scoring Atopic Dermatitis) [24] conducted at 12 and 30 months of age to determine extent and severity of eczema. Questions regarding wheeze were based on the International Study of Asthma and Allergies in Childhood (ISAAC) phase one questionnaire [25], with the exclusion of questions related to asthma diagnosis or effects on speech as these were largely not applicable to the age group being investigated.

Allergic sensitization was assessed by skin prick test (SPT) at 12 and 30 months of age using common food- and aero-allergen extracts (milk, peanut, house dust mite, cat, grass, mould; Hollister-Stier Laboratories, Spokane, WA) and whole egg, with a wheal diameter of >2 mm being considered positive. Histamine and glycerine were used as positive and negative controls, respectively.

Statistical analysis

Spearman correlations and Mann-Whitney U-tests were used to analyse non-parametric data. Differences in means for parametric data were compared using Student's t-test. Chi square tests were used for comparisons of categorical data between groups. Binary logistic regression and generalized estimating equations were used to calculate odds ratios for clinical outcomes based on 25(OH)D3 levels, while controlling for sex and season of birth. Seasons were categorized following definitions used by the Australian Bureau of Meteorology, for example winter is June to August. Cut-offs were applied for categorical analysis of 25(OH)D3 status following published examples (<50, 50-74.9, and > 75 nmol/L)[26]. To control for season when comparing the change in 25(OH)D3 status between birth and 6 months of age season-standardized quartiles were calculated [27], in addition to the use of partial correlation. All statistics were performed using SPSS v20 (IBM), except generalized estimating equations which were conducted using Stata 11 (StataCorp LP). Figures were generated using BioVenn [28] (Figure 1), Microsoft Excel (Windows 7 Professional) (Figure 2), and Prism v 6 (GraphPad Software Inc.) Figures 3 and 4).

Results

Study Population

Cord blood (CB) 25(OH)D3 was measured in 200 infants and peripheral blood 25(OH)D3 at 6 months was measured in 112 infants. The characteristics of the population included in present study (the n=225 with 25(OH)D3 measured at either time point) are presented in **Table 1**. This subset of infants did not differ significantly from the main cohort population.

25(OH)D3 levels at birth and 6 months

25(OH)D3 was substantially higher at 6 months of age than at birth (in CB) - with respective means and 95% confidence intervals (CI) of 93.1 (87.3-98.9) nmol/L and 57.9 (54.7-61.1) nmol/L. Seasonal variation in 25(OH)D3 levels for both neonatal and 6 month samples was highly significant. CB 25(OH)D3 levels collected in summer (72.6; 66.4-78.9 nmol/L) were significantly greater than in winter (52.0; 45.7-58.3 nmol/L) ($p<0.001$). For 6 month samples, 25(OH)D3 levels were similarly higher in summer (107.1; 96.7-117.6 nmol/L) than in winter (70.8; 56.2-85.3 nmol/L) ($p<0.001$). The mean (SD) vitamin D for each month of collection is presented in **Figure 2**. Fish oil supplementation had no significant impact on 25(OH)D3 levels ($p=0.418$).

There was no direct correlation between 25(OH)D3 at birth and 6 months of age (Pearson's $r=0.022$, $p=0.838$). To account for the possible role of season in this observation, subjects were categorised into quartiles of 25(OH)D3 based on season of collection (season-standardized quartiles) for both the birth and 6 months of age time point, and cross-classification of quartiles analysed. Season-standardized quartile of 25(OH)D3 at birth was not related to subsequent

classification at 6 months of age ($p=0.460$). An absence of association between 25(OH)D3 levels at the two time points was confirmed in partial correlation controlling for season ($r=0.163$, $p=0.136$), indicating that 25(OH)D3 status at 6 months of age is determined predominantly by environmental exposures rather than status at birth.

Immune response to allergens

Infants with higher 25(OH)D3 levels at birth tended to produce lower quantities of T helper (Th) 2 cytokines to food (OVA) and inhalant (HDM) allergens by 6 months of age. Specifically, CB 25(OH)D3 levels were inversely correlated with HDM IL-13 ($\rho=-0.57$; $p=0.0002$) and IL-5 ($\rho=-0.59$, $p=0.0001$) responses at 6 months, with similar negative correlations between CB 25(OH)D3 levels and IL-5 ($\rho=-0.29$; $p=0.009$) and IL-13 ($\rho=-0.21$; $p=0.061$) Th2 responses to OVA (**Figure 3**). Accordingly, when analysed according to category of 25(OH)D3 status HDM IL-5 and IL-13 was significantly higher in infants with CB 25(OH)D3 <50 nmol/L compared to those with levels >75 nmol/L ($p<0.001$ and $p=0.003$, respectively). Production of IL-5 to OVA was also significantly higher in those infants with CB 25(OH)D3 <50 nmol/L compared to >75 nmol/L ($p=0.032$).

Infants who had higher 25(OH)D3 levels at birth also had lower production of the inflammatory Th1 cytokines IFN γ ($\rho=-0.43$; $p=0.008$) (**Figure 3**) and TNF α ($\rho=-0.41$; $p=0.012$) in response to HDM at 6 months of age. Production of these cytokines in response to OVA were inversely, but non-significantly, correlated with CB 25(OH)D3 (IFN γ $\rho = -0.111$, $p=0.320$ [**Figure 3**] and TNF α $\rho=-0.130$, $p=0.243$). There was also an inverse correlation for IL-10 to HDM ($\rho=-0.35$; $p=0.034$) but not OVA ($\rho=-0.07$; $p=0.531$).

Interestingly, 25(OH)D3 levels at 6 months of age were not associated with the propensity for Th2 (IL-5 and IL-13) cytokine production to allergens (HDM IL-5 $\rho=-0.080$, $p=0.620$ and IL-13 $\rho=0.059$, $p=0.712$; OVA IL-5 $\rho=0.022$, $p=0.833$ and IL-13 $\rho=0.188$, $p=0.071$). Instead, levels were associated with an increased production of inflammatory cytokines in allergen cultures. Specifically 25(OH)D3 levels at 6 months were positively correlated with TNF α ($\rho=0.23$; $p=0.027$) and IL-17A ($\rho=0.24$; $p=0.023$) to OVA. There were similar trends for TNF α responses to HDM but this was not statistically significant ($p=0.067$).

There were no relationships between immune responses to milk allergen betalactoglobulin and 25(OH)D3 at birth or 6 months of age.

Innate immune response

Next we examined 25(OH)D3 status at birth and 6 months of age, in relationship to innate immune responses to ligands for TLR2 (LTA), TLR3 (Poly[I:C]), TLR4 (LPS) and TLR9 (CpG) at 6 months of age.

25(OH)D3 level in CB showed no significant correlation to innate responses at 6 months of age, while 25(OH)D3 levels at 6 months, however, were associated with increased cytokine responses to TLR2, TLR3, TLR4 and TLR9 activation (**Table 2**). TNF α and IL-12 in particular showed consistent positive correlation across ligands, and scatter plots of TNF α (TLR2, TLR3, TLR4 and TLR9), IL-12 (TLR2, TLR3, TLR9) and IL-10 (TLR3 and TLR9) are presented in **Figure 4**.

25(OH)D3 status and clinical outcomes

In other studies we have reported associations between CB 25(OH)D3 and clinical symptoms including wheezing, eczema and sensitization (SPT) at 12 months [11]. In this study we extended the analysis to include 25(OH)D3 measure at 6 months of age, and clinical outcomes at 6, 12 and 30 months of age. **Table 3** shows the odds ratios for developing wheezing and eczema with a 10 unit increase in 25(OH)D3 levels both a) at birth and b) at 6 months, estimated using binary logistic regression, adjusting for sex and season of birth. Incrementally higher CB 25(OH)D3 status was associated with reduced risk of eczema as early as at 6 months ($p=0.011$) which remained significant at 12 months ($p=0.034$).

When analysed longitudinally using a generalized estimating equation, children with CB 25(OH)D3 >75 nmol/L had a significantly lower risk of eczema during infancy and early childhood compared to those with CB 25(OH)D3 <50 nmol/L (OR=0.30, 95% CI: 0.14-0.63; $p=0.001$). Those children with a CB 25(OH)D3 level between 50 and 75 nmol/L did not have a significantly reduced risk of eczema in comparison to children with CB levels >75 nmol/L (OR=0.70, 95% CI: 0.39-1.24; $p=0.22$), although the linear trend across the categories was significant ($p=0.002$). We did not observe this same inverse association with eczema outcomes for peripheral blood 25(OH)D3 at 6 months.

Despite the relationships with the development of eczema symptoms at 6 and 12 months of age, 25(OH)D3 (at birth or 6 months of age) was not correlated with eczema severity as measured by SCORAD. However, in children with SCORAD measurements at 30 months of age ($n=45$) CB

25(OH)D3 levels were inversely correlated with SCORAD score on the day of assessment ($\rho=-0.32$; $p=0.033$) as well as their reported 'worst ever' SCORAD score ($\rho=-0.37$; $p=0.012$).

25(OH)D3 levels at birth or 6 months of age were not significantly associated with risk of parent reported wheeze at any point during follow up, although a non-significant ($p=0.073$) association between 25(OH)D3 at 6 months and wheeze at 30 months was observed.

Of those with 25(OH)D3 measures in CB and at 6 month of age, SPT results were available for $n=161$ (80.5%) and $n=108$ (96.4%) of children, respectively, at 12 months of age, and for $n=134$ (67%) and $n=93$ (83%), respectively, at 30 months of age. 25(OH)D3 levels at birth or 6 months of age did not differ by sensitization status at either of the follow-up visits (data not shown).

Immune responses in relation to clinical outcomes

The relationship between eczema and immune responses has already been published for this cohort [20]. Our findings here demonstrate that 25(OH)D3 was related in a consistent manner to both immune responses and the clinical outcomes. Briefly, $\text{IFN}\gamma$, IL-5 and IL-13 responses to HDM – each inversely associated with 25(OH)D3 levels at birth - were higher in infants with eczema at 6 months of age ($p=0.018$, $p=0.008$ and $p=0.031$, respectively) (**Figure 5**). Children with eczema at 12 months of age also had higher IL-13 ($p=0.011$) and IL-5 ($p=0.030$) responses to HDM (**Figure 5**). Infants with a history of reported wheeze by 6 months of age had higher IL-5 production to OVA ($p=0.027$) and $\text{IFN}\gamma$ responses to HDM ($p=0.050$), although this relationship did not persist at later follow-ups (**Figure 5**).

Discussion

We have previously described associations between immune function [20] and CB 25(OH)D3 status [11] in relation to clinical outcomes at 12 months of age in participants from this cohort. In the present study we have expanded our analysis to examine 25(OH)D3 measures at birth and 6 months of age in relation to innate and adaptive immune responses at 6 months of age, and clinical outcomes up to 30 months of age. Our findings reveal that infants with higher 25(OH)D3 levels at birth had lower production of Th2 (IL-5 and IL-13) cytokines in response to allergens by 6 months of age, suggesting a potential early protective effect against developing an allergic phenotype. Consistent with this we also observed an inverse association between CB 25(OH)D3 levels and eczema risk, as supported by Baiz and colleagues [10].

We did not observe differences in the risk of early childhood wheezing based on 25(OH)D3 level, in contrast to other studies [10, 14]. However, as these studies found no reduction in the risk of asthma the findings in regards to wheeze may be unrelated to allergic outcomes. This suggests that protective associations for CB 25(OH)D3 are strongest for eczema, as observed in the current study, and respiratory infections as reported by Camargo et al. [14]. Thus, the results presented herein support the hypothesis that 25(OH)D3 status very early in development is important in determining the patterns of T cell differentiation, and that suboptimal levels may increase the risk of excessive Th2 responses to allergens and subsequent emergence of allergic disease [9].

The relationship with Th1 function is less clear, as instead of a ‘classical’ reciprocal relationship with Th2 cytokines we saw a positive correlation between Th1 and Th2 cytokines. This has been noted in other studies, suggesting that in many children allergy is associated with mixed T cell hyper-responsiveness [4, 29, 30] that is not as selectively ‘Th2 skewed’ as first thought based on early animal models [31, 32].

As in previous studies, we were able to detect an increased propensity for Th2 responses to allergens as early as 6 months in subsequently allergic children [3, 4]. This suggests that the immunological events underlying the allergic phenotype are initiated very early in development, even before birth (reviewed in [33]). It may also explain why perinatal 25(OH)D3 levels were more strongly related to subsequent allergen-specific responses than 25(OH)D3 levels measured later in infancy.

While ‘adaptive’ responses reflect the earlier immunological milieu when the T-cells were ‘programmed’, innate responses are more likely to reflect the immediate environmental context. This is consistent with the observation that innate responses at 6 months of age were significantly related to 25(OH)D3 status at the time the samples were collected, rather than neonatal status. We have previously shown that normal postnatal immune maturation is associated with a progressive age-related increase in innate immune function to a spectrum of TLR ligands, including increased TNF α , IL-6 and IL-1 β production, and a parallel increase in Th1 responses [4]. In contrast, allergic children show attenuation of both their innate microbial responses and IFN γ Th1 adaptive responses [4]. In this study, 25(OH)D3 levels at 6 months were associated enhanced innate responses, although this was not associated with any parallel effect

on IFN γ production to allergens. Despite this variation, however, the consistency of the association between 25(OH)D₃, stronger innate responses and lower production of allergen-specific Th2 cytokines suggests that 25(OH)D₃ may be an important factor in normal immune maturation; a notion which is further supported by the reduced risk of clinical manifestations with increasing 25(OH)D₃ status.

There are a number of potential pathways through which 25(OH)D could feasibly influence developing immune function and protect from infant allergic disease. It is well recognized that the active metabolite of vitamin D, 1,25(OH)₂D, significantly influences the activity of immune cells, including effector and regulatory T (Treg) cells, and antigen presenting cells [34, 35]. It is also known that antigen presenting cells are capable of generating 1,25(OH)₂D to act as an autocrine and paracrine signal [36]. Jeffrey et al. [36] have demonstrated that Treg in the presence of DCs will respond equally when cultured with either 1,25(OH)₂D or 25(OH)D. This indicates that DCs are capable of producing adequate 1,25(OH)₂D from substrate to impact Treg responses. Hence, it could be proposed that when circulating concentrations of 25(OH)D are low, substrate availability is inadequate for optimal immune function and may predispose to an increased risk of immune related disorders. Aside from APC and T cell function, the role of 25(OH)D₃ and 1,25(OH)₂D₃ in antimicrobial peptide production [37] may be relevant to the decreased risk of eczema observed in the present study.

Patients with atopic dermatitis (AD) experience frequent bacterial skin infections which contribute to exacerbation of the condition [38]. The susceptibility to infection by pathogens is in part due to reduced production of the antimicrobial peptides cathelicidin (LL-37) and human

beta-defensin 2 (HBD-2) by epidermal keratinocytes in patients with AD [37, 39]. A recent paper by Kanda et al. [37] reported that LL-37 was positively correlated with 25(OH)D3 in both normal and AD participants, and that serum levels of both 25(OH)D3 and LL-37 are decreased in patients with AD. While such *in vitro* and observational data are suggestive of a therapeutic potential of vitamin D there are limited quality RCTs addressing supplementation in allergy prevention or treatment.

In two small RCTs improvements in eczema severity scores have been reported following supplementation with 1000 IU ergocalciferol (D2) for one month [40] or 1600 IU cholecalciferol (D3) for 60 days [41], although a third found no significant improvement after 21 days of supplementation with 4000 IU D3 [42]. One antenatal vitamin D supplementation RCT investigating prevention of respiratory and atopic conditions in the offspring was identified [43]. Supplementation with either 800 IU D2 daily from gestational week 27 until delivery, or a single 200,000 IU bolus, showed no effect on risk of wheeze, atopy or eczema by 3 years of age [43]. However, it should be noted that vitamin D deficiency was highly prevalent at baseline (45% with 25[OH]D <25 nmol/L) and supplementation was inadequate to achieve sufficient 25(OH)D levels at birth in a large portion of participants (8.3% achieved CB 25[OH]D >50 nmol/L) [44].

Limitations in trial duration, dose and sample size make it difficult to draw conclusions based on these data. That said, we identified two trials currently underway investigating antenatal and infant supplementation in relation to allergic disease: the Vitamin D Antenatal Asthma Reduction Trial (VDAART) (NCT00920621), examining the effects of higher dose (4400 IU vs 400 IU D3 daily) antenatal supplementation, and the Vitamin D in Allergy (VITAL) trial

(ACTRN12612000787886), comparing infant supplementation of 400 IU D3 daily to placebo from birth to 6 months of age. Such trials will help to determine whether vitamin D supplementation can independently influence developing immune function or allergic outcomes.

There are a number of strengths and recognised limitations of our study. While other studies have investigated 25(OH)D status at single time points, such as in pregnancy or at birth (CB) in relation to infant outcomes, the present study utilises a longitudinal design with 25(OH)D3 measures at two time points. We also complemented clinical outcomes with measures of immune function. However, our findings remain observational in nature and do not confirm causality. The original cohort from which these samples were derived was not designed to investigate the effects of 25(OH)D3, and lacks complete data for all subjects, such as 25(OH)D3 measures at both time points in addition to PBMC culture with all stimuli. In addition, it is becoming increasingly apparent that ultraviolet radiation (UVR), which stimulates endogenous vitamin D synthesis, has immunomodulatory effects independent of vitamin D production [45]. As UVR is the predominant source of vitamin D in the Australian population [46] it is possible that 25(OH)D3 is acting as a biomarker of UVR exposure, and we cannot exclude the possible contribution of non-vitamin D actions of UVR in the current findings.

In conclusion, we have demonstrated a link between 25(OH)D3 status, immune function and symptomatic allergic disease in high risk infants. The results suggest that a lower 25(OH)D3 level in early infancy is associated with the development of an immune profile that has previously been associated with an emerging allergic phenotype. In addition, our findings support earlier reports of an association between lower CB 25(OH)D3 and greater eczema risk.

Therefore, strategies aimed at preventing 25(OH)D3 insufficiency in early infancy may be effective in reducing the risk of eczema in infants with a hereditary risk of atopy. The results from vitamin D supplementation RCTs in pregnancy and infancy are eagerly awaited to determine whether modifying 25(OH)D3 status during these periods confers benefit in terms of allergy prevention.

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Conflict of interest: The authors have no conflicts of interests to declare.

Table 1. Comparison of characteristics between those participants with 25(OH)D3 and the total cohort		
	Present study	Total cohort
Maternal age (years), mean (SD)	33.0 (4.5)	32.8 (4.5)
Number of previous pregnancies, mean (SD)	2.3 (1.4)	2.3 (1.4)
Pets in the home, n (%) with pets	136/222 (61.2)	253/410 (61.7)
Infant sex, n (%) male	125/225 (55.6)	208/418 (49.8)
Birth weight (g), mean (SD)	3464 (461)	3475 (443)
Gestation at delivery (weeks), mean (SD)	39.2 (1.2)	39.3 (1.2)
Season of birth, n (%) - Summer (Dec – Feb)	41/225 (18.2)	85/419 (20.3)
- Autumn (Mar – May)	60/225 (26.7)	115/419 (27.4)
- Winter (Jun – Aug)	64/225 (28.4)	113/419 (27.0)
- Spring (Sept – Nov)	60/225 (26.7)	106/419 (25.3)
Ever had infant formula by 6 months, n (%)	127/188 (67.6)	214/323 (66.3)
Intervention group, n (%) receiving fish oil	114/225 (51.0)	218/420 (51.9)
Infants with eczema at 6 months, n (%)	100/188 (53)	178/323 (55.1)
Infants with eczema at 12 months, n (%)	70/185 (37.8)	129/323 (39.9)
Infants with eczema at 30 months, n (%)	53/152 (34.9)	87/254 (34.3)
Infants with wheeze at 6 months, n (%)	30/188 (16)	50/322 (15.5)
Infants with wheeze at 12 months, n (%)	52/186 (28)	95/325 (29.2)
Infants with wheeze at 30 months, n (%)	39/159 (24.5)	63/268 (23.5)
Infants sensitized at 12 months, n (%)	47/185 (25.4)	90/319 (28.2)
Infants sensitized at 30 months, n (%)	46/152 (30.7)	76/256 (29.7)

Table 2. Correlations between 25(OH)D3 and cytokine production to TLR ligands		
	Spearman's rank correlation coefficient (<i>p</i>)	
	CB 25(OH)D3	6 month 25(OH)D3
TLR2 - IL-1 β	0.058 (0.668)	0.00 (1.00)
- IL-6	0.047 (0.727)	0.141 (0.251)
- IL-10	-0.144 (0.286)	0.203 (0.097)
- IL-12	0.005 (0.975)	0.288 (0.031)
- TNF α	-0.173 (0.199)	0.285 (0.018)
- IFN γ	-0.108 (0.429)	0.145 (0.237)
TLR3 - IL-1 β	-0.041 (0.753)	0.109 (0.357)
- IL-6	0.056 (0.672)	0.234 (0.046)
- IL-10	-0.152 (0.245)	0.299 (0.010)
- IL-12	0.148 (0.316)	0.265 (0.045)
- TNF α	-0.053 (0.686)	0.191 (0.105)
- IFN γ	-0.090 (0.496)	0.142 (0.230)
TLR4 - IL-1 β	-0.386 (0.126)	0.230 (0.344)
- IL-6	-0.133 (0.610)	-0.368 (0.121)
- IL-10	-0.278 (0.279)	0.236 (0.331)
- IL-12	-	-
- TNF α	-0.036 (0.892)	0.494 (0.032)
- IFN γ	-0.036 (0.892)	0.184 (0.450)
TLR9 - IL-1 β	0.265 (0.075)	0.007 (0.959)
- IL-6	0.139 (0.356)	0.209 (0.123)
- IL-10	-0.036 (0.812)	0.204 (0.132)
- IL-12	0.047 (0.755)	0.232 (0.086)
- TNF α	0.216 (0.150)	0.291 (0.029)
- IFN γ	0.021 (0.892)	0.056 (0.680)

Table 3. OR from binary logistic regression for allergic outcomes associated with 10 nmol/L rise in 25(OH)D3, adjusted for sex and season of birth			
	OR	<i>p</i>	95% CI
a) 25(OH)D3 in cord blood			
Wheeze - 6 months (n=163)	0.97	0.74	0.78-1.19
- 12 months (n=164)	1.00	0.98	0.78-1.27
- 30 months (n=139)	0.95	0.66	0.77-1.18
Eczema - 6 months (n=163)	0.82	0.011	0.70-0.96
- 12 months (n=162)	0.84	0.034	0.72-0.99
- 30 months (n=139)	0.86	0.12	0.72-1.04
b) 25(OH)D3 in peripheral blood at 6 months			
Wheeze - 6 months (n=112)	0.95	0.66	0.76-1.19
- 12 months (n=108)	0.90	0.43	0.68-1.18
- 30 months (n=96)	0.83	0.073	0.67-1.02
Eczema - 6 months (n=112)	1.03	0.71	0.89-1.19
- 12 months (n=109)	1.03	0.68	0.89-1.20
- 30 months (n=96)	1.07	0.39	0.92-1.24

Figure legends

Figure 1. Proportional Venn diagram representing the number of study participants with CB or 6 month of age 25(OH)D3 measurements, and cell culture data.

Figure 2. Mean (SD) cord blood (light shaded bars) and 6 month of age peripheral blood (dark shaded bars) 25(OH)D3 by month of collection.

Figure 3. Scatter plots of cytokine production to HDM and OVA stimulation, in relation to 25(OH)D3 levels in CB. Significance *($p < 0.05$) **($p < 0.01$).

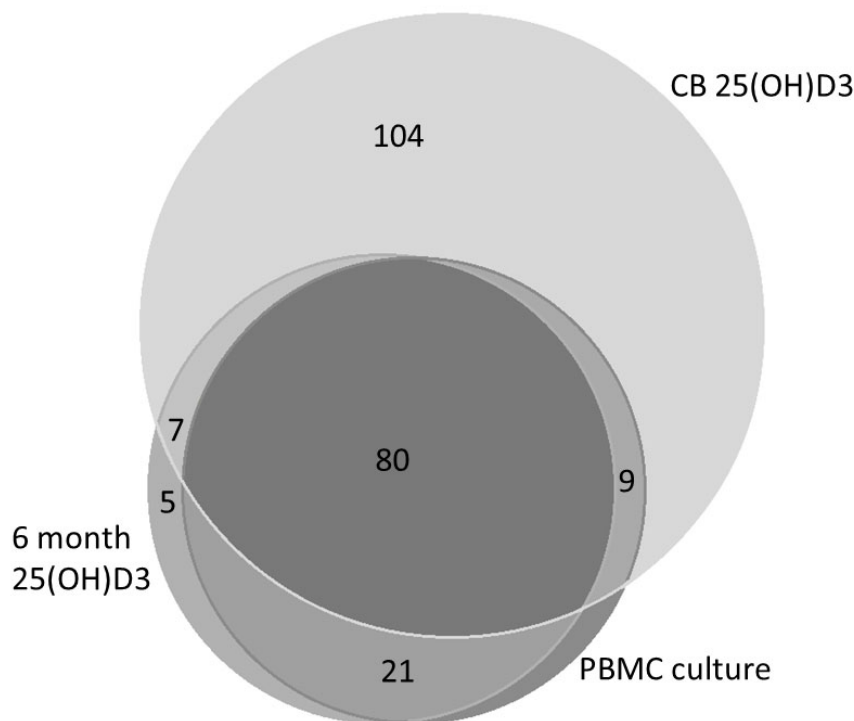
Figure 4. Scatter plots of cytokine production to TLR ligands, in relation to 25(OH)D3 levels at 6 months of age. Significance *($p < 0.05$).

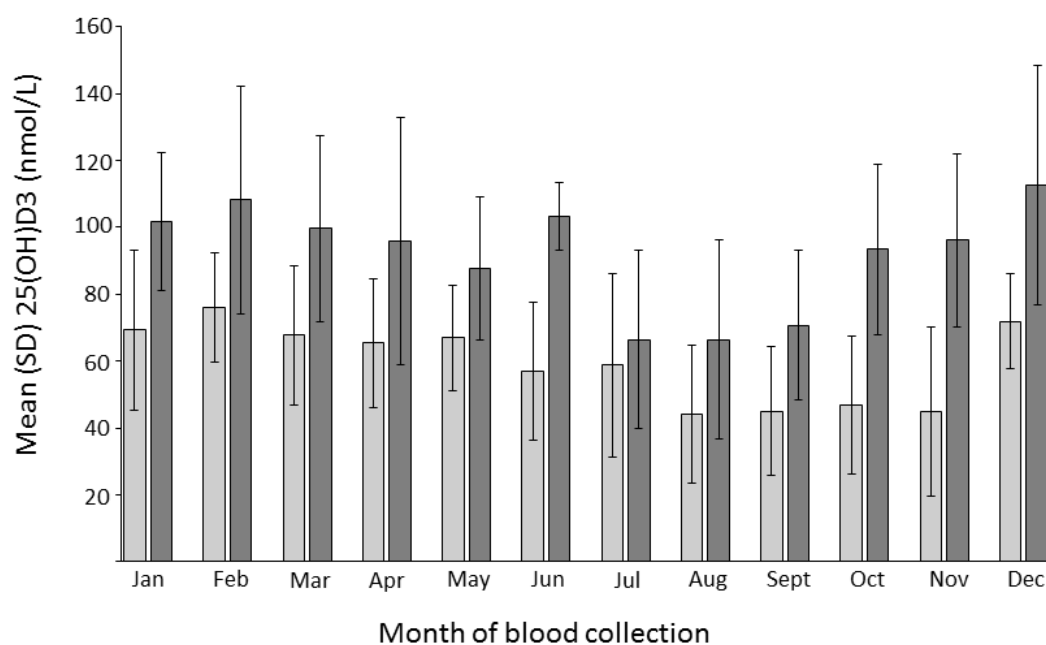
Figure 5. Tukey box and whisker plots of cytokine production to allergens in relation to eczema and wheeze status at 6, 12 and 30 months of age. Striped and dotted boxes represent participants positive or negative for the condition, respectively. Significance *($p < 0.05$) **($p < 0.01$).

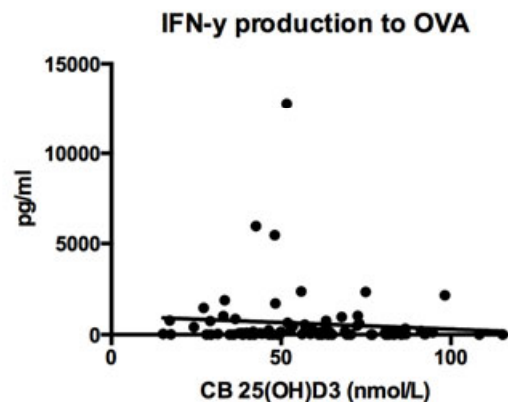
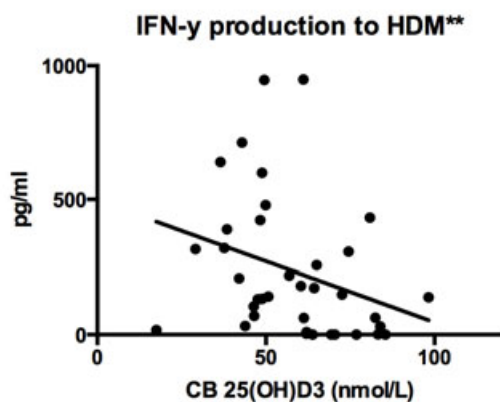
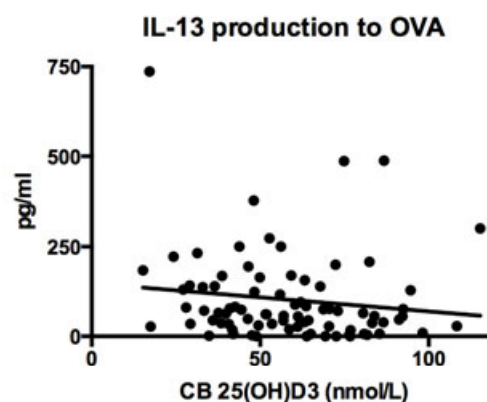
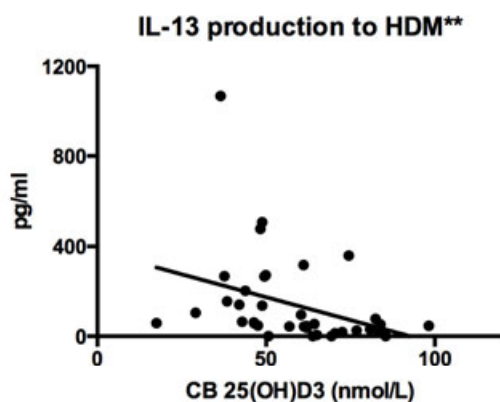
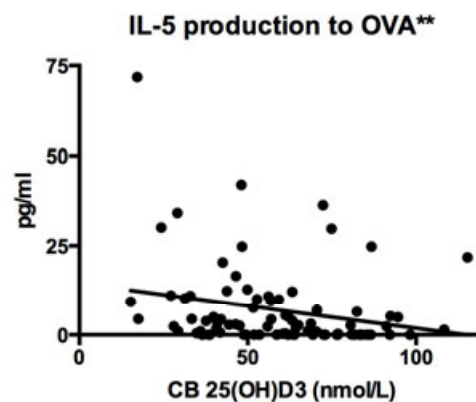
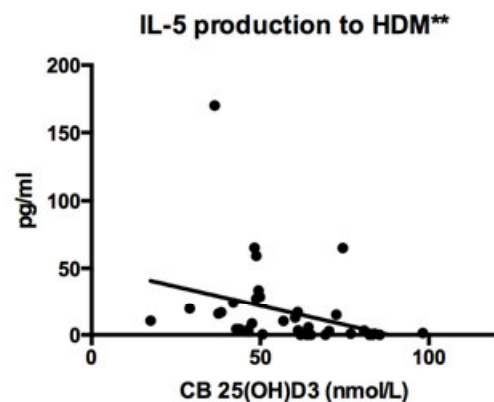
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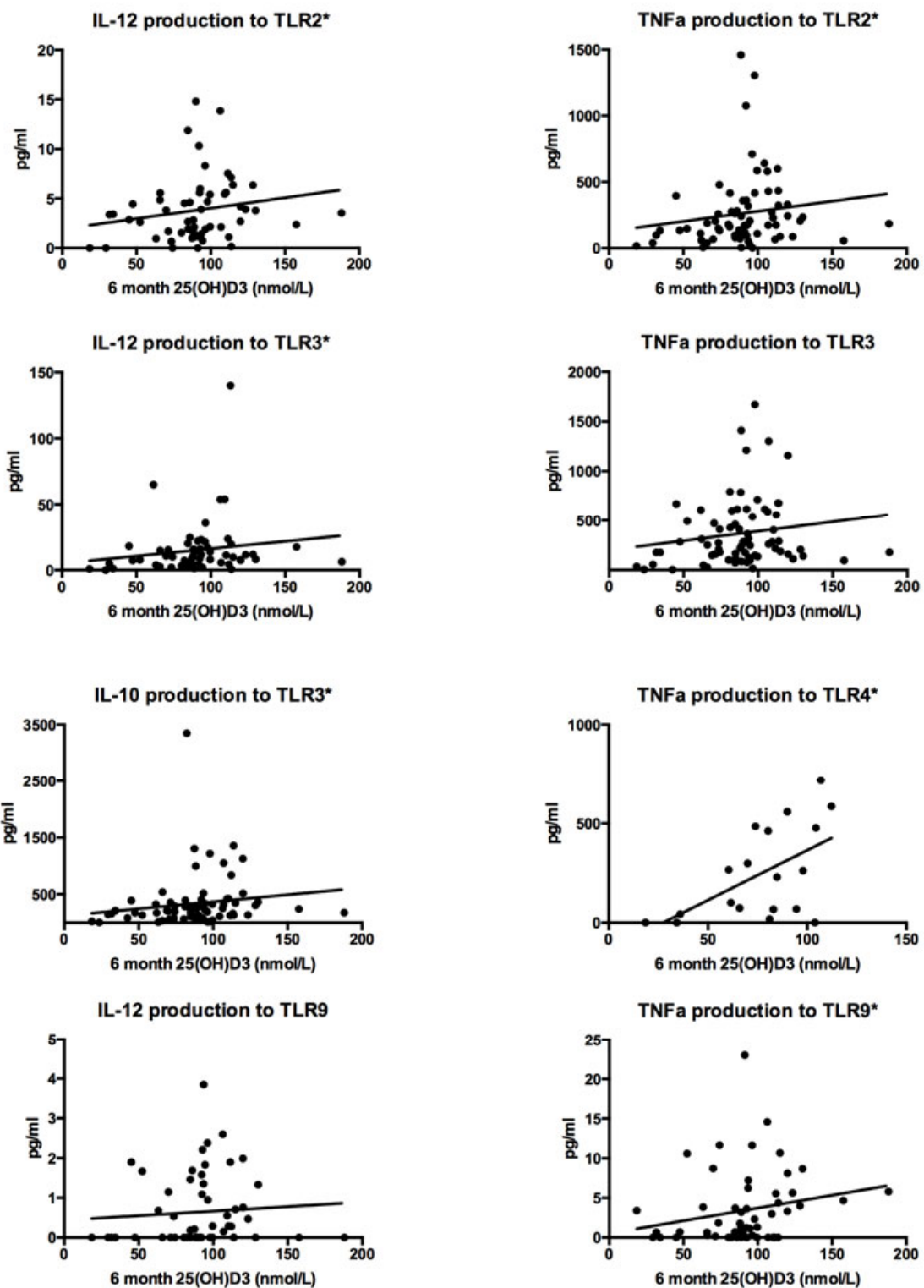
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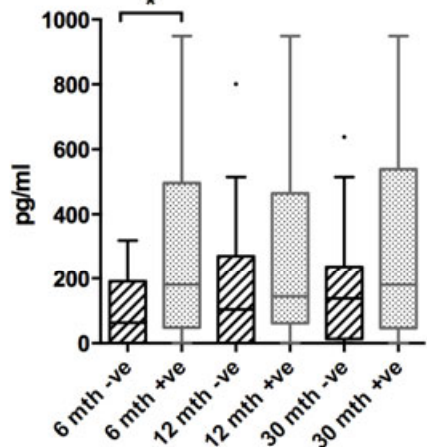
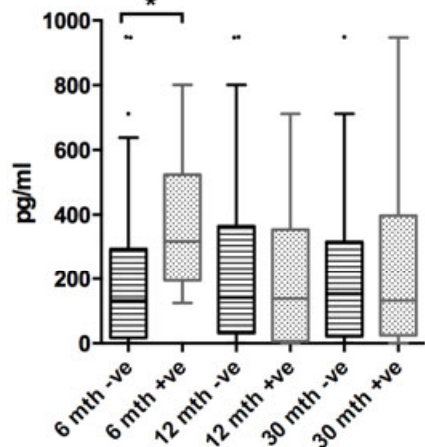
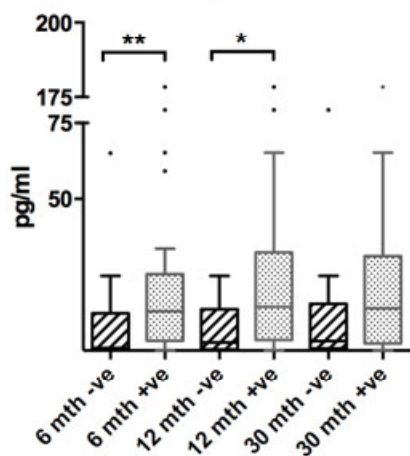
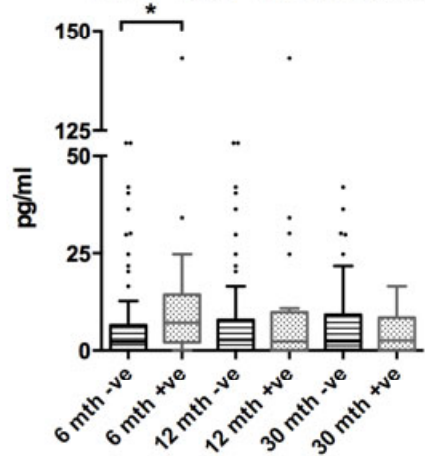
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HDM IFN- γ by eczema status**HDM IFN- γ by wheeze status****HDM IL-5 by eczema status****OVA IL-5 by wheeze status****HDM IL-13 by eczema status**